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SDF-1/CXCL12 Modulates Mitochondrial Respiration of Immature Blood Cells in a Bi-phasic Manner

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Abstract

SDF-1/CXCL12 is a potent chemokine required for the homing and engraftment of hematopoietic stem and progenitor cells. Previous Data from our group has shown that in an SDF-1/CXCL12 transgenic mouse model, Lineage⁻ Sca-1⁺ c-Kit⁺ (LSK) bone marrow cells have reduced mitochondrial membrane potential versus wild-type, These results suggested that SDF-1/CXCL12 may function to keep mitochondrial respiration low in immature blood cells in the bone marrow. Low mitochondrial metabolism helps to maintain low levels of reactive oxygen species (ROS), which can influence differentiation. To test whether SDF-1/CXCL12 regulates mitochondrial metabolism, we employed the human leukemia cell line HL-60, that expresses high levels of the SDF-1/CXCL12 receptor, CXCR4, as a model of hematopoietic progenitor cells *in vitro*. We treated HL-60 cells with SDF-1/CXCL12 for 2 and 24 hours. Oxygen consumption rates (OCR), mitochondrial-associated ATP production, mitochondrial mass, and mitochondrial membrane potential of HL-60 cells were significantly reduced at 2 hours and increased at 24 hours as compared to untreated control cells. These biphasic effects of SDF-1/CXCL12 were reproduced with lineage negative primary mouse bone marrow cells, suggesting a novel function of SDF-1/CXCL12 in modulating mitochondrial respiration by regulating mitochondrial oxidative phosphorylation, ATP production and mitochondrial content.

Keywords

SDF-1/CXCL12; Mitochondria; Oxygen Consumption; Blood Cells

Introduction

Stromal cell-derived factor 1 α (SDF-1), also known as CXCL12, is an important member of the CXC family of chemokines. SDF-1/CXCL12 is expressed in a wide array of different tissues and cell types, including immune cells, endothelial cells, stromal cells, fibroblasts, and cancer cells [1]. The gene encoding SDF-1/CXCL12 is located at 10q11.1 and has 6 exons encoding 68 amino acids. It has a molecular weight of 8 kDa, and its promoter

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contains binding sites for transcription factors such as SP1 [2]. Signal transduction induced by SDF-1/CXCL12 is mediated through the chemokine receptor CXCR4 [3–6]. Knockout of SDF-1/CXCL12 is perinatal lethal and mice lacking SDF-1/CXCL12 have severe defects in gastrointestinal vascularization, cerebral development, and hematopoietic defects [7–9]. CXCR4 knockout studies reveal a strikingly similar phenotype to that of SDF-1/CXCL12 knockout mice, suggesting that the SDF-1/CXCL12 and CXCR4 signaling axis is non-promiscuous [10].

SDF-1/CXCL12 is a potent chemotactic factor for hematopoietic stem (HSCs) and progenitor (HPCs) cells [11,12]. It plays an essential role in the maintenance of HSCs, including homing, engraftment and repopulating activity, as well as HSC quiescence and retention in the bone marrow [13–17]. It has been shown to enhance the survival of HSCs and HPCs, an effect increased in synergy with other cytokines [5,18–20]. Treatment of mouse bone marrow cells and human cord blood HPCs with soluble SDF-1/CXCL12 enhanced their replating efficiency, and bone marrow cells from mice expressing a human SDF-1/CXCL12 transgene exhibited increased replating capacity of single macrophage- and multipotent progenitor- derived colonies [21].

SDF-1/CXCL12 appears to be a key regulator of HSCs in the bone marrow microenvironment [22]. The niche provides signals regulating HSC functions, such as self-renewal and long term repopulating capability, as well as the ability to undergo multiline age differentiation. Several groups have shown in genetic studies that mesenchymal progenitor, endothelial, and stromal cell populations play a critical role in the maintenance of HSCs in the niche and depending on which niche cells HSCs interact with, helps to define the specific “sub-niche” in which HSCs may reside [23–30]. Deletion of SDF-1/CXCL12 from different types of niche cells leads to the reduction in HSC numbers, competitive repopulation, and increases in splenic HSCs, all of which indicate an essential role for SDF-1/CXCL12 in HSC function in the bone marrow microenvironment [23,25,27,29,30].

