The IKKα-Dependent NF-κB p52/RelB Noncanonical Pathway Is Essential To Sustain a CXCL12 Autocrine Loop in Cells Migrating in Response to HMGB1

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High-mobility group box 1 (HMGB1) is a nonhistone, chromatin architectural protein that is ubiquitously expressed by all mammalian cells, but it functions outside cells as a potent cytokine and chemoattractant. In vivo, HMGB1 is passively released by necrotic cells and actively secreted by immune effector cells (1–4). Extracellular HMGB1 signals through the receptor for advanced glycation end-products (RAGE), TLR2, and TLR4 (3–9). In this capacity HMGB1 acts as an alarmin or damage-associated molecular pattern that senses tissue damage and elicits a variety of proinflammatory responses (reviewed in 3, 4, 6, 10, 11). Moreover, the chemotactic activity of HMGB1 is an important initiating aspect of the wound healing response and how cells migrate to repair damaged tissues (12, 13).

Cell migration to HMGB1 requires the action of several interconnected signal transduction pathways. RAGE ligand-induced cell migration requires RAGE interaction with diaphanous-1, which is required for Rac-1 and Cdc42-regulated cell movement (14). We have previously shown that cellular chemotaxis toward HMGB1 in vitro requires canonical NF-κB activation in a variety of cell types (fibroblasts, mesoangioblasts, macrophages, and neutrophils) in vitro and also for the respective migration of neutrophils and mesoangioblasts in vivo mouse models of HMGB1-elicited peritonitis and muscle damage (15, 16). HMGB1 induction of canonical NF-κB signaling and fibroblast chemotaxis requires ERK activation (17) and Src family kinases, which reorganize the cellular cytoskeleton and induce Src, FAK, and paxillin phosphorylation (18). Time-lapse video microscopy experiments have revealed that the inhibitor of NF-κB kinase (IKK)β and IKKα-dependent NF-κB signaling pathways are essential for cells to become polarized to an HMGB1 gradient, which is indicative of critical functional roles in the initial steps of directed cell movement (16). Finally, we have also reported that the activity of IKKβ-dependent canonical NF-κB signaling is mechanistically essential for cells to maintain RAGE expression for their HMGB1 migratory response, whereas the IKKα-driven non-canonical NF-κB p52-ReLB signaling pathway is simultaneously critical for HMGB1-elicited chemotaxis for a different reason (16).

In this study, we have defined the mechanism of action of the IKKα-driven NF-κB ReLB/p52 signaling pathway for HMGB1 chemotaxis. Surprisingly, for cells to migrate in response to HMGB1, the NF-κB noncanonical pathway is solely required to...
maintain an autocrine loop of CXCL12, also known as stromal cell-derived factor 1 (SDF1). A neutralizing CXCL12 mAb completely blocks the HMGB1 migration responses of fibroblasts and macrophages. In addition, incubating IKKα or NF-κB p52-deficient cells with a limiting amount of recombinant CXCL12 rescued their directed migration response to HMGB1 and NF-κB p52 KO fibroblasts engineered to express nearly physiologic levels of CXCL12 migrate in response to HMGB1 akin to wild type (WT) cells. Moreover, AMD3100, a specific antagonist of the CXCL12 G-protein coupled receptor CXCR4 (18–20) and an anti-CXCR4 mAb both prevented HMGB1 migration responses, indicating that the CXCL12 receptor CXCR4 in addition to the HMGB1 receptor RAGE is also an essential requirement for cell migration toward HMGB1. Our results reveal that cell migration toward HMGB1 requires the IKKα/α/noncanonical NF-κB pathway to ensure that migrating cells continuously secrete CXCL12/SDF1, which would either interact or cosignal with HMGB1 via their respective receptors CXCR4 and RAGE for cells to migrate in response to HMGB1.

**Materials and Methods**

**Conditional IKKα KO mice**

Mice with IKKα alleles flanked by LoxP recombination sites (IKKα<sup>fl/fl</sup>) that express Cre recombinase under the control of the macrophage lysozyme (M<sub>lyso</sub>) promoter only in mature macrophages (MΦ) and neutrophils (IKKα<sup>fl/fl:MLysCre</sup>) mice have been described previously (16). All animal work was approved by Stony Brook University’s Institutional Animal Care and Use Committee in accordance with U.S. National Institutes of Health grant guidelines.

