

## **MIF-induced stromal PKC $\beta$ /IL8 is essential in human acute myeloid leukemia**

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### **Running Title**

MIF regulates AML microenvironment

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### **Conflict of interest statement**

The authors have declared that no conflict of interest exists

## **Abstract**

Acute myeloid leukemia (AML) cells exhibit a high level of spontaneous apoptosis when cultured *in vitro* but have a prolonged survival time *in vivo*, indicating that tissue microenvironment plays a critical role in promoting AML cell survival. *In vitro* studies have shown that bone marrow-mesenchymal stromal cells (BM-MSK) protect AML blasts from spontaneous and chemotherapy-induced apoptosis. Here we report a novel interaction between AML blasts and BM-MSK which benefits AML proliferation and survival. We initially examined the cytokine profile in cultured human AML compared to AML cultured with BM-MSK and found that macrophage-migration inhibitory factor (MIF) was highly expressed by primary AML, and that interleukin-8 (IL-8) was increased in AML/BM-MSK co-cultures. Recombinant MIF increased IL-8 expression in BM-MSK via its receptor CD74. Moreover, the MIF inhibitor ISO-1 inhibited AML-induced IL-8 expression by BM-MSK as well as BM-MSK-induced AML survival. Protein kinase C  $\beta$  (PKC $\beta$ ) regulated MIF-induced IL-8 in BM-MSK. Finally, targeted IL-8 shRNA inhibited BM-MSK-induced AML survival. These results describe a novel, bidirectional, pro-survival mechanism between AML blasts and BM-MSK. Furthermore, they provide biologic rationale for therapeutic strategies in AML targeting the microenvironment, specifically MIF and IL-8.

## **Introduction**

Survival of patients with acute myeloid leukemia (AML) is presently poor; two-thirds of young adults and 90% of older adults die of their disease (1). Even in patients who achieve remission with chemotherapy relapse is common and occurs from minimal residual disease sequestered in protective niches in the bone marrow microenvironment (2). Accordingly it is envisaged that improved outcomes will come from novel treatment strategies derived from an improved understanding of the biology of AML within the bone marrow microenvironment.

AML cells exhibit a high level of spontaneous apoptosis when cultured in vitro but have a prolonged survival time in vivo, indicating that the tissue microenvironment plays a critical role in promoting AML cell survival (3-6). Knowledge of the complexity of the bone marrow microenvironment is increasing especially with respect to the bone marrow mesenchymal stromal cells [BM-MSK] which are considered a major protective cell type (7). BM-MSK generate various factors whose primary functions are to influence tumor cell survival and homing (4, 8, 9). The apoptotic defect in AML is not cell-autonomous but highly dependent on extrinsic signals derived from their microenvironment. The complex cell-cell interactions between the AML tumor cells and their microenvironment are therefore essential for tumor growth and survival and thus present an attractive target for novel drug therapies.

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine which under normal conditions regulates cell-mediated immunity and inflammation (10). In cancer, MIF is overexpressed in a number of solid tumors including breast, prostate and colon cancers (11-13). MIF has also been shown to be overexpressed in various blood cancers including chronic lymphocytic leukemia (CLL) (14). In CLL, MIF is expressed by the malignant cells and induces protective IL-8 release in an autocrine-dependent manner. Blocking either MIF or IL-8 reduces survival of CLL. The increased secretion of IL-8 from tumor cells is thought to have wider significance to the tumor microenvironment. Serum IL-8 is known to be higher in patients with AML, myelodysplasia (MDS) and non-Hodgkin Lymphoma than in normal

controls and levels of IL-8 in these patients are similar to those found in patients with multiple organ failure of non-septic origin (15, 16). Furthermore leukemic blasts from the majority of patients with AML constitutively express IL-8 (17). In addition, inhibition of the IL-8 receptor, CXCR2 selectively inhibits proliferation of MDS/AML cell lines and patient samples (18). Together these studies suggest that MIF and IL-8 are functionally important in regulating the survival and proliferation of multiple tumors including AML. In the present study, we investigate how AML cells program BM-MSC via MIF to produce the survival cytokine IL-8 and characterize the signaling pathways underlying this inter-dependent cell-cell communication.

## **Materials and Methods**

### Materials

Anti-PKC, MAPK and AKT antibodies were purchased from Cell Signaling Technologies. Anti-CD74, anti-CXCR2, and anti-CXCR4 antibody were purchased from Miltenyi Biotec. All inhibitors were purchased from Tocris. The CD74 blocking antibody was purchased from BD Biosciences. Proteome Profiler Human XL array and recombinant human MIF were purchased from R&D Systems. MIF ELISA was purchased from BioLegend. IL-8 ELISA was purchased from eBiosciences. All other reagents were obtained from Sigma-Aldrich

### Cell culture

For primary cell isolation, heparinized blood was collected from volunteers and human peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque (Sigma-Aldrich) density gradient centrifugation. AML samples that comprised less than 80% blasts were purified using the CD34 positive selection kit. Cell type was confirmed by microscopy and flow cytometry. BM-MSC were isolated by bone marrow aspirates from AML patients. Mononuclear cells were collected by gradient centrifugation and plated in growth medium containing DMEM and 20% FBS and 1% L-glutamine. The non-adherent cells were removed after 2 days. When 60%-80% confluent, adherent cells were trypsinised and expanded for 3-5 weeks. BM-MSC were checked for positive expression of CD105, CD73, and CD90 (BM-MSC markers) and the lack of expression of CD45 and CD34 by flow cytometry. All patient information including genotype and WHO classification for AML and genotype and phenotype of AML BM-MSC are included in Supplementary Table 1 and Supplementary Table 2.

