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Research paper

Interleukin-8 blockade prevents activated endothelial cell mediated proliferation and chemoresistance of acute myeloid leukemia

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ABSTRACT

One of the greatest challenges in treating acute myeloid leukemia (AML) is chemotherapy refractory disease. Previously, we demonstrated a novel mechanism whereby AML-induced endothelial cell (EC) activation leads to subsequent leukemia cell adherence, quiescence and chemoresistance, identifying activated ECs as potential mediators of relapse. We now show mechanistically that EC activation induces the secretion of interleukin-8 (IL-8) leading to significant expansion of non-adherent AML cells and resistance to cytarabine (Ara-C). Through crystallography and computational modeling, we identified a pocket within IL-8 responsible for receptor binding, screened for small molecules that fit within this pocket, and blocked IL-8 induced proliferation and chemo-protection of AML cells with a hit compound. Results from this study show a new therapeutic strategy for targeting the sanctuary of an activated leukemia microenvironment.

1. Introduction

Despite achieving initial morphologic remission after chemotherapy, 60–80% of patients with acute myeloid leukemia (AML) suffer from disease relapse [1]. Even with recent drug approvals by the FDA, the majority of AML patients will die of relapsed or refractory leukemia. Thus, there is an urgent need for new therapeutics for people with AML.

AML is highly dependent on its microenvironment for its survival and growth [2]. Studies have shown that interaction with soluble factors, stromal cells and extracellular matrix (ECM) in the bone marrow (BM) is essential in maintaining AML [2–4]. Previously, we demonstrated that endothelial cells (ECs) serve as sanctuary sites for refractory AML [5,6].

Mechanistically, AML cells activate ECs and lead to a positive feedback loop involving the adhesion of a subset of AML cells to activated ECs, quiescence of AML cells, and resistance to chemotherapeutic agents such as cytarabine and anthracyclines that are cornerstones to clinical regimens [7]. Experiments with semipermeable membranes have also shown the ability of ECs to enhance the proliferation of non-adherent AML cells through paracrine action [8,9]. What has yet to be fully understood are the molecular mechanisms by which AML-activated ECs protect and enhance the growth of AML cells. By identifying these mechanisms, new therapeutic strategies can be devised.

EC activation is an inflammatory response that alters resting EC behavior resulting in the increased production of various soluble factors involved in the general immune response [10,11]. Interestingly, one of the most highly secreted factors is interleukin (IL)-8, a factor which has been shown to significantly affect AML cell proliferation [12–14]. In these studies, we directly demonstrate that AML-induced EC activation results in the increased production of IL-8 that enhances AML cell

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proliferation and chemoresistance. Studies aimed at blocking IL-8 signaling, utilizing crystallography and computational modeling, resulted in the identification of an IL-8 small molecule inhibitor able to abrogate enhanced growth and chemoresistance to Ara-C. Other studies have shown that IL-8 is overexpressed is AML patient samples and blockade of IL-8 receptor (CXCR2) activity through knockdown or pharmacologic approaches resulted in decreased proliferation of AML further suggesting an important role of this axis in AML progression [15]. Overall, these findings suggest that IL-8 production by EC activation plays a central role in: 1) the growth and survival of AML, 2) the supportive effects of ECs observed in AML and 3) identifies IL-8 inhibition as a potential method to augment chemotherapy.

2. Materials and methods

2.1. Cell lines and primary cells

Human umbilical vein endothelial cells (HUVECs; Lonza, Walkersville, MD; C2517A) were cultured using EGM-2 MV media (Lonza; CC-3202). KG-1 human leukemia cells (ATCC, Manassas, VA; CCL-246) were grown in IMDM (Hyclone, Fisher Scientific, Hanover Park, IL; SH30228.01) plus 20% FBS.

Bone marrow endothelial cells (BMECs) were freshly isolated from healthy human bone marrow (Lonza). Briefly, mononuclear cells were isolated from fresh bone marrow samples using Ficoll separation (GE Healthcare, Chicago, IL; GE17-1440-02) and plated in collagen-coated plates. Adherent cells were collected and cultured using EGM-2 MV media supplemented with 10% FBS to generate the BMECs.

2.2. AML-EC co-cultures

Co-cultures were established in 6 or 12-well plates to study the relationship between AML and ECs. HUVECs and BMECs were first grown to 60–80-% confluency then 0.5–1 $\times 10^6$ KG-1 cells were added. Co-cultures were maintained in base media supplemented with 10% FBS.

2.3. Conditioned media studies

Activated EC conditioned media (CM) was prepared by collecting the supernatant from contact co-cultures after 48-h of incubation. Non-activated CM was collected from HUVEC or BMEC alone cultures. CM was mixed with base EBM media (without supplements) at a 50/50 ratio by volume prior to use.

To test the effects of the CM on leukemia cell growth, 5×10^5 KG-1 cells were grown in EBM base media supplemented with the different CMs. As controls, KG-1 cells were also grown with non-activated CM alone or 100% EBM. To measure growth, cells were enumerated every other day over 8-days or exposed to proliferation assays.

2.4. Analysis of proliferative status and viability

To assess the proliferative status of AML cells in our co-cultures, BrdU uptake levels were quantified. Briefly, co-cultured cells were collected and incubated with BrdU as previously described [7]. Cells were then stained with anti-CD45-APC (555485) and anti-BrdU-FITC (51-33284X, both from BD Biosciences, San Jose, CA, USA) and analyzed by flow cytometry using a FACSCanto II (BD Biosciences) and FACSDiva software. Alternatively, proliferation studies were performed using CellTiter Blue (Promega, Madison, WI; G8080) or XTT cell proliferation assay (ATCC; 30–1011 K) as per the manufacturer's protocols.

To assess viability, AML cells were harvested after 24-h of treatment and stained with anti-CD45-V450 (560367), Annexin V-FITC (556419) and 7-AAD (555815) for flow cytometric analysis.

2.5. Drug treatments

Cells grown alone or in co-culture with ECs were treated with cytarabine (Ara-C) (Sigma-Aldrich, St Louis, MO; C6645) at a final concentration of 20 μM in 0.1% DMSO for a period of 24–48-h.

For *in vitro* IL-8 inhibitor screening, the top 19 compounds with the lowest energy scores were obtained from the National Cancer Institute/Developmental Therapeutics Program (NCI/DTP) Open Chemicals Repository 2007 plate. AML cells (in monoculture or co-culture) were treated with each compound for 24-h at a final concentration of 100 μM in 0.01% DMSO.

