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Lactate Maintains BCR/Abl Expression and Signaling in Chronic Myeloid Leukemia Cells Under Nutrient Restriction

Angela Silvano,*†[1] Giulio Menegazzi,*[1] Silvia Peppicelli,*[1] Caterina Mancini,* Alessio Biagioni,* Alessandro Tubita,* Ignazia Tusa,* Jessica Ruzzolini,* Matteo Lulli,* Elisabetta Rovida,* and Persio Dello Sbarba*

*Department of Experimental and Clinical Biomedical Sciences "Mario Serio," Careggi Hospital, Università degli Studi di Firenze, Florence, Italy
†Department of Health Sciences, Division of Obstetrics and Gynecology, Careggi Hospital, Università degli Studi di Firenze, Florence, Italy

This study was directed to deepen the effects of nutrient shortage on BCR/Abl$_{protein}$ expression and signaling in chronic myeloid leukemia (CML) cells. The backbone of the study was cell culture in medium lacking glucose, the consumption of which we had previously shown to drive BCR/Abl$_{protein}$ suppression, and glutamine, the other main nutrient besides glucose. In this context, we focused on the role of lactate, the main by-product of glucose metabolism under conditions of rapid cell growth, in particular as a modulator of the maintenance of CML stem/progenitor cell potential, a crucial determinant of disease course and relapse of disease. The results obtained indicated that lactate is a powerful surrogate of glucose to prevent the suppression of BCR/Abl signaling and is therefore capable to maintain BCR/Abl-dependent CML stem/progenitor cell potential. A number of metabolism-related functional and phenotypical features of CML cells were also determined. Among these, we focused on the effect of lactate on oxygen consumption rate, the dependence of this effect on the cell surface lactate carrier MCT-1, and the relationship of the lactate effect to pyruvate and to the activity of mitochondrial pyruvate carrier.

Key words: Chronic myeloid leukemia (CML); Nutrient shortage; Lactate; Stem cell potential; Acidosis; MCT-1

INTRODUCTION

Chronic myeloid leukemia (CML) is a hematopoietic disease induced by the t(9;22) translocation, which results in the expression of the BCR/Abl oncoprotein, a constitutively active tyrosine kinase[1]. Tyrosine kinase inhibitors (TKI) such as imatinib-mesylate (IM) are capable to block the ATP-binding site of BCR/Abl and thereby to inhibit its enzymatic activity, resulting in remission of disease and extended survival in chronic phase CML patients[2]. However, TKI therapy does not cure CML, as it reduces most often neoplastic burden to the so-called minimal residual disease (MRD), sustained by the persistence of a TKI-resistant leukemia stem cell (LSC) subset[3,4]. LSC, like normal hematopoietic stem cells (HSC), are believed to reside in bone marrow stem cell niches, which are sites physiologically subjected to metabolic constraints because of the reduced oxygen and nutrient supply. It was indeed demonstrated in our laboratory that incubation under low oxygen tension restrains HSC clonal expansion but not HSC maintenance[5], without blocking HSC cycling, thus favoring HSC self-renewal[6,7]. In CML, low oxygen tension inhibits the expression of BCR/Abl$_{protein}$, but not that of the relative transcript[8,9], thus selecting LSC that remain genetically leukemic but are naturally refractory to TKI because devoid of the drugs' molecular target. This LSC subset can drive relapse of disease once conditions permissive for BCR/Abl$_{protein}$ expression and thereby clonal expansion (i.e., nutrient supply) are established[10].

We also found that glucose exhaustion, favored by low oxygen tension (the "Pasteur effect"), is actually the pivotal factor for BCR/Abl$_{protein}$ suppression[9]. This suppression is also driven by glucose shortage in normoxia[11]. As glucose shortage does not affect the maintenance of

[1]These authors provided equal contribution to this work.
Address correspondence to Persio Dello Sbarba, Department of Experimental and Clinical Biomedical Sciences "Mario Serio," viale G.B. Morgagni 50, 50134 Firenze, Italy. E-mail: persio@unifi.it *or* Matteo Lulli, Department of Experimental and Clinical Biomedical Sciences "Mario Serio," viale G.B. Morgagni 50, 50134 Firenze, Italy. E-mail: matteo.lulli@unifi.it

stem cell potential in BCR/$Abl_{protein}$-negative CML cells, a key question is which source of energy these cells rely on. CML cells are characterized by a highly glycolysis-oriented baseline metabolic profile[12,13], which leads to the release into the environment of abundant lactate. A number of studies carried out with solid cancers showed that lactate released into the environment by the highly glycolytic majority of cancer cells may be taken up as an energy source (the "metabolic symbiosis") by a minority of cells that is metabolically adapted to take advantage of such a recycle. Actually, lactate is considered as a major energy fuel in tumors[14,15].

The question behind the study reported here is whether lactate is a player in the modulation of BCR/$Abl_{protein}$ expression and signaling and in the maintenance of BCR/Abl-independent CML stem/progenitor cell potential. The effects of lactate, alone or in combination with medium acidification to match the second main consequence of extensive glycolysis[16], were determined in the absence of glucose and glutamine, the latter being, besides glucose, the most important nutrient usually available to cells in tissue/cancer microenvironment[17]. The results obtained indicated that lactate is a powerful surrogate of glucose to prevent the suppression of BCR/Abl signaling. Thus, lactate emerged as a metabolite capable to maintain BCR/Abl-dependent, rather than BCR/Abl-independent, CML stem/progenitor cell potential.

MATERIALS AND METHODS

Cell Culture

K562 and KCL22 BCR/Abl-positive CML cell lines (German Collection of Cell Cultures Braunschweig, Germany) were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin (Euro-Clone, Paington, UK). The experiments were performed with RPMI-1640 medium without glucose and without glutamine (Biological Industries, Kibbutz Beit-Haemek, Israel), supplemented or not with sodium lactate (Sigma-Aldrich, St. Louis, MO, USA) at 10 mM final concentration. In some cultures, pH was adjusted to 6.7 ± 0.1 using 1 N HCl (VWR Chemicals, Radnor, PA, USA).

Cells were plated at 3×10^5 cells/ml of culture and incubated at 37°C in water-saturated atmosphere containing 5% CO_2 and 21% O_2; 24 h before plating, cells were transferred from maintenance cultures to fresh medium (at 5×10^5/ml). Viable cells were counted by trypan blue exclusion in a hemocytometer and evaluated by 7-amino-actinomycin D [7-AAD; BD Biosciences, San Jose, CA, USA; 10 min at room temperature (RT) after cell wash in phosphate-buffered saline (PBS)] exclusion in a flow cytometer (FACSCanto; BD Biosciences, Franklin Lakes, NJ, USA).

In some experiments, cultures were treated with the mono-carbon transporter 1/2 (MCT-1/2) inhibitor AR-C155858 (MedChemExpress, Monmouth Junction, NJ, USA) at 5 μM final concentration, or with the mitochondrial pyruvate carrier (MPC) inhibitor UK5099 (MedChemExpress) at 5 μM final concentration, or with methyl-pyruvate (Sigma-Aldrich) at 10 mM final concentration.

Culture Repopulation Ability (CRA) Assay

The CRA assay is an in vitro surrogate for the in vivo Marrow Repopulation Ability assay. The assay estimates the stem/progenitor cell potential of a native or experimentally manipulated cell population via cell transfer to cultures established in liquid medium rather than cell transplantation into animals[6,7,18,19]. Cells rescued at different times of incubation (day 10 and day 14) from cultures (LC1) to which the different experimental treatments are applied were washed and replated (3×10^4 cells/ml) into secondary liquid cultures (LC2) established with standard RPMI-1640 medium (i.e., containing glucose and glutamine). The number of viable cells was then counted at different times of incubation in LC2. It is worth pointing out that LC2 are meant to allow maximal cell growth and that in no case LC2 were subjected to experimental changes applied to LC1 (addition of lactate and/or low pH). The kinetics of cell number in LC2 provide an estimate of the CRA of LC1 cells. Culture medium was never renewed during LC1 or LC2.

