STAT3 modulates mitochondrial function and plays a critical role in the survival of leukemic stem cells

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Abstract
Signal transducer and activator of transcription 3 (STAT3) is a well-described transcription factor that mediates oxidative phosphorylation and glutamine uptake in bulk acute myeloid leukemia (AML) cells and leukemic stem cells (LSCs). STAT3 has also been shown to translocate to the mitochondria in AML cells, particularly when phosphorylated at the serine 727 (pSTAT3 S727) residue. Inhibition of STAT3 results in impaired mitochondrial function and decreased leukemia cell viability. We discovered a novel interaction of STAT3 with voltage-dependent anion channel 1 (VDAC1) in the mitochondria that provides a mechanism through which STAT3 modulates mitochondrial function and cell survival. Through VDAC1, STAT3 regulates calcium and reactive oxygen species (ROS) balance in the mitochondria. STAT3 inhibition also results in significantly reduced engraftment potential of LSCs, including primary samples resistant to venetoclax. These results implicate STAT3 as a therapeutic target in AML.

Introduction

Acute myeloid leukemia (AML) is a genetically heterogenous and highly aggressive myeloid neoplasm with poor prognosis.[1,2] Standard therapy for AML has historically consisted of induction chemotherapy with an anthracycline and cytarabine, followed by consolidation with either hematopoietic stem cell transplant or high dose cytarabine.[3] Recently, therapeutic options have broadened with the advent of novel targeted therapies.[4-7] However, despite high response rates, relapse is common.[6] Relapsed disease is believed to originate from a quiescent subpopulation of therapy-resistant leukemic stem cells (LSCs)[8] which are found in greater abundance at the time of relapse compared to diagnosis,[9-12] and negatively correlate with survival.[10,11] LSCs demonstrate a unique vulnerability in their preferential reliance on mitochondrial activity and oxidation phosphorylation (OXPHOS).[12-14] While Bcl-2 inhibition with venetoclax (Ven) in combination with the hypomethylating agent (HMA) azacitidine has demonstrated selectivity for LSCs via inhibition of OXPHOS,[13] resistance frequently develops via alterations in mitochondrial metabolism or activation of alternative antiapoptotic pathways.[15-19] Further, prior studies of patients who progress after frontline HMA/Ven have shown very poor outcomes, with a median survival following failure of HMA/Ven of 3 months or less.[20,21] New strategies targeting LSCs via their reliance on OXPHOS are of significant interest and have been described in several reports,[7,13,22] however further research is needed to elucidate the mechanisms underlying these observations.

Signal transducer and activator of transcription 3 (STAT3) has been shown to be important for leukemogenesis and is known to be highly expressed in many AML patient samples and cell lines.[23-26] Canonically, STAT3 is known to undergo phosphorylation at residue Tyr^{705} leading to dimerization and translocation to the nucleus where it functions as a transcription factor regulating cell development, renewal, proliferation, and cell death.[24,27-29] Our previous work additionally established that STAT3's transcriptional activity regulates mitochondrial function via a *MYC*-SLC1A5-mediated pathway.[26] Despite its well-described nuclear role as a transcription factor, STAT3 has also been discovered to localize to the mitochondria.[30,31] Prior work has suggested a variety of functions in the mitochondria, including modulation of electron transport chain (ETC) activity,[30-32] regulation of mitochondrial genes,[33] and regulation of mitochondrial calcium flux.[34,35] While phosphorylation of STAT3 at both Tyr^{705} (pSTAT3 Y705) and Ser^{727} (pSTAT3 S727) sites have been found in the mitochondria,[30-32,35,36] Ser^{727} phosphorylation is critical for modulation of

mitochondrial functions such as ETC activities.[30,31] These data suggest that STAT3 plays a critical role in the mitochondria, although this role in AML is not well characterized.

Here we show STAT3 plays a key role in mitochondrial function of AML cells, and that it interacts with several mitochondrial proteins including voltage-dependent anion channel 1 (VDAC1), playing a regulatory role of mitochondrial calcium and reactive oxygen species (ROS) flux.

Materials & Methods

Cell Culture

For human primary AML samples, base media of minimum essential medium and 5.5 mM of glucose was used, supplemented with physiologic levels of amino acids as previously reported[12,26] and 10 nM of human cytokines stem cell factor (SCF), interleukin-3, and Fms Related Receptor Tyrosine Kinase 3 (FLT3).

Patient Samples and LSC Enrichment

Primary human AML samples were obtained from apheresis products or bone marrow of patients with AML who gave written consent for sample procurement at the University of Colorado, according to the Colorado IRB Protocol #12-0173. LSCs were isolated using a ROS-low strategy.[37] For analysis of LSC-enriched fractions, specimens were processed as previously described.[12,38]

Viability Measured by Flow Cytometry

Cell viability was measured using Annexin V and Dapi stains and measured by flow cytometry. Ghost dye was also used to confirm cell death and measured by flow cytometry.

Single Cell RNA Sequencing

Clusters were annotated using clustifyr 1.9.1[39] and the leukemic/normal bone marrow reference dataset generated by Triana and colleagues.[40] Scanpy and Seurat 4.1.1 were then used to generate uniform manifold approximation and projections from the TotalVI embeddings and perform exploratory analysis, data visualization, etc.[41]

Flow Cytometry ImageStream

FIX & PERM cell fixation assay (ThermoFisher) was used to stain for intracellular markers pSTAT3 S727 and Tom20. Cells were then washed with FACS Buffer (1% FBS in 1X PBS) and ran on the ImageStreamX MkII (Cytek Biosciences). Samples were then analyzed using the Ideas 6.2 software threshold masking for mitochondrial staining which was utilized to determine the intensity per area of pSTAT3.