Despite work from several groups describing the role of SDF-1/CXCL12 in the maintenance of HSCs and HPCs in the various niches in the bone marrow [23,25,27,29,30], there is a paucity of information on the mechanism by which SDF-1/CXCL12 functions at the molecular level for immature blood cell function in the bone marrow. Regulation and restriction of mitochondrial metabolism has been shown to be critical in maintaining the quiescent state of HSCs in the bone marrow by preventing mitochondrial produced reactive oxygen species (ROS), which can promote differentiation and HSC attrition and potential dysfunction [31–36]. Recent work from our group has shown that SDF-1/CXCL12 can modulate mitochondrial activity and mitochondrial mass in murine bone marrow cells expressing a human SDF-1/CXCL12 transgene [37]. We therefore hypothesized that SDF-1/CXCL12 regulates mitochondrial respiration in early hematopoietic cells.

Materials and Methods

Oxygen Consumption Rates

Basal oxygen consumption rates (OCR) and mitochondrial-associated ATP production were obtained using the Seahorse Bioscience XF96 Extracellular Flux Analyzer from Seahorse

Bioscience, and measurements were performed according to the manufacturer's instructions and as described previously [37–39]. Mitochondrial-associated ATP production is the difference between the basal OCR and oligomycin-A repressed OCR [40].

Cell Culture and Lineage negative bone marrow cell isolation

HL-60 cells (ATCC CCL-240) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Iscove's Modified Dulbecco's Medium (IMDM) with 20% FBS. HL-60 cells were incubated in IMDM +20% FBS with and without 50 ng/ml SDF-1 (R&D, Minneapolis, MN) for two and 24 hours, respectively. This concentration of SDF-1 has been shown to elicit optimal responses in numerous of our assays [12,18,19,21,41]. C57Bl/6 strain mice were used to isolate lineage negative bone marrow cells. The Indiana University Committee on Use and Care of Animals approved the mouse studies. Mouse lineage negative cells were isolated using the Miltenyi Biotech (San Diego, CA) mouse Lineage Cell Depletion Kit. After lineage depletion, lineage negative cells were incubated in IMDM +10% FBS and stimulated with or without 50 ng/ml SDF-1 (R&D, Minneapolis, MN) for two and 24 hours, respectively.

Reagents and instruments

Anti-human CXCR4 APC conjugated antibody (Clone 12G5) and anti-human CXCR7 FITC conjugated antibody (Clone 358426) was from R&D, Minneapolis, Minnesota. Mitotracker Green FM and Mitotracker Red CMXRos were from Molecular Probes, Eugene, Oregon. Flow cytometry was performed with a FACS Calibre flow cytometer (BD Biosciences, Franklin Lakes, NJ). Flow cytometry data were analyzed using FlowJo (Ashland, Oregon). Oligomycin-A was purchased from Sigma-Aldrich (St. Louis, MO) and AMD3100 was a kind gift from AnorMed (Langley, BC, Canada).

Statistical analysis

Data were statistically analyzed and plotted using GraphPad Prism 6 (San Diego, CA). Differences were assessed with a 2-tailed Student t-test or one-way ANOVA with Tukey's post-hoc correction. P values ≤ 0.05 were considered significant.

Results and Discussion

To investigate the potential role that SDF-1/CXCL12 plays in controlling mitochondrial respiration, we employed the human promyelocytic leukemia cell line, HL-60, as a beginning of modeling human immature subsets of hematopoietic cells and then proceeded to confirm the results in lineage⁻ primary mouse bone marrow cells.

A) Effects on HL-60 cells

We maintained HL-60 cells in culture with IMDM +20% fetal bovine serum under standard cell culture conditions, then cultured them for two and 24 hours, respectively, with or without 50 ng/ml of SDF-1/CXCL12 to assess rapid and more delayed effects. We assessed oxygen consumption rates (OCR), a measure of mitochondrial respiration, for each group on a Seahorse Bioscience XF96 Extracellular Flux Analyzer (Seahorse XF96). Cells treated for two hours with SDF-1/CXCL12 had a significant reduction in OCR of $24.8 \pm 1.0\%$ (mean \pm

SEM) as compared to two-hour unstimulated control cells (Figure 1A_i). In contrast, HL-60 cells treated for 24 hours with SDF-1/CXCL12 had a significantly increased OCR of $96.7 \pm 13.3\%$ over unstimulated 24-hour control cells (Figure 1A_{ii}). This suggests that SDF-1/CXCL12 plays a potential role modulating mitochondrial respiration in a bi-phasic manner as indicated by changes in mitochondrial OCR.