Cell Lysis and Western Blotting

Cells were washed once with ice-cold PBS containing 100 μM Na_3VO_4. Total cell lysates were obtained in Laemmli buffer (62.5 mM tris/HCl, pH 6.8, 10% glycerol, 0.005% bromophenol blue, and 2% SDS). Protein concentration was determined by the BCA method (Pierce BCA Protein Assay Kit; ThermoFisher Scientific, Waltham, MA, USA), and 50 μg of sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred onto nitrocellulose membranes (Amersham Protran Premium Western blotting membranes; GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) by electroblotting. Primary antibodies used were raised against: c-Abl (K-12), rabbit polyclonal; MCT-1 (H-1), mouse monoclonal; lactate dehydrogenase (LDH)-A (ab85472), rabbit polyclonal; LDH-B (431.1), mouse monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA); CrkL, mouse monoclonal, to phospho-CrkL (Tyr207), rabbit polyclonal (Cell Signaling Technology, Danvers, MA, USA); vinculin (clone hVIN-1), mouse monoclonal (Sigma-Aldrich). Anti-c-Abl antibodies can be used to determine BCR/Abl expression given the great difference of molecular weight between the two proteins.

Crkl phosphorylation is used as an indicator of BCR/Abl kinase activity. Washed membranes were incubated for 1 h at RT in 1:1—Odyssey Blocking Buffer (OBB)/T-PBS containing an IRDye 800CW- or IRDye 680-conjugated secondary antibody. Antibody-coated protein bands were visualized by the Odyssey Infrared Imaging System (LI-COR Biosciences), as previously reported[20].

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was extracted from cells by using TRI reagent (Sigma-Aldrich). The amount and purity of RNA were determined spectrophotometrically. cDNA synthesis was obtained by incubating 1 μg of total RNA with iScript reverse transcription supermix (Bio-Rad, Milan, Italy) according to the manufacturer's instructions. qPCR was performed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The qPCR analysis was carried out in triplicate with a CFX96 Real-Time PCR System (Bio-Rad) with the default PCR setting: 40 cycles of 95°C for 10 s and 60°C for 30 s. The fold change was determined by the comparative Ct method using 18S and β-actin as reference genes. Primer sequences were as follows: β-actin (5′-TCGAGCCATAAAAGGCAACT-3′ forward and 5′-CTTCCTCAATCTCGCTCTCG-3′ reverse), 18S (5′-CGCCGCTAGAGGTGAAATTCT-3′ forward and 5′-CGAACCTCCGACTTTCGTTCT-3′ reverse), and *BCR/abl* (5′-GGAGCAGCAGAAGAAGTGTTT-3′ forward and 5′-TGGGTCCAGCGAGAAGGTTTT-3′ reverse).

Measure of Oxygen Consumption Rate (OCR) and Labeling of Mitochondria

To label mitochondria, cells were incubated for 30 min at 37C° with 5 nM MitoTracker Deep Red to determine mitochondrial mass (mitochondria labeling independent of membrane potential) or MitoTracker Orange to determine mitochondrial activity following the manufacturer's instructions (ThermoFisher Scientific), with a FACSCanto flow cytometer[21].

Basal OCR (pmolesO_2 consumed per min) was measured using a SeaHorse XF96 Extracellular Flux Analyzer (SeaHorse Bioscience, Billerica, MA, USA) following the manufacturer's instructions. Cells were counted and seeded (7×10^4 cells/well) in XF96 Seahorse microplates precoated with poly-D-lysin (ThermoFisher Scientific). Cells were suspended in XF Assay Medium supplemented with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose (Euro-Clone) and left to adhere for a minimum of 30 min at 37°C. The plate was left to equilibrate for 10 min in a CO_2-free incubator before being transferred to the Seahorse XF96 analyzer. The prehydrated cartridge was filled with the indicated compounds and calibrated for 30 min in the SeaHorse Analyzer. Normalization to protein content was performed after each experiment. The SeaHorse XF Report Generator automatically calculated the parameters from Wave data that have been exported to Excel.

Statistical Analysis

Data are presented as means ± standard error of the mean (SEM) or standard deviation (SD) of at least three independent experiments and were compared by Student's *t*-test or two-way analysis of variance (ANOVA) (when more than two samples were compared) using the GraphPad Prism software; *p* values lower than 0.05 were considered to reflect the statistical significance of differences.

RESULTS

Effects of Lactate on CML Cell Viability and Growth

The effects of lactate addition and/or pH reduction in the absence of glucose and glutamine were evaluated. Addition of 10 mM lactate alone did not change the pH of culture medium (data not shown). Neither in the presence of lactate nor under lactic acidosis (lactate/pH 6.7), the time 0 pH value changed throughout the incubation (data not shown). Figure 1 shows that, in control cultures of either K562 or KCL22 cells, cell viability decreased with respect to time 0 in function of incubation time (day 10 vs. time 0 and day 14 vs. day 10). This decrease was abolished in K562 cells in the presence of lactate as well as under lactic acidosis; low pH slightly reduced the positive effect of lactate addition on cell viability on day 14 of incubation but had no effect under all the other experimental conditions (Fig. 1A). In KCL22, like in K562 cell cultures, lactate largely prevented the time-dependent decrease in viable cell number on day 14 of incubation; low pH did not interfere with the effect of lactate or the behavior of control cultures (Fig. 1B).

The kinetics of viable cell number in culture was then determined. Figure 1C shows that, in control K562 cell cultures, the number of viable cells, following a transient increase (day 3), decreased progressively in the course of incubation. In the presence of lactate, the number of viable cells was significantly higher than in control cultures from day 7, to attain on day 10 more than a doubling with respect to time 0 and a more than fourfold increase with respect to control. Medium acidification had no effect under control conditions but reduced markedly the growth-promoting effect of lactate, in keeping with what shown for cell viability in Figure 1A. In control KCL22 cell cultures (Fig. 1D) viable cell number underwent a more marked and sustained growth than in K562 cell cultures, so that KCL22 cells were on day 14 still more than 50% of time 0 level, whereas K562 cells were fivefold reduced. No experimental change had effect on KCL22 cells until day 10 of incubation, whereas on day 14 the numerical decrease in viable KCL22 cells with respect to time 0 in control cultures was overridden in the