Seahorse Cell Mito Stress Test

The extracellular flux assay XF96 kit was used to measure oxygen consumption rate (OCR). Drug-treated or siRNA-treated primary AML cells or LSCs were plated on Cell-Tak–coated XF96 cell culture microplates at 200,000 cells/well in 5 replicates and measured according to the manufacturer's protocol. OCR was measured at basal level and after injection of 5 µg/mL of oligomycin, 2 µmol/L of Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), 5 µmol/L of antimycin A, and 5 µmol/L of rotenone.

Metabolomic Experiments
Metabolomics analyses were performed on 10 µl of sample extracts via ultra-high pressure liquid chromatography coupled to high resolution mass spectrometry (Vanquish – QExactive – Thermo Fisher, San Jose, CA, USA) using a high-throughput 5 min gradient-based method.

siRNA Transfections
siRNA sequences targeting STAT3 (siSTAT3) and a scrambled control (siSCR) were purchased directly from Horizon Discovery's ON-TARGETplus siRNA Reagents collection. The lyophilized siRNA products were resuspended in RNAse-free water at 5 µmol/L, which was used as a stock solution. $2x10^6$ cells were suspended in 80 µL of Buffer T, and 20µL of siRNA stock solution was added. These cells were then electroporated using the Neon Electroporation Transfection System (Thermo) according to the manufacturer's protocol using the following settings: 1,600 V, 10 ms, 3 pulses.

Electron Microscopy
MOLM-13 cells were treated with DMSO or Stattic 5µM for 12 hours and subsequently fixed prior to submission to the Electron Microscopy Core at University of Colorado for subsequent imaging. Mitochondrial number and area were measured using the ImageJ software.

Immunoprecipitation Assay
Immunoprecipitation assays were performed using the mitochondrial protein IP kit from Abcam (catalog # ab239710) according to the manufacturer's protocol. Eluent was then submitted to the University of Colorado proteomics core.

Mitochondrial Calcium Assay
100,000-500,000 primary human AML ROS-Low cells or MOLM-13 cells were used for analysis of mitochondrial calcium content. For mitochondrial calcium measurements, cells were incubated with Rhod2AM (Invitrogen, R1245MP; 500nM) for 30 minutes at 37°C. Cells were then washed with calcium free, magnesium free 1X PBS (Corning, 21-031-CV) twice and resuspended in 1X calcium free, magnesium free PBS supplemented with 2% FBS (Atlas Biologicals, no. F-500-D) and analyzed by flow cytometry (PE channel). Detection for Rhod-2 AM was done on 552/581 channel. Viability Measured by Flow Cytometry Cell viability was measured using Dapi and measured by flow cytometry. Cells were stained in PBS supplemented with 2% FBS.

Mouse Studies
Human primary AML cells were treated with either vehicle control or 5µM of Stattic overnight. On the same day, NSG-S mice were conditioned with 25 mg/kg busulfan via intraperitoneal injection. On the second day, AML cells were washed with FACS buffer and resuspended in saline with the addition of anti-human CD3 antibody (OKT3 BioXCell) at a final concentration of 1 $\mu g/10^6$ cells and incubated for 15 minutes prior to injection as a means to reduce graft versus host disease. 8-9 mice per group were injected with 2.5×10^6 cells/mouse. Mice were sacrificed after 8-12 weeks and femurs were collected and flushed with FACS buffer. Flow cytometry was then performed after staining with mouse and human specific CD45 antibodies. All animal experiments were approved by the Rocky Mountain Regional VA Medical Center (Institutional Animal Care and Use Committee) under protocol number CD2114M.

Results

STAT3 is highly expressed in acute myeloid leukemia cells and its phosphorylation facilitates mitochondrial localization

To investigate the role of STAT3 in human AML cells, we utilized both the AML cell line Molm-13 and primary AML samples donated by patients at University of Colorado. First, we measured expression of total STAT3 and pSTAT3 S727, a known marker of STAT3 activation. Single cell RNA sequencing from 15 human primary AML samples showed STAT3 was prominently detected in several cell clusters, including those known to contain LSCs (**Figure 1A).** Further quantification from western blots of 4 primary AML samples enriched for LSCs showed higher expression of pSTAT3 S727 in LSCs compared to other bulk leukemia cells (**Figure 1B**). This is in accordance with our previously published work demonstrating significantly higher STAT3 expression in AML patient samples compared to normal cord blood mononuclear cells.[26] Previous work has shown that phosphorylated STAT3 can localize to the mitochondria and modulate function and ETC activity.[30-32,42] Next, ImageStream was used to assess the localization of pSTAT3 S727 to the mitochondria in human AML cells. As shown in **Figure 1C**, pSTAT3 is present in the mitochondria in the majority of cells in two primary AML samples. To further study the role of STAT3 in the mitochondria, we used the AML cell line Molm-13. Similar to primary AML samples, Molm-13 cells show high expression of STAT3 and pSTAT3 S727 (**Figure 1D**). To inhibit mitochondrial STAT3, we used a potent STAT3 inhibitor, Stattic. Stattic has been shown to inhibit STAT3 dimerization, but it has also been shown to inhibit phosphorylation at S727, which is critical for STAT3 mitochondrial localization and function.[43] To find the optimal dose to study STAT3 inhibition in these cells, we first treated Molm-13 cells with increasing doses of Stattic. As shown in **Supplemental Figure 1A**, 5μM of Stattic results in significant cell death at 24 hours compared to vehicle control. However, this dose did not cause significant cell death until 16 hours (**Supplemental Figure 1B**), allowing a window to understand the effects of STAT3 inhibition prior to cell death. We next tested how Stattic inhibits STAT3 in Molm-13 cells. As shown in **Figure 1E**, Stattic treatment resulted in significant reduction of pSTAT3 S727 after 9 hours in culture, while only resulting in a minimal reduction of total STAT3 (**Supplemental Figure 1C**). Since phosphorylation of S727 has been linked to localization and activity of mitochondrial STAT3, we further confirmed that culture with Stattic resulted in decreased localization of STAT3 to the mitochondria (**Figure 1F**), including its phosphorylated form pSTAT3 S727 (**Figure 1G**).