### RNA extraction and real-time PCR

Total RNA was extracted from  $5 \times 10^5$  cells using the Nucleic acid Prep Station from Applied Biosystems (Paisley, UK), according to the manufacturer's instructions. Reverse transcription was performed using the RNA polymerase chain reaction (PCR) core kit

(Applied Biosystems). Relative quantitative real-time PCR used SYBR green technology (Roche) on cDNA generated from the reverse transcription of purified RNA. After pre-amplification (95°C for 2 minutes), the PCRs were amplified for 45 cycles (95°C for 15 seconds and 60°C for 10 seconds and 72°C for 10 seconds) on a 384-well LightCycler 480 (Roche). Each mRNA expression was normalized against GAPDH mRNA expression using the standard curve method.

#### Western immunoblotting and ELISAs.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot analyses were performed. Briefly, whole cell lysates were extracted and sodium dodecyl sulphate-polyacrylamide gel electrophoresis separation performed. Protein was transferred to nitrocellulose membrane and Western blot analysis performed with the indicated antisera according to their manufacturer's guidelines. To examine MIF and IL-8 secretion into media we used LEGEND MAX™ Human Active MIF ELISA Kit (BioLegend) and Human IL-8 ELISA Ready-SET-Go (eBiosciences).

#### shRNA silencing of CD74, PKC $\beta$ and IL-8

Five Mission shRNA targeted lentivirus particles (Sigma-Aldrich) for each target were obtained.  $2 \times 10^4$  cells BM-MSC were infected with each lentivirus. For all gene expression experiments the cells were incubated for 72 hours post transfection before RNA extraction.

#### Flow cytometry

Flow cytometry for measuring AML cell number was performed on the Cube 6 (Sysmex-Partec). For the AML/BM-MSC co-cultures AML cell viability was measured using flow cytometry. After exclusion of BMSC by electronic gating using forward scatter, AML cells were counted using CD34 gating.

#### Cytokine array expression analysis

Primary AML blasts  $0.25 \times 10^6$  were cultured alone or co-cultured on confluent primary BM- MSC. Conditioned medium was then collected from these cultures as well as from BM- MSC culture and analysed using the Proteome Profiler Human XL Cytokine Array following the manufacturer's instructions. Quantification of cytokine optical densities were obtained with the HImage++ software (WesternVision).

#### Statistical analyses

The Mann-Whitney U test was used to compare test groups where stated. Results where  $P < 0.05$  were considered statistically significant. Results represent the mean  $\pm$  standard deviation of 4 or more independent experiments. We generated statistics with Graphpad Prism5 software (Graphpad, San Diego, CA, USA). For Western blotting, data are representative images of 3 independent experiments.

#### Study approval

AML cells and BM- MSC were obtained from AML patient bone marrow or blood following informed consent and under approval from the UK National Research Ethics Service (LRECre07/H0310/146).

## **Results**

### **BM-MSC support AML survival.**

The microenvironment supports AML survival and proliferation (4, 5, 19). To study the cell-cell communication between BM-MSC and AML cells, we established a co-culture system using primary AML cells and BM-MSC derived from treatment-naïve AML patients. Here we show a significant difference in primary AML survival when cultured on BM-MSC for 6 days and 14 days compared to AML blast survival when cultured in basal media alone (Figure 1A and supplementary figure 1). Supplementary figure 2 shows the different combinations of AML and BM-MSC in all experiments.

To determine what factors are responsible for improved primary AML blast survival on BM-MSC we analyzed the profile of cytokines and chemokines present in primary AML cultures, BM-MSC cultures and primary AML blasts cultured in combination with BM-MSC. Cytokine array profiles of the three culture conditions (Figure 1B) show a consistent up-regulation of interleukin-8 (IL-8) in the co-culture sample media (Figure 1C). Moreover we also observed high levels of MIF in all AML supernatants, low levels of MIF in all BM-MSC supernatants and high levels of MIF in the AML/BM-MSC co-cultures (Figure 1B and Figure 1D). To verify these observations we carried out IL-8 and MIF specific ELISAs. IL-8 concentrations peak at 8 and 24 hours in the AML/ BM-MSC co-culture supernatants (Figure 1E), whereas MIF concentrations were high and at similar levels in AML culture supernatants and AML/ BM-MSC co-culture supernatants (Figure 1F).

### **AML derived MIF induces IL-8 expression in BM-MSC.**

Next we looked to determine whether BM-MSC needed direct contact with AML to increase IL-8 expression. RT-PCR showed that IL-8 mRNA from BM-MSC incubated with AML increased by 57 fold when in direct contact (DC) and by 50 fold when in indirect contact (IC) with AML blasts (Figure 2A). This confirms that direct tumor cell to stromal cell contact is not necessary for AML to induce increased IL-8 expression by BM-MSC.



Next we examined the mRNA expression levels of MIF in primary AML (n=5) and BM-MSC (n=5) cultures. RT-PCR showed that primary AML cultures but not BM-MSC cultures express high levels of MIF mRNA under normal basal conditions (Figure 2B). Since MIF expression has been shown to be increased in AML patients compared to normal patients (20) and the ability of MIF to induce IL-8 production by primary CLL (14), we hypothesized that MIF from AML was responsible for the increased IL-8 expression in BM-MSC. To test this hypothesis we stimulated BM-MSC with 100ng/mL recombinant human MIF and assayed for IL-8 mRNA and protein expression over a period of 24 hours. We show that IL-8 mRNA and protein increased (Figure 2C and 2D) in response to MIF. To confirm that MIF secreted from AML cells regulates IL-8 expression we used ISO-1, a nontoxic inhibitor of MIF which functions by binding to bioactive MIF at its N-terminal tautomerase site (21). MIF stimulated BM-MSC pretreated with ISO-1 showed a decrease in IL-8 mRNA levels compared to untreated BM-MSC (Figure 2E). Moreover, AML survival was inhibited when cultured with BM-MSC in the presence of ISO-1 compared to control AML-BM-MSC cultures (Figure 2F). Together these data confirm that MIF secreted by AML cells induces IL-8 expression in BM-MSC.

