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Nuclear ERK5 inhibits progression of leukemic monocytes to macrophages by regulating the transcription factor PU.1 and heat shock protein HSP70

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Abstract

Differentiation therapy can supplement the therapy of APL, but other subtypes of AML are treated principally with cytotoxic agents, with few lasting remissions. While the induction of monocyte followed by macrophage differentiation by vitamin D derivatives (VDDs) is dramatic in cultured AML cells of all subtypes, attempts to translate this to the clinic have not been effective. Thus, better understanding of the mechanisms underlying VDD-induced differentiation may improve this approach. The key events in this form of differentiation include increased expression of CD11b, and the transcription factor PU.1 is known to be a part of this process. We show here that in the transition of monocytes to macrophages induced by a VDD, ERK5, a member of the MAPK family of signaling molecules, prevents PU.1 expression. However, upon ERK5 inhibition PU.1 protein is stabilized by HSP70. Thus, ERK5 may be a target for manipulation of the immunoregulatory actions of macrophages in cancer.

Keywords

AML; differentiation; 1, 25-dihydroxyvitamin D₃; ERK5; heat shock protein

INTRODUCTION

ERK5 belongs to the MAPK family which consists of several parallel signaling cascades, including Raf-MEK1/2-ERK1/2, MEK2/3-MEK5-Cot1-ERK5, MEKK1-MEK4/7-JNK and MKK3/6-MAPK p38, which are involved in a variety of cellular activities, such as proliferation, differentiation, survival, and death [1–4]. Some pathways in the MAPK family share the same extracellular stimuli as well as some upstream components, so there is considerable functional redundancy. However, each of these pathways also has distinctive functions and activation of one pathway may not activate the parallel pathways that contain the same components [4–6]. Understanding these pathways is especially important in the treatment of malignancies. For instance, the upregulation of MAPK pathways in melanoma,

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lung cancer, colorectal cancer and non-small cell lung carcinoma provides potential targets for tumor chemotherapy. MAPK pathways are also involved in differentiation of multiple cell types [7–14].

MEK5 activates ERK5 by phosphorylation, and the known upstream regulators are MEKK2 and MEKK3. ERK5 in turn activates various cellular substrates including the myocyte enhancer factor 2 (MEF2) family members [15–17]. Like ERK1/2, MEK5 and ERK5 have also been found upregulated in various neoplasms including prostate and breast cancers [18–22]. In addition, studies have demonstrated that ERK5 upregulation could be responsible for chemo-resistance in breast cancer [23]. Thus, controlling ERK5 activity alone or together with other anti-cancer reagents during chemotherapy may be a new therapeutic strategy, or may enhance the therapeutic effect of the current cancer treatments.

Structurally, ERK5 is a much larger protein than other members in the ERK family; it contains two domains, a kinase domain in the N-terminus, which is homologous to ERK2, and a non-kinase domain in the C-terminus. MEK5 is the only known upstream activator of ERK5; it phosphorylates ERK5 at the TEY (*Thr218/Tyr220*) motif in the kinase domain which triggers the auto-phosphorylation in multiple C-terminal residues, including *Thr732* and *Ser769/773/775* [24, 25]. The non-kinase domain can interact with and activate transcription factors such as MEF2C [25]. Binding between the C-terminus and N-terminus domains results in ERK5 retention in the cytoplasm, whereas C-terminus domain phosphorylation disrupts this binding, leading to nuclear translocation of ERK5 [26]. Inhibition of the C-terminus auto-phosphorylation blocks nuclear entry of ERK5 following MEK5 activation [17, 27]. A recent study reported that non-kinase domain deletion results in increased activity of the kinase domain, implicating an inhibitory role of the non-kinase domain [28].

Pharmacological inhibitors target different levels of the MEK5/ERK5 pathway, including XMD8-92, which specifically prevents the C-terminus auto-phosphorylation of ERK5 [17]. Recently, our laboratory reported that in 1, 25-dihydroxyvitamin D3 (1,25D)-induced monocytic differentiation of human AML cells, inhibition of ERK5 with XMD8-92 results in further maturation of monocytes to macrophages, which is characterized by decreased CD14 expression and increased CD11b expression, as illustrated in Figure 1 [30, 31].