STAT3 inhibition results in mitochondrial dysfunction and alters OXPHOS

To assess the effects of pharmacologic inhibition of STAT3 on mitochondrial function, we first used Seahorse MitoStress testing to measure oxygen consumption rates (OCR) in the presence or absence of Stattic. Stattic treatment resulted in a significant decrease in OCR after 12 hours compared to control samples (**Figure 2A**). We next sought to determine if STAT3 inhibition results in altered TCA cycle activity. Mass spectrometry-based metabolomic assays demonstrated that STAT3 inhibition with Stattic resulted in decreased TCA metabolites (**Figure 2B**), and significantly decreased ATP production (**Figure 2C**). Further, glutamine and its downstream metabolites were decreased upon STAT3 inhibition (**Supplemental Figures 2A-C**), while glycolytic metabolites were elevated (**Supplemental Figure 2D-E**). We have previously shown that STAT3 regulates glutamine influx via transcriptional regulation of *MYC* and subsequent *MYC*-dependent modulation of the glutamine transport via solute carrier 1A5 (SLC1A5).[26] Here we demonstrate that Stattic treatment results in decreased levels of *MYC* (**Supplemental Figure 2F**).

Taken with the above findings of decreased glutamine, these findings suggest that Stattic additionally inhibits STAT3's action as a transcription factor, resulting in decreased levels of *MYC* and subsequent reduced glutamine uptake. In addition to regulation of TCA cycle activity, we sought to determine whether STAT3 regulates mitochondrial dynamics. Using MitoTracker green, we found that pharmacologic inhibition of STAT3 with Stattic results in decrease mitochondrial size (**Figure 3A**). Similarly, transcriptomic inhibition of STAT3 with small interfering RNA (siSTAT3) resulted in decreased levels of STAT3 (**Supplemental Figure 3A**) and subsequent decreased mitochondrial size (**Supplemental Figure 3B**) when compared to control (siSCR). This was further confirmed by Electron Microscopy (EM) in Molm-13 cells (**Figure 3B**) treated with Stattic or vehicle control for 12 hours. While Stattic-treated cells showed no significant difference in mitochondrial number per cell (**Figure 3C**), they did show significantly decreased mitochondrial size based on quantification of mitochondrial area (**Figure 3D**). To understand whether proteins related to mitochondrial fusion or fission are affected by STAT3 inhibition, we performed western blots of Molm-13 cells treated with Stattic for 12 hours. This showed increased levels of Dynamin-like Protein 1 (DRP1) (**Figure 3E**), a protein involved in mitochondrial fission. Protein levels of mitofusin2 (MFN2) and mitofusin1 (MFN1) are also mildly decreased (**Figure 3E and Supplemental Figure 3C**). Taken together, these changes suggest that STAT3 inhibition alters mitochondrial dynamics leading to mitophagy, some of which may be mediated through its transcriptional regulation of *MYC*.

STAT3 interacts with VDAC1 in the mitochondria and alters mitochondrial calcium flux

Having established that STAT3 localizes to the mitochondria and that inhibition of STAT3 results in mitochondrial dysfunction, we next sought to understand what STAT3 interacts with in the mitochondria. Using an immunoprecipitation assay followed by mass spectrometry **(Figure 4A**) on mitochondrial fraction isolates from Molm-13 cells, we identified several STAT3-associated mitochondrial proteins (**Figure 4B**), including voltage-dependent anion channel 1 (VDAC1). Subsequent western blot assays revealed reduction in VDAC1 levels in mitochondrial fraction isolates of Molm-13 cells following STAT3 inhibition with Stattic at 9 hours (**Figure 4C**). Prior work has demonstrated that both STAT3 and VDAC1 play roles in mitochondrial calcium regulation.[34,35,44,45] Additionally, mitochondrial calcium homeostasis has been shown to be important for LSCs in AML.[19] To determine the potential effects of STAT3 inhibition on mitochondrial calcium flux in AML, we performed calcium assays with Rhod-2 AM staining. We found that pharmacologic (Stattic) inhibition of Molm-13 cells significantly decreased levels of mitochondrial calcium (**Figure 4D** and **Figure 4E**). We recapitulated these findings with transcriptomically (siSTAT3) inhibited Molm-13 cells. After genetic knockdown of STAT3, we showed decreased VDAC1 levels in the mitochondrial fraction (**Supplemental Figure 4A and 4B**), leading to decreased mitochondrial calcium (**Supplemental Figure 4C)**, in the absence of cell death (**Supplemental Figure 4D**). Next, we assessed whether STAT3 inhibition affects mitochondrial ROS, given VDAC1 has been shown to regulate ROS formation.[46] As shown in **Figure 4F**, mitochondrial ROS is increased in Molm-13 cells treated with Stattic for 9 hours. Together, these studies suggest that STAT3 binds to and regulates the function of VDAC1.