2.6. E-selectin expression analysis

Co-cultures were established and after 24–48-h, ECs were harvested and stained using anti-E-selectin-APC (551144) and anti-CD105-PE (560839; both from BD Biosciences) then analyzed by flow cytometry. The levels of E-selectin expression were determined and used to quantify EC activation [10].

2.7. Cytokine analysis

Cell culture supernatants were analyzed for the concentration of IL-8 utilizing the VersaMAP Custom Multi-Analyte Profiling Development System (R&D Systems, Minneapolis, MN) and BioPlex array reader equipped with Bio-Plex software (Bio-Rad, Hercules, CA). Values were extrapolated from a standard curve.

2.8. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed on HUVECs that were activated by co-culture with KG-1 for 24-h. Following activation, HUVECs were sorted using a BD FACSAria (BD Biosciences) based on EC specific staining with CD105 [5]. Isolated HUVECs were then subjected to RNA extraction and first strand synthesis using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The reactions were performed using TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA). The following primers were used: IL-8 (Hs00174103_m1, Thermo Fisher Scientific). Data detection was performed using the Strategene qRT-PCR instrument software (Agilent Technologies, Santa Clara, CA). All data was calculated based on β-actin endogenous control levels.

2.9. Protein expression analysis

Protein expression was performed using Western blot analysis. Briefly, cells were lysed in RIPA buffer including Halt protease inhibitor (Fisher Scientific, Hanover Park, IL; 87785) and subjected to electrophoresis using 12% polyacrylamide gels (Bio-Rad, Hercules, CA; 4568044). Proteins were transferred onto a 0.45 μm polyvinylidene difluoride (PVDF) membrane (Fisher Scientific, Hanover Park, IL; IPVH00010). Membranes were blocked in 5% BSA and immunoblotted with Akt (Cell Signaling Technologies, Danvers, MA; 9272), 4E-BP1 (Cell Signaling Technology; 9644), p-4E-BP1 (Cell Signaling Technologies; 9451), p-Akt (Ser473) (Affinity; AF0016), and GAPDH (Life Technologies; 398600). HRP-conjugated secondary antibodies (Cell Signaling Technologies; 7074) were used, and protein levels were visualized using enhanced chemiluminescence (ECL) (Bio-Rad; 1705060).

2.10. Crystallization of human interleukin IL-8

Recombinant IL-8 isolated and purified from Pichia pastoris [16] was concentrated to 10 mg/ml with equal volumes of Hampton Crystal Screen Cryo 1 (HR2-121) and 2 (HR2-122) (Hampton Research, CA). Large crystals formed in 0.2 M Ammonium acetate, 0.085 M Sodium

citrate tribasic dihydrate pH 5.6, 30% w/v Polyethylene glycol 4000 and 15% v/v Glycerol. Single crystals were flash cooled and stored in liquid nitrogen prior to data collection at the National Synchrotron Light Source beamline X6A.

2.11. Data reduction and structure determination of human interleukin IL-8

X-ray data was reduced with DENZO and SCALEPACK. The 2.0 Å crystal structure of human IL-8 expressed in *E. coli*, PDB 3IL8, was used for molecular replacement with X-ray data from *pichia pastoris* rIL-8 crystals. SHELXL was used to refine the molecular replacement model to 1.0 Å, PDB 5D14 and 0.95 Å, PDB 4XDX.

2.12. Molecular docking to select IL-8 binding compounds

We mapped the site of IL-8 presumed to be involved in receptor binding based on previous studies with IL-8/CXCR2 binding [17]. This site was localized at a solvent accessible pocket formed at the interface of two IL-8 subunits that form the dimer. We used molecular docking to select compounds with the potential to bind this site. To prepare the site for docking, all water molecules were removed and protonation of IL-8 was done with SYBYL (Tripos). The molecular surface of the structure was explored using sets of spheres to describe potential binding pockets. The sites selected for molecular docking were defined using the SPHGEN program (generates a grid of points that reflect the shape of the selected site) and filtered through CLUSTER. The CLUSTER program groups the selected spheres to define the points that are used by DOCK6 to match potential ligand atoms with spheres. Intermolecular AMBER energy scoring, contact scoring, and bump filtering were implemented in the DOCK program algorithm. Atomic coordinates for 139,735 small molecules in the National Cancer Institute Developmental Therapeutics Program 2007 library (NCI/DTP) of drug-like compounds were positioned in each structural pocket in 1000 different orientations and scored based on predicted polar and nonpolar interactions. The most favorable orientation and scores (contact and electrostatic) were calculated. PYMOL was used to generate graphic images.

2.13. Statistics

Statistical differences were calculated using the Student *t* test. The reported values represent the mean ± SEM. A p value ≤ 0.05 was considered to be significant.

3. Results

3.1. Leukemia cell mediated endothelial cell activation affects the proliferative status of leukemia cells in vitro

We and others have demonstrated the ability of AML cells to initiate EC activation [7,18]. To confirm the ability of AML cells to activate ECs, co-cultures of KG-1 cells with

HUVECs were prepared. The results indicated a significant increase in E-selectin, a potent biomarker of EC activation, expression on ECs in co-cultures compared to monoculture ECs (Fig. 1A and B). We previously observed that AML cells that adhere to activated ECs adopt a quiescent state [7]. In these studies, we observed a similar outcome based on BrdU uptake (Fig. 1C), however; the proliferative status of the non-adherent AML cells remained active (Fig. 1D). Having shown the effects of EC activation on adhesion and quiescence of AML cells as well as its potential role in leukemia relapse, we now focused our efforts on examining the role of EC activation on the growth of the non-adherent AML cell sub-population.

3.2. EC activation enhances AML cell growth

Previous studies have shown that soluble factors released by ECs enhance the growth and survival of leukemia cells *in vitro* [8], however; the mechanism initiating this response has not been elucidated. To determine if EC activation is responsible for initiating this synergistic intercellular response, experiments were performed wherein AML cells were grown in media supplemented with CM generated from both activated and non-activated ECs. Our results clearly demonstrated that supplementing base media (EBM) with CM from activated ECs (50/50 ratio by volume) resulted in significantly increased KG-1 cell growth when compared to KG-1 cells grown in EBM supplemented (also 50/50) with non-activated CM (Fig. 2A). KG-1 cells grown with 100% CM from non-activated ECs or with base EBM media alone also did not grow as well as cultures with activated CM (Fig. 2A). Interestingly, cells grown with non-activated CM with or without EBM grew better than EBM alone intimating that non-activated ECs can produce factors that enhance KG-1 proliferation but not as significantly as activated ECs. The data clearly demonstrate that CM from AML activated ECs contains key soluble factors capable of significantly enhancing AML cell growth and that EC activation is a necessary trigger to initiate the synergy between AML and ECs.