**Reagents**

Full-length, LPS-free recombinant HMGB1 protein was obtained from HMGBiotech (Turin, Italy) and was rigorously tested to be LPS free by the manufacturer. Recombinant murine SDF1 was from PeproTech (Rocky Hill, NJ). Other reagents were as follows: 8-μm pore size cellulose nitrate filters (for macrophages) and polyvinylpyrrolidone-free polycarbonate filters (for fibroblasts) for 48-well microchemotaxis (Boyden-type) chambers (Neuroprobe, Cabin John, MD); fibronectin (Roche); human recombinant PDGF (R&amp;D Systems, Minneapolis, MN); human recombinant complement component 5a (C5a), 4-hydroxytamoxifen, and AMD3100 (Sigma, St. Louis, MO); Alexa Fluor 488-conjugated streptavidin (Molecular Probes, Eugene, OR); K15C mAb that specifically targets an amino terminal epitope in secreted by HMGB1-responding cells was neutralized using the SDF1 for their migratory response to HMGB1. CXCL12 actively produced by cells was essential for their HMGB1 migration response. To test

**Results**

**Neutralizing the CXCL12/SDF1 actively produced by cells completely blocks their migration response to HMGB1**

Recently, we reported conclusive in vitro and in vivo evidence that the IKKβ-dependent canonical and IKKα-dependent noncanonical signaling pathways were simultaneously essential for cells to migrate toward HMGB1 (16). Multiple lines of evidence point to RAGE, HMGB1’s ubiquitously expressed receptor, as the required target of the IKKβ-dependent RelA/p65 pathway, but the critical target or targets of the IKKα-dependent RelB/p52 pathway remained undefined. Basal and induced transcription of CXCL12/SDF1 directly depends on NF-κB p52/RelB heterodimers (25, 26), and we found that CXCL12 mRNA was modestly induced by HMGB1 with a dependency on IKKα (16). Therefore, we investigated the possibility that cells need to actively produce CXCL12/SDF1 for their migratory response to HMGB1. CXCL12 actively secreted by HMGB1-responding cells was neutralized using the K15C mAb that specifically targets an amino terminal epitope in CXCL12 (27). As shown in Fig. 1, K15C completely blocked both fibroblast and primary macrophage chemotactic responses to HMGB1. As expected K15C also completely blocked cell migration to CXCL12 itself as a positive control, but it had no effect on MEF and macrophage chemotaxis to their respective positive controls PDGF or C5a (Fig. 1). Moreover, cell migration assays performed with an irrelevant mouse IgG2a Ab as an isotype-matched negative control showed no effect on cell migration in response to HMGB1 or CXCL12/SDF1 (Fig. 1A).

**CXCL12/SDF1 rescues the defective HMGB1 migration responses of either IKKα- or p52-deficient cells up to the levels of WT cells**

Next, in light of the above results, we asked whether CXCL12/SDF1 was the sole IKKα/p52/RelB target whose production by cells was essential for their HMGB1 migration response. To test
this idea, we mixed recombinant CXCL12/SDF1α with either IKKα or p52 KO cells and assessed their migration response to an HMGB1 gradient. Indeed, supplementing IKKα conditional KO primary macrophages that were differentiated from the bone marrow progenitors of IKKαf/f;MLysCre mice (Fig. 2A) or IKKα and p52 KO MEFs (Fig. 2B) with only 5 ng/ml of CXCL12/SDF1 (one-tenth the necessary concentration for CXCL12/SDF1 migration assays) rescued their HMGB1 chemotactic responses. In addition, a dose response experiment with IKKα conditional KO macrophages showed that as little as 0.5 ng/ml of CXCL12/SDF1 was sufficient to rescue their HMGB1 migratory response (Supplemental Fig. 2). Although this type of ectopic addition experiment provides reasonable suggestive evidence that CXCL12 may be the target of the IKKα/noncanonical NF-κB pathway that is required for cell migration to HMGB1, this strategy alone is not sufficient to make this conclusion for two main reasons: 1) the low level of CXCL12 produced by WT cells in the context of short term cell migration assays performed in serum free media precludes its quantification by ELISA to determine whether this is comparable to the effective ectopic doses of CXCL12, and 2) the localized concentration of CXCL12 produced by WT cells is likely to be considerably higher at the cell surface before it diffuses into the surrounding media, and this cannot be replicated by ectopic CXCL12 addition to cells.