STAT3 inhibition decreases viability and engraftment potential of leukemic cells and can overcome venetoclax resistance

To determine the impact of STAT3 inhibition on leukemic cells *in vitro*, we cultured Molm-13 cells with Stattic 5μM or vehicle control for 24 hours, followed by viability flow cytometry assays. As

shown in **Figure 5A**, there was significant cell death of Stattic treated cells. Similarly, we saw significant cell death in Molm-13 cells treated with 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), a VDAC1 inhibitor, compared to vehicle control (**Supplemental Figure 5A**). Interestingly, culturing Molm-13 cells with both Stattic and DIDS resulted in similar cell death, suggesting they are both in the same pathway. (**Supplemental Figure 5A**). We then sought to understand whether LSCs from primary AML samples would be sensitive to STAT3 inhibition. To do this, we treated two AML patient samples *ex-vivo* with Stattic 5μM or vehicle control for 16 hours, followed by xenotransplantation into NSG-S mice pre-treated with Busulfan. After 8-12 weeks, we found almost complete eradication of LSCs treated with Stattic, reflected by no engraftment compared to vehicle controls (**Figure 5B and 5C**). Given the unmet need to develop therapies that can overcome venetoclax and HMA resistance, we sought to understand whether STAT3 inhibition can effectively eliminate venetoclax resistant LSCs. To do so, we used LSCs isolated from three different AML patient samples that were resistant to venetoclax and azacitidine. We then treated LSCs *in vitro* with Stattic 5μM or vehicle control for 16 hours, followed by viability assays. As shown in **Figure 5D**, we saw a significant decrease in viability compared to vehicle control. LSCs were also independently treated with venetoclax as a positive control to ensure resistance. We then utilized one of the venetoclax-resistant samples to perform patient derived xenografts. As shown in **Figure 5E**, *ex-vivo* treatment of venetoclax-resistant AML cells with Stattic resulted in a significant reduction of engraftment in NGS-S mice. To further understand how STAT3 inhibition could potentially overcome venetoclax resistance, we performed single cell RNA sequencing in a primary AML sample that was resistant to venetoclax and azacitidine. As shown in **Figure 5F**, STAT3 expression was higher in the leukemia stem cell cluster (circled) at day 28 after treatment initiation compared to the diagnostic sample, suggesting LSCs with high STAT3 expression are selected for after Ven/HMA treatment. Together, these data suggest STAT3 could be an effective target in AML, particularly in the setting of venetoclax-resistance.

Discussion

STAT3 is a well-establish transcription factor which is known to play important roles in cancer cell transformation and expansion.[24,47,48] In LSCs, STAT3 has been shown to regulate both MCL-1 expression[25] and glutamine flux,[26] which are critical pathways for LSC survival. STAT3 phosphorylation at S727 has recognized importance in both the function of mitochondrial STAT3, and localization of STAT3 to the mitochondria.[30] However, it has been shown that pSTAT3 S727 is required for modulation of mitochondrial function, even in the absence of mitochondrial localization.[42] While recent research has shown that mitochondrial STAT3 also plays a role in regulating ETC activity[30-32] and mitochondrial calcium flux,[34,35] this function has not been studied in the context of myeloid malignancies.

In this study, we confirm prior reports showing STAT3 in highly expressed in AML patient samples and cell lines, and that pSTAT3, in particular serine 727 phosphorylation, is associated with mitochondrial localization and function of STAT3. We demonstrate that STAT3 plays a critical role in mitochondrial function. We show that transcriptomic and pharmacologic inhibition of STAT3 results in decreased mitochondrial size, decreased TCA cycle metabolites, and impaired OXPHOS. While the role of STAT3 in TCA cycle activity has been demonstrated given its regulation of *MYC* and downstream glutaminolysis,[26] we also discovered a novel role of mitochondrial STAT3 via its

interaction with VDAC1. VDAC1 has a variety of mitochondrial roles, including regulating mitochondrial calcium[45,49] and ROS flux.[50-52] VDAC1 has also been shown to interact with Bcl-2 family proteins and regulate apoptosis.[53,54] In this study, we show that STAT3 inhibition leads to decreased VDAC1 in the mitochondrial cell fractions, followed by a decrease in mitochondrial calcium content and increased ROS. However, VDAC1's role in apoptosis appears to be independent of STAT3 as the development of apoptosis does not appear to be affected by STAT3 inhibition. Further, VDAC1 inhibition effectively results in cell death of AML cells, suggesting the STAT3-VDAC1 interaction is a critical component of the cell's survival. Importantly, we show that STAT3 inhibition effectively eradicates LSCs, thereby impairing their engraftment potential. We additionally demonstrate that STAT3 inhibition is an effective modality to overcome venetoclax resistance, which aligns with prior work that has demonstrated the importance of mitochondrial calcium in LSCs.[19]

In conclusion, we show STAT3 is highly expressed in AML cells, and inhibition of STAT3 results in decreased OXPHOS, decreased mitochondrial calcium, decreased cell viability, and impaired engraftment potential. We additionally describe a novel role of STAT3 which interacts with VDAC1 in the mitochondria. These important functions of STAT3 represent potential therapeutic strategies in targeting AML, including LSCs. Given the promising therapeutic implications of targeting STAT3, inhibitors of this protein are currently being investigated in AML (NCT05986240) as well as other cancers (NCT03195699), with thus far no reports of drug limiting toxicities.