3.3. EC activation results in increased production of soluble factors that promote AML proliferation

Previous studies have reported elevated levels of several soluble factors in the serum of leukemic patients many of which have been linked to AML cell growth *in vitro* [8,19–23]. Interestingly, among these factors is IL-8 which is also specifically produced during the normal EC activation immune response [10]. Initial studies were performed to determine the direct effect of IL-8 on AML proliferation. Following exposure to IL-8 for 24-h, KG-1 cells were analyzed using a proliferation assay which showed that IL-8 supplementation was able to significantly enhance AML cell proliferation (Fig. 2B). Next, to directly demonstrate that AML induced EC activation results in the production of IL-8, we quantitatively measured its production by activated and non-activated ECs using ELISA. The results demonstrated a significant presence of IL-8 in CM from AML activated ECs in comparison to CM from non-activated ECs (Fig. 2C). Fresh media and CM from KG-1 cells alone had undetectable levels of IL-8 (Fig. 2C). While it has been shown that primary AML cells and various AML cell lines can produce IL-8 *in vitro* [8,24], the KG-1 cell line produces low to non-detectable quantities of this factor making them an ideal control leukemia cell in these experiments [25,26]. These findings demonstrate that AML-induced EC activation is necessary for the production of IL-8. The observation that non-activated ECs did not produce this supportive factor further highlights the importance of EC activation as an essential initial step to enable ECs to support the growth of AML [5,8,27–31].

To confirm our ELISA analysis and demonstrate that IL-8 production was targeted to activated ECs, we next measured mRNA expression of this soluble factor in purified activated and non-activated ECs using qRT-PCR. Immediately prior to analysis, activated ECs were sorted from KG-1 containing co-cultures using flow sorting based on CD105 and CD45 expression patterns (Fig. 2D). The results confirmed our findings with ELISA demonstrating increased mRNA expression of IL-8 in activated ECs in comparison to non-activated EC controls (Fig. 2E). Analysis of purified KG-1 cells from co-cultures was also performed to determine if AML cells were a source of IL-8. Our data demonstrated that KG-1 cells do not express detectable levels of IL-8 intimating that activated ECs were the main source of this factor in our system (Fig. 2E).

3.4. Small molecule inhibitor to inhibit IL-8 – receptor interactions

There is increasing interest in the design and testing of IL-8 inhibitors since IL-8 signaling has been shown to be involved in cancer cell proliferation, tumor angiogenesis and metastasis [32]. Since AML activated ECs secrete IL-8, potentially explaining enhanced AML cell growth, we hypothesized that compounds that block IL-8's ability to

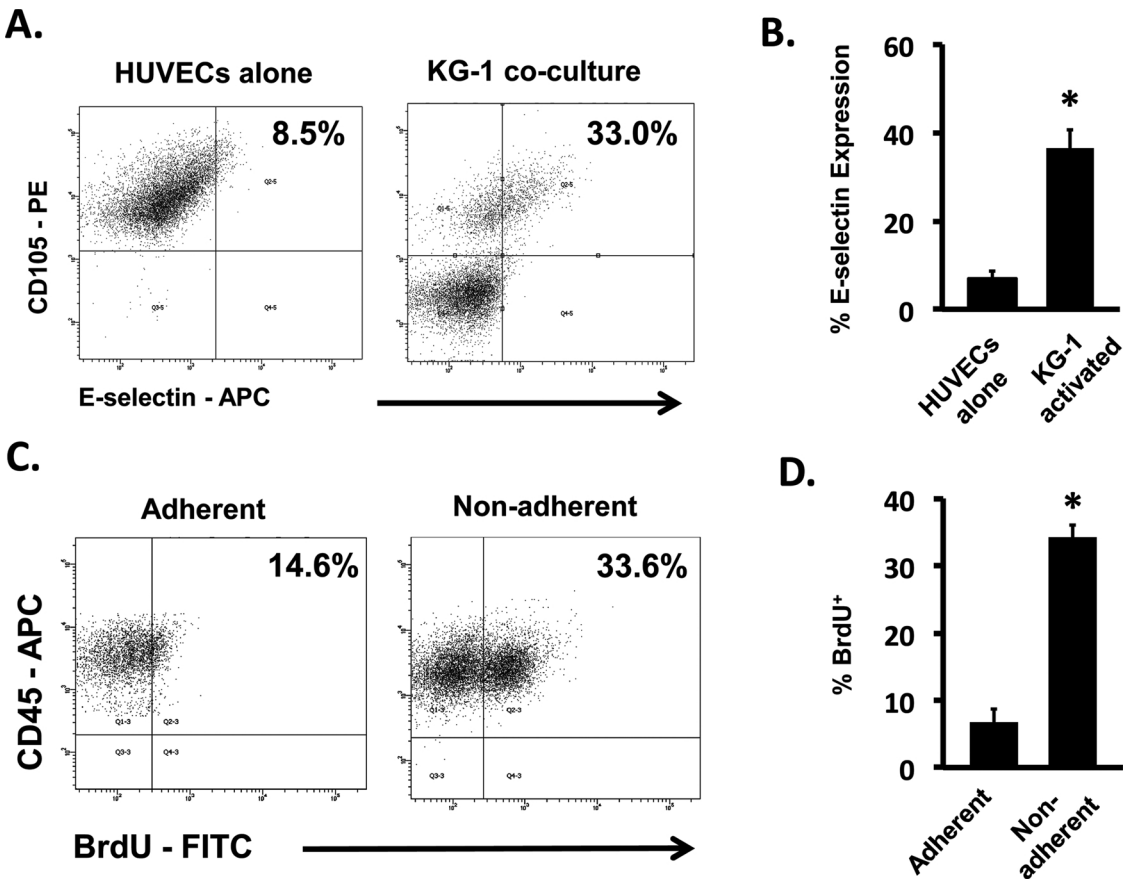

Fig. 1. Leukemia cells activate resting ECs resulting in altered leukemia cell proliferation. **(A)** Representative flow cytometry plots showing E-selectin levels on KG-1 activated HUVECs and non-activated HUVEC controls. **(B)** The levels of E-selectin expression on the surface of ECs showed significant increases when activated with KG-1 cells in co-culture. * $p < 0.05$ **(C)** Representative flow cytometry plots showing BrdU uptake by adherent and non-adherent AML cells in contact co-cultures of HUVECs and KG-1 cells. **(D)** BrdU uptake in non-adherent KG-1 cell populations was significantly higher in comparison to adherent populations indicating a proliferative phenotype. * $p < 0.05$.

interact with its receptors would abrogate these responses. The 2.0 angstrom (Å) crystal structure and NMR solution structures of IL-8 were determined and provided insight into the structural framework of IL-8 and the surfaces involved in receptor binding (Fig. 3Ai). The IL-8 monomer consists of three antiparallel β-strands connected to a long alpha helix corresponding to carboxy terminal residues 57–72 (Fig. 3Aii). IL-8 has been shown to be dimeric in solution and in the crystal structure, stabilized by residues in the first β-strand (residues 23–29) in each molecule, forming a 6 stranded β-sheet in the dimer. Although the sites on IL-8 involved in receptor binding are not completely understood, site directed mutagenesis studies implicated positions at the homodimer interface between two IL-8 subunits (Fig. 3Aiii). To determine if the structural pocket was targetable, we used the 0.95 Å crystal structure of human IL-8 as the basis for selection of candidate small molecules by molecular docking.