#### **MIF induced IL-8 upregulation is mediated through CD74.**

Depending on the cellular context and the disease involved, MIF signaling is mediated by its receptors CXCR2 (Interleukin 8 receptor, ILR8) and/or CXCR4 (Stromal-derived factor 1 receptor), and/or CD74 (22, 23). BM-MSC have been reported to express all three receptors (24-26). Using CD105 as a BM-MSC marker to confirm mesenchymal cell phenotype, we show that CD74 and CXCR4 are expressed but CXCR2 is not expressed on all primary BM-MSC isolated from AML patients (Figure 3A). BM-MSC were further characterized using CD73 and CD90, and lack of CD45 expression.

We used specific inhibitors of CXCR2, CXCR4 and CD74 to determine which receptor/s were responsible for MIF induced IL-8 up-regulation. Inhibition of CXCR2 and CXCR4 using pertussis toxin (a GPCR inhibitor) had no effect on MIF induced IL-8 mRNA expression (Figure 3B). However, the anti-CD74 blocking antibody inhibited MIF induced IL-8 expression in BM-MSC (Figure 3C). These results suggest that CD74 is the dominant receptor in regulating MIF induced IL-8 expression in AML patient derived BM-MSC. To further characterize this interaction we used lentiviral viral mediated knockdown (KD) of CD74 in AML patient derived BM-MSC, confirming reduced mRNA and protein expression of CD74 after transduction with control KD or CD74 KD lentivirus (Figure 3D). Furthermore we demonstrate that CD74 knockdown inhibits MIF induced IL-8 mRNA expression in AML patient derived BM-MSC (Figure 3E).

#### **Pharmacological inhibition of PKC $\beta$ inhibits MIF-induced IL-8 induction in BM-MSC**

We next investigated the signaling cascade in AML patient derived BM-MSC downstream of MIF induced CD74 activation. It has been shown that MIF binding to CD74 activates downstream signaling through the phosphatidylinositol 3-kinase/protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) signaling pathways and promotes cell proliferation and survival (27). In addition, Lutzny et al. recently described the activation of a PKC pathway in murine stromal cells co-cultured with chronic lymphocytic leukemia (B-CLL) (28). We treated AML stimulated BM-MSC with LY294002 (a PI3K/Akt inhibitor), PD098059 (a MAPK kinase (MEK) 1 inhibitor) or Ro-31-8220 (a PKC pan inhibitor) to determine which pathway/s regulate AML induced BM-MSC IL-8 mRNA induction. We show that Ro-31-8220, the PKC inhibitor, was able to significantly inhibit IL-8 expression by approximately 80%, while LY294002 and PD098059 had little or no effect (Figure 4A). Similarly, we found that Ro-31-8220 was able to inhibit IL-8 expression by circa 90% in experiments where BM-MSC were directly activated using rhMIF (rather than AML cells) (Figure 4B). However in addition we observed that PD98059, was able to moderately inhibit rhMIF induced IL-8 mRNA induction in BM-MSC by approximately 30% (Figure 4B).

To clarify whether PKC, MAPK or both are activated in response to MIF, we performed Western blot analysis on BM-MSK for specific phosphorylation of PKC isoforms, MAPK or AKT in response to MIF activation. BM-MSK were activated by AML for 15 minutes or MIF treatment (100ng/ml) for various times. We initially show that MIF and AML both induce phosphorylation of PKC  $\alpha/\beta$  and PKC  $\beta$  in BM-MSK (Figure 4C). BM-MSK from four patient samples treated with MIF had no increase in phosphorylation of AKT and MAPK (Figure 4D). Next, the PKC isoform specific inhibitors Go6976 (PKC $\alpha/\beta$ ) and enzastaurin (PKC $\beta$ ), were used to block MIF induced IL-8 expression in BM-MSK. Both inhibitors showed inhibition of MIF induced IL-8 up-regulation (Figure 4E). Finally we used lentiviral viral mediated knockdown (KD) for PKC $\beta$ , confirming reduced mRNA expression of PKC $\beta$  after transduction of BM-MSK with control KD or PKC $\beta$  KD virus (Figure 4F). We then demonstrate that knockdown of PKC $\beta$  inhibits MIF induced IL-8 mRNA expression (Figure 4G). Together these results confirm that MIF induced IL-8 expression in AML patient derived BM-MSK requires PKC $\beta$ .

### **Targeting the MIF-PKC $\beta$ -IL-8 axis disrupts BM-MSK induced protection of primary human AML blasts**

Finally to examine the effect of blocking IL-8 on BM-MSK protection and survival of primary AML blasts we co-cultured primary AML blasts derived from treatment-naïve AML patients with BM-MSK (either control KD or IL-8 KD). Firstly we used lentiviral viral mediated knockdown (KD) for IL-8. Figure 5A and 5B shows the mRNA expression and protein expression of IL-8 after transduction of BM-MSK with control KD or IL-8 KD virus. Figure 5C demonstrates that knockdown of IL-8 inhibits MIF induced IL-8 mRNA expression. Next we show that KD of IL-8 in BM-MSK significantly inhibits AML survival when in co-culture compared to control KD BM-MSK (Figure 5D). Finally, blocking the IL-8R using SB225002 inhibited AML survival when cultured with BM-MSK (Figure 5E). Taken together, these results identify a novel pro-tumoral regulatory pathway in the AML microenvironment.

## **Discussion**

AML is primarily a disease of the elderly with a median age at diagnosis in the Swedish Acute Leukemia Registry of 72 years (29). Outcomes for the 75% of patients who get AML over the age of 60 remain generally poor, largely because the intensity and side effects of existing curative therapeutic strategies (which are commonly used to treat younger fitter patients) coupled with patient co-morbidities, frequently limit their use in this older less fit population (30). Accordingly, there is an urgent need to identify pharmacologic strategies to tackle AML, which are not only effective but can also be tolerated by both older and less well patients. It is envisaged that treatments which target the tumor microenvironment may well help realize this goal.