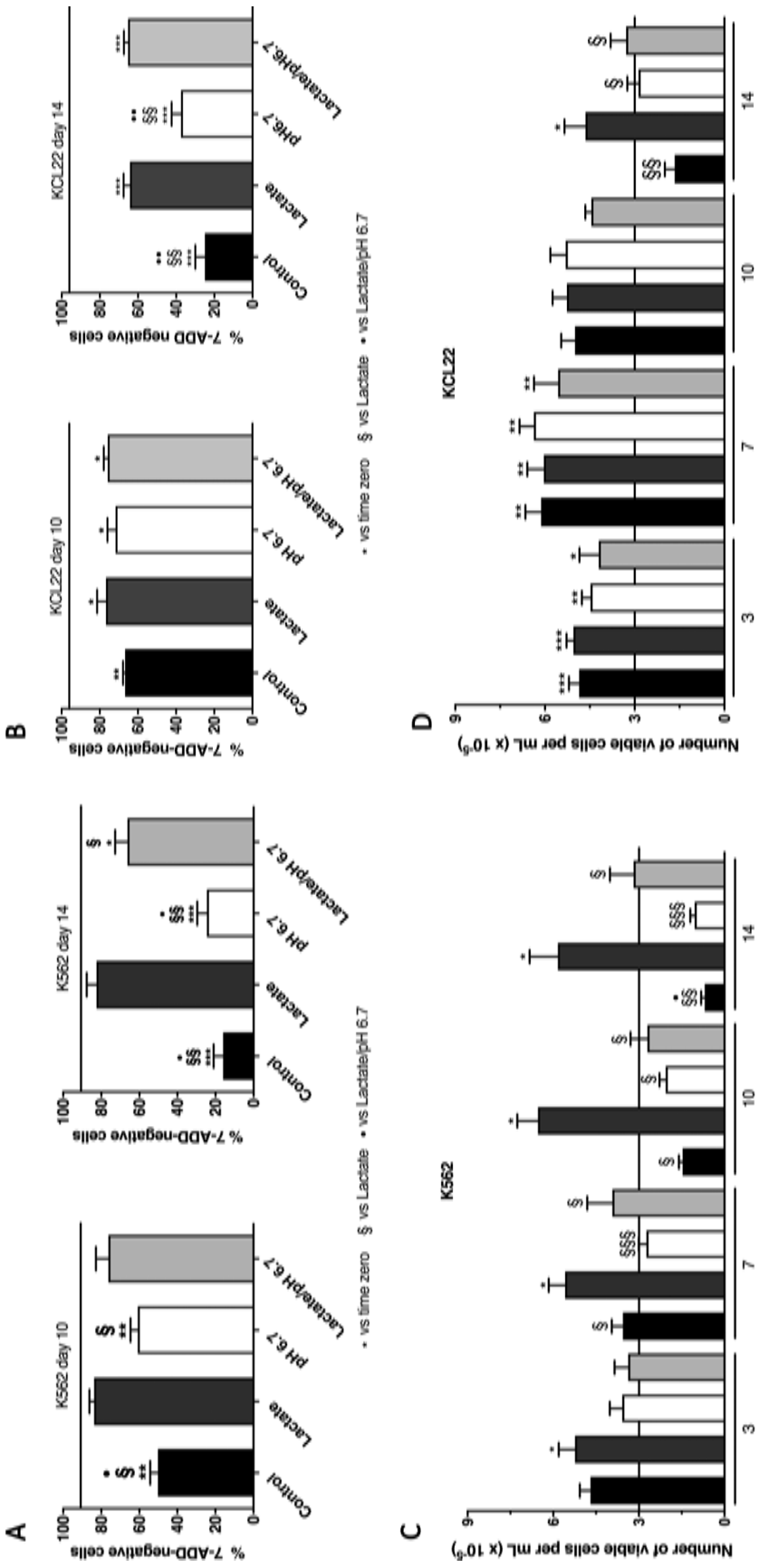

Figure 1. Effects of lactate and/or low pH on chronic myeloid leukemia (CML) cell survival and growth in culture. K562 (A, C) or KCL22 (B, D) cells were seeded at 3×10^5 cells/ml in glucose/glutamine-free culture medium. Lactate (10 mM) and/or 1 N HCl to reduce medium pH to 6.7 ± 0.1 were added or not at time 0 of incubation. 7-Amino-actinomycin D (7-ADD)-negative (A, B) or trypan blue-negative (C, D) cells were counted on the indicated days of incubation. Horizontal lines indicate the time 0 values. Histograms represent means ± standard error of the mean (SEM) of data obtained from three (A, B) or five (C, D) independent experiments. The statistical significance of the differences indicated in (A) and (B) was determined by analysis of variance (ANOVA). Single symbols, $p < 0.05$; double symbols, $p < 0.01$, triple symbols, $p < 0.001$.

presence of lactate, matching the K562 results, as well as under lactic acidosis.

Effects of Lactate on BCR/Abl$_{protein}$- and BCR/Abl-Dependent Signaling

The effects of lactate and/or acidosis on the expression of BCR/Abl$_{protein}$ and on BCR/Abl-dependent signaling were then determined at time 0 and on days 10 and 14 of incubation (Fig. 2). In control CML cell cultures, BCR/Abl$_{protein}$ expression and especially Crkl phosphorylation, commonly used as a read-out of BCR/Abl kinase activity[22], were progressively suppressed in the course of incubation, more rapidly in K562 than in KCL22 cells. Crkl expression on day 14, on the contrary, was found identical in all experimental variants. In the presence of lactate, BCR/Abl$_{protein}$ expression and Crkl phosphorylation were maintained at the levels of time 0 (or even slightly enhanced) throughout incubation of either cell line. Medium acidification did not determine appreciable effects on either BCR/Abl$_{protein}$ expression or Crkl phosphorylation in lactate-treated cultures. In the absence of lactate, Crkl phosphorylation was weakly stimulated at pH 6.7 with respect to control.

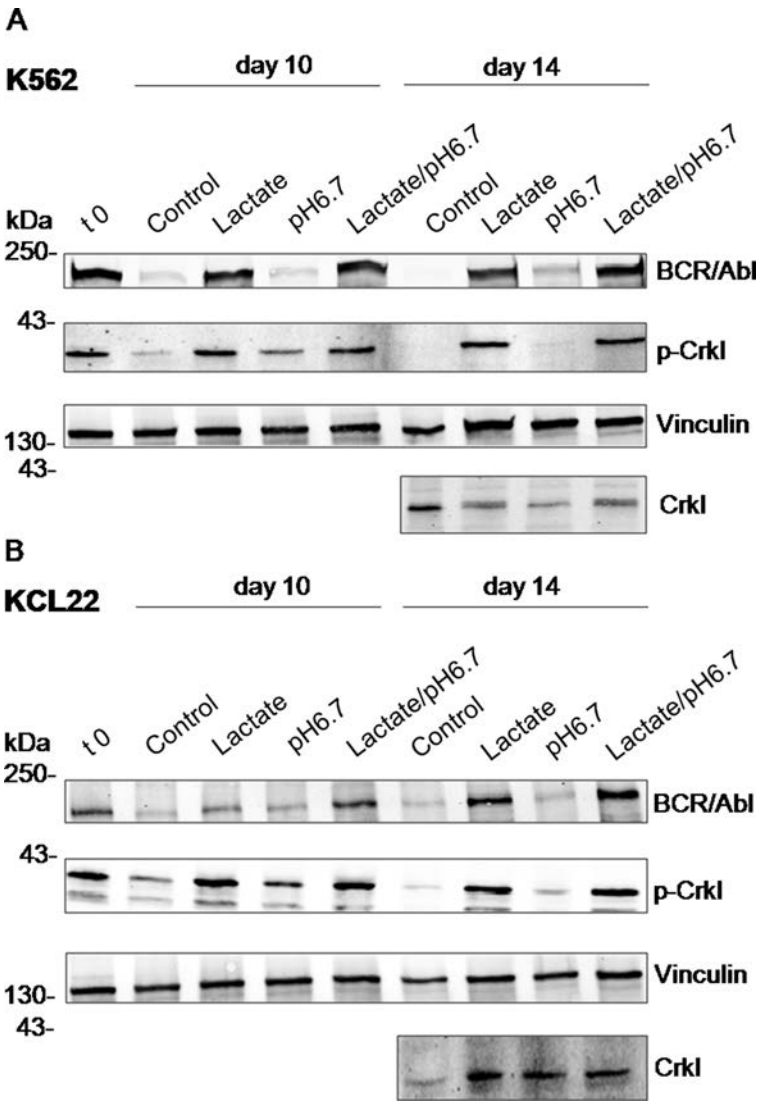

Figure 2. Effects of lactate and/or low pH on BCR/Abl expression and signaling in CML cells. K562 (A) or KCL22 (B) cells were cultured for the indicated times as described in the legend of Figure 1. Total cell lysates in Laemmli buffer were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with antibodies raised against c-Abl, Crkl, or p-Crkl. Anti-vinculin antibody was used to verify the equalization of protein loading. Time 0: t 0. One representative experiment out of three with similar outcome is shown. Averaged densitometric values obtained on day 14 of incubation from the three independent experiments performed are reported in Supplementary Figure S1 (available at https://www.sbsc.unifi.it/vp-363-supplementary-material-rev.html).