Next, as a more physiologically rigorous experiment that does not have the inherent caveats of adding CXCL12 to cells, we

**FIGURE 1.** HMGB1 Chemotaxis of MEFs and primary mature macrophages requires their production of CXCL12. Migration assays with primary WT MΦ (A) and immortalized WT MEFs (B) were performed in 48-well microchemotaxis chambers as described previously (16) in response HMGB1 (50 ng/ml), PDGF (10 ng/ml; MEF migration positive control), or C5a (2 nM; MΦ migration positive control) for 3 h. Neutralizing mAb against CXCL12 (K15C) was added to the wells where indicated at 30 μg/ml, which effectively blocks CXCL12 activity (27). Migration results with 30 μg/ml of a mouse IgG2A isotype control Ab are also shown in (A). Bars are distances migrated through filter pores (WT MΦ in A) or net cell movement per high-power field (HPF) after subtracting basal migration in serum free media (WT MEFs in B). Experiments were repeated three to five times each in duplicate. All error bars are SEM. Mean values of WT MΦ basal migration distances toward serum-free media controls were 35 ± 2.6 and 35 ± 3.3 μm with and without K15C, respectively, and 31 ± 1.6 μm for mouse IgG2a controls. Mean values of WT MEF basal migration toward serum-free media controls were 26 ± 1.1 and 22 ± 1.4 cells per HPF with and without K15C, respectively. The p values were determined by two-way (A) or one-way (B) ANOVA with Tukey post test. ***p < 0.001.

**FIGURE 2.** Supplementing IKKα or p52 KO cells with recombinant CXCL12/SDF1α rescues their migration responses to HMGB1. IKKα conditional KO primary MΦ (A) or immortalized IKKα or p52 KO MEFs (B) with or without supplementation by 5 ng/ml of recombinant CXCL12/SDF1 were subjected to HMGB1 migration assays as indicated, and PDGF served as a positive migration control for the KO cells. Experiments were performed three times for WT and five times for all KO samples, each in duplicate. Mean values of WT and IKKα KO MΦ basal migration distances toward serum-free media controls were 30 ± 1.0 and 31 ± 2.5 μm, respectively. Mean values of IKKα KO and p52 KO MEF basal migration toward serum-free media controls were 11 ± 4.0 and 6 ± 2.0 cells per HPF, respectively. Basal migration in serum-free media with independent preparations of WT MΦ and MEFs routinely varied between 20–25 μm and 15 to 20 cells per HPF, respectively, over the 3-h chemotaxis assay. Error bars are SEM. The p values were determined by one-way ANOVA with Tukey post test. *p < 0.05, ***p < 0.001. ns, Not significant.
enforced constitutive CXCL12 expression in p52 KO MEFs by stably transducing them with a murine Moloney retrovirus harboring a CXCL12-IRE5-GFP bicistronic expression cassette (21). We used a diluted stock of CXCL12-GFP retrovirus, so that p52 KO target cells would be infected by one retroviral particle per cell to avoid overexpressing the CXCL12 transgene. Under these limiting retroviral infection conditions ~4% of p52 KO cells were clearly GFP positive, and the latter cells were FACS purified for our migration assays (Supplemental Fig. 1). Next, we used a quantitative CXCL12 ELISA assay to compare the relative levels of CXCL12 produced by WT, p52 KO, and p52 KO CXCL12-GFP cells. To accurately quantify the levels of CXCL12 produced by each cell type, we prepared cell supernatants conditioned for 18–19 h from 2 × 10⁵ cells seeded in multiple wells of six-well plates. These quantitative ELISA assays showed that FACS-purified p52 KO CXCL12-GFP–positive cells produced CXCL12 at levels only somewhat higher than WT control MEFs, whereas p52 KO MEFs were completely deficient for CXCL12 secretion (Fig. 3A). We used the above strategy to validate that our engineered p52 KO CXCL12-GFP cells produced comparable levels of CXCL12 as WT MEFs, because the very low level of the cytokine constitutively produced by WT cells precluded its direct quantification by ELISA in the context of our cell migration assays, which were initiated with freshly prepared cells only 30–60 min after their resuspension in serum-free media. Importantly, p52 KO/CXCL12-GFP cells migrated toward HMGB1 with an efficiency that was statistically comparable to WT MEFs, whereas their p52 KO counterparts were completely negative for HMGB1-induced migration (Fig. 3B). Taken together, the above experiments definitively demonstrate that CXCL2/SDF1 is the sole target of the IKKα/noncanonical NF-κB pathway that is essential for cell migration in response to HMGB1.