Authorship Contributions

K.G and M.L.A designed the research, K.G, J.B, R.P, A.I, J.R, A.G, W.S, A.D, performed experiments and analyzed the data. K.G and M.L.A wrote the manuscript with input from A.G, A.D, C.M and D.A.P.

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FIGURE 1

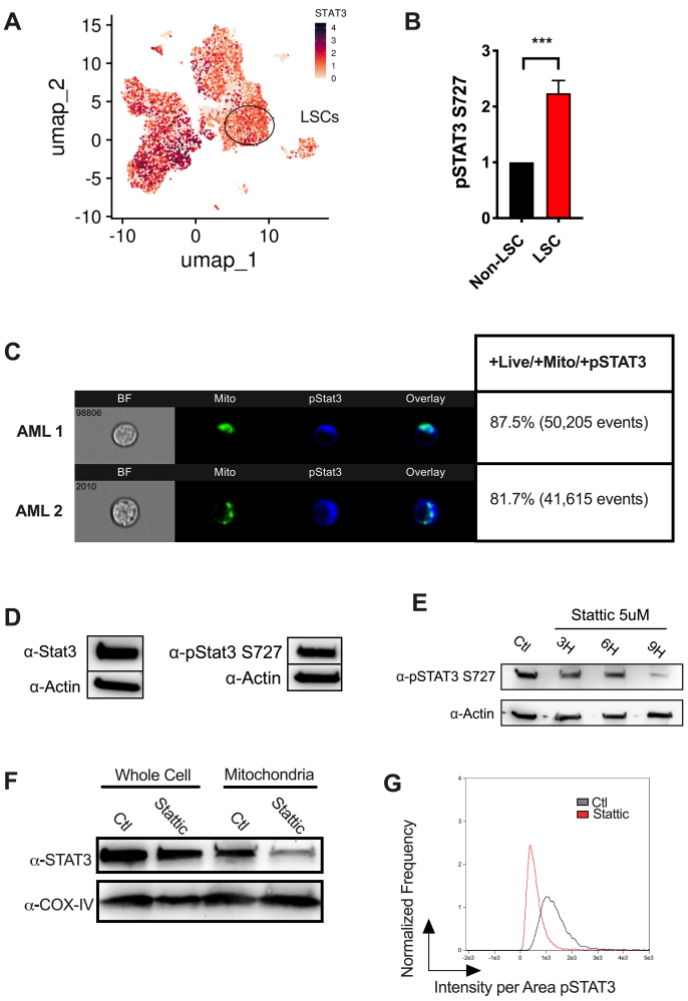

Figure 1. STAT3 is highly expressed in acute myeloid leukemia, and it localizes to the mitochondria. (A) Single cell RNA sequencing of 15 AML samples. Leukemia stem cell cluster is circled. (B) Western blot quantification of pSTAT3 at S727 comparing leukemia stem cells to bulk cells of 4 primary AML samples. (C) Representative images from two AML patient samples staining for mitochondria, pSTAT3 S727 and their respective overlays. (D) Western blots showing total STAT3 and pSTAT3 S727 in Molm-13 cells. (E) Western blot showing pSTAT3 S727 in Molm-13 in the presence of Stattic 5μM at 3, 6 or 9 hours compared to vehicle control. (F) Western blot showing total STAT3 levels in whole cell or mitochondrial fractions of Molm-13 cells treated with Stattic 5μM or vehicle control for 12 hours. (G) Quantification by intensity per area of pSTAT3 S727 in the mitochondria of Molm-13 cells treated with Stattic 5μM for 12 hours compared to vehicle controls based on flow cytometry and Image Stream. Statistical analyses were performed using a Student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