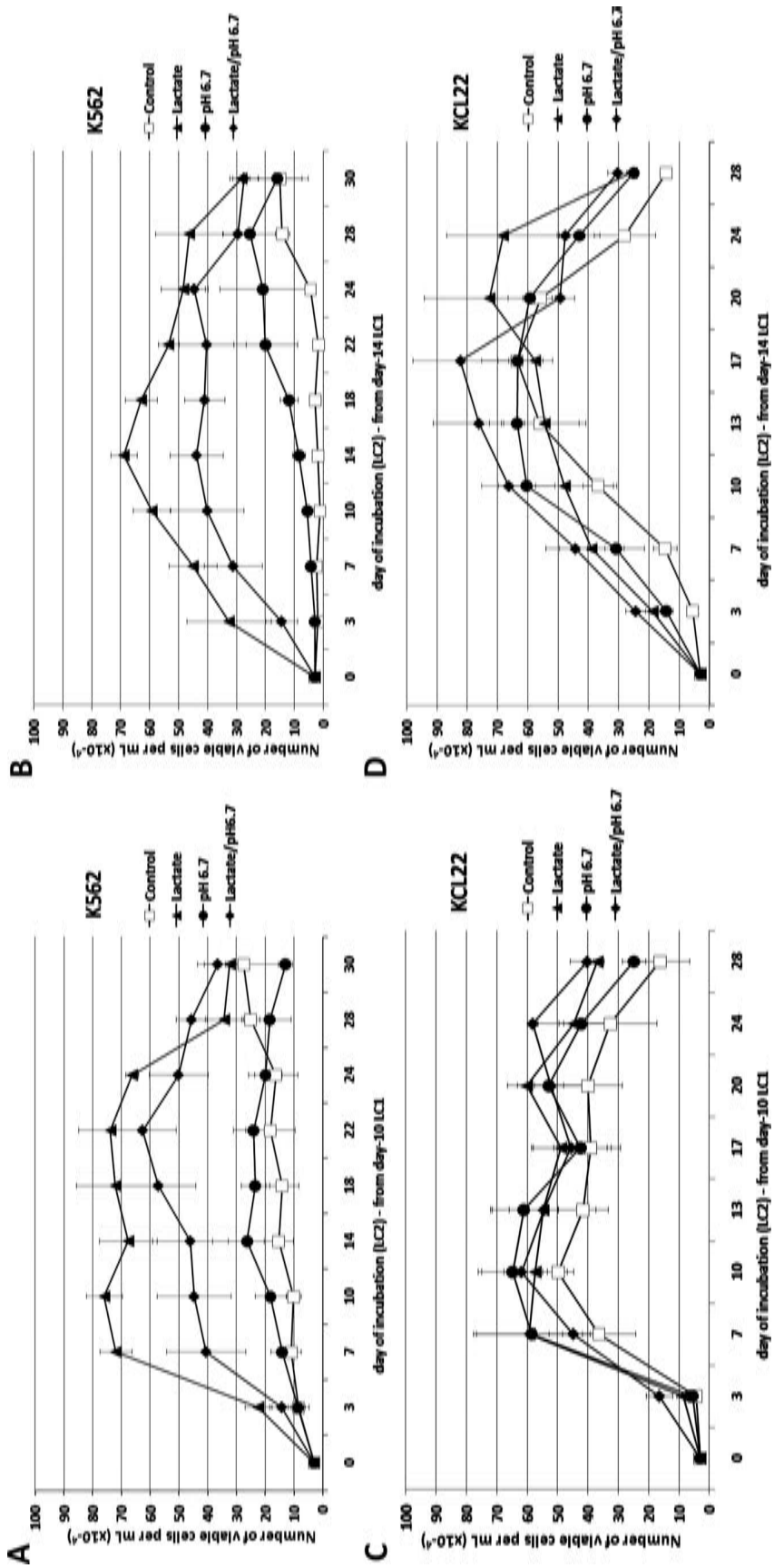

Figure 3. Effects of lactate and/or low pH on the maintenance of CML stem/progenitor cell potential. K562 (A, B) or KCL22 (C, D) cells were cultured as described in the legend of Figure 1 and subjected to the indicated experimental treatments for 10 (A, C) or 14 (B, D) days (LC1). Cells were then rescued from LC1 and replated at 3×10^4 cells/ml into "expansion" secondary cultures (LC2) established in standard medium (glucose/glutamine-supplemented, lactate-free, pH 7.4) to ensure maximal growth. Trypan blue-negative cells were counted at the times of incubation in LC2 indicated in abscissa. Values are means ± SEM of data obtained from five independent experiments. The statistical significance of differences relative to K562 cells (A, B), as determined by ANOVA, is reported in Supplementary Table S1; in the case of KCL22 cells, differences were never significant for any comparison at any incubation time (C, D).

Averaged densitometric values obtained on day 14 from three independent experiments including the one shown in Figure 2 are reported in Supplementary Figure S1 (this and the additional supplementary material are available at https://www.sbsc.unifi.it/vp-363-supplementary-material-rev.html). Thus, lactate is a booster of BCR/Abl signaling under glucose and glutamine shortage. This effect of lactate was found due at least in part to transcriptional upregulation of *BCR/abl* expression (Supplementary Fig. S2).

Effects of Lactate on the Stem/Progenitor Cell Potential of CML Cells

The effects of lactate addition and/or pH reduction on the maintenance of stem/progenitor cell potential were then evaluated by CRA assay. Cells recovered on day 10 or 14 from cultures subjected to the different experimental treatments (LC1) were transferred to secondary cultures (LC2) ensuring conditions permissive for maximal cell growth (presence of glucose and glutamine), and the kinetics of viable cell number in LC2 was determined (Fig. 3). Lactate addition to, but not acidification of, LC1 markedly enhanced LC2 repopulation by K562 cells rescued from either day 10 or day 14 LC1, differences being significant from day 7 or day 3 on, respectively (Fig. 3A and B). Acidification of lactate-supplemented LC1 attenuated the effects of lactate, differences being significant for days 7 and 10 in LC2 established with day 10 LC1 cells (Fig. 3A) and never in LC2 established with day 14 LC1 cells (Fig. 3B). As far as KCL22 cells are concerned, no significant differences were observed among all four experimental variants, for both times of transfer (days 10 and 14) of LC1 cells to LC2 (Fig. 3C and D).

Effects of Lactate on the Metabolic Profile and Phenotype of CML Cells

The results summarized so far suggested to deepen the effects of lactate on cells' metabolic profile as well as metabolism-related phenotypical markers. Mitochondrial function was assayed first by flow cytometry following cell staining with MitoTracker Orange or MitoTracker deep red. K562 and KCL22 cells exhibited a similar basal mitochondrial activity; however, KCL22 cells were found endowed with a fourfold higher mitochondrial mass than K562 cells, so that the latter showed a fourfold higher mitochondrial activity to mass ratio than KCL22 cells (Supplementary Fig. S3).

The effects of lactate were then tested on cells' metabolic profile and the expression of lactate metabolism-related markers of cell phenotype (Fig. 4). Lactate markedly enhanced cell OCR in either K562 or KCL22 cell cultures, as determined by SeaHorse technology (Fig. 4A and C). The phenotypical markers taken into consideration (Fig. 4B and D) were MCT-1, which facilitates lactate and pyruvate upload[23,24], the lactate exporter MCT-4, LDH-A, which catalyzes the conversion of pyruvate to lactate, and LDH-B, which works the opposite way, catalyzing the conversion of lactate to pyruvate[25]. No significant change was observed on day 10 of incubation for any of the markers in either cell line. On day 14 of incubation of either cell line, the expression of all markers was markedly enhanced in lactate-supplemented cultures with respect to control, with the exception of MCT-4 in KCL22 cells. Medium acidification in the absence of lactate enhanced, much less markedly, the expression of MCT-1, MCT-4, and LDH-A in K562, and of MCT-1 in KCL22 cell cultures. The enhanced lactate-induced expression of both MCT-1 and LDH-B, shared by the two cell lines, is likely to represent a positive feedback favoring the utilization of lactate as a nutrient.