We next analyzed mitochondrial-associated ATP production by using the Seahorse XF96 (Figure 1B_i and 1B_{ii}). Mitochondrial-associated ATP production is the difference between the OCR and oligomycin-A (0.6 μ M) repressed OCR [34]. Mitochondrial-associated ATP production was significantly reduced in the two-hour SDF-1/CXCL12 treated group compared to the unstimulated control cells ($-29.2 \pm 2.9\%$), while the 24-hour treated group was significantly increased versus control ($+120.1 \pm 20.3\%$). These results show that as OCR is reciprocally decreased or increased over time by SDF-1/CXCL12 treatment of HL-60 cells, there are closely related changes in ATP production suggesting that these processes are tightly coupled and regulated in a biphasic manner *in vitro*.

Next we sought to confirm that the changes in mitochondrial respiration and ATP production in HL-60 cells are mediated through the SDF-1/CXCL12-CXCR4 axis. We first measured surface expression of human CXCR4 and CXCR7, both receptors for SDF-1/CXCL12, using fluorochrome conjugated antibodies. (Figure 2A). Once we determined that the HL-60 cells expressed CXCR4 with minimal or no expression of CXCR7, we used the CXCR4 antagonist AMD3100 [42] to attempt to block the SDF-1/CXCL12-CXCR4 mediated changes in mitochondrial respiration. HL-60 cells were pretreated for 30 minutes with 100 ng/ml of AMD3100 and then stimulated for 2 and 24 hours, respectively, with 50 ng/ml of SDF-1/CXCL12. HL-60 cells treated with SDF-1/CXCL12 for two hours have significantly reduced OCR as compared to untreated control cells, while both the AMD3100 alone group and the SDF-1/CXCL12 + AMD3100 group were not significantly different from untreated control (Figure 2B_i). Cells treated with SDF-1/CXCL12 for 24 hours had significantly increased OCR as compared to untreated control and cells pretreated with AMD3100 alone or pretreated with AMD3100 and then stimulated with SDF-1/CXCL12 for 24 hours had significantly reduced OCR as compared to untreated control cells (Figure 2B_{ii}). These results show that blocking CXCR4 with AMD3100 oblates the SDF-1/CXCL12 mediated biphasic changes in OCR, suggesting that these metabolic effects of SDF-1/CXCL12 are mediated through the CXCR4 receptor.

To further investigate the mechanism by which SDF-1/CXCL12 mediates changes in mitochondrial respiration, we investigated changes in mitochondrial mass and mitochondrial membrane potential (ψ_m). We hypothesized that changes in mitochondrial respiration and ATP production should be accompanied by concomitant changes in mitochondrial mass and ψ_m . To assess these changes we used the mitochondrial specific dyes MitoTracker Green FM (50nM) and MitoTracker Red CMXRos (50 nM). MitoTracker Green FM is a mitochondrial specific dye that is mitochondrial membrane potential independent and is an indicator of mitochondrial mass, while Mitotracker Red CMXRos is membrane potential dependent and used as an indicator of ψ_m (a measure of mitochondrial electron transport chain function). HL-60 cells were cultured for two and 24 hour, respectively, with 50 ng/ml of SDF-1/CXCL12. After each time point, cells were harvested and stained with

MitoTracker Green FM or MitoTracker Red CMXRos and mitochondrial mass and ψ_m was measured by flow cytometry. Both the mitochondrial mass and ψ_m for the two-hour SDF-1/CXCL12 stimulated group was significantly decreased versus unstimulated control (Figure 3A_i and 3B_i). There was a trend toward increased mitochondrial mass at 24 hours (Figure 3A_{ii}). The ψ_m of the 24-hour SDF-1/CXCL12 stimulated group was significantly increased versus control (Figure 3B_{ii}). Much like the OCR and ATP production, SDF-1/CXCL12 is also producing biphasic changes in mitochondrial mass and ψ_m , suggesting that it may be regulating several different aspect of mitochondrial respiration through regulation of electron transport, oxidative phosphorylation, and mitochondrial biogenesis.