FIGURE 2

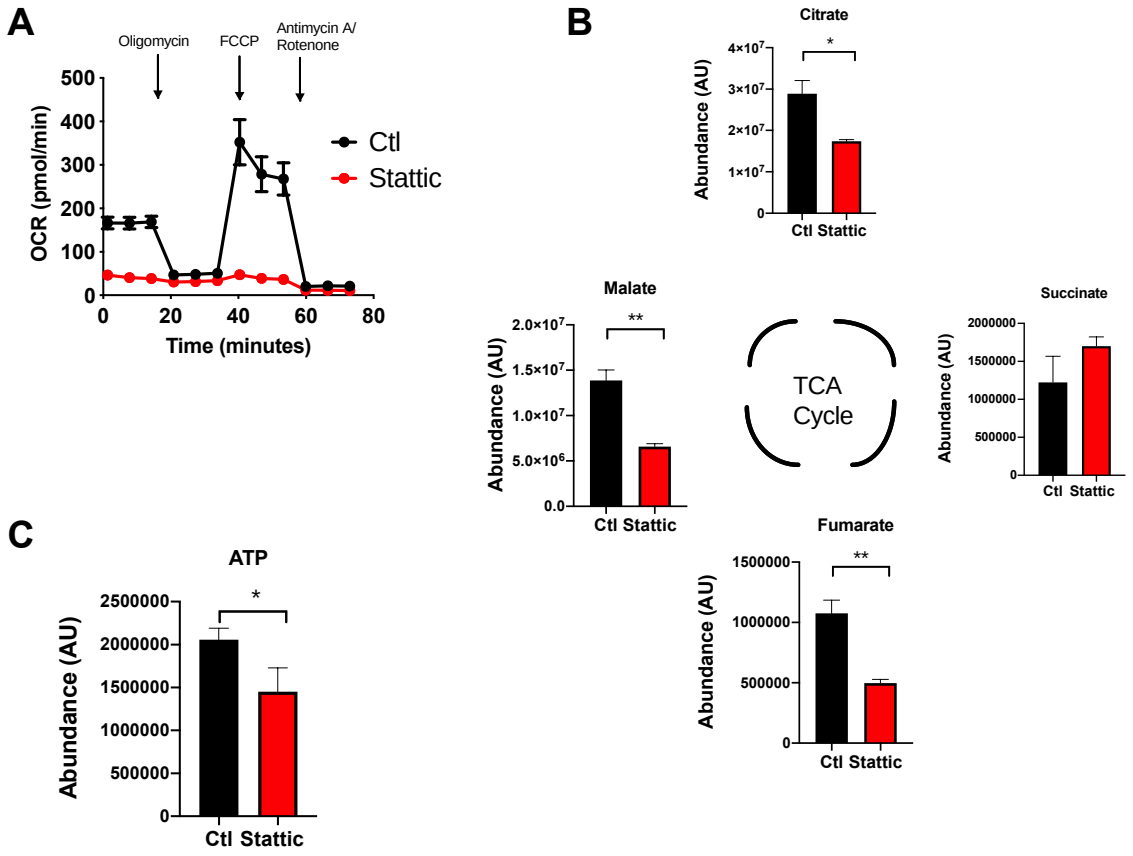

Figure 2. STAT3 inhibition results in decreased mitochondrial function and decreased mitochondrial metabolites. (A) Seahorse Mito Stress Test in MOLM-13 in the presence or absence of Stattic 5μM after 12 hours in culture. (B) Metabolomics assay showing quantification of TCA cycle metabolites citrate, succinate, fumatare and malate in MOLM-13 cells after 12 hour incubation of Stattic 5μM or vehicle control. (C) Metabolomics assay showing quantification of ATP in Molm-13 cells after 12-hour incubation of Stattic 5μM or vehicle control. Statistical analyses were performed using a Student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