Interference With the Effects of Lactate Addition

The results of the experiments of Figure 4 led to test whether the treatment with the MCT-1/2 inhibitor AR-C15585, added to cultures at time 0, interfered with the lactate-dependent increase in cell number. AR-C15585 was preliminarily shown to be effective at 5 μM and devoid of aspecific toxic effects up to the 40 μM final concentration, in either control or lactate-supplemented cultures of either cell line (data not shown). Figure 5 shows that, on day 10 of incubation, the number of viable cells was more than 10-fold in K562 and almost 3-fold in KCL22 cells higher in lactate-supplemented than in control cultures. MCT-1/2 inhibition reduced the number of viable K562 cells incubated in the presence of lactate to levels below those of time 0 (Fig. 5A, left panel). MCT-1/2 inhibition resulted in a less marked effect in lactate-supplemented KCL22 cell cultures, where the number of viable cells was significantly reduced, but to levels well above those of time 0 (Fig. 5B, left panel). MCT-1/2 inhibition suppressed the lactate-dependent increase in BCR/Abl protein expression and Crkl phosphorylation in either cell line (Fig. 5A and B, right panels).

To address the question whether lactate metabolism is needed for its effects on BCR/Abl expression and signaling, we tested the effects of MPC inhibition, under the assumption that, via LDH-B-dependent conversion to pyruvate, lactate would be funneled to oxidative metabolism in the mitochondria. MPC inhibition indeed abolished the lactate-dependent stimulation of cell proliferation, maintaining the number of viable cells after 10 days of incubation at the levels of time 0 in cultures of either cell line (Fig. 5C and D, left panels). The effects on cell count were paralleled by those on BCR/Abl protein expression and signaling determined by Western blotting (Fig. 5C and D, right panels).

The effects of MPC prompted us to ask whether pyruvate can mimic the effects of lactate, and in particular whether pyruvate can rescue the effects of the block of lactate uptake

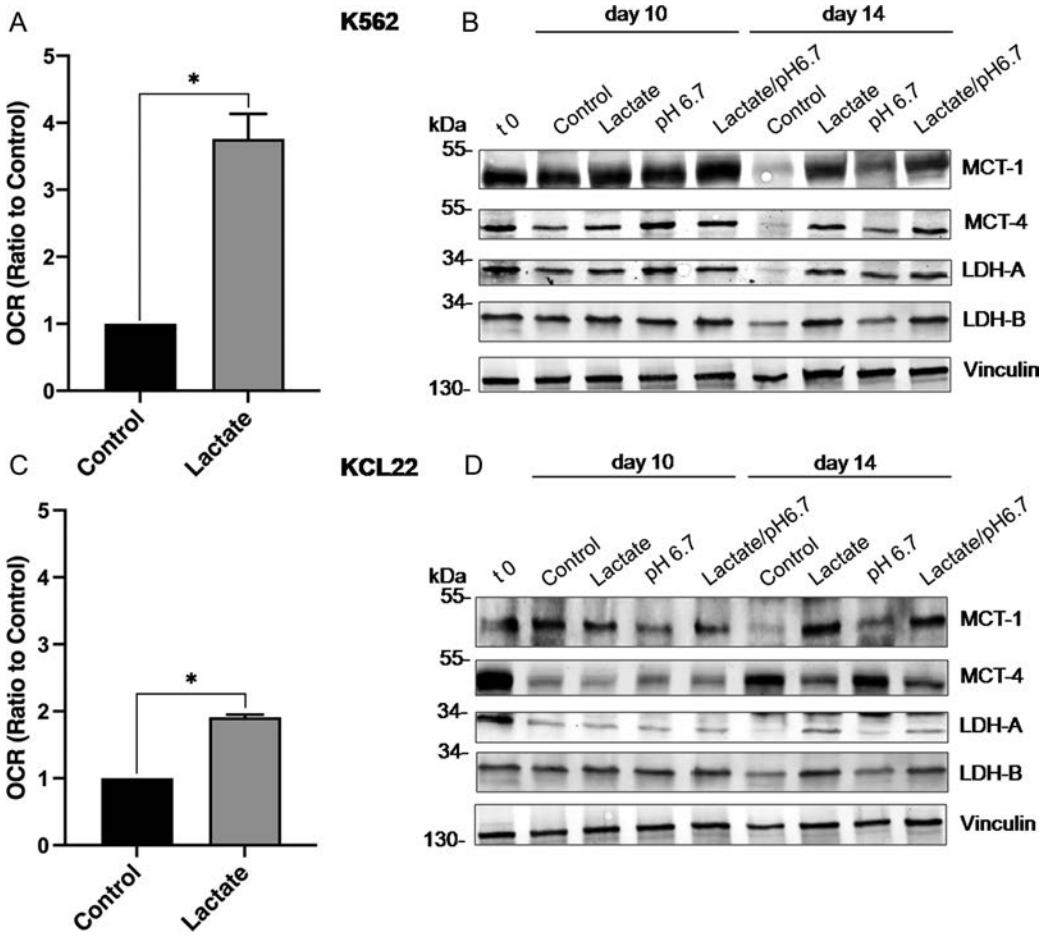

Figure 4. Effects of lactate and/or low pH on the metabolic profile and markers of CML cells. K562 (A, B) or KCL22 (C, D) cells were cultured for the indicated times as described in the legend of Figure 1. Basal oxygen consumption rate was determined by SeaHorse technology on day 10 of incubation according to the manufacturer's instructions; the statistical significance of differences (three independent experiments) was determined by the Student's *t*-test for paired samples; $*p < 0.05$ (A, C). Total cell lysates in Laemmli buffer were subjected to SDS-PAGE and immunoblotting with antibodies raised against the indicated proteins. Anti-vinculin antibody was used to verify the equalization of protein loading. Time 0: t 0. One representative experiment out of three with similar outcome is shown (B, D).

due to MCT-1 inhibition. Cultures were supplemented with methyl-pyruvate, a cell-permeable form of pyruvate that therefore, unlike lactate, does not need cell surface carriers such as MCT-1 to be taken up by cells. Methyl-pyruvate addition rescued completely (KCL22 cells) or partially (K562 cells) the negative effects of AR-C155858 treatment on the lactate-dependent increase in cell number in culture (Fig. 6A and C). That the lactate-dependent enhancement of BCR/Abl expression and signaling was maintained in the presence of methyl-pyruvate despite AR-C155858 treatment is shown in Figure 6B and D.

Interference With the Effects of Endogenous Lactate

Finally, we tried to confirm the above-described effects of lactate addition to glucose-free cell cultures using instead a standard culture medium containing glucose and glutamine. Under these conditions, as expected[9,26], glucose concentration in culture medium on day 4 of incubation was found markedly reduced with respect to time 0 (Fig. 7A and B), apparently as a consequence of cell growth (Fig. 7C and D). MCT-1 inhibition reduced the number of viable cell number in cultures of either cell line (Fig. 7C and D, controls). This reduction was paralleled by a complete or almost complete suppression of BCR/Abl protein expression and a reduction in Crkl phosphorylation (Fig. 7E and F). As glucose exhaustion from culture medium has been found linked to BCR/Abl protein suppression[9], the fact that on day 4 of incubation BCR/Abl is suppressed following MCT-1 inhibition seems to indicate that at this time point the maintenance of BCR/Abl expression relies on lactate endogenously generated in parallel to glucose consumption rather than on glucose itself.