Cellular chemotactic responses to HMGB1 are blocked by AMD3100, a specific CXCR4 antagonist and also by a monoclonal anti-CXCR4 Ab

CXCR4, a ubiquitously expressed G-protein coupled receptor, uniquely promotes CXCL12-mediated chemotaxis (28–31). In addition, it was reported previously that in addition to HMGB1’s ubiquitously expressed receptor RAGE, cellular chemotaxis toward HMGB1 also requires the activity of at least one G protein-coupled receptor, because pertussis toxin treatment abolishes the HMGB1 chemotactic response (12). Our data clearly show that noncanonical NF-κB–regulated CXCL12 production is critical for cell migrations toward HMGB1; therefore, we next began to investigate the potential role of CXCR4 in HMGB1–mediated chemotactic responses. To this end, we performed HMGB1 migration assays in the presence of AMD3100, a highly specific bicyclam nonpeptide antagonist of CXCR4 that has no inhibitory effects on other G protein-coupled receptors at concentrations up to 50 μg/ml (18–20). AMD3100 (10 μg/ml = 12.5 μM) completely ablated the HMGB1 migration responses of primary macrophages (Fig. 4A) and MEFs (Fig. 4B). As expected, this same dose of AMD3100 extinguished cell migration to CXCL12/SDF1 (as a positive control), but it had no effect on cell migration to the positive controls PDGF or C5a (Fig. 4A, 4B). To further demonstrate that the inhibitory effect of AMD3100 was specific for CXCR4, we performed additional experiments with several other independently prepared batches of in vitro-differentiated primary mature macrophages. First, we exposed WT macrophages to different concentrations of AMD3100 and found that a drug dose as low as 0.1 μg/ml (0.125 μM) was sufficient to inhibit cell migration to either HMGB1 or CXCL12/SDF1 to the same degree (Fig. 5A). Importantly, 0.125 μM is just above the IC₅₀ (IC₅₀) of AMD3100 previously shown to inhibit CXCL12/SDF–induced calcium flux in a variety of different cell types (20).

Finally, WT macrophage chemotaxis assays were also performed in the presence of a rat anti-mouse CXCR4 neutralizing mAb, which completely blocked migration responses to either HMGB1 or CXCL12/SDF1, but had no effect on their migration to C5a (Fig. 5B). In addition, cell migration assays performed with an irrelevant rat IgG2b Ab as an isotype-matched negative control showed no effect on cell migration in response to HMGB1 or CXCL12/SDF1 (Fig. 5B).

**Discussion**

Cell migration is a sophisticated, multistep phenomenon that is critical for animal development, innate and adaptive immunity, and the recognition and repair of damaged tissue. Inappropriate or dysregulated cell migration contributes to the progression of cancers, poor wound healing, inflammatory-induced tissue injury, and several other maladies (reviewed in 32, 33). In cell nuclei,
HMGB1 functions as an essential chromosome architectural protein, but outside cells it acts as a cytokine and chemoattractant. Moreover, extracellular HMGB1 allows the body to initially recruit inflammatory cells, which are followed by cells with the ability to orchestrate tissue regeneration and scar formation (3, 4, 13, 34).

HMGB1-mediated receptor engagement activates several signaling pathways, including NF-κB, Erk, and Src, that are each required to induce and maintain cell migration toward HMGB1 (15–17); however, the downstream targets of these pathways, whose activities are required for HMGB1 chemotactic responses, remain unclear. Although many targets of the NF-κB signaling pathways encode cytokines, chemokines, and receptors, which are systemically important for cell migration responses involved in immunity (reviewed in (35–37), NF-κB activity is not intrinsically necessary inside cells for their directed movement toward a variety of chemotactic stimuli (15, 16). As a result, the cell signaling machinery and regulation of HMGB1 chemotactic responses are expected to be considerably more complex than observed for most if not all other chemotaxants, because both major arms of NF-κB signaling (IKKβ-driven canonical and IKKα-driven noncanonical) are simultaneously critical for eliciting HMGB1-mediated cell migration responses. In this study, we have shown that sustained production of CXCL12/SDF1 by the IKKα-driven noncanonical NF-κB signaling pathway is essential for cell movement toward HMGB1. Thus, HMGB1 chemotactic responses are unique in requiring the IKKβ-driven canonical NF-κB pathway to maintain expression of HMGB1’s receptor RAGE by responder cells (16); and also as shown herein, the IKKα/noncanonical NF-κB regulated, functional interplay between cellularly produced CXCL12 and extracellular HMGB1.