Here we report a novel survival pathway within the human AML microenvironment which functions as a feedback/autocrine loop involving the constitutive expression of the chemokine MIF by the AML blasts which in turn induces IL-8 expression in BM-MSC. Interestingly, another group showed that the repertoire of constitutive in vitro chemokine release from AML shows variation between different AML patient samples (31). We find that although baseline expression of MIF by AML varies between patient samples tested, all samples analyzed expressed MIF. Moreover in co-culture experiments AML patient derived BM-MSC's were found to be ubiquitously responsive to AML derived MIF which resulted in an increase in IL-8 expression by the BM-MSCs. This is in keeping with similar reports on other cytokine pathways which have shown that BM-MSC can constitutively express various chemokines (32), and AML cells are able to respond to these chemokines (32, 33). In this study we also examined the genotype of six BM-MSC used for the experiments and found three of six to be normal and the other three the genotyping failed (supplementary table 2). This is apparently in contrast to Huang et al who found that three out of four BM-MSC from AML patients tested had cytogenetic abnormalities within the stromal cells (34). Presently therefore the incidence and functional consequences of cytogenetic mutations within stromal

cells remains undefined. Nevertheless taken together, despite the established heterogeneity in AML we find the MIF/IL8 autocrine loop a constant finding across the samples we tested which makes this an attractive drugable target.

IL-8 is a pro-inflammatory chemokine whose primary function is to activate two cell surface G protein coupled receptors, CXCR1 and CXCR2 which promote neutrophil migration and degranulation (35-37). Elevated IL-8 secretion in tumor biology is well characterized with a number of studies showing the importance of this chemokine in AML. In 1993 Tobler et al described the constitutive expression of IL-8 and its receptor in human myeloid leukemia and more recently Schinke et al have reported that inhibition of the IL-8 receptor CXCR2 selectively inhibits immature hematopoietic stem cells from MDS/AML samples (17, 18). In other malignancies IL-8 has been characterized in endothelial cells and tumor associated macrophages, suggesting that IL-8 has a function in the liver and prostate tumor microenvironments (38, 39). As rodents lack a direct homologue of IL-8 we purified BM-MSCs extracted from patient bone marrow aspirates at the time of diagnosis of AML. Our study describes for the first time how AML stimulates the production of IL-8 from BM-MSCs and inhibiting this process prevents AML survival.

Extensive studies of MIF function have revealed its central role in innate and adaptive immunity (10). More recently, the ability of this cytokine to support tumor progression has been highlighted and revealing MIF as a potential target for anti-cancer therapies in melanoma and colon cancer (40). MIF occurs in immunologically distinct conformational isoforms, reduced MIF and oxidized MIF (oxMIF) with the latter predominantly expressed in patients with inflammatory diseases (41), and is highly expressed by various cancer cell lines (42). This has led to the evaluation of an oxMIF blocking antibody (imalumab) in early phase clinical studies of selected solid tumors (<https://clinicaltrials.gov/ct2/show/NCT01765790>). Our findings provide a biological rationale for the clinical assessment of imalumab or other MIF inhibitors in AML patients.

Activation of PKC signaling pathway has been characterized in cancer cells. In hematologic malignancies different PKC isoforms have been identified as key players in the leukemia microenvironment. In multiple myeloma, pharmacological inhibition of activated PKC $\beta$ II using enzastaurin inhibited growth factors and cytokines secreted by MM derived bone marrow stromal cells(28). In CLL, PKC $\beta$  is immediately downstream of the B cell receptor and has been shown to be important to CLL cell autocrine survival and proliferation in-vivo (43). PKC $\beta$  is also essential for the development of CLL in the TCL1 transgenic mouse model, making it a valid therapeutic target in this malignancy (44) Furthermore induction of PKC $\beta$ II in stromal cells is required for the survival of leukemic B cells and stromal PKC $\beta$ II is upregulated in samples from CLL, ALL and MCL patients (28). Our results demonstrate that in primary samples from AML patients at diagnosis PKC $\beta$  is phosphorylated in the BM-MSc in response to MIF stimulation. This leads us to hypothesize that this pathway may commonly be activated in other hematological malignancies and moreover the cancer cell is inducing this activation. In summary this study links secretion of MIF from primary AML to a specific BM-MSc pathway utilizing PKC $\beta$  to feedback survival signals including IL-8 secretion to AML. In doing so we have identified in-vitro the potential efficacy of targeting any one of these molecules to disrupt AML/BM-MSc pro-survival interactions.

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## Legends

**Figure 1. BM-MSC support AML survival.** (A). AML blasts ( $0.25 \times 10^6$ ) were co-cultured with primary BM-MSC on a 12 well plate for 6 days ( $n=20$ ), AML blast number was assessed using a trypan blue exclusion hemocytometer-based counts and CD34+ staining using flow cytometry. (B) Cell free supernatants from 7 individual AML patients blasts ( $0.25 \times 10^6$ ) were co-cultured with primary BM-MSC for 24 h and a representative cytokine antibody arrays of each of the cell culture conditioned media using the Human cytokine proteome profiler array. (C) Fold induction of cytokines between BM-MSC and AML cultured on BM-MSC. Results from 7 different primary AML on 4 different BM-MSC. Bars show mean and SEM. Significantly up-regulated cytokines are included in the graph. (D) Quantification of cytokine optical density of AML only arrays (7 individual AML samples). Graph shows the top 8 cytokines expressed by AML. Bars show mean and SEM. (E) IL-8 ELISA of each of the cell culture conditioned media from various time points (3 individual AML samples). (F). MIF ELISA of each of the cell culture conditioned media from various time points (3 individual AML samples). The Mann-Whitney U test was used to compare between treatment groups (\* =  $p < 0.05$ ).