3.5. NCI34255 inhibits IL-8 and reduces the growth of AML cells

Using the site identified as the receptor binding site on IL-8, we screened 139,735 small molecule compounds from the NIH NCI/DTP. Following docking studies to measure affinity between the compounds and the receptor binding site, these compounds were ranked based on overall energy scores. Proliferation studies using the top 19 compounds (Supplemental Table 1) revealed that 5 of the 19 compounds tested could significantly reduce KG-1 proliferation (Fig. 3B). Of these the compound with the highest inhibitory activity was 1,5-dihydroxy-1,5-pentanedisulfonic acid (NCI34255). NCI34255 interfered with IL-8-receptor interaction by binding at the homodimeric interface of two IL-8 sub-units (Fig. 3C) with a highly favorable docking score of −28.9 as computed by AUTODOCK. To test the effect of the IL-8 inhibitor NCI34255 on AML cells, KG-1 cells that were grown in activated EC CM were exposed to NCI34255 at different concentrations (50 and 100 μM) and proliferation assays were performed. The results demonstrated that NCI34255 significantly abrogated IL-8 effects resulting in significantly decreased KG-1 proliferation at both concentrations tested (Fig. 3Di). To further confirm these effects, we also performed experiments using an additional, more clinically relevant, EC source that was derived from human bone marrow (BMECs). In similar studies with BMEC CM, we observed that NCI34255 was able to decrease KG-1 cell proliferation in the presence of BMEC CM in comparison to non-treated controls (Fig. 3Dii). Finally, experiments were then conducted to test NCI34255 using our co-culture system. Here, BMECs were cultured in the presence of KG-1 cells to allow for EC activation and subsequent IL-8 production. Co-cultures were then treated with NCI34255 and non-adherent KG-1 cells analyzed for proliferation. The results show that NCI34255 was able to significantly decrease KG-1 growth even in the presence of activated ECs that were a continual source of IL-8 (Fig. 3E). The data suggests that NCI34255 is an effective inhibitor of the IL-8 pathway and can prevent enhanced AML cell growth due to activated EC-derived IL-8.

3.6. Inhibition of IL-8 significantly increases AML response to cytarabine

We next investigated how NCI34255 affected chemotherapy treatment. Interestingly, initial studies demonstrated that co-culture of KG-1 cells with BMECs conferred chemoprotective effects on non-adherent