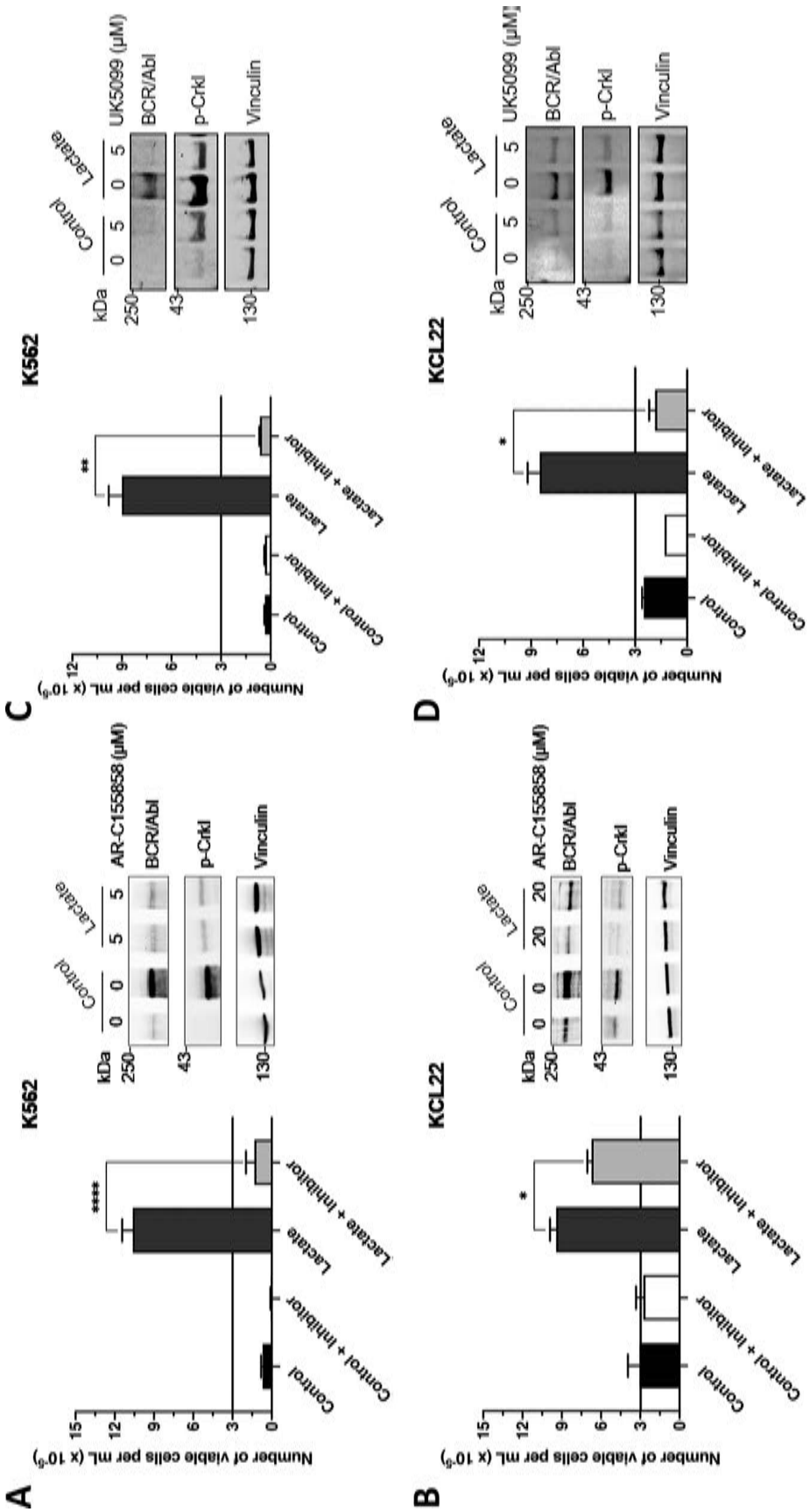

Figure 5. Effects of mono-carbon transporter 1 (MCT-1) or mitochondrial pyruvate carrier (MPC) inhibition on BCR/Abl expression/signaling and cell growth in the presence of lactate. K562 (A, C) or KCL22 (B, D) cells were cultured for 14 days as described in the legend of Figure 1, in the presence or absence of the MCT-1 inhibitor AR-C155858 (A, B) or the MPC inhibitor UK5099 (C, D) from time 0 of incubation. Left panels: Histograms represent counts of trypan blue-negative cells and are means ± SEM of data obtained from three independent experiments; the statistical significance of differences was determined by the Student's *t*-test for paired samples; $*p < 0.05$; $**p < 0.01$; $****p < 0.001$; horizontal lines indicate time 0 values. Right panels: Cells were lysed in Laemmli buffer and total cell lysates subjected to SDS-PAGE and immunoblotting with antibodies raised against c-Abl or p-Crkl; anti-vinculin antibody was used to verify the equalization of protein loading; one representative experiment out of three with similar outcome is shown.

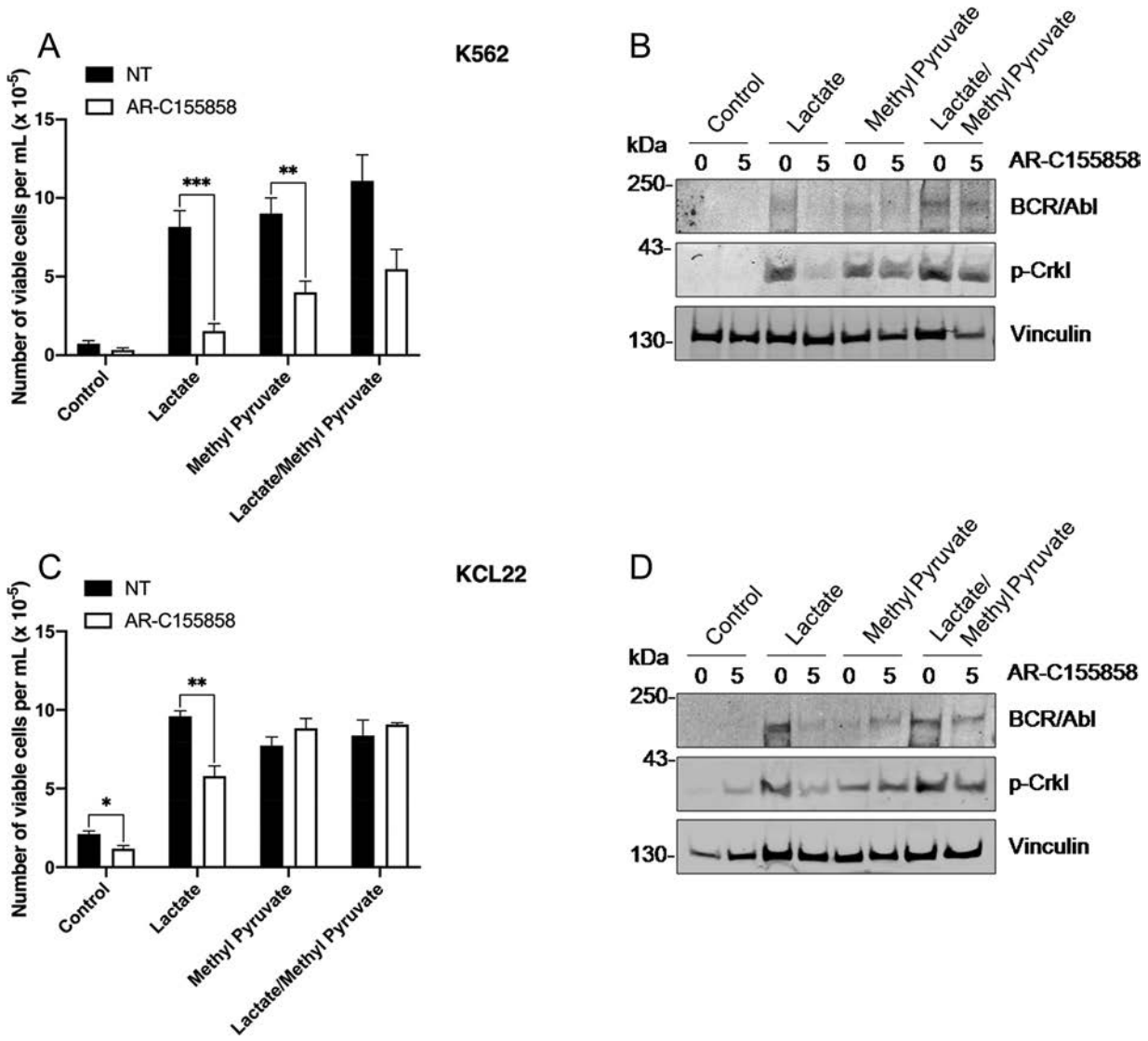

Figure 6. Rescue by methyl-pyruvate addition of the effects of MCT-1 inhibition on BCR/Abl expression/signaling and cell growth in the presence of lactate. K562 (A, B) or KCL22 (C, D) cells were cultured for 14 days as described in the legend of Figure 1, in the presence or the absence of lactate and/or methyl-pyruvate and/or the MCT-1 inhibitor AR-C155858 from time 0 of incubation. Histograms represent counts of trypan blue-negative cells and are means ± SEM of data obtained from three independent experiments; the statistical significance of differences was determined by the Student's *t*-test for paired samples; $*p < 0.05$; $**p < 0.01$; $***p < 0.005$ (A, C). Cells were lysed in Laemmli buffer and total cell lysates subjected to SDS-PAGE and immunoblotting with antibodies raised against c-Abl or p-Crkl; anti-vinculin antibody was used to verify the equalization of protein loading; one representative experiment out of three with similar outcome is shown (B, D).