**Figure 2. AML derived MIF induces IL-8 expression in BM-MSC.** (A). AML blasts from 5 patients ( $0.25 \times 10^6$ ) were co-cultured with primary BM-MSC either in direct contact (DC) or indirect contact (ID – transwell insert) for 24 h. IL-8 mRNA in BM-MSC was then assessed by real-time PCR. mRNA expression was normalized to GAPDH mRNA levels ( $n=5$ ). (B) BM-MSC and primary AML from 5 patients were cultured alone and measured for MIF mRNA levels ( $n=5$ ). mRNA expression was normalized to GAPDH mRNA levels. (C) BM-MSC from 5 patients were treated with recombinant human MIF (100ng/ml) for indicated times and then extracted RNA was assessed for IL-8 mRNA by real-time PCR. mRNA expression was normalized to GAPDH mRNA levels ( $n=5$ ). (D). BM-MSC from 5 patients were treated with recombinant human MIF (100ng/ml) for indicated times and then media

was assessed for IL-8 protein expression by ELISA. (n=5). (E) BM-MSC from 4 patients were pretreated with ISO-1 (10  $\mu$ M) for 5 mins before treatment with recombinant human MIF (100ng/ml) for 4 h then assessed for IL-8 mRNA expression (n=4). (F) BM-MSC were pretreated with ISO-1 (10  $\mu$ M) for 5 mins before the addition of primary AML blasts from 10 patient samples for 48 h. AML blast number was assessed using a trypan blue exclusion hemocytometer-based counts (n=10). The Mann-Whitney U test was used to compare between treatment groups (\* = p<0.05).

**Figure 3. MIF-induced IL-8 upregulation is mediated through CD74.** (A) BM-MSC were assessed for CD105, CD74, CXCR2 and CXCR4 using flow cytometry. (B and C) BM-MSC from 4 patient samples were pretreated with the GPCR inhibitor pertussis toxin (100ng/ml) or for CD74 ( $\alpha$ CD74 ab 10 $\mu$ g/ml) for 30 minutes before being stimulated with MIF for 4 h. RNA was extracted and assessed for IL-8 mRNA by real-time PCR. mRNA expression was normalized to GAPDH mRNA levels (n=4). (D) BM-MSC from 4 patient samples were infected with control shRNA or CD74 shRNA for 72 h and analyzed for CD74 mRNA expression by RT-PCR and protein expression by flow cytometry. (E) BM-MSC from 4 patient samples were infected with control shRNA or CD74 shRNA for 72 h then treated with recombinant MIF and analyzed for IL-8 mRNA expression by RT-PCR. The Mann-Whitney U test was used to compare between treatment groups (\* = p<0.05).

**Figure 4. Inhibition of PKC $\beta$  regulates AML derived MIF-induced IL-8 mRNA induction in BM-MSC.** (A) BM-MSC from 4 patients samples were pretreated with Ro-31-8220 (1 $\mu$ M), PD98059 (10  $\mu$ M) and LY294002 (10  $\mu$ M) and then incubated with primary AML blast for 4 h. RNA was extracted and assessed for IL-8 mRNA by real-time PCR. mRNA expression was normalized to GAPDH mRNA levels (n=4). (B) BM-MSC from 4 different samples were pretreated with Ro-31-8220 (250 nM), PD98059 (10  $\mu$ M) and LY294002 (10  $\mu$ M) and then incubated with MIF for 4 h. RNA was extracted and assessed for IL-8 mRNA by real-time

PCR. mRNA expression was normalized to GAPDH mRNA levels (n=4). (C) BM-MSC were cultured with AML for 15 mins or recombinant MIF (100ng/ml) for various times. Protein was extracted and Western blotting performed. Blots were probed for pPKC $\alpha$ / $\beta$ II, pPKC $\beta$ , PKD/PKC $\mu$ , PKC $\delta$ , PKC $\delta$ / $\theta$ . Blots were then reprobed for  $\beta$ actin to show equal sample loading. (D) Four different BM-MSC with recombinant MIF (100ng/ml) at various times. Protein was extracted and Western blotting performed. Blots were probed for pAKT and pMAPK as well as total AKT and total MAPK. Blots were then reprobed for  $\beta$ actin to show equal sample loading. (E) Four different BM-MSC were pretreated with Go 6976 (1 $\mu$ M) and enzastaurin (1  $\mu$ M) and then incubated with MIF for 4 h. RNA was extracted and assessed for IL-8 mRNA by real-time PCR. mRNA expression was normalized to GAPDH mRNA levels (n=4). (F) Four different BM-MSC were infected with control shRNA or PKC $\beta$  shRNA for 72 h and analyzed for PKC $\beta$  mRNA expression by RT-PCR. (G) Four different BM-MSC were infected with control shRNA or PKC $\beta$  shRNA for 72 h then treated with recombinant MIF and analyzed for IL-8 mRNA expression by RT-PCR. The Mann-Whitney U test was used to compare between treatment groups (\* = p<0.05).

**Figure 5. Targeting MIF-PKC-IL-8 axis in AML disrupts BM-MSC derived protection.** (A and B) BM-MSC from six samples were infected with control shRNA or IL-8 shRNA for 72 h and analyzed for IL-8 mRNA expression by RT-PCR and protein expression by ELISA. (C) BM-MSC from six samples were infected with control shRNA or IL-8 shRNA for 72 h then treated with recombinant MIF and analyzed for IL-8 mRNA expression by RT-PCR. (D) BM-MSC were infected with control shRNA or IL-8 shRNA for 72 h and co-cultured with AML blasts from seven samples for 48 h. AML blast number was assessed using a trypan blue exclusion hemocytometer-based counts (n=7). (E) BM-MSC were pretreated with SB225002 (100 nM) for 30 mins before the addition of primary AML blasts from 10 samples for 48 hours. AML blast number was assessed using a trypan blue exclusion hemocytometer-based counts. The Mann-Whitney U test was used to compare between treatment groups (\* = p<0.05).

