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Reciprocal leukemia-stroma VCAM-1/VLA-4-dependent activation of NF-κB mediates chemoresistance

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Short Title: NF-κB mediates chemoresistance in leukemia.

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Key Points:

- VCAM-1/VLA-4 triggers reciprocal NF-κB activation in leukemia and stromal cells and mediates crosstalk between leukemia and stromal cells.
- VCAM-1/VLA-4 and NF-κB signaling plays a pivotal role in the development of leukemia chemoresistance.

Abstract

Leukemia cells are protected from chemotherapy-induced apoptosis by their interactions with bone marrow (BM) mesenchymal stromal cells (BM-MSC). Yet the underlying mechanisms associated with this protective effect remain unclear. Genome-wide gene expression profiling of BM-MSC revealed that co-culture with leukemia cells upregulated the transcription of genes associated with NF-κB signaling. Moreover, primary BM-MSC from leukemia patients expressed NF-κB target genes at higher levels than their normal BM-MSC counterparts. The blockade of NF-κB activation via chemical agents or the overexpression of the mutant form of IκBα in BM-MSC markedly reduced the stromal-mediated drug resistance in leukemia cells *in vitro* and *in vivo*. In particular, our unique *in vivo* model of human leukemia BM microenvironment illustrated a direct link between NF-κB activation and stromal-associated chemo-protection. Mechanistic *in vitro* studies revealed that the interaction between VCAM-1 and VLA-4 played an integral role in the activation of NF-κB in the stromal and tumor cell compartments. Together, these results suggest that reciprocal NF-κB activation in BM-MSC and leukemia cells is essential for promoting chemoresistance in the transformed cells, and targeting NF-κB or VLA-4/VCAM-1 signaling could be a clinically relevant mechanism to overcome stroma-mediated chemoresistance in BM-resident leukemia cells.

Introduction

Experimental evidence gathered over the last two decades has demonstrated that bone marrow (BM) mesenchymal stromal cells (BM-MSC) can prevent spontaneous as well as chemotherapy-induced apoptosis in acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and other types of leukemia. [1-4] Undoubtedly, this chemoresistance-enhancing effect has profound clinical significance, since it promotes post-therapy residual disease that retains a greater potential for relapse.

Within the BM microenvironment, BM-MSC produce cytokines and chemokines and initiate cell adhesion-mediated signals that tightly regulate normal and malignant hematopoietic cell survival and appear to drive the chemoresistance-promoting effect of the BM microenvironment.[5-9] Cell–cell adhesion between BM-MSC and leukemia blasts follows a normal physiological process involving adhesion receptors on the leukemia cell surface (such as integrins β1, β2, and the very late antigen-4 [VLA-4]) interacting with stromal ligands such as vascular cell adhesion molecule 1 (VCAM-1).[10-12] This type of adhesive interaction triggers the activation of pro-survival and proliferative pathways in both the blasts and stromal cells that are critical for blast survival.[13]

Co-culture models of ALL cells and BM-MSC have been used to study the complex and dynamic networks of various growth factors and cytokines in which leukemic blasts and stromal cells crosstalk and reciprocally regulate their cytokine expression.[14,15] However, the process by which leukemia-stroma interactions confer chemoresistance to leukemia cells is not fully understood, particularly concerning the requisite changes that occur in BM-MSC. Such changes are likely, given that leukemia cells promote changes in their BM microenvironment that suppress normal hematopoiesis and enhance leukemia progression.[16] Related examples where tumor cells modify their surrounding stroma come from studies in solid tumors reporting that tumor cells can recruit vascular endothelial cells, MSC, and fibrovascular tumor associated fibroblasts (TAFs) from nearby tissues as well as from the BM.[17-20] Once they are in the tumor microenvironment, these “normal” cells aid in the promotion of tumor extracellular matrix remodeling, motility, and metastasis.[21,22]

Recent reports have described NF-κB activation in tumor-surrounding stroma upon interaction with tumor cells. [23-25]Classical activation of NF-κB occurs by factors that stimulate the IκB kinase complex to phosphorylate and degrade IκB, leading to NF-κB nuclear translocation and subsequent target gene expression.[26] In this report, we used co-culture model systems of human BM-MSC with human leukemia cells to identify changes induced by their interaction that contribute to the stroma-mediated chemoresistance of leukemia cells. The results presented here demonstrate that the leukemia-stroma interactions induce in these cells reciprocal NF-κB activation along with the ubiquitous upregulation of VCAM-1 in the BM-MSC unveiling a possible mechanism that involves integrin engagement and soluble factor-mediated signaling as responsible for this phenomenon.

Methods

Please refer to Supplemental Methods (see the Blood Web site) for detailed descriptions of the methods and reagents used.

Chemicals, reagents, and antibodies

MLN120B (provided by Millennium Pharmaceuticals, Inc.) was dissolved in DMSO and used at a final concentration of 10 μmol/L. CDDO-Me, the C-28 methyl ester derivative of the novel synthetic triterpenoid 2-cyano-3, 12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), was kindly provided by Dr. Edward Sausville (National Cancer Institute, Bethesda, MD) under the Rapid Access to Interventional Development program and by Dr. Michael Sporn (Dartmouth Medical College, Hanover, NH) and was used at a concentration of 50 ng/mL. The VLA-4 blocking antibody (CD49d, Cat#555501; BD Biosciences) was used at a concentration of 10 μg/0.5 × 10^6 cells. Recombinant human IL-1 receptor antagonist (IL-1RA, Cat# 200-01RA, PeproTech) was used at concentration of 200 ng/mL. VCR and Doxo were used at final concentrations of 75 ng/mL, and 50 ng/mL, respectively, unless otherwise indicated. Cytarabidine (AraC) was used at a final concentration of 1 μmol/L. Allophycocyanin (APC)-conjugated anti-human CD90 (Thy-1) and phycoerythrin (PE)-conjugated anti-human CD45 antibodies were obtained from BD-Biosciences. DAPI (4,6-diamidino-2-phenylindole) was obtained from Sigma-Aldrich (St. Louis, MO).

Leukemia cell lines and primary samples

Human OCI-AML3 cells and the preB-ALL REH cells, NALM6-luciferase-CopGFP, and RS4;11 were maintained in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine at 37°C in 5% CO_2. Samples of BM or peripheral blood were obtained for *in vitro* studies from patients with newly diagnosed

or recurrent ALL or AML who had a high (more than 40%) blast count. Informed consent was obtained following institutional guidelines. Clinical data for patient samples are presented in Supplemental Table S1. Mononuclear cells were separated by Ficoll-Hypaque (Sigma Chemical) density-gradient centrifugation.

Isolation and Culture of Primary BM-MSC

BM-MSC were isolated from BM of consented AML patients undergoing diagnostic BM aspiration and from healthy donors who were undergoing BM harvest for use in allogeneic BM transplantation. A complete description of the isolation and culture procedures can be found in Supplemental Methods.

Isolation and expansion of endothelial colony-forming cells (ECFCs)

Human ECFCs were isolated and expanded as previously described.[27] Endothelial growth medium (EGM) was prepared as suggested by the manufacturer except that FBS was replaced with 10% pHPL.

Co-culture isolation and RNA extraction

Normal BM-MSC were co-cultured with leukemic cell lines or leukemic cells from patient samples and processed as indicated in Supplemental Methods.

mRNA hybridization and gene-expression profiling.