DISCUSSION

Lactate emerged from this study as a metabolite capable to influence survival and growth, as well as BCR/Abl signaling, in CML cell cultures. Lactate, unlike low pH alone and irrespectively of pH, enhanced viability of K562 cells from day 7 and of KCL22 cells on day 14 only (Fig. 1A and B). These differences were due apparently to a higher resistance of KCL22 cells to the harsh incubation conditions of our experimental system. Likewise, control KCL22 compared to K562 cells exhibited enhanced growth/slower decay of viable cell number with respect to time 0 (Fig. 1C vs. 1D). On the other hand, when lactate was added to culture medium, cell growth was pushed to higher levels in K562 than in KCL22 cell cultures. This suggests that, in comparison to KCL22 cells, K562 cells have a limited endogenous *reservoir* of lactate but are well equipped to respond survival- and growth-wise to the supply of exogenous lactate.

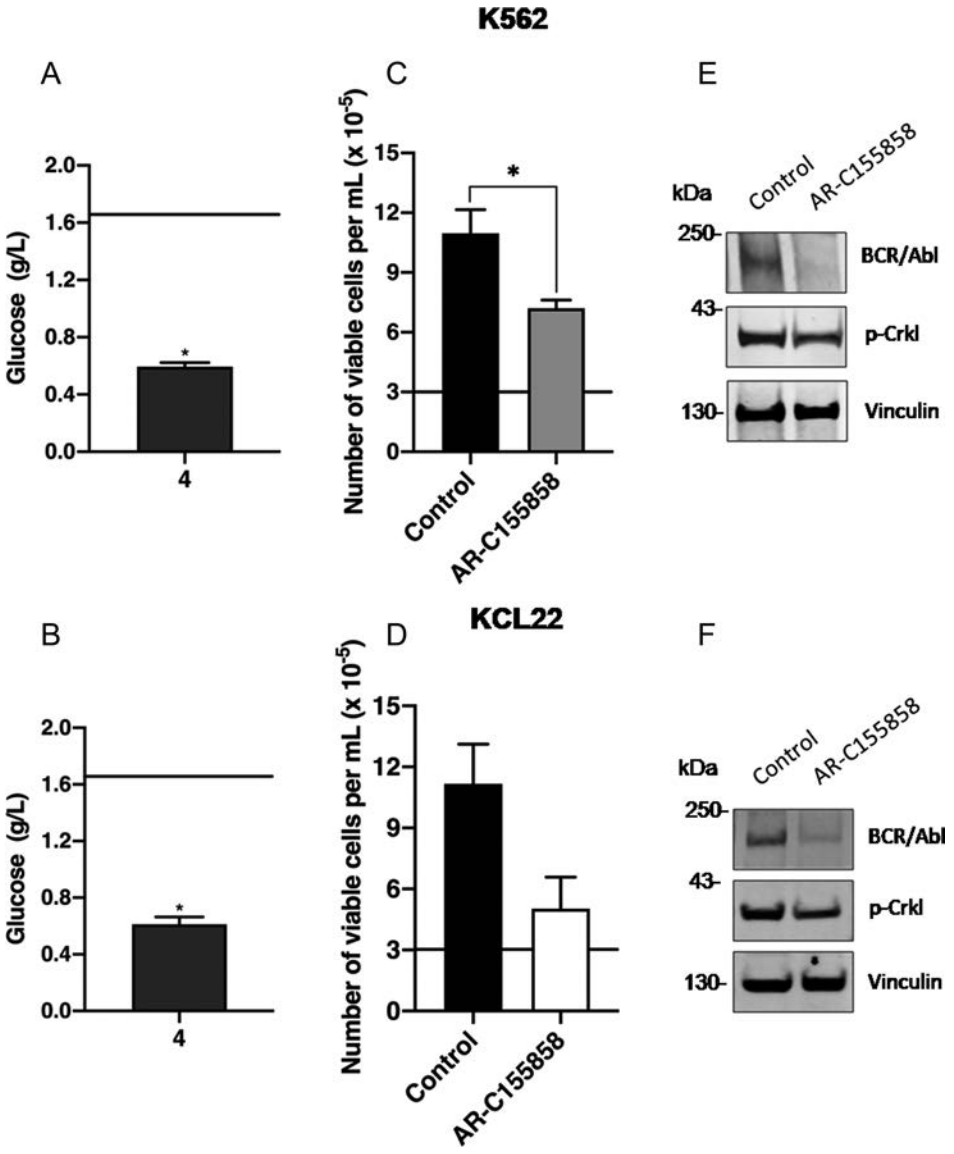

Figure 7. Effects of MCT-1 inhibition on BCR/Abl expression/signaling and cell growth in standard glucose-containing medium. K562 (A, C, E) or KCL22 (B, D, F) cells were seeded at 3×10^5 cells/ml in standard culture medium containing glucose and glutamine, in the absence (A, B) or in the absence or presence (C–F) of the MCT-1 inhibitor AR-C155858. Glucose concentration in culture medium was measured at time 0 (line) and day 4 (A, B). Trypan blue-negative cells were counted at time 0 (line) and day 4 of incubation; histograms represent means ± SEM of data obtained from three independent experiments (C, D). Cells were lysed in Laemmli buffer and total cell lysates subjected to SDS-PAGE and immunoblotting with antibodies raised against c-Abl or p-Crkl; anti-vinculin antibody was used to verify the equalization of protein loading; one representative experiment out of three with similar outcome is shown (E, F). The statistical significance of differences was determined by the Student's *t*-test for paired samples; $*p < 0.05$ (A–D).

The results obtained for BCR/Abl expression and signaling (Fig. 2) supported those relative to cell viability and growth. The maintenance in KCL22 cells even in the absence of lactate of relatively high levels of Crkl phosphorylation, an indicator of BCR/Abl enzymatic activity, is well in keeping with the higher resistance of KCL22 when compared to K562 cells to the harsh incubation conditions of our experimental system. Taken together, the data shown in Figures 1D and 2B, day 10, for control and low-pH KCL22 cell cultures indicate that there is a threshold level of Crkl phosphorylation above which BCR/Abl signaling, although reduced, was sufficient to sustain cell growth to levels unsuitable to be increased by the addition of lactate. Likewise, in day 14 KCL22 cell cultures, the level of Crkl phosphorylation in low pH, although markedly reduced (Fig. 2B), was apparently sufficient to maintain cell number around the time 0 levels (Fig. 1D), whereas this was not the case in control

cultures, where pCrkl band was significantly weaker than in low pH cultures. As far as K562 cells are concerned, pCrkl levels in control or low pH cultures (Fig. 2A) were lower than those of KCL22 cells on both day 10 and day 14 and were already insufficient on day 10 to sustain the maintenance of viable cell number at the time 0 value (Fig. 1C). This different behavior between K562 and KCL22 cells, short of representing a problem, actually contributes to strengthen the relationship between level of Crkl phosphorylation and CML cell growth.