It is generally accepted that cell fate is ultimately determined by the expression of lineage-determining transcription factors at a specific time, combination, and expression levels in response to colony stimulating factors, cytokines, differentiation inducers, etc. [32]. PU.1 is an important transcription factor (TF) for hematopoiesis [32], which has effects on granulocyte or monocyte development depending on the cell context [33, 34]. Regarding differentiation marker expression, PU.1 is believed to directly bind to the promotor region of CD11b to regulate its expression [35], and can either function alone or cooperate with other TFs, such as AP1, c-Jun, ATF4, C/EBP β , AML1, to regulate their target genes [36, 37].

Cytoplasmic chaperones, including heat shock proteins, are known to regulate protein levels, including the cellular abundance of transcription factors [38, 39]. Heat shock proteins were first identified as a group of proteins induced by heat shock or other physical and chemical

stresses, and later found to be chaperones in charge of protein folding, assembling, dis-assembling, trafficking and degradation [40, 41, 42]. Heat shock protein transcription factor 1 (HSF1) is required for the expression of HSPs [43]. Besides induced expression of HSPs in stressed conditions, there are also constitutively expressed HSPs which are usually found in multi-protein complexes [44]. HSPs interact with a wide range of substrates including kinases, steroid receptors and lipids, regulating cell proliferation, survival, differentiation, etc. [45–50]. ERK5 is one of the client proteins of HSP90 and the association of these two proteins requires the dual phosphorylation of the TEY motif in the kinase domain of ERK5 [47, 50]. HSP70, another member in the HSP family, has been recently demonstrated to interact with PU.1 to prevent its degradation in monocyte to macrophage maturation in response to M-CSF cytokine stimulus [51]. Though the exact molecular mechanisms of HSPs binding and release of their client proteins are not fully understood, in the case of HSP70 and HSP90, the binding of ATP and its hydrolysis are required for this process. Most of the HSP70 and HSP90 inhibitors target the ATP binding site to block their chaperone functions [52–54].

Tumor associated macrophages (TAMs) are a type of macrophages found near or within the malignant tissues [55]. The functions of TAMs are controversial, as they can be either pro-tumoral by promoting tumor growth and metastasis or anti-tumoral by killing the tumor cells or inhibiting their growth [55, 56]. Reducing the number of TAMs or re-differentiating TAM precursors to anti-tumor macrophages are two important strategies in cancer immune-therapy [55, 57]. However, the mechanisms underlying macrophage differentiation are not well understood and a study of the mechanisms involved in macrophage maturation should help to manipulate the functions of macrophages in malignant diseases.

Here, we investigated more detailed mechanisms of 1,25D-induced monocyte progression to macrophage while ERK5 is inhibited. We monitored the maturation of monocytes using two differentiation markers: an increased level of CD11b, and a decreased level of CD14 (as illustrated in Figure 1). In a previous study, we have demonstrated that MEF2C signal is required for the increased CD14 expression in 1,25D-induced monocytic differentiation by regulating C/EBP β [59]. Here we focus on the mechanisms of the upregulation of CD11b levels. We found that PU.1 is required for the ERK5 inhibition-potentiated CD11b expression in an AML cell model. Further, an increased HSP70 protein level is a possible cause of upregulated PU.1. This study shows that manipulating ERK5 activity leads to a change in the HSP70 protein levels, consistent with a critical role of heat shock proteins in 1,25D-induced cell differentiation. Thus, our study provides an improved understanding of the mechanisms involved in monocyte to macrophage transition. Importantly, ERK5 and HSP70 may be targets for therapeutic blockade of macrophage maturation, or targets for differentiation of monocytes to macrophages with antitumor functions [56].

MATERIALS AND METHODS

Reagents

1,25D was purchased from Sigma Aldrich. BIX02189 and Ver155008 were purchased from Selleckchem and XMD8-92 was from Tocorris. PU.1, HSP70, HSP90, HSF1 and Calregulin antibodies, the lentiviral particles containing PU.1 or Control shRNAs were obtained from

Santa Cruz Biotechnology (Dallas, TX). anti-CD11b MO-FITC and anti-CD14 My4-RD-1 for flow cytometry were from Beckman Coulter Inc. (Brea, CA). cDNA Archive Kit and SYBR Green Master Kit were obtained from Applied Biosystems of Life Technologies (Grand Island, NY). HL60 and U937 cell lines were from American Type Culture Collection (Manassas, VA).