Total RNA was amplified and hybridized to Illumina HT12 version 4 human whole-genome arrays as described previously. [28]

***In Vivo* Extramedullary Bone Formation and Murine Leukemia Model**

Extramedullary bone formation was performed as previously described [29]. Detailed description can be found in Supplemental Methods.

Statistical Analyses

For the gene analysis study, significance testing for differentially-expressed probes (DEPs) was determined by the Wilcoxon rank-sum test applied to individual processed bead values, with FDR significance values (q) established by the method of Benjamini and Hochberg.[30] For the other data, the results are expressed as the mean ± SEM for triplicate independent experiments. The Student's paired *t* test was used for statistical comparisons between groups. Differences with P values ≤ 0.05 were considered statistically significant.

Study Approval

Animal protocols were approved by the Animal Care and Use Committee of the MD Anderson Cancer Center. Leukemia patient and healthy donor BM samples were obtained following written informed consent in accordance with tissue procurement protocols approved by the Institution Review Board of the MD Anderson Cancer Center. This study was conducted in accordance with the Declaration of Helsinki.

Results

Co-culture of human-derived BM-MSC and leukemia cells induces gene expression in BM-MSC that is consistent with NF-κB activation

To elucidate molecular mechanisms by which leukemia–stroma interactions within the BM microenvironment could confer chemoresistance to leukemia cells, we used genome-wide gene expression profiling (GEP) to examine human normal BM-MSC that had been co-cultured with the pre-B ALL REH cells and then been separated by FACS to isolate each individual cell type. As controls, BM-MSC and REH cells were cultured alone (monocultures) and examined. After 48 hours of co-culture, BM-MSC were sorted and separated from the leukemia cells using the specific markers CD90 and CD45, respectively (Supplemental Fig. 1A and 2). After sorting, a sample of each purified cell population was checked by flow cytometry and the cell purity was assessed at > 98.5%. Total RNA from four cell populations (REH or BM-MSC, each cultured alone or co-cultured and then purified) was generated in three independent experiments and taken through standard labeling procedures for hybridization to microarrays for GEP (GEO accession number: GSE55533). Using strict significance criteria (*P* value < 0.01, false discovery rate *q* statistic < 0.1) for three independent experiments, we found 160 differentially expressed probes (DEPs) for 154 genes consistently upregulated in co-cultured BM-MSC as well as 166 DEPs for 159 downregulated genes (Supplemental Tables 1 and 2). Based on an analysis of GEP data for REH cells (please see the Supplemental Methods section), none of the DEPs that were upregulated in BM-MSC were attributable to contamination of the co-cultured and purified BM-MSC by the REH cells.

Among the upregulated DEPs induced by co-culture in BM-MSC, there were a variety of cytokines and chemokines including IL8, IL6, CCL2 and the VLA-4 ligand VCAM-1 as well as other recognized target genes of the NF-κB pathway (Supplemental Fig. 1B). Gene set enrichment analyses (GSEA) identified 180 gene sets from category C2 of the Molecular Signatures Database that were significantly enriched in co-cultured samples (*q* value < 0.1). Table 1 lists the most enriched gene sets, for

which more information is available at http://www.broadinstitute.org/gsea/msigdb/search.jsp. The GSEA indicated an activation of NF-κB and NF-κB-related pathways in the co-cultured BM-MSC (Supplemental Fig. 3 and Supplemental Tables 3 and 4).

To corroborate the GEP results, we performed a quantitative reverse-transcription (qRT)-PCR validation assay for a group of well-known target genes of NF-κB. Upregulation of these genes by co-culture with REH cells was confirmed, in most cases in the range of 2- to 4-fold (Fig. 1A). To exclude the possibility that the observed upregulation of NF-κB target genes in BM-MSC was cell line-dependent, we co-cultured normal BM-MSC (N-MSC) with several primary leukemia samples of ALL and AML (Supplemental Table 5). After 48 hours, we isolated BM-MSC from co-cultures by FACS and used qRT-PCR to analyze changes in the expression of selected genes as compared to BM-MSC cultured alone. Despite some variability between patient samples, the primary leukemia cells significantly upregulated the expression of the examined NF-κB target genes in N-MSC, following the same pattern as observed in the REH cells (Fig. 1B). In addition, we isolated BM-MSC from 8 normal donors and from 12 AML patients (Supplemental Table 6) and cultured them in the absence of other BM-derived cells for 4 to 8 weeks before RNA extraction. qRT-PCR analysis consistently showed upregulation of NF-κB target genes in AML-derived BM-MSC (AML-MSC) compared to N-MSC (Fig. 1C). The GEP and qRT-PCR data suggested that NF-κB activation in BM-MSC is a common consequence of the leukemia–stroma interaction.

To confirm these data we sought direct evidence for the activation of the canonical NF-κB pathway in BM-MSC by interaction with leukemia cells. Such activation causes members of the IκB family of inhibitors, particularly IκBα, to be phosphorylated by the IκB kinase (IKK) complex. This activation results in the ubiquitination and proteasomal degradation of the IκB family proteins, and the release and nuclear translocation of the NF-κB heterodimers containing p65 (RelA) or c-Rel.[26] Western blot analysis of cytosolic and nuclear fractions from BM-MSC co-cultured with REH cells or OCI-

AML3, an AML cell line, showed increased p65 protein level in nuclear extracts from co-cultured BM-MSC compared to BM-MSC cultured alone (Fig. 1D). These results were confirmed by confocal microscopy of BM-MSC co-cultured with REH cells (Fig. 1E) and OCI-AML3 cells (Supplemental Fig. 4).

Chemical Inhibition of NF-κB Activation Blocks Stroma-mediated Resistance of Leukemia Cells to Chemotherapeutic Agents

Given that many of the NF-κB target genes (e.g., *IL-8* and *IL-6*) that were upregulated in BM-MSC by co-culture have paracrine effects, we hypothesized that the stromal NF-κB activation may contribute to the chemoresistance of leukemia cells. To investigate this hypothesis, we first used the small-molecule IKKβ inhibitor MLN120B[31] to block classical NF-κB activation. Treatment of REH cells cultured alone with 10 μmol/L MLN120B did not have an obvious effect on the proliferation or viability of the leukemia cells. However, the toxicity (measured by % of annexin V+DAPI-positive cells) of a sub-lethal dose (i.e., 75 ng/mL) of vincristine (VCR), a standard chemotherapeutic agent used in ALL therapy, increased from ~ 35% when used alone to ~ 80% when combined with MLN120B (Fig. 2A and Supplemental Fig. 5A). The co-culture with stroma reduced the cytotoxicity of VCR in the leukemia cells (i.e., ~ 50% less apoptosis than observed in the VCR-treated REH cells cultured alone), which was consistent with a microenvironmental protective effect. [4] Moreover, treatment with MLN120B blocked the ability of BM-MSC to induce chemoresistance to VCR in co-cultured REH cells (i.e., there was no significant difference between the percentages of apoptotic cells in the monocultured versus the co-cultured leukemia cells).