As far as the interference of lactate with the terminal mechanisms of regulation of BCR/Abl expression is concerned, a thorough investigation of the issue was beyond the scopes of this study. Indeed, while characterizing the effects of oxygen and/or glucose shortage on those mechanisms, we found a very complex scenario, where BCR/Abl protein suppression followed a number of different pathways, among which regulation of transcription played a marginal role[11]. On the contrary, in this study, lactate addition resulted unexpectedly in a marked enhancement of *BCR/abl* mRNA levels (sevenfold for K562 cells and fourfold for KCL22 cells; Supplementary Fig. S2), suggesting that transcriptional regulation is the main mechanism of response to lactate. Whether such an outcome is specific for lactate or reflects different strategies the cells adopt while responding to nutrient shortage (oxygen and glucose) or supply (lactate) would require further investigation.

The main target of this project was actually to test the power of lactate to sustain the maintenance of stem/progenitor cell potential in CML cell populations, being this issue relevant to the control of tissue homing of cell subsets capable to maintain and drive relapse of disease. As shown and commented extensively elsewhere[9,18,27], when experimentally manipulated LC1 cells are transplanted into growth-permissive LC2, the repopulation of the latter occurs according to two different prototypical kinetics. In one case, the increase in cell number in LC2 is preceded by a lag phase usually long about 2 weeks, whereas in the other one LC2 are promptly repopulated, without a lag phase. The latter pattern is typical of LC1 cells where BCR/Abl signaling is active at the time of, or is rescued rapidly upon, transfer into LC2[9]. In this respect, K562 and KCL22 cells exhibited, once again, a different behavior: control K562 cells repopulated LC2 slowly (cells transferred from day 10 LC1) (Fig. 3A) or after a more than 3-week-long lag phase (cells from day 14 LC1) (Fig. 3B), whereas KCL22 cells repopulated LC2 promptly to reach the peak of repopulation relatively early. The latter outcome is in keeping with the enhanced resistance to incubation conditions of KCL22 cells in comparison to K562 cells with respect to reduction in cell number (Fig. 1D vs. 1C) and suppression of BCR/Abl signaling (Fig. 2B vs. 2A) we discussed above. Thus, in lactate-free KCL22 cells, either the levels of BCR/Abl expression and Crkl phosphorylation at the end of LC1 (Fig. 2B), although significantly reduced, remained sufficient to ensure a prompt LC2 repopulation, or BCR/Abl signaling was rapidly rescued upon transfer to LC2. This said, one must point out again that the main interest of our study was to characterize the effects of lactate, in particular on the maintenance of stem cell potential. In this respect, K562 cells yielded the maximal response to lactate addition as for either BCR/Abl signaling (Fig. 2A) or stem cell potential (Fig. 3A and B). It is worth noting that the peak values of LC2 repopulation by cells from lactate-supplemented LC1 were for K562 cells equal or higher than those obtained for KCL22 cells. This indicates that exogenous lactate is powerful enough to override the native differences of metabolic asset between the two cell lines. Overall, when glucose/glutamine shortage would result in the suppression of BCR/Abl signaling in the course of incubation, lactate is capable to prevent this suppression and thereby boost the maintenance of BCR/Abl-dependent stem cell potential.

The data summarized above need to be evaluated under the light of the metabolism-controlled CML stem/progenitor niche model we put forward[10]. Previous work showed that the overall metabolic profile of CML cells is glycolysis oriented[12,13] and that glucose consumption drives the suppression of BCR/Abl protein and signaling, attained indeed when glucose is almost exhausted in the environment[9]. The fact that in a "glycolytic" environment glucose consumption results mainly in the conversion of pyruvate, the terminal metabolite of glycolysis, to lactate commanded the investigation of the role of the latter in the context of the CML stem/progenitor niche model. It was necessary to determine in particular whether lactate could represent a fuel for CML cells capable to interfere with BCR/Abl protein expression and signaling. The novelty of results reported here stays in the demonstration that lactate is a good surrogate of glucose in the maintenance of BCR/Abl signaling, so that the abundant lactate released extracellularly in highly glycolytic tissue environments may prevent, or delay significantly, BCR/Abl suppression. The use of glucose-deprived cultures and the addition of exogenous lactate indeed enabled us to reach the conclusion that BCR/Abl expression level is kept constant while the effects of decreasing glucose are progressively compensated for by those of increasing lactate. The possibility of an alternation between glucose and lactate in sustaining BCR/Abl expression was indirectly confirmed in the experiments shown in Figure 7, where the treatment with the MCT-1 inhibitor of cultures incubated in the presence of glucose succeeded in reducing BCR/Abl protein expression. Overall, these findings indicate, on the one hand, that the presence of lactate in

the environment may help cells that store less glucose intracellularly, and thereby run short of it more quickly, to maintain BCR/Abl signaling longer, and on the other hand, that not only glucose but also lactate needs to be exhausted in the environment before BCR/Abl signaling is suppressed. Thus, the absence of lactate emerged as a metabolic condition within the CML stem/progenitor niche appropriate for the selection and maintenance of cells that represent a *reservoir* of TKI-resistant disease.

On the basis of all the above, lactate uptake appears as a key metabolic feature of cells capable to maintain BCR/Abl signaling despite the shortage of glucose. In this respect, the only limit is the capacity of cells to take in and metabolize lactate. We found that lactate, besides working as a fuel, acts to overcome this limit by enhancing the expression of MCT-1, driver of lactate uptake, and LDH-B, converter of lactate to pyruvate (Fig. 4). That MCT-1 and LDH-B are functional to the positive effects of lactate on BCR/Abl signaling and cell growth was indicated by the effects of inhibition of lactate uptake (Fig. 5A and B) or pyruvate transport into the mitochondria (Fig. 5C and D). The key role of pyruvate in the lactate-dependent maintenance of BCR/Abl signaling was confirmed on the other hand by the rescue of the effects of the block of lactate uptake in cultures treated with the cell membrane-permeable methyl-pyruvate (Fig. 6). The partial effect obtained for K562 cells seems to indicate that in these cells methyl-pyruvate is internalized in part via MCT-1, like lactate[23,24]. The contribution of the axis lactate>pyruvate as an energetic fuel to mitochondrial respiration was revealed by SeaHorse experiments showing a significant enhancement of OCR in lactate-supplemented cultures (Fig. 4A and C). The downstream connections between lactate/pyruvate fueling of mitochondrial respiration and the terminal mechanisms of regulation of BCR/Abl expression will be the object of further studies.

The relationship between the effects of lactate and those of low pH, i.e., whether lactate acts via its effect on intracellular pH (pHi) and/or has pH-independent mechanisms of action is a question very complex to answer. MCT-1 symports lactate with H+, and it is known that cytosolic pH and lactate concentration cannot easily be varied independently. However, the pH dependence of lactate transport may not hold for high intracellular lactate concentrations[28], and effects of lactate independent of pHi changes have been actually observed, so that decreases of pHi as lactate increases can explain some, but not all, of the effects of lactate[29]. This may be due to the fact that cells, and cancer cells in particular, exhibit the growth-stimulating upregulation of acid extruders or buffers (CO_2/HCO_3^-, Na^+/H^+ exchanger, Na^+/HCO_3^- symporter, and V-type ATPase), which may well compensate for the effects on pHi of MCT-1-dependent lactate uptake[30]. Although to explore in detail this issue was beyond the scopes of our study, we verified that the exogenous lowering of extracellular pH, which results in intracellular acidification[31], did not mimic (nor interfere significantly with) the effects of lactate addition on BCR/Abl expression and signaling (Fig. 2 and Supplementary Fig. S1). Even more importantly, low pH actually reduced the positive effects of lactate on growth (Fig. 1C) and maintenance of BCR/Abl-dependent stem/progenitor cell potential (Fig. 3A) in K562 cell cultures. As tissue environments where abundant lactate is made available by high levels of glycolysis are usually subjected to marked acidosis[32], these findings, although limited to K562 cells, need to be discussed appropriately in the context of the CML stem/progenitor cell niche model. One may speculate that acidity, reducing the growth-promoting action of lactate, contributes to spare stem/progenitor cell potential within lactate-rich zones of the niche. This hypothesis, well in keeping with the finding that extracellular acidosis contributes to induce the dormancy of tumor cells[16], is the object of current work.

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