As RAGE is the receptor for HMGB1, our results suggest that CXCL12 interplay with HMGB1 provides cells with an essential accessory or coreceptor signal via its receptor CXCR4 to enable directed cell movement toward HMGB1. Interestingly, secretion of HMGB1 by macrophages and dendritic cells was recently shown to enhance their chemotaxis toward recombinant CXCL12/SDF1 (38), suggesting that the combined action of HMGB1 and CXCL12/SDF1 signaling collaborate to enhance specific cell migration responses. Moreover, multiple reports have shown that in addition to evidence of HMGB1 interacting with CXCL12/SDF1 (38), HMGB1 also forms specific, functional complexes with a select number of endogenous and exogenous effectors (e.g., CpG-ODN5, LPS, IL-1β, nucleosomes) to enhance proinflammatory cytokine production by HMGB1-responding cells (39–46). Our results with AMD3100, a highly specific CXCR4 antagonist, and a neutralizing anti-CXCR4 mAb both reveal that the activity of CXCL12’s receptor CXCR4 is critical for HMGB1-mediated cell migration responses (Figs. 4, 5). Recently, we demonstrated that IKKβ KO macrophages, MEFs, and neutrophils were incompetent for HMGB1 migration and that this defect could be fully corrected by enforced overexpression of RAGE, a direct target of IKKβ-dependent NF-κB p65/p50 signaling (16), indicating that IKKβ-driven canonical NF-κB signaling was necessary to maintain RAGE expression for cells to migrate toward HMGB1. Interestingly, we find that IKKβ KO-RAGE MEFs, akin to their WT counterparts, require CXCR4 activity to migrate in response to HMGB1, because AMD3100 blocks their HMGB1-induced migration (Supplemental Fig. 3A). Other experiments with IKKβ KO-RAGE MEFs show that the K15C anti-CXCL12 mAb also blocks their HMGB1 migration (data not shown). Thus, even RAGE overexpression is not sufficient to supplant the need for the critical, additional requirement of CXCL12-CXCR4 en-

![FIGURE 4.](image-url) AMD3100, a CXCR4 antagonist, blocks WT MEF and MΦ migration in response to HMGB1. WT MΦ (A) and WT MEF (B) migrations were performed with or without the CXCR4 inhibitor AMD3100 (10 μg/ml) as indicated. Experiments were performed four times in (A) and three times in (B), each in duplicate. Mean values of WT MΦ basal migration distances toward serum-free media controls were 28 ± 3.0 and 29 ± 3.0 μm with and without AMD3100, respectively. Mean values of WT MEF basal migration toward serum-free media controls were 21 ± 3.0 and 16 ± 2.0 cells per HPF with and without AMD3100, respectively. All error bars are SEM. The p values were determined by one-way ANOVA with Tukey post test. **p = 0.0035, ***p < 0.001, ****p < 0.0001.

![FIGURE 5.](image-url) Exposure of WT MΦ to either low doses of AMD3100 or to an anti-CXCR4 neutralizing Ab show that CXCR4 activity is essential for cell migration in response to HMGB1. (A) Effects of different doses of AMD3100 on WT MΦ migrations as indicated. (B) WT MΦ migrations were done in the presence and absence of a neutralizing anti-CXCR4 mAb (15 μg/ml) as indicated. Migration results with 15 μg/ml of a rat IgG2b isotype control Ab are also shown in (B). Experiments were performed three to five times, each in duplicate. Mean values of WT MΦ basal migration distances toward serum-free media controls were 24 ± 4.0 μm and 30 ± 1.2 μm for rat IgG2b controls. All error bars are SEM. The p values were determined by one way ANOVA with Tukey post test. ***p < 0.001.
gagement for cell migration to HMGB1. However, unlike RAGE, enforcing CXCR4 in IKKβ KO cells failed to rescue their HMGB1 migration response (Supplemental Fig. 3B), and CXCR4 overexpression did not alter the barely detectable levels of cell surface RAGE on IKKβ KO cells (Supplemental Fig. 3C, 3D). On the basis of our collective data, we propose that CXCL12-mediated engagement of CXCR4 works as an essential coreceptor signal for RAGE receptor-dependent HMGB1 migration responses. Interestingly, CXCL12/SDFI binding to CXCR4 can also functionally couple to the output of other receptors. For example, CXCL12-CXCR4 engagement is also known to induce CXCR4-TCR heterodimerization, which is required for a variety of signaling outcomes in T cells, including enhanced gene transcription and cytokine production, increased calcium ion concentrations in conjunction with CXCR4 endocytosis, and CXCL12-mediated cell migration (47–49). Although we cannot formally rule out the possibility that CXCL12 engagement of CXCR4 might have other indirect effects such as enhancing HMGB1 binding to RAGE, we consider this latter possibility less likely because HMGB1 has been shown previously to directly bind to RAGE with higher affinity than other RAGE ligands (5, 50). Rather, we envision that the essential second cell signal delivered by CXCL12-CXCR4 engagement may either be necessary to collaborate with HMGB1-mediated RAGE signaling for cells to sense and move toward an HMGB1 gradient, or cell-secreted CXCL12 may be interacting with HMGB1 in a “piggy-backing” fashion to facilitate secreted CXCL12’s delivery to cell surface CXCR4, thereby permitting cell migration toward HMGB1. Future work will be directed toward the further elaboration of the remaining details of this novel mechanism.