FIGURE 3

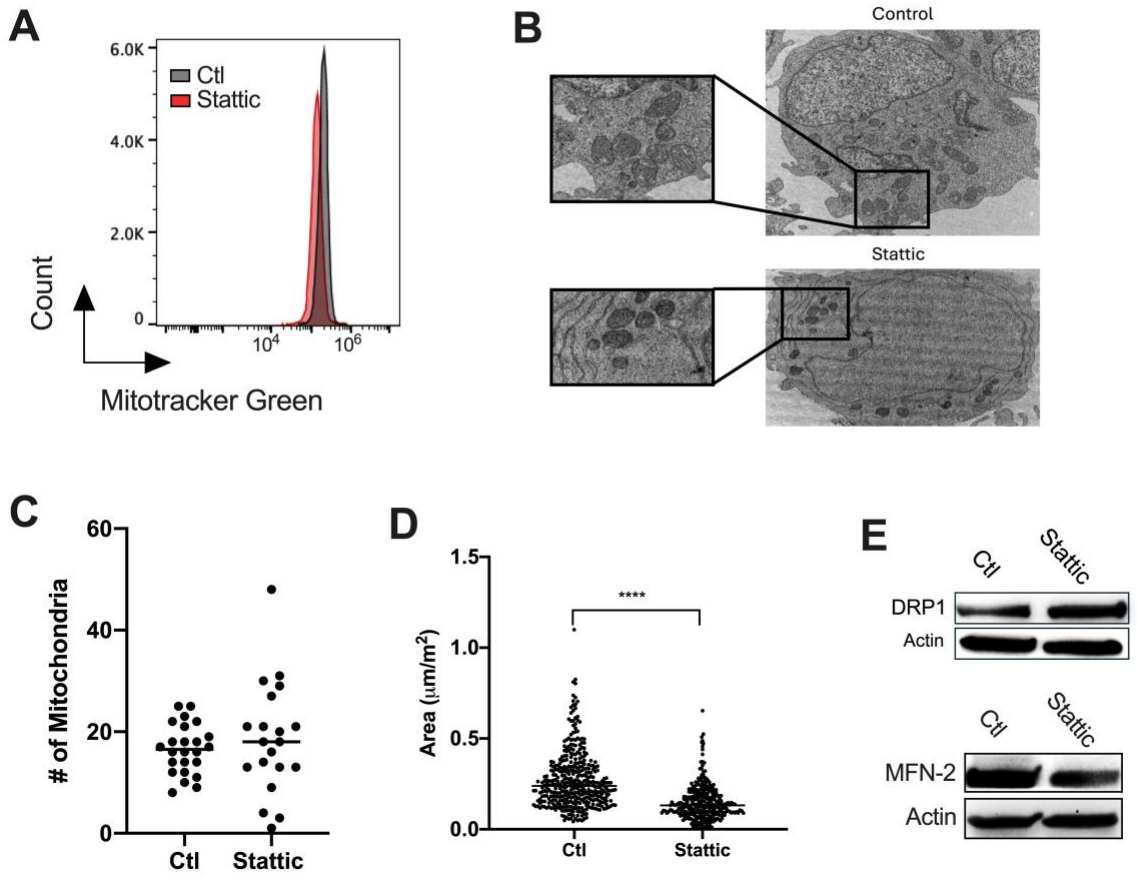

Figure 3. STAT3 inhibition leads to mitochondrial fusion. (A) Mitotracker green was used to measure mitochondrial mass by flow cytometry in the presence of Stattic 5μM or vehicle control for 12 hours. (B) Representative electron microscopy images of Molm-13 cells treated with of Stattic 5μM for 12 hours or vehicle control. (C) Quantification of number of mitochondria imaged by electron microscopy in Molm-13 cells treated with of Stattic 5μM for 12 hours or vehicle control. (D) Quantification of mitochondrial area of Molm-13 cells treated with of Stattic 5μM for 12 hours or vehicle control. (E) Western blot showing protein expression level of DRP-1 and MFN-2 in Molm-13 cells treated with of Stattic 5μM for 12 hours or vehicle control. Statistical analyses were performed using a Student's t-test. P values are represented as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

FIGURE 4

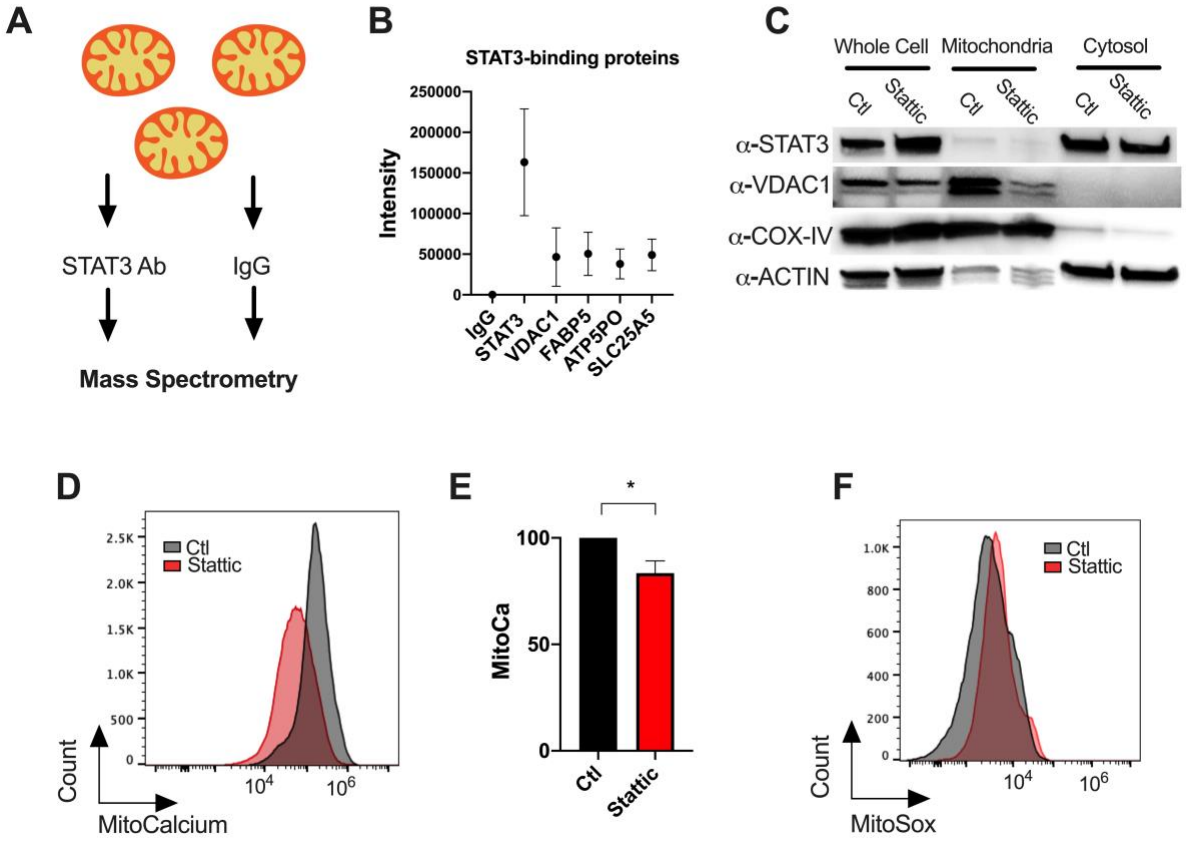

Figure 4. STAT3 interacts with VDAC1 and regulates its function. (A) Cartoon representation of the immunoprecipitation experimental design. (B) Intensity based absolute quantification of proteins bound to STAT3 as measured by mass spectrometry. IgG serves as a negative control and STAT3 as a positive control. (C) Western blot showing protein expression of STAT3 and VDAC1in whole cell, mitochondrial or cytosolic fractions of Molm-13 cells treated with Stattic 5μM or vehicle control for 9 hours. COX-IV and Actin antibodies serve as mitochondrial and cytosolic controls, respectively. (D) Mitochondrial calcium as measured by flow cytometry in Molm-13 cells treated with Stattic 5μM or vehicle control for 9 hours. (E) Quantification of 3 technical replicates of mitochondrial calcium in Molm-13 cells treated with Stattic 5μM or vehicle control for 9 hours. (F) Mitochondrial ROS as measured with flow cytometry in Molm-13 cells treated with Stattic 5μM or vehicle control for 9 hours. Statistical analyses were performed using a Student's t-test. P values are represented as follows: * $p \leq 0.05$.