**Figure 1**

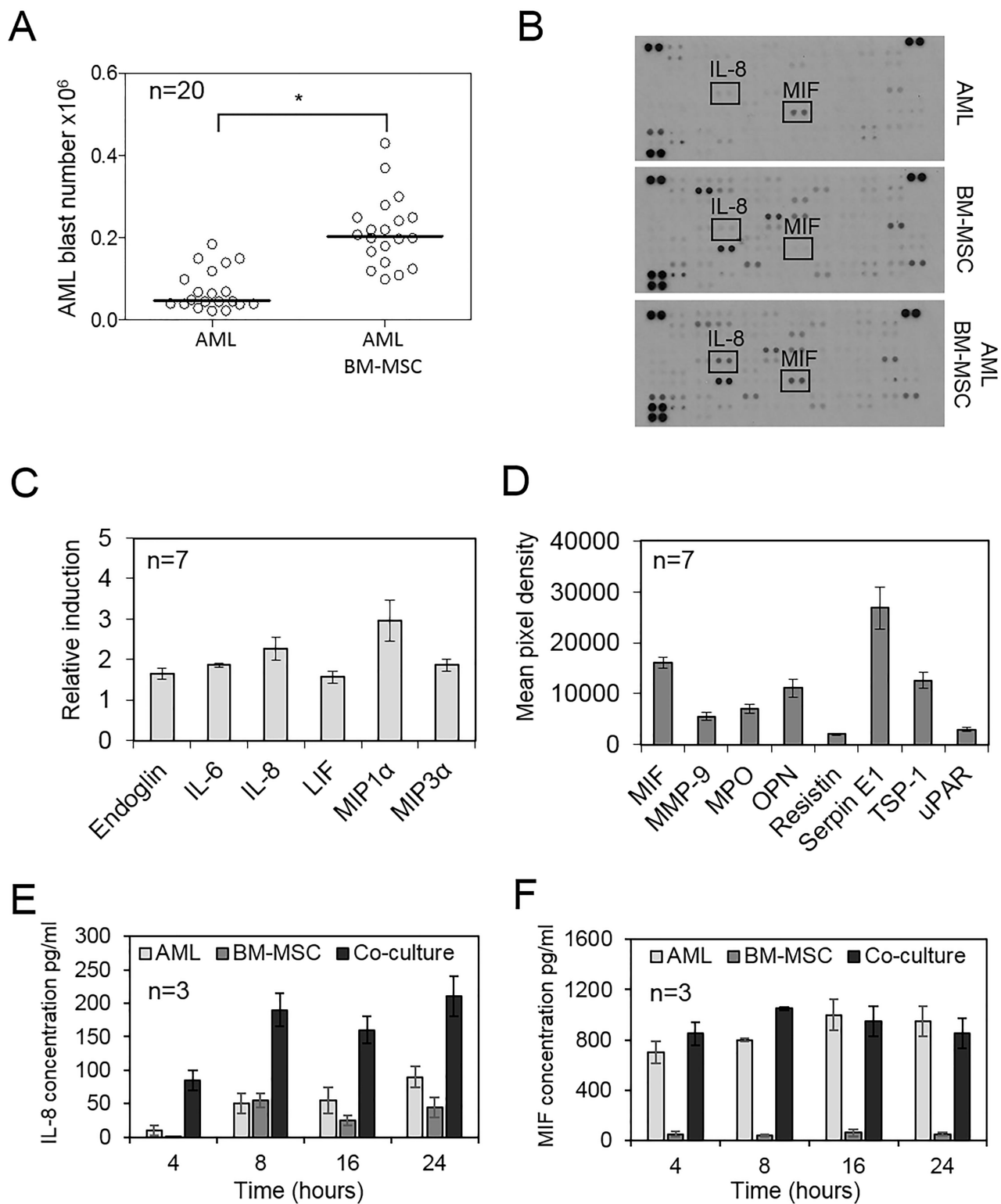
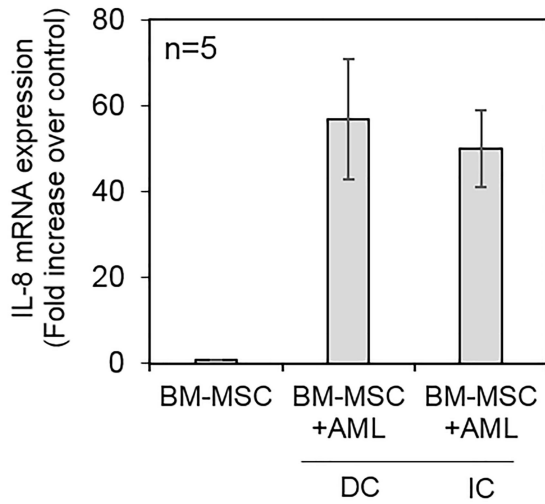
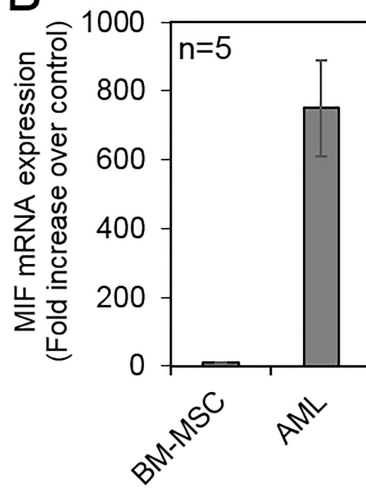