Similar results were obtained when RS4;11 cells were treated with MLN120B and VCR (Fig. 2B and Supplemental Fig. 5B) and when OCI-AML3 cells were treated with the chemotherapeutic agent doxorubicin (Doxo, 50 ng/mL) in combination with MLN120B (Fig. 2C and Supplemental Fig. 5C). A

comparable reduction in BM-MSC–mediated chemoresistance was observed when 50 ng/mL CDDO-Me, another putative NF-κB inhibitor,[32,33] was used in combination with 50 ng/mL of Doxo in both REH (Fig. 2D and Supplemental Fig. 5D) and RS4;11 cells (Supplemental Fig. 5E and 5F). Inhibition of stroma-mediated chemoresistance was also observed in blasts from two out of three AML patients (Patients 13 and 15) treated with the combination of AraC and MLN-120B (Supplemental Fig. 6 and Supplemental Table 5). Together, these results indicated that blocking NF-κB activation in both leukemia cells and the stroma can not only abrogate the stroma-mediated chemoresistance of leukemia cells, but also increase the apoptotic effects of chemotherapeutic agents, particularly those that induce NF-κB pro-survival pathways.[34,35]

Overexpression of IκBα "Super Repressor" in BM-MSC Blocks NF-κB Activity and Impairs the BM-MSC−mediated Chemoresistance in Leukemia Cells

Although the use of chemical inhibitors to block NF-κB activation suggested the involvement of the NF-κB pathway in the cytoprotective response of the leukemia cells to various chemotherapeutic agents, it cannot dissect the relevance of the blocking effects in each particular compartment (i.e., BM stromal cells or leukemia cells) and it could certainly produce off-target effects. To investigate whether inhibition of canonical NF-κB activation in BM-MSC alone is sufficient to reduce stroma-mediated chemoresistance in leukemia cells, we expressed a "super repressor" form of IκBα, (IκBα-SR) in BM-MSC via a lentiviral vector (Supplemental Fig. 5A). This mutated form of IκBα cannot be phosphorylated by the IKK complex and consequently inhibits canonical NF-κB activation. The overexpression of IκBα-SR did not markedly affect the proliferation (not shown) or differentiation potential of the stably transduced BM-MSC (Supplemental Fig. 5B). However, it effectively blocked the nuclear localization of p65 after TNF-α stimulation (Fig. 3A), demonstrating the ability of this mutant IκBα to inhibit canonical NF-κB activation. The overexpression of IκBα-SR in the BM-MSC also blocked or decreased the co-

culture−induced upregulation of NF-κB target genes (Fig. 3B), indicating that NF-κB activation is indeed involved in this process. Most importantly, IκBα-SR overexpression significantly impaired the ability of BM-MSC to protect the REH cells from apoptosis induced by VCR (75 ng/mL, Fig. 3C), illustrating that NF-κB activation in BM-MSC is involved in the chemoresistance of the leukemia cells conferred by their interaction with the stromal cells.

Blockade of NF-κB Activation in BM Stroma in the Humanized Extramedullary BM Model Reduces Leukemia Burden Following Chemotherapy *In Vivo*

To assess the contribution of NF-κB activation to the stroma-mediated chemoresistance of leukemia cells *in vivo*, we used an approach recently developed in our laboratory to generate ectopic human-derived extramedullary bone and BM in immunodeficient mice. [29] Briefly, primary human BM-MSC are mixed with primary human endothelial precursors (ECFCs) and Matrigel and injected subcutaneously in mice to generate the humanized extramedullary bone marrow niches hosting human hematopoietic and/or xenotransplanted leukemic cells. We used BM-MSC that had been stably transduced with either empty vector or IκBα-SR, injecting them with ECFCs and Matrigel subcutaneously into contralateral flanks of NOD/SCID/IL-2rγ-null mice (Fig. 4A).

Within 8 weeks after implantation, we observed the development of well-defined, vascularized bone-like tissues in both flanks of all mice. *In vivo* staining with OsteoSense 750, a fluorescent agent that targets hydroxylapatite, confirmed high osteoblast activity originating from the BM-MSC−ECFC−Matrigel-derived tissues in the flanks of the mice (Supplemental Fig. 8). After the formation of the humanized extramedullary bone, we intravenously injected the preB-ALL cell line NALM6-luciferase-CopGFP into each mouse and monitored tumor burden by bioluminescence imaging with the IVIS system. After 10 days of NALM6-luciferase-CopGFP cell engraftment, luciferase activity was observed not only in the calvaria and vertebral column but also in the flanks of mice where the BM-

MSC−ECFC−Matrigel mixtures had been implanted (Fig. 4B), suggesting that the leukemia cells engrafted not only into murine bone marrow reservoirs but also into both control- and IκBα-SR−transduced BM-MSC−derived extramedullary bones.

Immediately after confirmation of leukemia cell engraftment, the mice were administered VCR (150 μg/kg) every 3 days for a total of 10 days. Whereas there were no obvious differences between the luciferase signals in both flanks before treatment (Fig. 4B and D), at the end of the treatment (i.e., 20 days after the transplantation) there was a statistically significant reduction in leukemia cell burden in extramedullary bones derived from the IκBα-SR–transduced BM-MSC (Fig. 4B, lower panel; Fig. 4C and D). These observations were confirmed by hematoxylin and eosin staining and by immunohistochemical staining with anti-luciferase antibody (Fig. 4E). These results indicated that the NF-κB activity in BM-MSC is an integral part of the stroma-mediated chemoresistance imparted by the BM microenvironment to the leukemic cells.

The Transcriptional Activation of NF-κB Target Genes in BM-MSC is Linked to Interaction Between VCAM-1 and VLA-4.

Both cDNA array and qRT-PCR data indicated that leukemia-stroma interaction induced the transcriptional upregulation of NF-κB target genes in BM-MSC. Notably, one of the consistently upregulated genes was *VCAM-1*. The VCAM-1 protein is an integrin receptor cell adhesion molecule that acts as the primary ligand for VLA-4; an interaction that participates in leukocyte-endothelial cell signal transduction. A previous study demonstrated that the interaction between endothelial cells and monocytes via the VCAM-1-VLA-4 engagement caused the activation of NF-κB in both cell types. The activating mechanism also apparently involved IL-1β and the IL-1 receptor (IL-1R).[36]

To test whether VCAM-1-VLA-4 signaling could play a role in leukemia cell-mediated NF-κB activation in cells constituting the microenvironment, we utilized VLA-4 blocking antibody (VLA-4 bAb). Pre-incubation of REH or OCI-AML3 cells with the VLA-4 bAb prior to co-culture significantly reduced expression of selected NF-κB target genes in BM-MSC (Figs. 5A and 5B, respectively). Furthermore, and in agreement with previous data, [36] IL-1 blockade using the IL-1 receptor antagonist (IL1RA) had an even more pronounced inhibitory effect. Accordingly, the inhibition of the VLA-4 and VCAM-1 interaction also impaired the autocrine *IL-1α* mRNA expression in co-cultured REH (Fig. 5C) and OCI-AML3 (Fig. 5D), albeit the effect on *IL-1β* was modest. This result suggested that BM-MSC co-culture could induce the reciprocal activation of NF-κB and IL-1α production in the leukemic cells. Indeed, Western blot analyses revealed that co-culture with BM-MSC induced p65 translocation to the nuclear fraction of both REH and OCI-AML3 cells when compared to the same cells cultured alone (Fig. 5E), though to a lesser extent to that observed for BM-MSC (i.e., compared to Fig. 1D). Moreover, and in agreement with the qRT-PCR data (Fig. 5A and B), blockade of VCAM-1-VLA-4 signaling by pre-incubating REH and OCI-AML3 cells with VLA-4 bAb significantly reduced p65 nuclear translocation in co-cultured BM-MSC (Fig. 5F).

Discussion

Many studies have illustrated the ability of BM stromal cells, in particular BM-MSC, to protect leukemia cells from spontaneous and chemotherapy-induced apoptosis. Moreover, increasing evidence indicates that "crosstalk" between leukemia cells and BM stromal cells results in reciprocal modulation of each other's functions[13,37-39] to create a leukemia-promoting "soil" that is able to nurture the malignant "seed" cells.[23] In this regard, most of the previous studies have focused on characterizing changes in leukemia cells that were caused by interaction with stromal cells. In this study, we focused instead on

changes in BM-MSC caused by leukemia cells, through which the leukemia cells essentially modify their own microenvironment.