B) Effect on Mouse Primary Bone Marrow Lineage⁻ Cells

To determine if SDF-1/CXCL12 effects on HL-60 cells could be mimicked in primary cells, we isolated lineage negative (Lin⁻) bone marrow cells (enriched for immature blood cells) from C57Bl/6 mice and cultured them with or without 50 ng/ml SDF-1/CXCL12 for two and 24 hours, respectively. Cells were then collected after each time point and OCR rates of each group were measured on the Seahorse Bioscience XF96 Extracellular Flux Analyzer. As already noted for the human HL-60 cells (Figure 1), the OCR for the two-hour SDF-1/CXCL12 stimulated group was significantly reduced versus unstimulated two-hour control (Figure 4A_i). The OCR of the 24-hour SDF-1/CXCL12 stimulated group, however, was significantly increased (Figure 4A_{ii}). These results are similar to the changes in OCR of the SDF-1/CXCL12 treated HL-60 cells. Next we analyzed mitochondrial-associated ATP production using the Seahorse Bioscience XF96 Extracellular Flux Analyzer (Figure 4B_i and 4B_{ii}). Treatment of Lin⁻ cells with SDF-1/CXCL12 produced a significant decrease in the mitochondrial-associated ATP production versus two-hour unstimulated control and treatment of Lin⁻ cells with SDF-1/CXCL12 for 24 hours produced a significant increase in the mitochondrial-associated ATP production as compared to control cells. These results demonstrate that ex vivo treatment of primary mouse immature blood cells in Lin⁻ bone marrow cells with SDF-1/CXCL12 regulates mitochondrial respiration in a biphasic manner similar to that seen in HL-60 cells.

Conclusions

Regulation of mitochondrial respiration is essential to HSC and HPC maintenance and proper function [31–36]. Disregulation of mitochondrial function can lead to various disorders including leukemia or bone marrow failure. Many groups have shown an essential role for SDF-1/CXCL12 in HSC maintenance, but its role in mitochondrial regulation had yet to be established. Our findings in HL-60 and primary Lin⁻ bone marrow cells suggest that SDF-1/CXCL12 regulates mitochondrial OCR, ATP production as well as ψ_m and mitochondrial mass in a biphasic fashion and that these effects are mediated through CXCR4. These results suggest that SDF-1/CXCL12 plays a potential role in regulating mitochondrial respiration in immature blood cell types. Mitochondrial regulation in a biphasic manner also suggests that time-dependent changes in SDF-1/CXCL12, as well as changes in the expression of SDF-1/CXCL12 by various niche cells, may differentially effect hematopoietic cells in the bone marrow and potentially influence HSC maintenance and differentiation. It is clear that mitochondrial metabolism of HSCs and HPCs is important

[33–37] and these results may have important implications for the regulation of mitochondrial metabolism of HSCs and HPCs in the bone marrow microenvironment. By continuing to study the role of SDF-1/CXCL12 in mitochondrial regulation in more defined HSC and HPC populations, studies may elucidate the role that it plays in regulating HSC and HPC function by various SDF-1/CXCL12 expressing niche cells.

Acknowledgments

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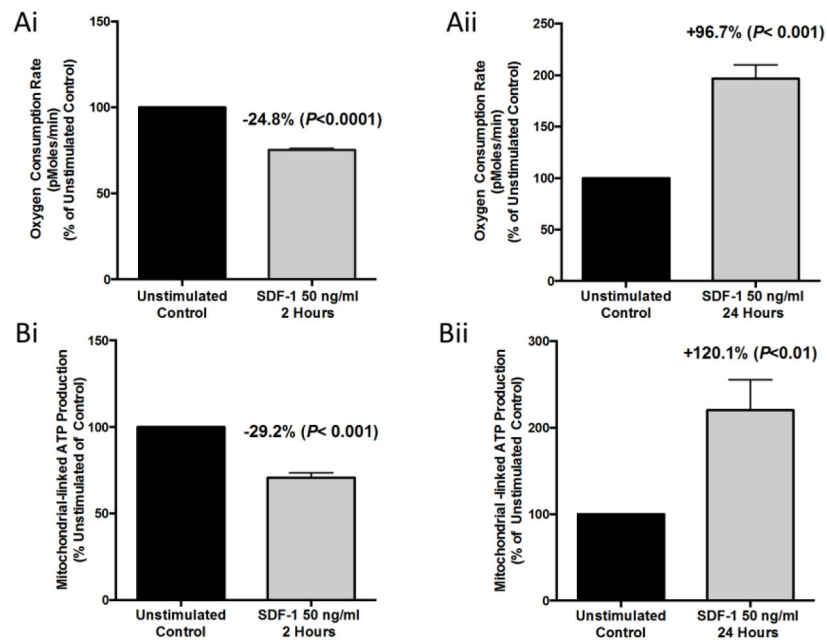