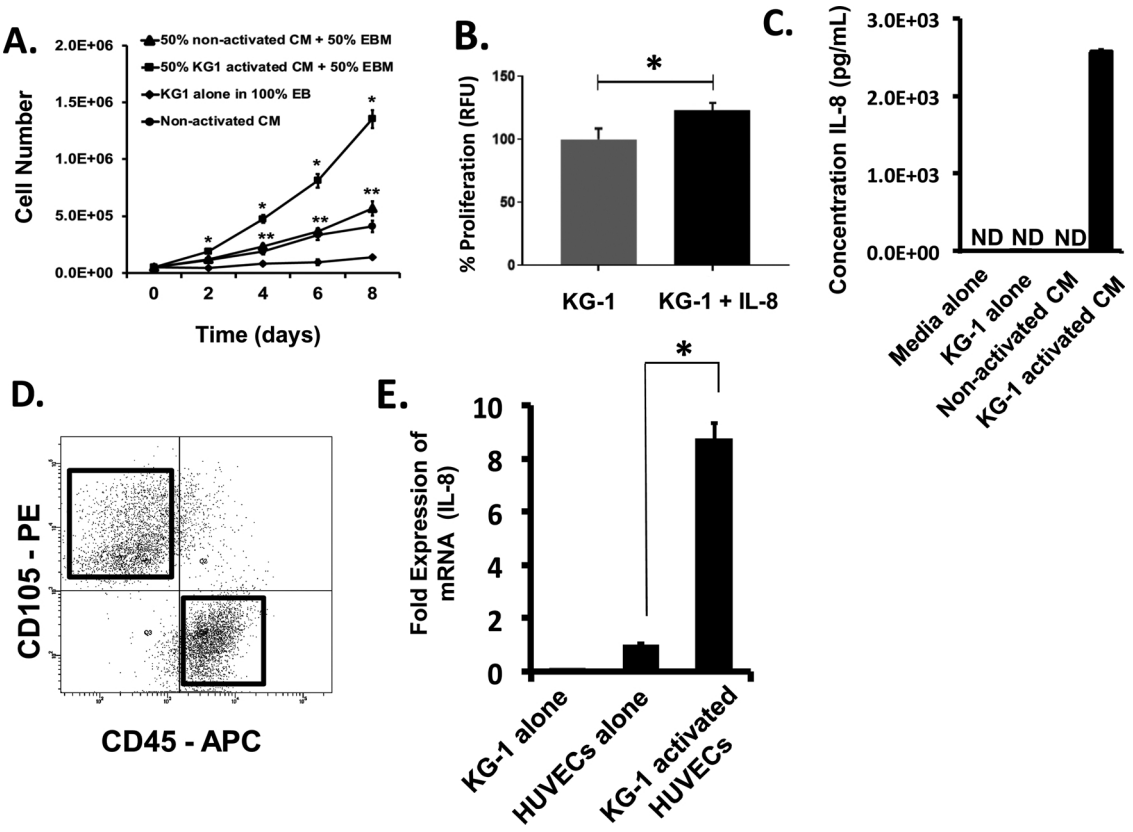

Fig. 2. Leukemia activated ECs secrete IL-8 which enhances leukemia cell expansion. **(A)** Growth curves of KG-1 cells grown in different media are shown. Supplementing base EBM media with 50% (by volume) activated CM induces significant levels of KG-1 cell growth in comparison to all other cultures tested including those supplemented with non-activated CM. Cells from each culture cohort were enumerated every 2-days over an 8-day culture period. * $p < 0.05$ *versus* all other cultures; ** $p < 0.05$ *versus* 100% EBM cultures. **(B)** KG-1 proliferation was significantly enhanced when media was supplemented with IL-8. **(C)** Fresh, unfrozen supernatants from non-activated and activated co-cultures were evaluated for the production of IL-8 by ELISA. Higher IL-8 concentrations were observed in activated CM. As controls, supernatants from KG-1 alone cultures and pure EGM-2 media were analyzed. Values were extrapolated from standard curves with linear detection limits of 10–3300 pg/mL. ND indicates non-detectable levels. **(D)** Flow cytometry-based sorting was used to isolate ECs from activating co-cultures. Flow cytometry plots identify gates established for sorting. Representative plots are shown. ECs were isolated based on CD105 (PE) expression while KG-1 cells were identified using CD45 (APC). **(E)** Sorted ECs were analyzed for mRNA expression levels for IL-8 using qRT-PCR. Non-activated ECs and KG-1 cells alone were analyzed as negative controls. * $p < 0.05$ *versus* ECs alone.

AML cells (Fig. 4A). While Ara-C treatment was still able to decrease cell viability in all cohorts, the numbers of viable KG-1 cells was significantly higher in co-cultures in comparison to KG-1 alone monocultures. To determine if the protective effects were modulated by activated EC-generated IL-8, we next tested whether or not NCI34255 treatment could enhance Ara-C induced killing. Here KG-1 cells were co-cultured with ECs and treated with Ara-C alone, NCI34255 alone or in combination. The results showed that the combination of Ara-C and NCI34255 induced significantly higher cytotoxicity than Ara-C or NCI34255 alone (Fig. 4B). As expected, Ara-C alone induced significant apoptosis of KG-1 cells while NCI34255 alone did not at the concentrations tested. This data supports the conclusion that EC generated IL-8 confers chemoprotective effects and that IL-8 inhibition through the use of NCI34255 can significantly improve AML response to Ara-C.

3.7. NCI34255 effects on AML proliferation and resistance to Ara-C is through Akt signaling

To further understand how NCI34255 decreases AML proliferation and increases AML response to Ara-C, we next performed studies to analyze the effects of NCI34255 on downstream IL-8 signaling. In these experiments, NCI34255 effects on the Akt pathway were analyzed due to the known dual effects of this pathway on cell proliferation and chemoresistance and the fact that this pathway is known to be constitutively active in 50–70% of AML cases [33]. Here, co-cultures with KG-1 and BMECs were first established and then subsequently treated with NCI34255. Following treatment, non-adherent KG-1 cells were collected and analyzed by Western blotting. The results showed a significant decrease of phosphorylated Akt (S743) in treated KG-1 cells in comparison to non-treated controls indicating that IL-8 inhibition using NCI34255 prevented activation of AKT (Fig. 4C). Next, we performed analysis on specific downstream effectors of Akt involved in both cell proliferation and apoptosis. Akt signaling through mTOR (specifically mTORC1) is known to affect cell proliferation [34]. To test if decreased Akt activity could affect this pathway, we measured the levels of phosphorylated 4E-BP1, a downstream effector of mTORC1, and showed a significant decrease in phosphorylation compared to controls (Fig. 4D). This decrease may explain the low levels of KG-1 proliferation measured in NCI34255 treated cultures. To determine how NCI34255 was able to enhance response to Ara-C treatment, we analyzed its effect on the pro-apoptotic protein Bax as Bax is known to be directly inhibited by Akt [35]. Analysis of total Bax expression levels showed no change in comparison to untreated cells (data not shown), however; there was a significant increase in the amount of truncated Bax (18 kDa; p18Bax) generated after treatment (Fig. 4E). This is an interesting finding since p18Bax has been shown to sensitize cellular response to apoptosis, thereby enhancing the overall effects of chemotherapy in a variety of cancer models [36,37]. Overall, these results indicate that inhibition of IL-8 induced signaling through Akt is a possible mechanism of the dual effects NCI34255 has on decreasing AML