Cell Culture and Differentiation Assay

The cell lines HL60 (promyeloblasts from an acute promyeloblastic leukemia patient) and U937 (monocytes from histiocytic lymphoma) were grown in RPMI-1640 medium with 10% of calf bovine serum at 37°C with 5% of CO₂. To induce monocytic differentiation, 1,25D was added to either non-transfected or shRNA-transfected cells at a final concentration of 10 nM and incubated for 72 hrs. MEK5 and ERK5 inhibition was achieved by incubating the cells with 10 µM BIX02198 in a preliminary experiment or 5 µM XMD8-92 1 hr prior to the 1,25D addition, and the inhibitors remained in the culture together with 1,25D for additional 72 hrs. For HSP70 inhibition, Ver155008 was added at the same time as XMD8-92 and remained in the medium until cells were harvested.

Flow Cytometry Analysis

After three washes with PBS, the harvested cells were incubated with 1 ml of PBS containing 0.5 µg anti-CD11b MO-FITC and 0.1 µg anti-CD14 My4-RD-1 antibodies for 45 min followed by another 2 washes with PBS. The labelled cells were then fixed with 1% paraformaldehyde in PBS overnight at 4°C then washed twice with ice cold PBS before being analyzed using BD FACS Caliber flow cytometry (BD Biosciences).

Western Blotting

Protein expression levels were determined by western blots using whole cell lysates prepared with RIPA buffer. Equal amount of protein was loaded to 10% acrylamide gels for electrophoresis then transferred to PVDF membranes. The membranes were incubated with the specific first antibody overnight at 4°C and HRP conjugated secondary antibody at room temperature for 1 hr. Protein bands were visualized using ECL substrate (Bioexpress). Optical densities (ODs) of the bands were analyzed with ImageJ, an image processing and analyzing system (down loaded from NIH website). ODs were normalized to the band of the untreated control group, and then normalized to the loading controls.

RT-PCR and Real Time PCR

RNA levels were measured using RT-PCR followed by quantitative Real Time PCR. First, total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and then reverse transcribed to cDNA using random hexamers as primers with ABI cDNA Archive kit. Real time PCR was done using ABI SYBR Green master kit and ABI7500 thermal cycler. mRNA levels of target genes were calculated relative to RNA polymerase II (RPII) using the comparative C(T) (threshold cycle) method in ABI7500 analysis software. PU.1 primers: forward: 5'-GTT CTC GTC CAA GCA CAA GG-3', reverse: 5'-CTT CGC CGC TGA ACT GGT AG-3', RPII primers: forward: 5'-GCA CCA CGT CCA ATG ACA T-3'; reverse: 5'-GTG CGG CTG CTT CCA TAA-3'.

shRNA Transduction with Lentiviral Particles

First, the cells were incubated with polybrene for 1 hr to remove the charges on the cell surface. Then the virus particles carrying either PU.1 or Control shRNAs were added to the cell cultures. Incubating the cells at 37 °C for 48 hrs allowed the integration of shRNAs to the genome and the puromycin resistant protein to express. Puromycin (0.5 µg/ml based on dose response experiment) was then added to select the transduced cells. All the non-transduced cells were killed by puromycin within 7 days. The cells carrying shRNAs continued to be cultured and puromycin was removed from the media.

Statistical Analysis

Each experiment was repeated 3 to 5 times. The results are presented as the mean ± standard error. Statistical significance of the difference between two mean values in all the experiments was determined by a two-tailed paired t-test in Microsoft EXCEL, and $p < 0.05$ is considered to be significant.

RESULTS

Progression of AML Cells with Monocytic phenotype to Macrophages Is associated with Increase of PU.1 Protein, but not PU.1 mRNA Levels

This laboratory previously reported that when ERK5 activity is inhibited in the process of 1,25D-induced monocytic differentiation of human leukemia cells HL60 and U937, the monocyte-like cells further differentiate to cells with macrophage-like phenotype, which is characterized by upregulated CD11b levels with concomitantly down-regulated CD14 levels (Figure 1) [31]. This process was further examined here with a focus on the inhibition of ERK5 auto-phosphorylation using the pharmacological inhibitor XMD8-92 [17, 31]. While both the inhibition of ERK5 phosphorylation by BIX02189 or XMD8-92 significantly increased cell surface CD11b levels while decreasing CD14 levels compared to treatment with 1,25D alone, the direct inhibition of ERK5 activity by XMD8-92 produced a more marked macrophage-like phenotype than BIX02189 (Figure 2A). The effect of BIX02189 or XMD8-92 alone on cell surface expression of CD11b and CD14 is negligible.