Figure 1. SDF-1/CXCL12 Regulates mitochondrial respiration of HL-60 cells in a biphasic manner

The human leukemia cell line, HL-60, was treated with 50 ng/ml of SDF-1 for 2 and 24 hours respectively. After each time point, cells were collected and their oxygen consumption rates (OCR) was measured (A) on the Seahorse Bioscience Extracellular Flux Analyzer. (B) Mitochondrial-linked ATP production of SDF-1 treated HL-60 cells was also measured using the Seahorse Bioscience Extracellular Flux Analyzer. Results are the mean \pm SEM of four independent experiments with significant differences as compared to unstimulated control shown in the figure.

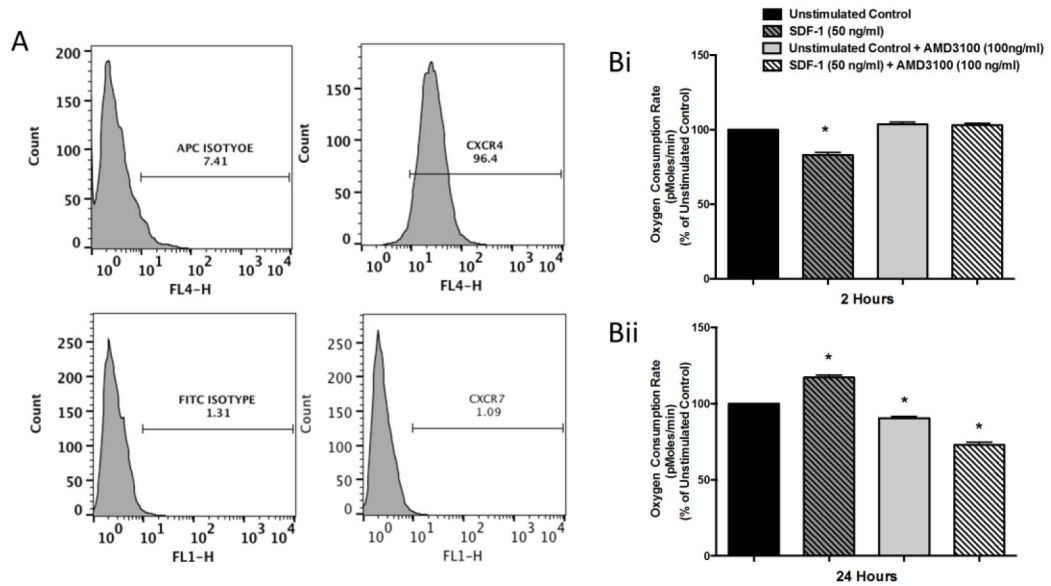


Figure 2. SDF-1/CXCL12 mediated effects on HL-60 cells are CXCR4 specific
 (A) HL-60 cells were stained with anti-hCXCR4-APC and anti-hCXCR7-FITC conjugated antibodies and the level of surface expression of each receptor was determined by flow cytometry. (B) HL-60 cells were pre-treated with 100ng/ml of the CXCR4 antagonist, AMD3100, for 30 minutes. After pre-treatment, cells were treated with 50ng/ml of SDF-1 for 2 and 24 hours respectively. After each time point, cells were collected and their oxygen consumption rates (OCR) (B) were measured on the Seahorse Bioscience Extracellular Flux Analyzer. Results are the mean \pm SD of one experiment with triplicates of each group. *, $p < 0.05$ comparing each group to unstimulated control.