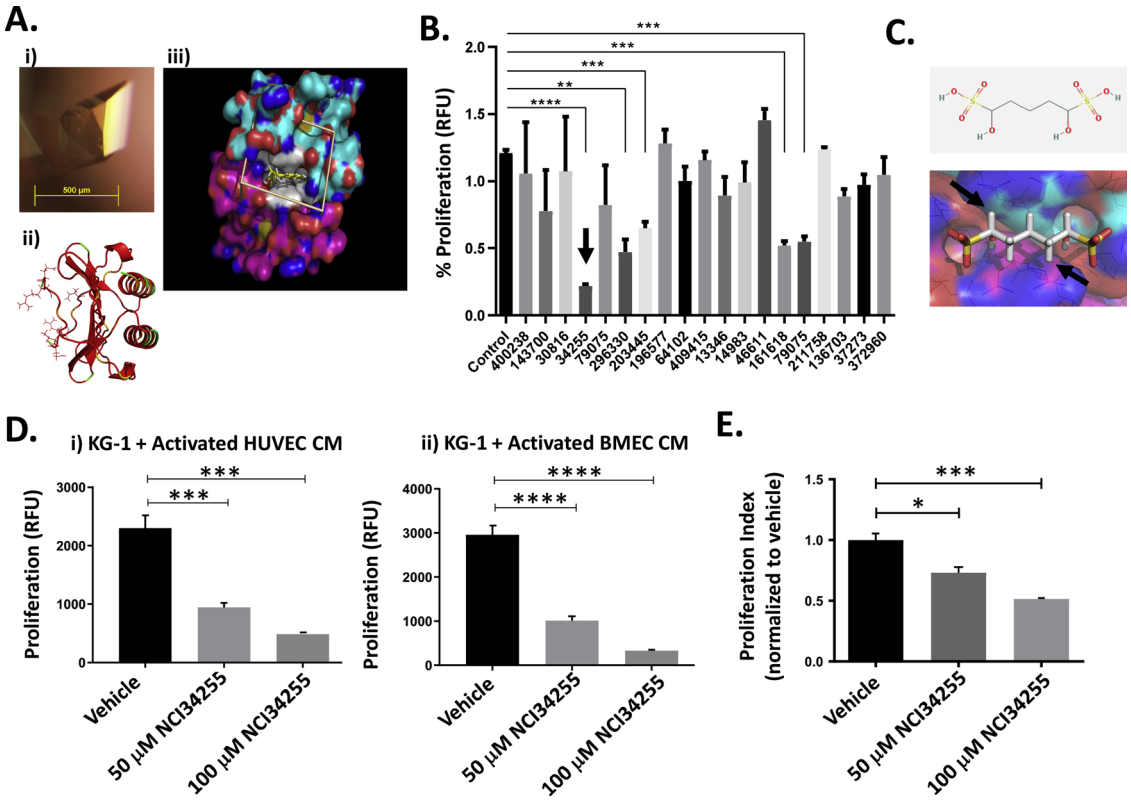

Fig. 3. Identification of small molecule inhibitor of IL-8 that can significantly reduce leukemia cell expansion. **(A)** Representative IL-8 crystal grown using the vapor diffusion hanging drop method. IL8 crystal yielding complete X-ray diffraction data set to 0.95 Å grown in 0.17 M Ammonium acetate, 0.085 M Sodium citrate tribasic dihydrate pH 5.6, 20% w/v Polyethylene glycol 4000 and 15% v/v Glycerol (i). The IL-8 dimer is shown as a ribbon diagram in red. Side chains are shown for residues implicated in receptor binding based on mutagenesis studies (ii). The molecular surface of the IL-8 dimer is shown with blue for nitrogen, red for oxygen, cyan for carbon in the (top IL-8 subunit) and magenta for carbon (bottom IL-8 subunit). Residues implicated in receptor binding are shown in white. The scoring grid for molecular docking is shown as a box. 139,735 NCI/DTP compounds were docked into the binding site *in silico* to predict the binding affinity for IL-8 (iii). **(B)** Docking studies identified 19 small molecules capable of binding IL-8's receptor binding site. Treatment of KG-1 cells with these molecules identified 5 that were able to significantly decrease KG-1 proliferation. The best hit molecule is identified with an arrow. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ **(C)** The best hit molecule was NCI34255 which interferes with binding at the homodimeric interface of two IL-8 sub-units (see arrows). **(D)** KG-1 cells were grown in CM from KG-1 activated ECs (HUVECs and BMECs) supplemented with NCI34255. The presence of NCI34255 was able to significantly decrease IL-8 induced cell proliferation contrary to the enhanced proliferation observed in previous CM studies. *** $p < 0.001$; **** $p < 0.0001$ **(E)** Supplementation of co-cultures comprising KG-1/BMECs with NCI34255 showed significant decreases in non-adherent KG-1 cell proliferation indicating the ability of NCI34255 to overcome activated EC-generated IL-8 signaling. * $p < 0.05$; *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proliferation and enhancing KG-1 response to Ara-C.

4. Discussion

In this study we demonstrate that AML cell-induced EC activation is essential to initiate the synergistic effects seen between AML cells and ECs. Once ECs were activated by AML cells, they secreted IL-8 resulting in enhanced proliferation and chemoresistance of AML cells. Without EC activation or in the presence of an IL-8 inhibitor (NCI34255) these supportive effects were abrogated highlighting the importance of IL-8 in these processes. Our studies agree with others who have shown the role of EC-secreted factors in enhancing the proliferation and survival of AML cells *in vitro* [5,8,27–31]. Data from our study extend our understanding of the AML microenvironment by showing a novel mechanism wherein AML cell-induced EC activation is the initiating step and identifying the important role of IL-8 in these supportive processes.

We observed that AML-induced EC activation leads to significantly greater production of IL-8 by ECs. IL-8 was originally observed in AML in the 1990s [38]. Critical experiments subsequently demonstrated the pro-proliferative and anti-apoptotic effects of IL-8 on AML cells [12–14]. *In vitro* studies showed that stromal cells support leukemic myeloblast growth by stimulating the production of IL-8, while enhanced angiogenesis *via* IL-8 signaling has also been shown to be important for disease development and modulating therapeutic outcomes [14,39–41]. In AML patients, transcript expression of IL-8 or one of its receptors (CXCR2) has prognostic importance [15,42]. However, AML-EC cross-talk biology was assumed to be unidirectional with AML cells on the receiving end. Not appreciated until now is that AML cells instigate ECs to produce unwanted IL-8 through contact activation. Given these results, the poor prognosis associated with IL-8 may be caused by disease geography (*i.e.* AML/vascular association) and suggest that the IL-8 pathway represents a potential therapeutic target in the treatment of AML. Targeting the IL-8/CXCR1/2 axis is being tested in other cancers. In a phase Ib study (NCT02001974), patients with HER-2 negative metastatic breast cancer who had received more than three lines of cytotoxic chemotherapy and not known to be refractory to paclitaxel were treated with the CXCR1/2 inhibitor, reparixin, plus weekly paclitaxel [43]. Thirty-three patients were enrolled and there were no dose limiting toxicities. Of evaluable patients, 8/27 (30%) achieved a confirmed RECIST response. Most of the responding patients received the highest dose tested (1200 mg TID). Two patients showed durable responses of greater than 12 months. Repiraxin is now being tested in a randomized, double-blind, placebo-controlled phase 2 study of paclitaxel in combination with reparixin compared to paclitaxel alone as front-line therapy for metastatic triple-negative breast cancer (FRIDA trial; NCT02370238).