Initially, to elucidate the underlying mechanisms we examined changes in the levels of the lineage-determining transcription factor, PU.1, which is known to play an important role in monocyte/macrophage maturation [33, 36, 37]. Treatment with 1,25D alone modestly increased the protein levels of PU.1 compared to the untreated control cells (Figure 2B), and the addition of XMD8-92 to 1,25D substantially increased the PU.1 protein level further ($p < 0.05$, 1,25D +XMD8-92 vs. 1,25D alone) (Figure 2B). PU.1 protein was barely detectable on Western blots of extracts of cells treated with XMD8-92 alone.

We then measured PU.1 mRNA levels following the same treatments at 4, 24, 48 and 72 hrs to determine whether the upregulated PU.1 protein is due to increased transcription. As shown in the diagrams, with 1,25D+XMD8-92, PU.1 mRNA remained the same level as in the untreated controls at all the time points except for at 24 hrs in U937 cells. At this time point, 1,25D+XMD8-92 treated U937 cells had lower PU.1 mRNA compared to untreated controls or 1,25D alone treated cells (Figure 2C). The discrepancy between protein and

mRNA levels of PU.1 indicates that PU.1 is not regulated at transcriptional level when ERK5 activity is inhibited in the presence of 1,25D. Collectively, the above data suggest that in the presence of 1,25D the CD11b expression induced by ERK5 inhibition is associated with increased PU.1 protein level (Figure 2), which is likely to be post-transcriptionally regulated.

PU.1 Is Required for the Optimal Increase in CD11b Surface Expression Following Inhibition of ERK5

Since the role of PU.1 in monocyte/macrophage differentiation and CD11b expression is well recognized in other systems [32, 35–37], we investigated whether PU.1 is involved in the potentiation of macrophage differentiation by ERK5 inhibition. We stably transfected HL60 and U937 cells with short hairpin RNAs (shPU.1 or shControl) to knockdown the expression of PU.1. Flow cytometry demonstrated that following exposure to 1, 25D +XMD8-92, HL60 or U937 cells in which PU.1 was knocked down expressed significantly less CD11b compared to shControl cells (Figure 3A). PU.1 knockdown had little effect on CD11b expression in untreated control cells and XMD8-92 alone treated cells. In HL60 cells treated with 1,25D alone, CD11b level decreased modestly but significantly when PU.1 was knocked down, but no significant decrease in CD11b expression in U937 cells with the same treatment. In all these treatment groups, PU.1 knockdown resulted in no observable effect on CD14 levels (Figure 3A). Western blots confirmed the decreased expression of PU.1 protein in the PU.1 knock-down cells treated with 1,25D+XMD8-92 (Figure 3B). These data suggest that upregulation of PU.1 is needed for the potentiation of CD11b expression by ERK5 inhibition in 1,25D-treated AML cells. Thus, the data show that in these cells ERK5 represses CD11b expression through PU.1.

ERK5 Inhibition Increases the Levels of HSP70 in AML Cells

As shown in Figure 2, mRNA and protein levels of PU.1 are differentially regulated in AML cells treated with 1,25D and the ERK5 inhibitor XMD8-92, suggesting that the changed protein levels are the result of post-transcriptional regulation. A recent study demonstrated that HSF1 plays a dual role on PU.1 expression: on one hand, HSF1 upregulates PU.1 transcription, and on the other hand, it can increase HSP70 expression, which in turn is able to prevent PU.1 degradation through inhibition of proteasome function, as illustrated in Figure 4A [51]. Heat shock protein HSP90 has also been reported to be involved in cell differentiation and to interact with vitamin D receptor [48]. Accordingly, we measured the levels of HSF1, HSP70 and HSP90 in this system. As shown in Figure 4B, in both cell lines the HSF1 and HSP70 protein levels increased following 1,25D+XMD8-92 treatment. However, HSP90 failed to show any significant change following this protocol. Although PU.1 protein level increased significantly following the 1,25D+XMD8-92 exposure of the cells, PU.1 mRNA level stayed the same as in the untreated control (Figure 2), making it unlikely that PU.1 is regulated by HSF1 directly at transcriptional level. XMD8-92 alone showed a minimal effect on HSF1 and HSP70 protein expression compared to 1,25D +XMD8-92. So, a likely mechanism is that 1,25D and ERK5 inhibition lead to HSP70 upregulation which in turn prevents PU.1 degradation. The levels of HSP90 showed no changes in this protocol, so it is less likely to participate as a chaperone for PU.1.