The whole-genome gene expression profiling we carried out in this study identified consistent transcriptional changes suggesting NF-κB activation in the BM-MSC, which was apparently caused by leukemia cells (Fig. 1A,B,C and Supplemental Fig. 1B and 3). Our subsequent experiments confirmed that the co-culture with leukemic cells activated NF-κB in BM-MSC, at least in part through the canonical pathway, as demonstrated by the nuclear translocation of p65/RelA (Fig. 1D and E and Supplemental Fig. 4). These data are consistent with our observation that phosphorylated p65 (Ser276) is localized in the nuclei of CD90-positive BM stroma cells in BM biopsies of patients with ALL (Supplemental Fig. 9) and that AML-derived BM-MSC display higher transcriptional activity of NF-κB target genes even after several passages suggesting that these leukemia-induced changes can persist for prolonged time even in the absence of leukemia cells (Fig. 1C).

We further showed that the blockade of NF-κB activation in BM-MSC, either by chemical inhibition or by the overexpression of a "super-repressor" form of IκBα, could notably impair their ability to confer chemoresistance to the leukemia cells (Fig. 3 through Fig. 5). This mechanism could, at least in part, contribute to the increased efficacy of the proteasome inhibitor bortezomib, which is known to block NF-κB signaling, in combination with standard chemotherapy in patients with advanced ALL.[40] Bortezomib is used in the treatment of multiple myeloma,[41] and, in combination with other agents, has been the focus of several clinical trials for the treatment of AML.[42-44]

In recent years, studies conducted in mice have shown NF-κB activation in tumor-surrounding stroma upon interaction with tumor cells. In a mouse model of squamous skin carcinoma, carcinoma cells were shown to "educate" normal fibroblasts into tumor-promoting stromal cells through the activation of NF-κB.[24] In a recent report and using a murine model of chronic myeloid leukemia (CML), Schmidt et al. showed that CML cells can induce the expression of placental growth factor by BM-MSC in a NF-κB-

dependent manner. [23] Unfortunately, little is known about the role of activated NF-κB in human BM-derived MSC. Our results extend this data by showing for the first time in a *bona-fide* human BM model of acute leukemia a direct link between NF-κB activation and stromal-associated chemo-protection. Our *in vivo* model allowed us to illustrate that the blockade of NF-κB activation with IκBα-SR in BM-MSC increased the induction of apoptosis in leukemia cells by chemotherapy (Fig. 4). Furthermore, *in vitro* chemical inhibition of NF-κB in both stromal and leukemia cells increased the toxicity of standard chemotherapeutic agents, suggesting that the inhibition of NF-κB in combination with traditional chemotherapy, especially those known to activate NF-κB–mediated pro-survival pathways, could improve the chances of eradicating circulating as well as residual BM leukemia cells.

A role for adhesion-mediated signals in the activation of NF-κB was shown in monocytes whereby binding of monocyte-associated VLA-4 to VCAM-1 expressed on the surface of vascular endothelial cells induces NF-κB activation in both cell types. [36] Our data demonstrate the upregulation of VCAM-1 in co-cultured BM-MSC, and our subsequent observations indicated that direct contact between leukemia cells and BM-MSC is needed to achieve maximal NF-κB activation in BM-MSC (Supplemental Fig. 10). Specifically, the expression of VLA-4 on the leukemic cells and VCAM-1 on BM-MSC has been implicated in the maintenance of residual disease in AML and in resistance of stroma-adherent AML cells to chemotherapy. [7] Interestingly, experiments conducted using human recombinant VCAM-1–coated dishes indicated that this adhesion molecule is sufficient to stimulate NF-κB activation in leukemic cells and such activation can be blocked with MLN-120B (Supplemental Fig. 11A). In spite of the NF-κB activation, immobilized VCAM-1 failed to confer resistance to chemotherapy treatment (Supplemental Fig. 11B) suggesting the need for stromal cells capable of responding to activated leukemic cells. In agreement with this data, only conditioned medium from co-cultured BM-MSC and not from monocultured BM-MSC was capable of protecting leukemic cells from chemotherapy-induced apoptosis (Supplemental Fig. 11C-D). These data indicate that direct contact between leukemic cells and BM-MSC

(likely through VCAM-1/VLA4 interaction) is needed for a reciprocal activation of NF-κB in both cellular compartments and to trigger the stroma-mediated chemoresistance. In addition, our results using VLA-4 blocking antibody indicated that the interaction between VLA-4 and VCAM-1 is, at least in part, responsible for the activation of NF-κB in the BM-MSC (Fig. 5 A, B and F). Notably, a recent report showed dramatic improvement of survival of mice transplanted with primary human ALL cells, upon combination of Natalizumab, an anti-functional VLA-4 antibody, with chemotherapy. [45] Our data further indicated that co-culture with BM-MSC can also induce NF-κB activation in leukemia cells (Fig. 5E) and that blockade of the VLA-4-VCAM-1 interaction during co-culture can impair levels of *IL-1α*, and to a less extent *IL-1β*, mRNA expression in the REH and OCI-AML3 leukemia cells (Fig. 5C and D). In addition, disruption of the VLA-4-VCAM-1 interaction during co-culture impaired the nuclear translocation of NF-κB in BM-MSC (Fig. 5F). We also found that interference of IL-1α and IL-1β signaling using IL-1RA resulted in enhanced blockade of NF-κB activation in BM-MSC (Fig. 5A and B) and on the transcriptional upregulation of *IL-1α* and *IL-1β* mRNA in REH and OCI-AML3 cells (Fig. 5C and D). This data is in agreement with a recent publication from Weinberg's group showing that carcinoma cell–derived IL-1 signals through the IL-1R on tumor-associated MSC inducing the expression of prostaglandin E_2 and production of cytokines including IL-6 and IL-8.[46] This integrin-mediated signaling involving the VLA-4 and VCAM-1 interaction could be one of the mechanisms cooperating to establish communication between the leukemia and stromal cells. We envision that a combination of cell adhesion-mediated signals (VLA-4/VCAM-1) and soluble factor-mediated signals (IL-1) is needed for this "stroma education" phenomenon to occur (Fig. 6). These findings suggest future avenues exploring targeted inhibition of selective pathways with the goal to abrogate microenvironment-mediated chemoresistance in leukemias.

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Table 1. Gene sets differentially regulated in BM-MSC co-cultured with REH leukemia cells compared to BM-MSC in monoculture.