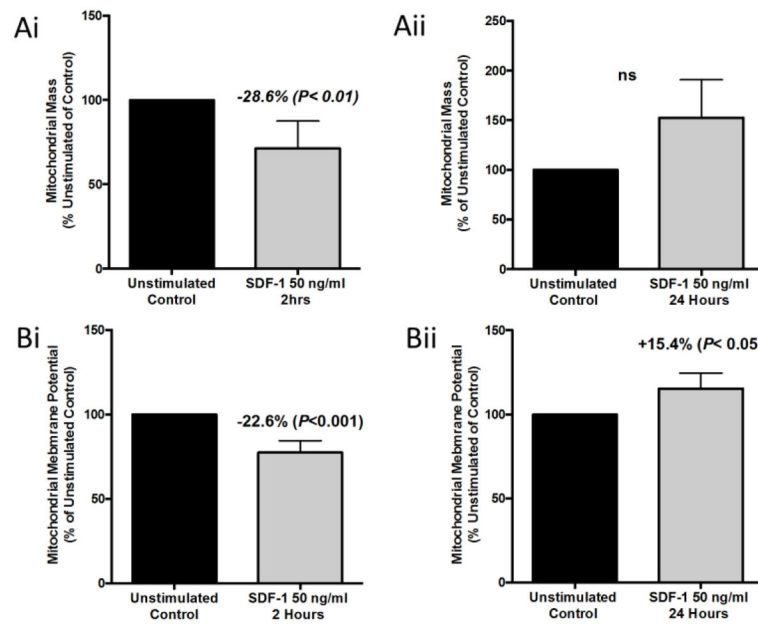


Figure 3. SDF-1/CXCL12 Regulates mitochondrial mass and mitochondrial membrane potential of HL-60 cells in a biphasic manner

(A & B) HL-60 cells were treated with 50ng/ml of SDF-1 for 2 and 24 hours respectively. After each time point, cells were collected and stained with 50 nM MitoTracker Green (A) to measure mitochondrial mass or 50 nM MitoTracker Red CMXRos (B) to measure mitochondrial membrane potential. Mitochondrial mass and membrane potential were analyzed by flow cytometry. Results are the mean \pm SEM of five independent experiments with significant differences as compared to unstimulated control shown in the figure.

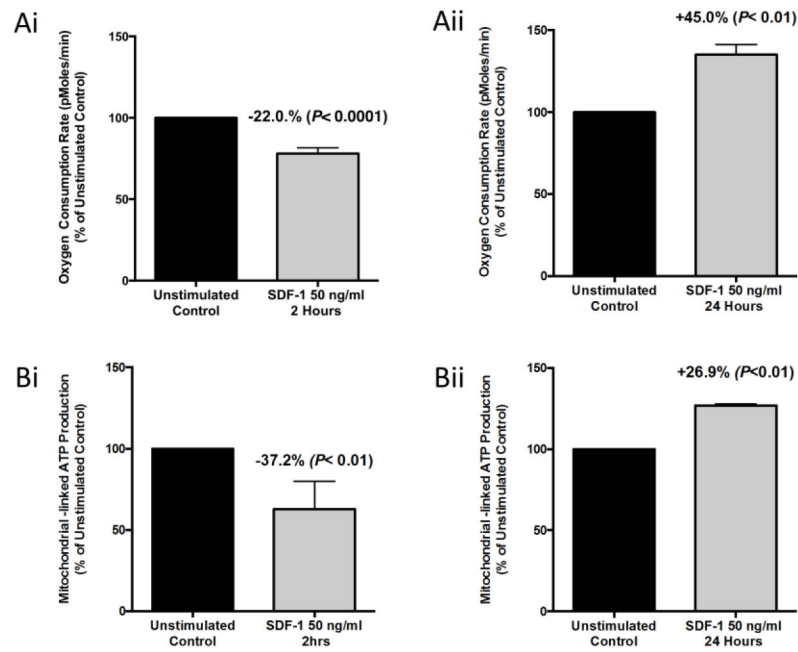


Figure 4. SDF-1/CXCL12 Regulates mitochondrial respiration of lineage negative bone marrow cells in a biphasic manner

Lineage negative bone marrow cells were isolated from C57Bl/6 mice and treated with 50ng/ml of SDF-1 for 2 and 24 hours respectively. After each time point the the OCR (A) and mitochondrial-linked ATP production (B) were measured on the Seahorse Bioscience Extracellular Flux Analyzer. Results are the mean \pm SEM of three independent experiments with significant differences as compared to unstimulated control shown in the figure.