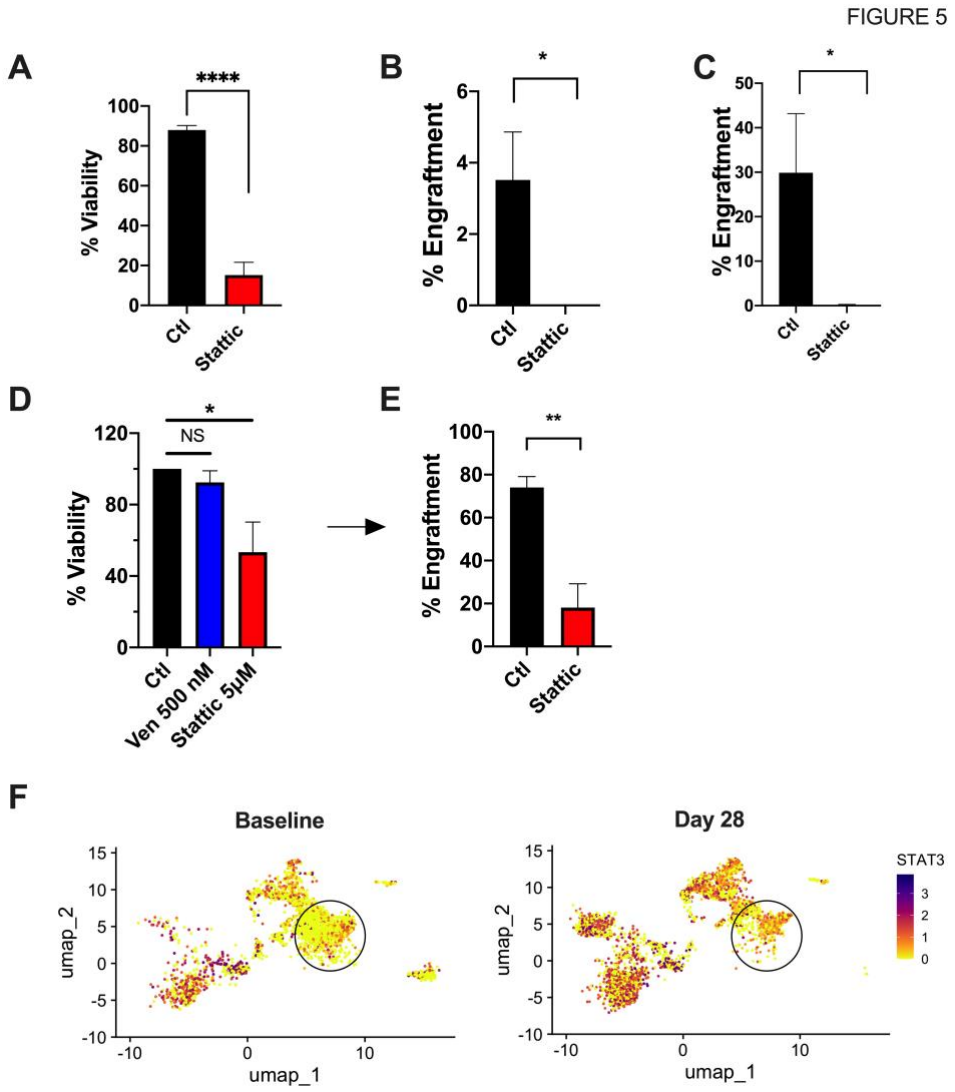

Figure 5. STAT3 inhibition decreases viability of leukemia stem cells in vitro and ex-vivo and overcomes venetoclax-resistance. (A) Viability as measured by flow cytometry of Molm-13 cells treated with Stattic 5μM or vehicle control for 24 hours. (B-C) Engraftment as measured by human CD45 positivity in PDX mice injected with bone marrow cells from two primary AML samples treated ex-vivo with Stattic 5μM or vehicle control for 16 hours (n = 5 and n =6 mice per group, respectively). (D) Viability as measured by flow cytometry LSCs isolated from three AML patient samples and treated with Stattic 5μM, venetoclax 500nM or vehicle control for 16 hours. (E) Engraftment as measured by human CD45 positivity in PDX mice injected with bone marrow cells from one primary AML samples treated ex-vivo with Stattic 5μM or vehicle control for 16 hours (n = 6 mice for control and n = 8 for Stattic treated) (F) Single cell RNA sequencing showing measuring STAT3 expression in the LSC population (circled) from bone marrow biopsies of a patient refractory to venetoclax and azacitidine at baseline and at day 28 after the first cycle of therapy.