Figure 2

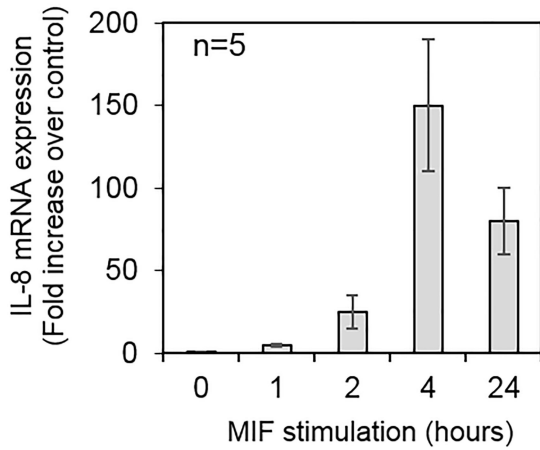
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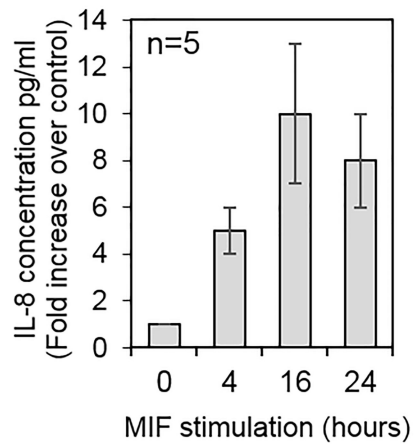
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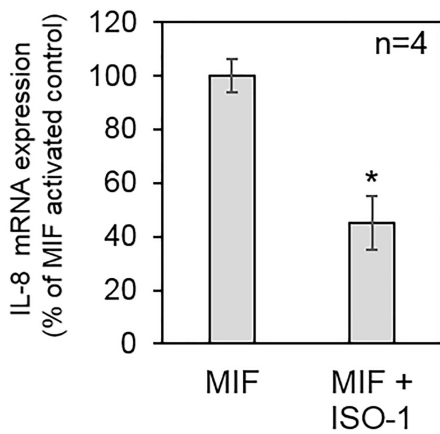
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**D**



**E**



**F**

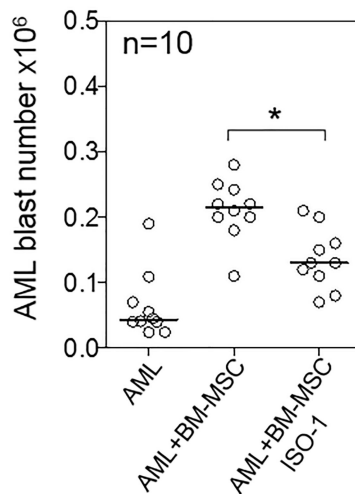
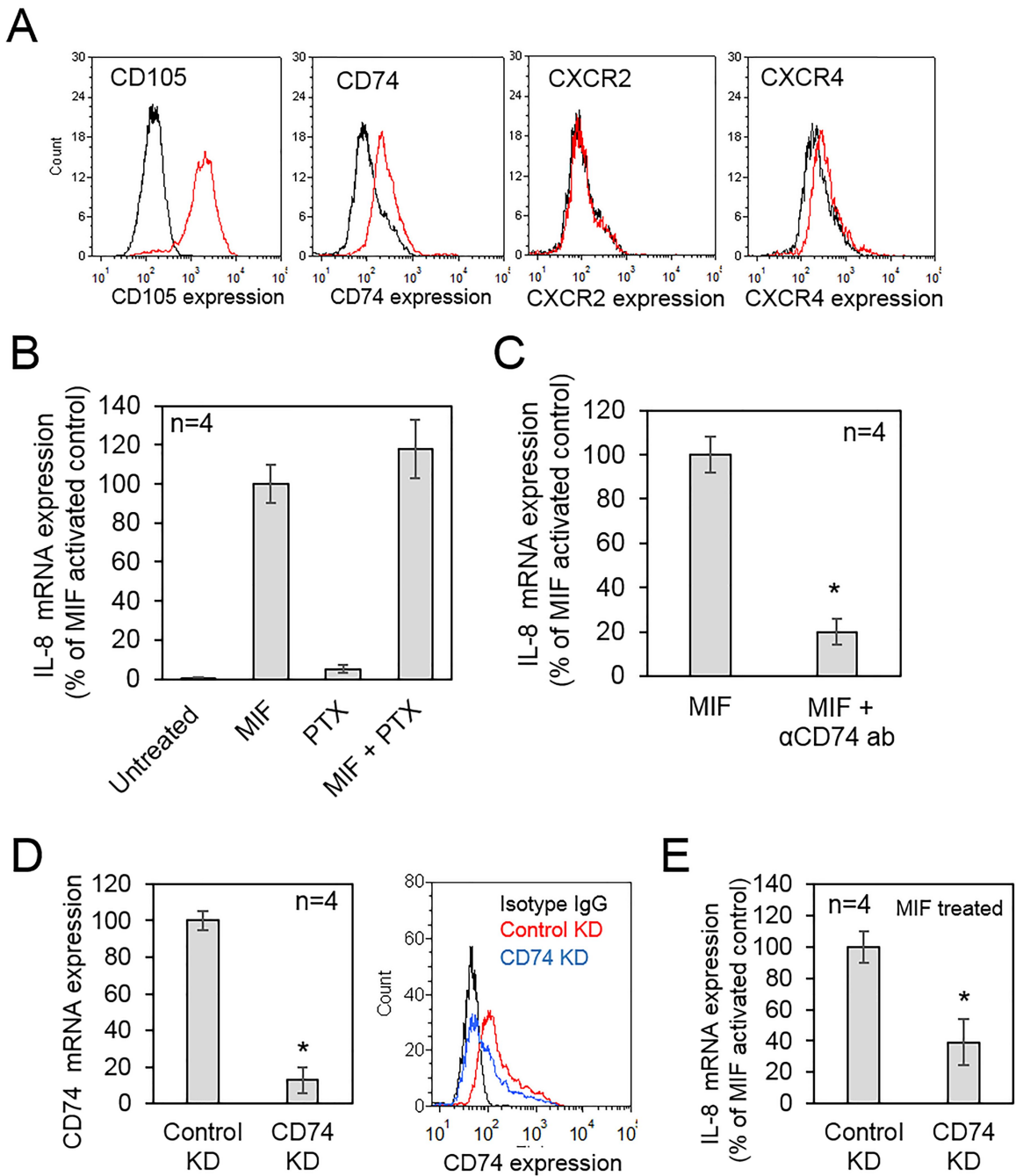