NAME	SIZE	ES	NES	FDR q-val	FWER p-val	RANK AT MAX
HINATA_NFKB_TARGETS_KERATINOCYTE_UP	44	0.74373627	2.763994	0	0	910
ZHANG_RESPONSE_TO_IKK_INHIBITOR_AND_TNF_UP	114	0.59999883	2.6790879	0	0	864
AMIT_EGF_RESPONSE_60_MCF10A	33	0.7639718	2.678291	0	0	490
HINATA_NFKB_TARGETS_FIBROBLAST_UP	46	0.6953736	2.640253	0	0	540
KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY	29	0.77374154	2.575111	0	0	763
KIM_WT1_TARGETS_UP	159	0.5234185	2.4591517	4.38E-04	0.002	2290
MCLACHLAN_DENTAL_CARIES_UP	92	0.57450825	2.4543602	3.75E-04	0.002	306
KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	47	0.6524064	2.453981	3.28E-04	0.002	1268
ZUCCHI_METASTASIS_DN	17	0.8249239	2.4468715	4.36E-04	0.003	520
GRAHAM_CML_DIVIDING_VS_NORMAL_QUIESCENT_DN	49	0.6465402	2.4402559	5.20E-04	0.004	625
SANA_TNF_SIGNALING_UP	37	0.68556935	2.4303193	7.04E-04	0.006	562
SEKI_INFLAMMATORY_RESPONSE_LPS_UP	44	0.66059566	2.4077451	9.58E-04	0.009	864
DASU_IL6_SIGNALING_UP	48	0.6300051	2.3814805	0.001368899	0.014	969
LIEN_BREAST_CARCINOMA_METAPLASTIC_VS_DUCTAL_UP	47	0.6460621	2.3720858	0.00127112	0.014	952
REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	36	0.666963	2.3602338	0.001439457	0.017	490
MCLACHLAN_DENTAL_CARIES_DN	105	0.53308874	2.33769	0.002061751	0.026	306
KEGG_RIG_I_LIKE_RECEPTOR_SIGNALING_PATHWAY	28	0.6970441	2.2951434	0.0034468	0.046	1268
TENEDINI_MEGAKARYOCYTE_MARKERS	27	0.6971226	2.2726986	0.004659514	0.065	624
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	78	0.55096024	2.2703986	0.00447959	0.066	1258
KEGG_BLADDER_CANCER	29	0.6838998	2.2639232	0.004569982	0.07	1057

Figure Legends

Figure 1. Co-culture with leukemia cells induces NF-κB activation in BM-MSC. **A**, A group of array-identified upregulated genes in co-cultured BM-MSC was validated by qRT-PCR. The bars represent qRT-PCR data from three independent experiments and results are expressed as fold difference expression (± SEM) in the co-cultured BM-MSC versus the monocultured BM-MSC. **B**, A total of five ALL and seven AML patient samples were independently co-cultured with BM-MSC and processed as in Fig. 1 **A**. Data from qRT-PCR analysis are expressed as fold difference expression in co-cultured BM-MSC versus the monocultured BM-MSC. Mean expression value for each gene is shown as a bar. **C**, qRT-PCR analysis showing expression levels of a selected group of NF-κB target genes in AML-derived BM-MSC (AML-MSC, n = 12) compared to normal donor BM-MSC (N-MSC, n = 8). A total of twelve AML-derived and eight normal donor-derived primary MSC cultures were analyzed. Bars represent the mean value (± SEM) Log2 expression levels relative to ABL (housekeeping control) expression levels. **D**, Western Blot analysis of cytosolic (CF) and nuclear (NF) fractions of lysates from BM-MSC cultured alone (–) or co-cultured with REH or OCI-AML3 cells for 1 hour. Each well correspond to 5 µg of total protein. Membranes were probed with rabbit monoclonal anti-p65, mouse monoclonal anti-PARP1 (nuclear fraction loading control) and mouse monoclonal anti-GAPDH (cytosolic fraction loading control). **E**, Immunofluorescence staining for p65/RelA showing p65 translocation into BM-MSC nuclei upon interaction with leukemia cells in co-culture conditions. BM-MSC were cultured alone (monoculture) or co-cultured with REH cells for 24 hours and then fixed with 4% PFA. As controls, BM-MSC cultured alone were treated with vehicle or TNF-α (20 ng/mL) for 30 minutes. Nuclei were counterstained with DAPI. Scale bar: 10 µm. Arrows point at absence (monoculture panel) or presence (co-culture panel) of p65 in nuclei.

Figure 2. Blockade of NF-κB activation enhances pro-apoptotic effects of standard chemotherapy. REH (**A** and **D**), RS4;11 (**B**) and OCI-AML3 (**C**) cells were cultured alone (monoculture) or co-cultured with BM-MSC as indicated in Methods. Monocultured and co-cultured cells were treated for 72 hours with

either vincristine (VCR, **A** and **B**) or doxorubicin (Doxo, **C** and **D**) as monotherapy or in combination with one of the IKKβ inhibitors MLN120B (MLN, **A**, **B** and **C**) or CDDO-Me (**D**). The percentage of apoptotic cells (annexinV^+/$DAPI^+$) was assessed by flow cytometry using annexin V^+/$DAPI^+$ staining and counting beads. Data for the absolute number of viable cells are shown in the Supplemental Fig. S5. Results are expressed as the mean of the percentage of annexin V^+/$DAPI^+$ (± SEM) of three independent experiments. The symbol (*) indicates a statistically significant difference at $P \leq 0.05$. AnnV: annexinV; MC: monocultured; CC: co-cultured.

Figure 3. The effects of overexpression of IκBα "super repressor" (IκBα-SR) in BM-MSC. **A,** Immunofluorescence staining for p65/RelA in BM-MSC transduced with either empty-control (MSC-Control) or IκBα-SR virus (MSC−IκBα-SR)**.** p65/RelA nuclear translocation was determined by confocal microscopy analysis in cells treated with DMSO (vehicle) or TNFα (20 ng/mL) for 30 minutes before fixation with 4% PFA. Approximately 50 fields with 2 to 3 cells/field were analyzed per condition. The nuclei were counterstained with DAPI. Scale bar: 10 μm. **B**, MSC-Control or MSC−IκBα-SR were cultured alone (monoculture) or co-cultured with REH cells for 48 hours as indicated. Total RNA was extracted and qRT-PCR was carried out to detect the expression of a selected group of NFκB target genes. Results of three independent experiments are expressed as the mean fold difference expression (± SEM) in different culture conditions over the expression levels in the monocultured MSC-Control. **C**, REH cells were cultured alone or co-cultured with either MSC-Control or MSC−IκBα-SR and then treated with vincristine (VCR) for 72 hours. As described for Fig. 2, the percentage of apoptotic cells (left panel) and absolute number of viable cells (right panel) was assessed by flow cytometry using annexin V^+/$DAPI^+$ staining and counting beads. Results are expressed as the mean of the percentage of annexin V^+/$DAPI^+$ (± SEM) and the mean of the absolute number of viable cells (± SEM) of two independent experiments. (*) *P* value ≤ 0.05.

Figure 4. Blockade of NF-κB activation in BM-MSC reduces *in vivo* leukemia burden. **A**, A mixture of Matrigel, endothelial colony-forming cells (ECFCs) and either control BM-MSC (MSC-Control) or IκBα-SR−transduced BM-MSC (MSC−IκBα-SR) were injected subcutaneously into contralateral flanks of NOD/SCID/IL-2rγnull mice (a representative example of two independent experiments with 4 mice n = 8 is shown). NALM6-luciferase-CopGFP leukemia cells were intravenously transplanted into the mice and tumor burden was monitored throughout the experiment by bioluminescence imaging with the IVIS system. Ten days after leukemia cell engraftment, mice were administered vincristine (VCR) for another 10 days. **B**, Animals were imaged and analyzed for luciferase signal right before chemotherapy treatment started (day 10; upper panel) and on the last day of treatment (day 20; lower panel). **C**, Both extramedullary bones from each mouse were surgically removed at the end of the experiment (upper panel) and the intensity of signal irradiating from them was quantified and plotted (lower panel). **D**, Average radiance measurement of luciferase signal corresponding to extramedullary bone areas derived from BM-MSC−Control or BM-MSC−IκBα-SR before and after ten days of treatment with VCR indicating a decrease in leukemia burden in extramedullary bones derived from BM-MSC−IκBα-SR. Average radiance is expressed as photons per second per centimeter squared per steradian (p/s/cm2/sr). The symbol (*) indicates a statistically significant difference at $P \leq 0.05$. **E**, Immunohistochemical analysis of artificial BM sections stained with H&E or anti-luciferase antibody.