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Disclosures

The authors have no financial conflicts of interest.

References

**Supplementary Figure Legends:**

**Suppl. Fig. 1. Effects of IKKα KO MΦ migration towards HMGB1 by different doses of SDF-1/CXCL12**

IKKα KO MΦ were supplemented with the indicated doses of CXCL12/SDF-1 and then tested for their ability to migrate towards HMGB1 (50 ng/ml). Mean values of IKKα KO MΦ basal migration distances towards serum free media controls were 23 ± 2 μm. Results are derived from one representative experiment. Error bars are standard deviation.

**Suppl. Fig. 2. FACS purification of retroviral transduced p52KO/CXCL12-GFP cells.**

NF-κB p52 KO MEFs were stably transduced by a 1:5 diluted stock of a murine moloney retrovirus expressing a bi-cistronic CXCL12-GFP expression cassette. Four days post infection GFP positive cells were FACS purified. GFP-Hi cells were ~4% of the entire cell population (upper panel). A cell scatter plot clearly identifies the GFP positive sub-population of cells (center panel); and a histogram of GFP-Low and GFP-Hi cells (bottom panel) indicates the gates used to purify the latter GFP-Hi cells, which were used for the CXCL12 ELISA assays and cell migration experiments in text Figure 3.
Suppl. Fig. 3. RAGE over-expressing cells require CXCR4 activity for HMGB1 migration; and CCXR4 over-expression does not affect levels of cell surface RAGE

(A) IKKβ KO-RAGE MEF migration to either HMGB1 or PDGF was performed in the presence or absence of AMD3100 (10 μg/ml) as indicated. Results shown are the averages of 2 independent experiments. Mean values of serum free media migration controls were 3 ± 1.0 and 4 ± 1.0 cells per HPF for IKKβ KO-RAGE cells with and without AMD3100 respectively. Error bars are standard error of the mean.

(B) WT and IKKβ KO-CXCR4 MEF migrations to either HMGB1 or PDGF were performed as indicated. Data are the averages of 2 independent experiments. Mean values of serum free media migration controls were 2.2 ± 1.4 and 1.1 ± 1.1 per HPF for WT and IKKβ KO-CXCR4 MEFs respectively. (C) Relative cell surface levels of RAGE on IKKβ KO, IKKβ KO-RAGE and IKKβ-KO-CXCR4 MEFs. MEFs were detached from tissue culture dishes by exposing them to PBS + 5 mM EDTA for 10 minutes at 4 ºC. All released cells were pelleted and re-suspended at 5 X 10⁶ cells/ml and stained with biotin-anti-RAGE (R&D systems) followed by alexa flour 488 conjugated streptavidin (Molecular probes). MEFs were also independently stained goat IgG followed by alexa flour 488 conjugated strepavidin to control for background staining. Next, cells were fixed and analyzed by flow cytometry. Positive cells were gated and RAGE MFI was determined with FlowJo (Tree Star Inc.). Data are presented as net RAGE MFI (RAGE MFI – IgG control MFI) for IKKβ KO, IKKβ KO-RAGE and IKKβ-KO-CXCR4 MEFs from one representative experiment. (D) CXCR4 cell surface expression by IKKβ KO and IKKβ KO-CXCR4
cells. MEFs were detached from tissue culture plates with PBS + 5 mM EDTA for 10 minutes at 4 °C. Cells were centrifuged and re-suspended at 5 X 10^6 cells/ml and stained with alexa flour 647 conjugated anti-CXCR4 antibody (BioLegend). Next, cells were fixed and submitted to flow cytometry. FACS data were analyzed with FlowJo (Tree Star Inc.) and are presented as dot plots of antibody isotype control (black), IKKβ KO (blue), and IKKβ KO-CXCR4 (red) MEFs of one representative experiment.