Figure 3



# Figure 4

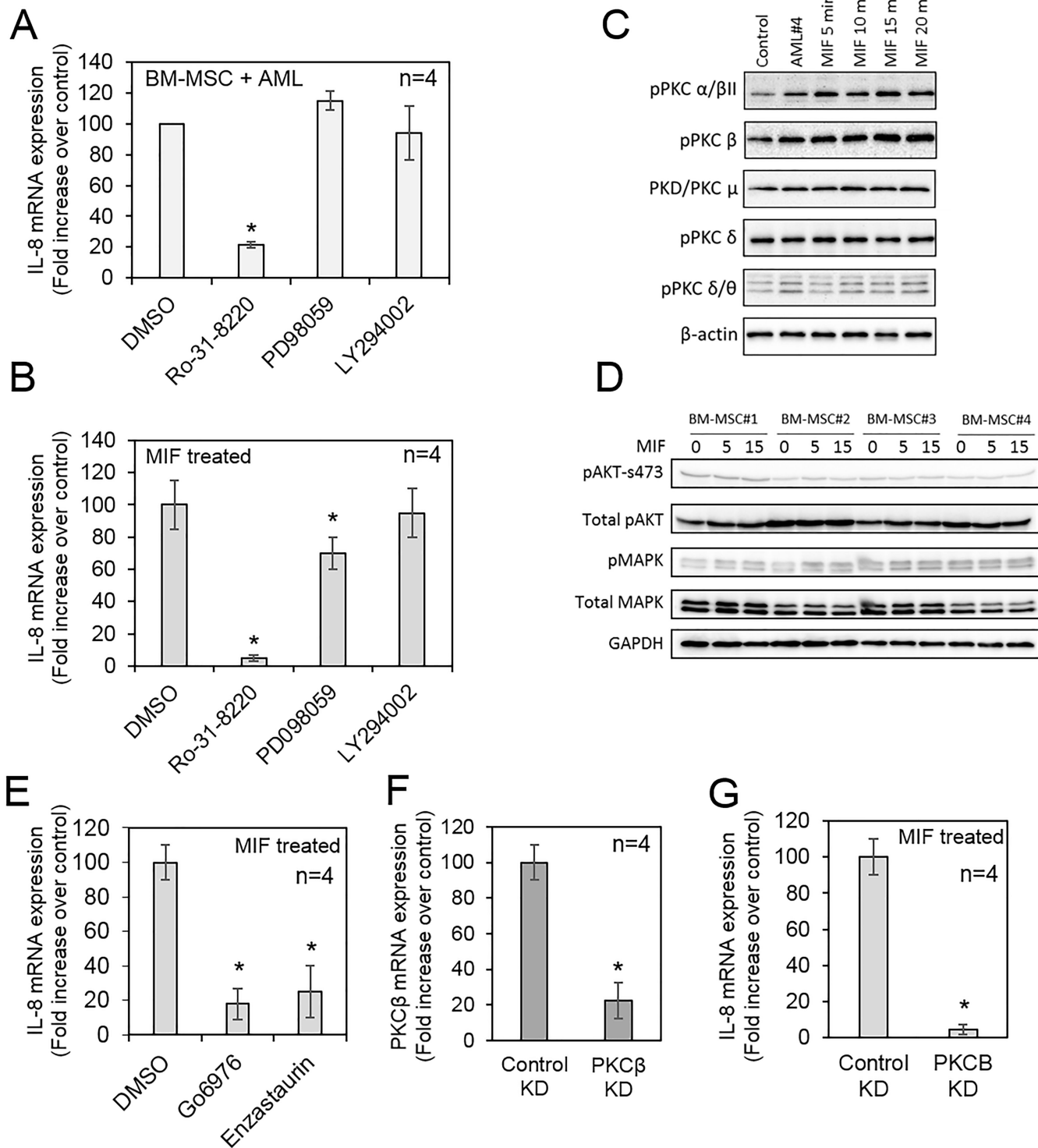
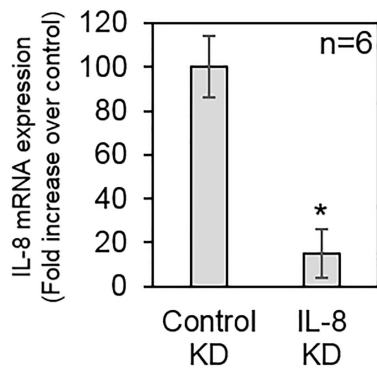
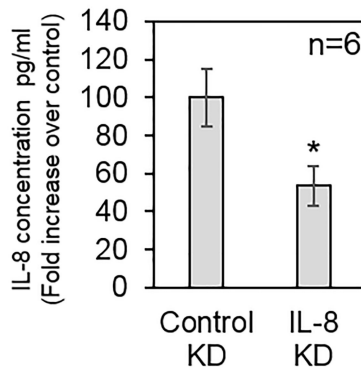


Figure 5

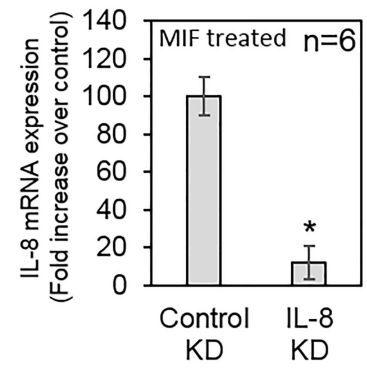
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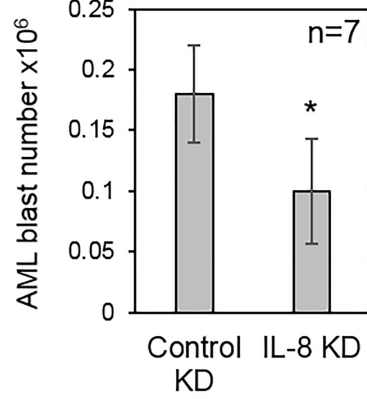
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