HSP 70 Protein Is Necessary for the CD11b and PU.1 Upregulation Induced by ERK5 Inhibition

Since transcriptional regulation of PU.1 is unlikely in this system (Figure 2C), we focused on the involvement of HSP70 in increasing protein stability of PU.1. To confirm that HSP70 is essential for PU.1 and CD11b upregulation in macrophage-like phenotype changes induced by 1,25D+XMD8-92 in AML cells, a pharmacological inhibitor, Ver155008, was used to suppress HSP70 kinase activity. Most heat shock protein inhibitors inhibit ATP binding to HSPs including the one used in this study, Ver155008 [60, 61]. Incubation for 72 hrs with Ver155008 of cells treated with 1,25D and XMD8-92 significantly reduced CD11b expression. This resulted in the same CD11b levels as in 1,25D alone-treated cells, but had no observable effect on CD14 expression (Figure 5A). Ver155008 alone showed no effect on CD11b and CD14 expression (Figure 5A). Western blots confirmed that PU.1 levels were reduced with Ver155008 in 1,25D+XMD8-92 treated cells, but Ver155008 alone did not affect PU.1 protein levels in both cell lines (Figure 5B). These data suggest that HSP70 is responsible, at least in part, for the upregulated CD11b and PU.1 protein levels, most likely by reducing PU.1 degradation when 1,25D and the ERK5 auto-phosphorylation inhibitor are present.

DISCUSSION

In this study we explored the downstream mechanisms of macrophage maturation induced by the exposure of AML cells to 1,25D together with XMD8-92, an inhibitor of ERK5 [17, 31]. PU.1 is identified as an essential transcription factor involved in the increased CD11b levels in the macrophage differentiation. We also found that that HSP70 regulates PU.1 protein level in this process (Figures 5). This report not only provides information on mechanisms of 1,25D-induced monocyte/macrophage differentiation in AML cells, but may also be useful for the studies of mechanisms of tumor-associated macrophage differentiation.

PU.1 is already a well-documented transcription factor for monocyte/macrophage maturation during normal hematopoiesis [32]. Irradiated mice transplanted with PU.1 knockout HSCs fail to generate monocytes, and in fetal liver progenitor cells, a high PU.1 level favors macrophage differentiation [62, 63]. PU.1 can also bind to the promoter region of CD11b and control its expression directly [35]. In the system we used, PU.1 was required for CD11b upregulation in AML cells treated with 1,25D in combination with the ERK5 auto-phosphorylation inhibitor XMD8-92. CD14 which decreases during macrophage maturation was not affected by PU.1, as with PU.1 knockdown, there still was a decrease of cell surface CD14 in AML cells treated with 1,25D+XMD8-92. However, as in other differentiation systems, other transcription factors are likely to be important to macrophage maturation.

Heat shock proteins HSP90 and HSP70 have been reported to be involved in differentiation of several cell types, such as erythrocytes, monocytes and neuronal cells [64]. A recent study showed that HSP70 promotes macrophage maturation by protecting PU.1 from proteasome degradation in macrophage colony-stimulating factor induced differentiation [51]. The data in the current study demonstrate that, similar to the previously reported regulatory

mechanisms of PU.1 by HSP70, also take place in 1,25D+ERK5 inhibitor-induced macrophage-like phenotype changes.

This is the first report that HSP70 is involved in 1,25D-induced monocyte/macrophage differentiation. How inhibition of ERK5 auto-phosphorylation in the presence of 1,25D results in increased HSP70 levels is not yet clear; thus the detailed mechanism of this increase will need further investigation.

In summary, this study increases our understanding of the signaling pathway required for the 1,25D-induced maturation of AML cells. Potential therapeutic targets are identified that may help to improve the use of vitamin D derivatives, alone or in combination with other compounds, for therapy of AML, and perhaps of other types of malignancy.

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Abbreviations

APL	acute promyelocytic leukemia
ERK5	extracellular-signal-regulated kinase 5
MAPK	mitogen-activated protein kinases
MEK	MAPK/ERK Kinase
JNK	Jun amino-terminal kinase
AP1	activator protein 1
AML1	acute myeloid leukemia 1
C/EBPβ	CCAAT-enhancer-binding protein β
ATF4	activating transcription factor 4
M-CSF	macrophage colony-stimulating factor
TF	transcription factor

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Figure 1. A schematic illustration of the proposed model used in this study

1,25D-induced monocytic phenotype changes are accompanied by increased PU.1 protein levels; inhibition of nuclear translocation of ERK5 leads to macrophage-like changes with further increase of PU.1 level. cERK5= cytoplasmic ERK5, nERK5= nuclear ERK5.