Figure 5. Blockade of VLA-4/VCAM-1 signaling impairs NF-κB activation and transcription of NF-κB downstream target genes. **A**, Normal BM-MSC were co-cultured for 24 hours with REH (A) or OCI-AML3 (**B**) cells that were either untreated or pre-incubated with VLA-4 blocking antibody (VLA-4 bAb) for 1 hour. When indicated, 200 ng/mL of IL-1 receptor antagonist (IL-1RA) was added to the co-culture medium. BM-MSC were cultured alone (monocultured) as controls. After separating the cells (as indicated in the methods section), total RNA from BM-MSC and leukemic cells was extracted. Expression of selected NF-κB target genes in co-cultured BM-MSC was determined by qRT-PCR (A, B). Bars represent qRT-PCR data from triplicate samples and results are expressed as fold difference

expression (± SEM) in each co-culture condition versus the monocultured BM-MSC. (C, D) Total RNA from REH (**C**) and OCI-AML3 (**D**) cells (co-cultured with MSC in experiments shown in **A** and **B**, respectively) was extracted and IL-1α and IL-1 β mRNA expression levels were quantified by qRT-PCR. Bars represent qRT-PCR data from triplicate samples and results are expressed as fold difference expression (± SEM) in each co-culture condition versus the monocultured OCI-AML3 cells. **E**, Western Blot analysis of cytosolic (CF) and nuclear (NF) fractions of lysates from REH and OCI-AML3 cultured alone (–) or co-cultured with BM-MSC (+) for 1 hour. **F**, Western Blot analysis of cytosolic (CF) and nuclear (NF) fractions of BM-MSC lysates cultured alone (–) or co-cultured with OCI-AML3 or REH cells for 1 hour. Leukemic cells were pre-incubated with VLA-4 bAb when indicated (+) before co-culture. Each well correspond to 5 µg of total protein. Membranes were probed with rabbit monoclonal anti-p65, mouse monoclonal anti-PARP1 (nuclear fraction loading control) and mouse monoclonal anti-GAPDH (cytosolic fraction loading control).

Figure 6. Schematic representation of leukemic cells−BM-MSC crosstalk. Interactions of leukemic cells with BM-MSC promote transcriptional changes in the BM stroma that ultimately impact leukemic cells proliferation and survival. Activation of NF-κB and transcription of NF-κB downstream target genes (*IL-8, IL-6, CCL2, VCAM-1*, etc.) are triggered in BM-MSC by these interactions. Communication between these two cell types can be achieved by integrins and their receptors (i.e., VCAM-1/VLA-4) or by soluble factors that are directly secreted to the extracellular milieu (such as IL-1). VCAM-1/VLA-4 interaction between leukemia cells and BM-MSC may provide a possible mechanism for the activation of NF-κB in both, BM-MSC and in leukemia cells.

Figure 1

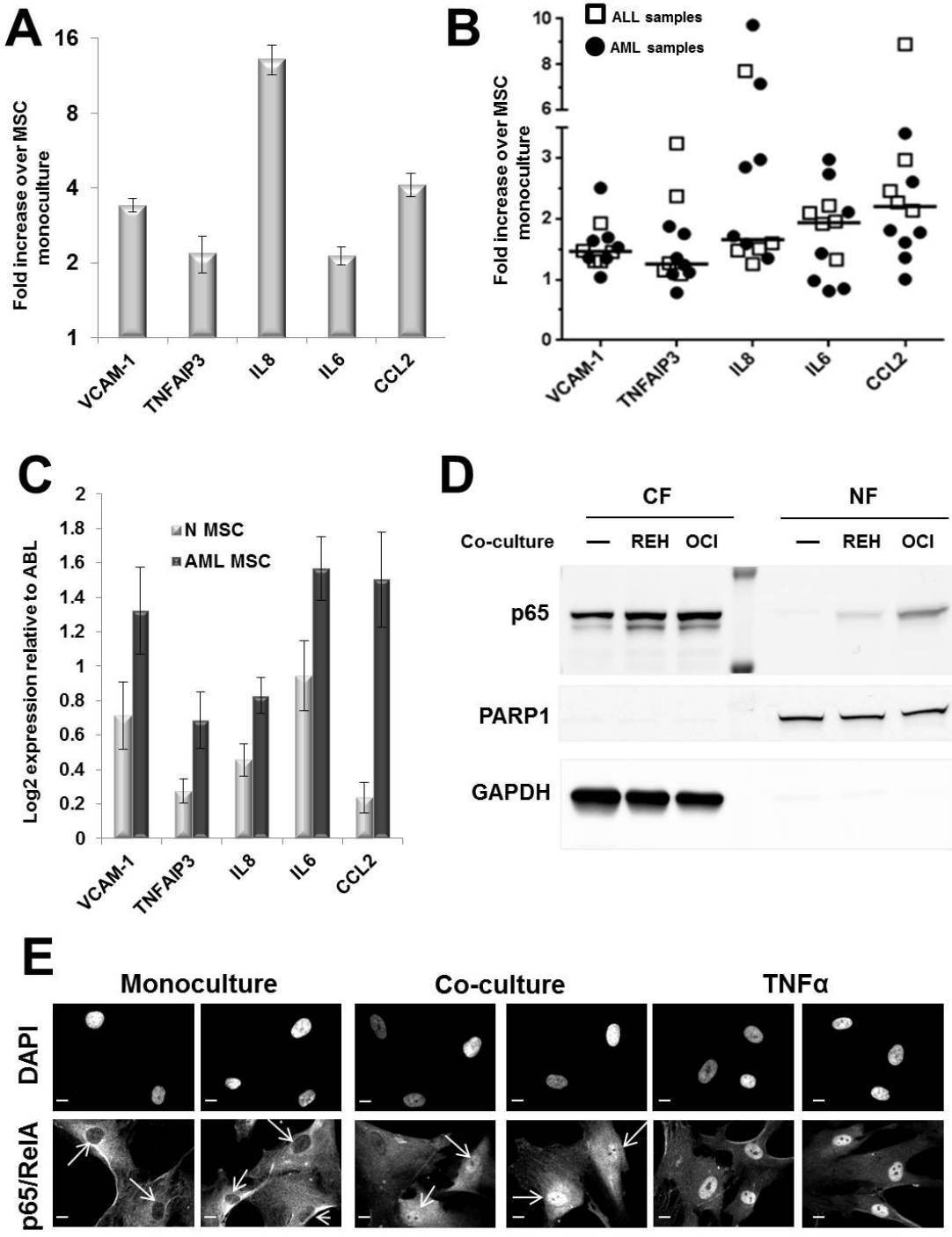
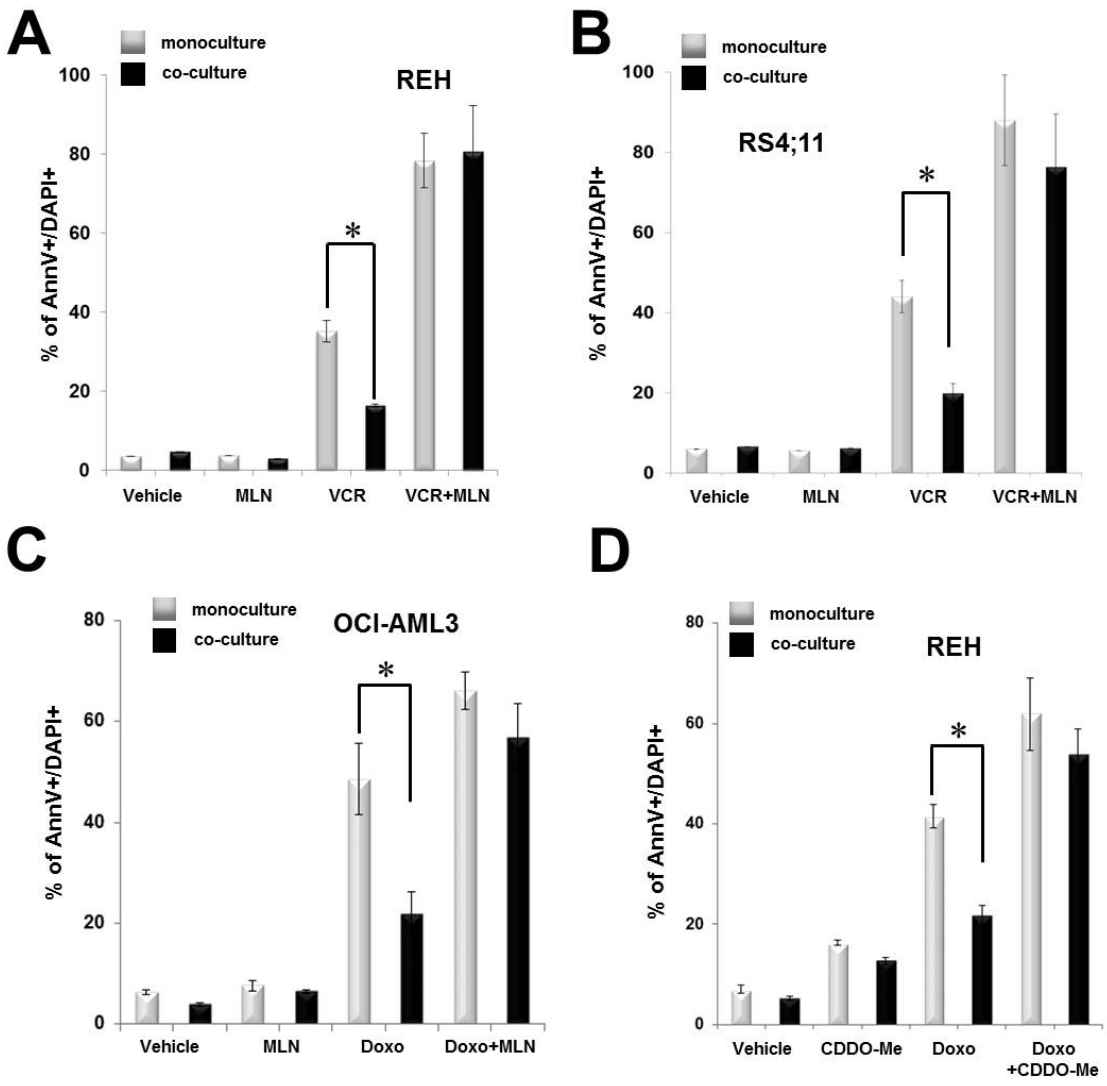