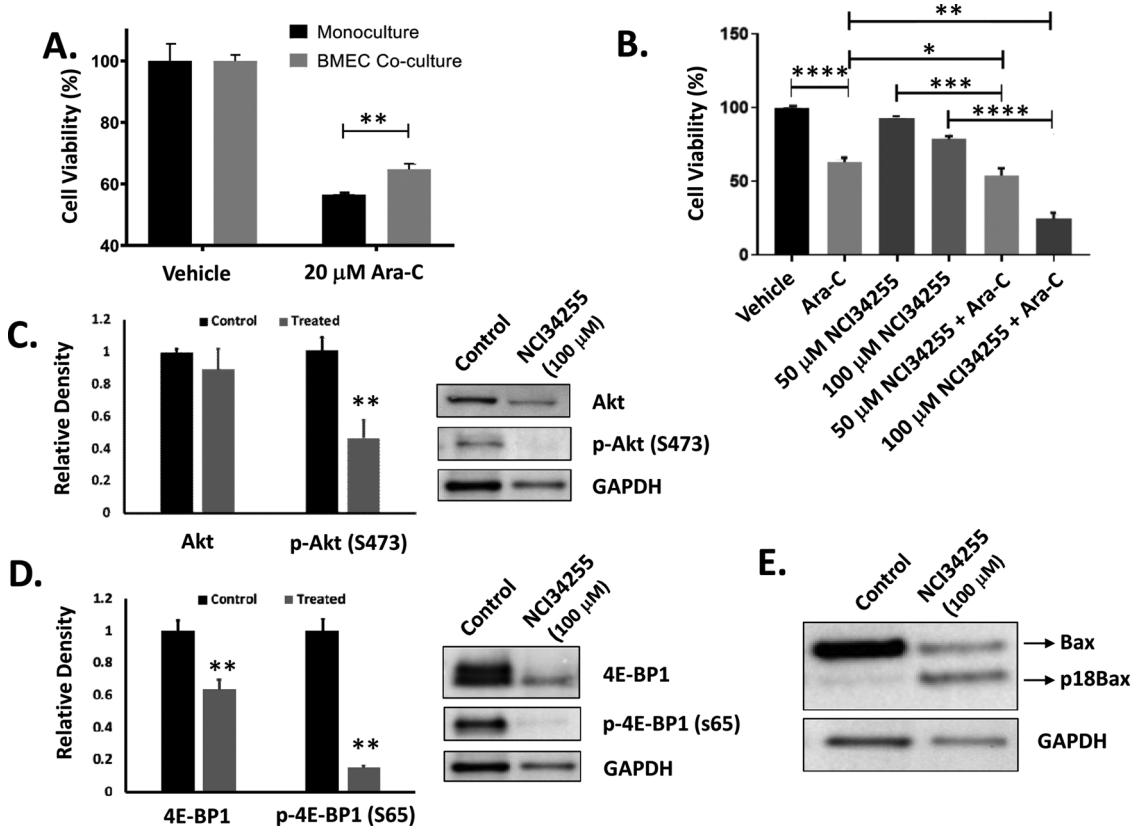

Fig. 4. IL-8 induces chemoresistance which is abrogated using NCI34255. **(A)** The response of KG-1 cells in monoculture or non-adherent KG-1 cells in co-culture to Ara-C was tested and showed that KG-1 cells are significantly less sensitive to Ara-C when cultured with ECs. ** $p < 0.01$ **(B)** Co-cultures were treated with Ara-C, NCI34255 or a combination of both. Significant decreases in cell viability was observed when Ara-C alone treated groups were compared to Ara-C + NCI34255 treated groups demonstrating that NCI34255 was able to augment Ara-C and enhance apoptotic responses. NCI34255 alone treated groups did affect cell viability in comparison to vehicle controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ **(C, D)** Analysis of Akt activity in non-adherent KG-1 cells collected from EC co-culture treated with NCI34255 showed significantly decreased levels of phosphorylated Akt in comparison to non-treated controls (C). A similar analysis also showed a significant decrease in phosphorylated 4E-BP1 a downstream target of mTORC1 in the same cell population (D). ** $p < 0.01$ **(E)** Bax expression in KG-1 cells isolated from co-cultures exposed to NCI34255 treatment was assessed. The results showed that Bax is cleaved in response to NCI34255 forming the apoptosis enhancing truncated form, p18Bax.

To overcome the seemingly critical role of IL-8 in AML, we experimented with a strategy aimed at disrupting its receptor binding. Whereas past research focused on knocking-down or blocking CXCR2 activation in AML [15], we were cognizant of the fact that IL-8 is known to agonize both CXCR2 and CXCR1 receptors, both of which have been implicated in the progression of various cancers [44]. Thus, given IL-8's receptor promiscuity, we sought to directly neutralize IL-8. However, our initial attempts to inhibit soluble IL-8 directly with antibodies did not prove successful (data not shown), likely due to the immense secretion of IL-8 by activated ECs. To overcome these obstacles, we carefully examined the IL-8 molecule with high-resolution crystallography and found an invagination within the protein representing a potential site wherein IL-8 physically interlocks with its receptors. Using high throughput computational methods and docking studies, we identified a series of small molecules (19 in total) that best fit inside within the IL-8 binding pocket. We then used *in vitro* assays to rank-order the compounds based on their ability to reduce AML cell proliferation. Interestingly, the best hit compound, NCI34255, displayed dual effects where treatment not only decreased AML proliferation but also enhanced AML cytotoxicity in the presence of Ara-C.

To mechanistically understand these findings, we investigated the Akt pathway, which is activated by IL-8 and has known effects on AML [33]. We initially found that AML cells treated with NCI34255 displayed a significant decrease in phosphorylated Akt at serine 473 (fully activated Akt [45]) and a concomitant decrease in phosphorylated 4E-BP1. In this state, 4E-BP1 is able to bind eIF-4E and prevent cell proliferation through inhibition of numerous proteins such as c-myc, MMP9, Bcl-2, and Mcl-1 [34,46,47]. These observations provide a mechanistic explanation for the decrease in AML cell proliferation upon treatment with NCI34255.

Akt has also been shown to directly prevent apoptosis by inhibiting pro-apoptotic molecules such as Bax, while enabling the function of anti-apoptotic proteins [48,49]. Interestingly, analysis of Bax protein expression demonstrated that NCI34255 treatment produced a cleaved version of Bax. Several studies have shown that Bax can undergo cleavage *via* calpains to form an 18 kDa truncated version of Bax (p18Bax) [36,50]. p18Bax has been shown to be more potent than full-length Bax (21 kDa) in the induction of apoptosis in part through increased efflux of cytochrome c [36]. Through such mechanisms, p18Bax enhances sensitivity of cancer cells to chemotherapy by essentially preparing the cells to enter apoptosis, a phenomenon referred to as mitochondrial priming [51,52]. The observed increase in p18Bax levels in NCI34255 treated AML cells intimates that IL-8 inhibition 'primes' AML cells for apoptosis thus explaining why the combination of NCI34255 and Ara-C resulted in higher cell death in comparison to Ara-C alone. Surprisingly, we also observed that the level of total Bax was not significantly affected by NCI34255 treatment, even with IL-8 blockade and decreased Akt activity. This could be due to the milieu of other soluble factors produced in the activated EC co-cultures that may affect Bax. For example, activated ECs are known to produce high levels of the pro-inflammatory factor IL-6 [10]. Since IL-6 has been shown to affect Bax expression in various cell types through JAK/STAT [53,54],