Figure 2. Progression of AML cells with monocytic phenotype to macrophage phenotype is associated with increase of PU.1 protein levels

A. Leukemia cell lines HL60 and U937 were treated with 1,25D (10 nM) and/or the ERK5 inhibitors (BIX02189, 10 μ M; XMD8-92, 5 μ M) for 72 hrs, and differentiation (CD11b and CD14 expression) was assessed by flow cytometry. Leukemia cells increased maturation of monocytes to macrophages (increased CD11b and decreased CD14 levels) when ERK5 was

inhibited in the presence of 1,25D (*, $p < 0.05$, 1,25D + XMD8-92/BIX02189 vs. 1,25D alone in terms of CD14 levels, $n \geq 3$; #, $p < 0.05$, 1,25D+XMD8-92/BIX02189 vs 1,25D in terms of CD11b levels, $n \geq 3$; BIX and XMD represent BIX02189 and XMD8-92 respectively). BIX02189 or XMD8-92 alone did not have any significant effect on CD11b expression or CD14 expression ($n > 3$).

B. ERK5 inhibition by XMD8-92 markedly increased protein levels of PU.1 in the presence of 1,25D (*, $P < 0.05$, 1,25D+XMD8-92 vs 1,25D alone, $n > 3$). PU.1 protein was barely detectable with XMD8-92 alone.

C. The PU.1 mRNA levels were determined by RT-PCR and real time PCR at 6, 24, 48 and 72 hrs. The PU.1 mRNA levels in 1,25D +XMD8-92 treated cells were comparable to 1,25D alone at all these time point except for at 24 hrs in U937 cells. At this time point, PU.1 mRNA level in U937 cells was significantly (*, $p < 0.05$, $n = 3$) lower in 1,25D +XMD8-92 treated cells compared to 1,25D alone or untreated control cells.

Figure 3. PU.1 is required for the optimal increase in CD11b levels following ERK5 inhibition in AML cells

A. PU.1 was stably knocked down by shRNAs in HL60 and U937 cells. The PU.1 knockdown decreased CD11 expression in 1,25D+XMD8-92 treated cells in both cell lines, and it also decreased CD11b expression in 1,25D alone-treated HL60 cells, but not in U937 cells. The PU.1 knockdown did not affect CD11b expression in control cells and XMD8-92 alone treated cells, nor CD14 expression in all these treatment groups. (*, $p < 0.05$, $n = 3$).

B. Western blots shows decreased PU.1 protein levels in the PU.1 knockdown group compared to the control group in 1,25D+XMD8-92 treated cells (*, $p < 0.05$, $n = 3$).

Figure 4. ERK5 inhibition changes the levels of heat shock proteins in AML cells

A. A scheme of regulation of PU.1 by HSF1 and HSP70 as documented in the literature [51].

B. Protein levels of HSF1, HSP70 and HSP90 in HL60 and U937 cells in response to 1,25D alone, XMD8-92 alone and 1,25D+XMD8-92. HSF1 and HSP70 protein levels increased significantly in cells treated with 1,25D+XMD8-92 compared to 1,25D alone or XMD8-92 alone (*, $p < 0.05$; **, $p < 0.01$, $n=3$).

Figure 5. HSP 70 kinase activity inhibition attenuates CD11b and PU.1 potentiation by ERK5 inhibition in the presence of 1,25D

The requirement of HSP70 kinase activity in CD11b and PU.1 protein upregulation induced by 1,25D and ERK5 inhibitor (XMD8-92) was shown by the addition of Ver155008 (25 μ M), a specific HSP70 kinase inhibitor. AML cells were treated with 1,25D alone, XMD8-92 alone, Ver155008 alone or Ver155008 together with 1,25D+XMD8-92 for 72 hrs. Flow cytometry results and western blots demonstrate that Ver155008 attenuated CD11b (A) and PU.1 upregulation (B) resulting from 1,25D+XMD8-92 exposure (*, $p < 0.05$, $n \geq 3$). Ver155008 (25 μ M, 72 hrs) alone showed minimal effect on CD11b, CD14, as well as PU.1 protein expression.

Figure 6. The overall scheme of the mechanisms of PU.1 upregulation by ERK5 described in this study

Overall scheme of the mechanisms for this study: the ERK5 inhibitor XMD8-92 together with 1,25D leads to upregulation of HSP70 in leukemia cells. HSP70, in turn, prevents proteasomal degradation of PU.1, resulting in increased CD11b expression.