Figure 2



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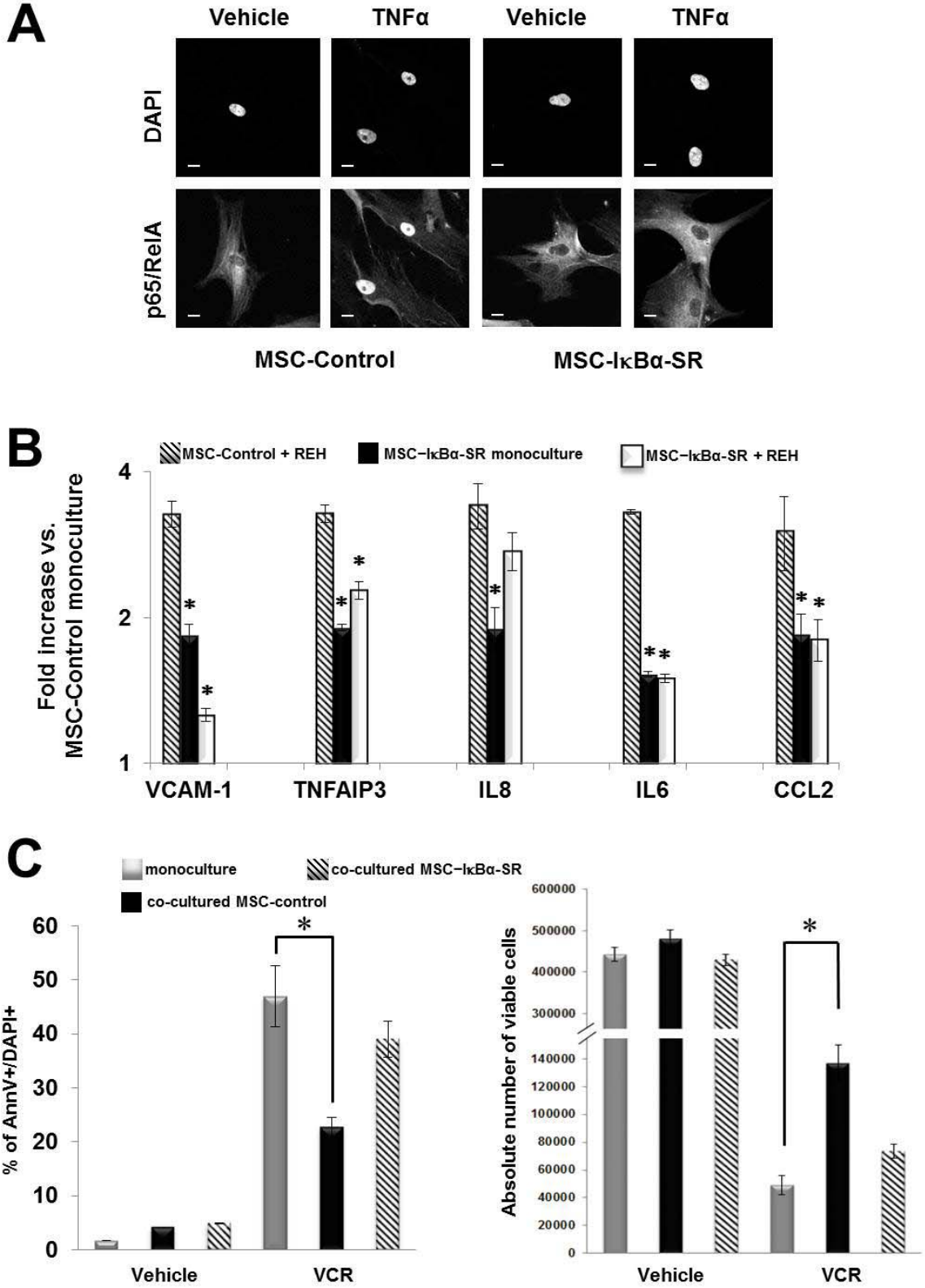