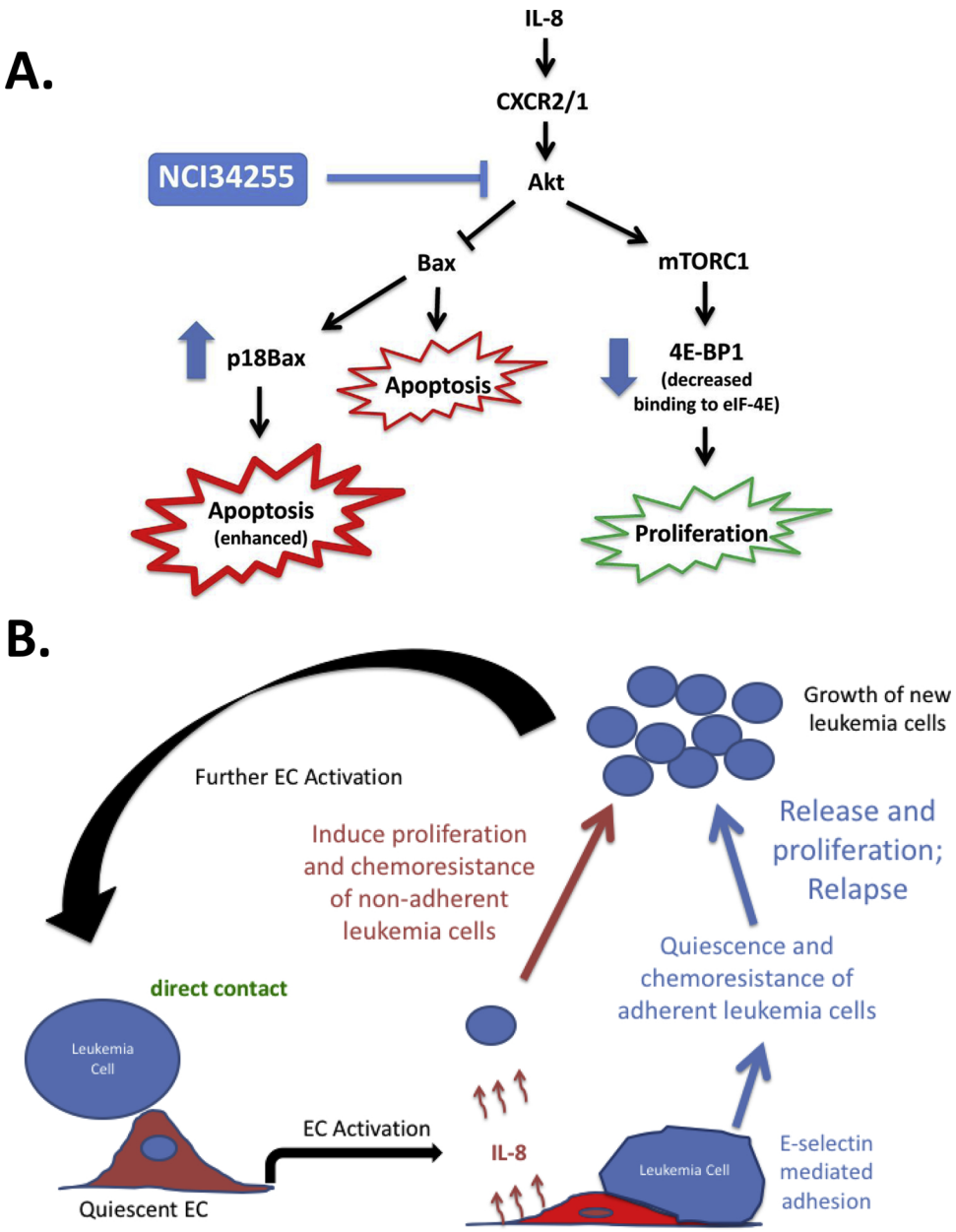

Fig. 5. (A) Schematic summarizing observed effects of NCI34255 on Akt induced proliferation and survival of AML cells. Treatment with NCI34255 reduces the effects of EC activation through Akt signaling (blue lines) resulting in decreased proliferation and enhanced apoptosis (in the presence of Ara-C) of AML cells. **(B)** The impact of EC activation on adherent and non-adherent AML cell populations is shown. Adherent cells become quiescent and chemoresistant implicating them in relapse. Non-adherent cells show significant expansion and chemoresistance following EC activation identifying them as the cellular source for enhanced leukemia cellularity. Therapies aimed at targeting this process may provide new avenues for the optimal treatment of patients with AML. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

we speculate that signaling through alternate pathways may have prevented observable changes in total Bax expression in response to NCI34255 treatment. Interestingly, maintenance of Bax expression may have allowed for the observed formation of truncated p18Bax. These results support the conclusion that inhibition of Akt pathway constituents using NCI34255 overcomes the AML promotive effects of IL-8 resulting in decreased cellular proliferation and enhancing sensitivity to chemotherapy (Fig. 5A).

There is evidence that IL-8 blockade may also be useful for the treatment of specific AML subpopulations. Recently, the FDA has approved new drugs that target AML gene mutations IDH1 and IDH2. Mutations in IDH1/2 produce the oncometabolite R-2-hydroxyglutarate (R-2HG), which enhances NF-kB-dependent expression of IL-8 [55]. Thus, it is possible that IDH1/2 mutant AML may be more responsive to anti-IL-8 therapeutic strategies, including the strategy we demonstrate in this study. Interestingly, among AML patients, those with FLT3-ITD mutations have the highest IL-8 mRNA expression [42]. High IL-8 gene expression is also a prognostic marker of inferior survival outcomes in AML patients with FLT3-ITD. Thus, it is also conceivable that an anti-IL-8 treatment strategy, may be well suited for the treatment of the AML FLT3-mutant subpopulation.

In concert with our previous studies, we can now postulate a mechanism wherein EC activation governs the proliferation, survival and relapse of AML (Fig. 5B). Here, AML cells initiate EC activation. Activated-ECs then induce the adhesion of a population of leukemia cells, which become quiescent and chemoresistant identifying these cells as potential mediators of relapse. The present results add a second scenario wherein activated ECs also produce soluble factors (IL-8) that enhance the proliferation and chemoresistance of non-adherent leukemia cells resulting in increased AML cellularity. Therefore, EC activation acts as a double-edged sword by generating microenvironments that support chemoresistance and relapse while concomitantly enhancing proliferation and growth. Now that we have identified IL-8 as a reactive cytokine from activated ECs and a cytokine that can be pharmacologically targeted, our next effort will be to scale the system to primary AML cells that secrete IL-8. A follow-up question will be whether the additional mass of IL-8 produced by both activated ECs and AML cells responds in a dose-dependent manner.

Overall, these findings identify EC activation-based processes as potential targets for the development of next generation therapies. In this context, the present results show that activated EC-generated IL-8 is an essential component in AML and directly blocking IL-8 may be a new therapeutic strategy to enhance AML patient outcomes.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2019.106180.

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