Figure 4

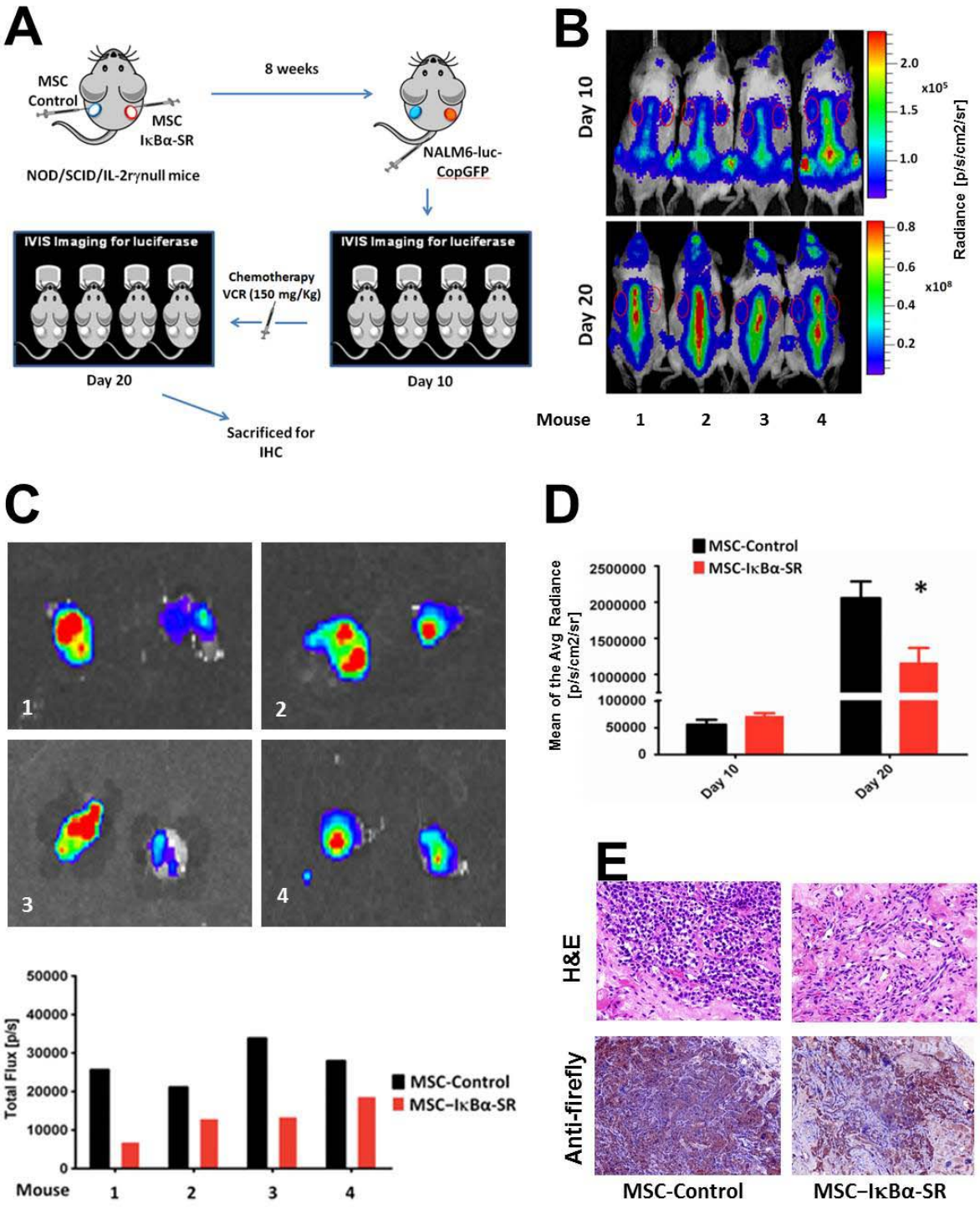

Figure 5

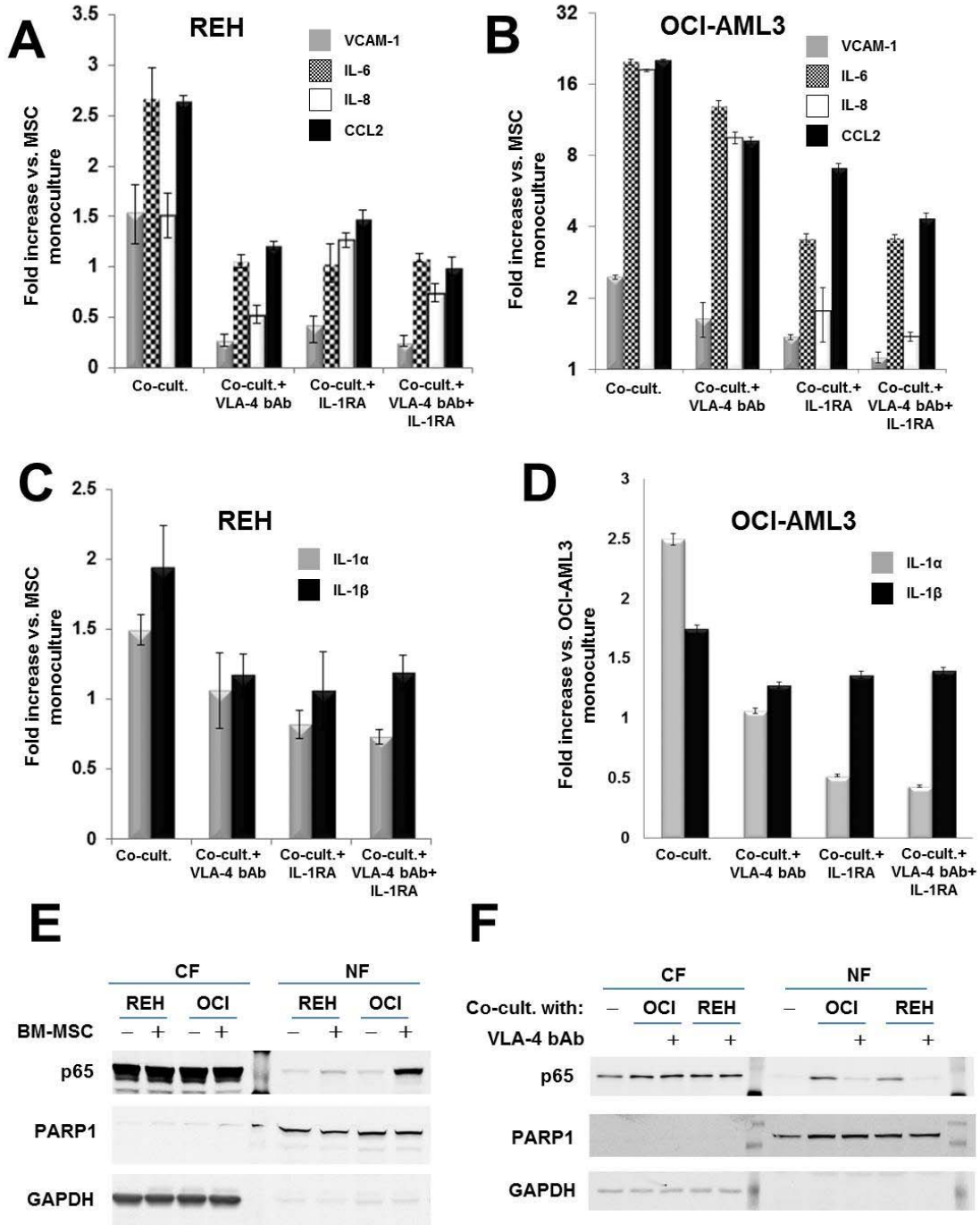

Figure 6