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Cell migration to CXCL12 requires simultaneous IKKα and IKKβ-dependent NF-κB signaling

Marianna Penzo[*,†], **David M. Habiel**[‡,§,#], **Mahalakshmi Ramadass**[‡,§,#], **Richard R. Kew**[#‡,§], and **Kenneth B. Marcu**[#†,‡,§]

[*]CRBA Laboratory and Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy

[†]Biochemistry and Cell Biology Dept., Stony Brook University, Stony Brook, New York 11794-5215, USA

[‡]Molecular and Cellular Biology Graduate Program, Stony Brook University, Stony Brook, NY, 11794 USA

[§]Department of Pathology, Stony Brook University Medical Center, Stony Brook, New York 11794, USA

[#] These authors contributed equally to this work.

Abstract

CXCL12 and its unique receptor CXCR4, is critical for the homing of a variety of cell lineages during both development and tissue repair. CXCL12 is particularly important for the recruitment of hemato/lymphopoietic cells to their target organs. In conjunction with the damage-associated alarmin molecule HMGB1, CXCL12 mediates immune effector and stem/progenitor cell migration towards damaged tissues for subsequent repair. Previously, we showed that cell migration to HMGB1 simultaneously requires both IKKβ and IKKα-dependent NF-κB activation. IKKβ-mediated activation maintains sufficient expression of HMGB1's receptor RAGE, while IKKα-dependent NF-κB activation ensures continuous production of CXCL12, which complexes with HMGB1 to engage CXCR4. Here using fibroblasts and primary mature macrophages, we show that IKKβ and IKKα are simultaneously essential for cell migration in response to CXCL12 alone. Non-canonical NF-κB pathway subunits RelB and p52 are also both essential for cell migration towards CXCL12, suggesting that IKKα is required to drive non-canonical NF-κB signaling. Flow cytometric analyses of CXCR4 expression show that IKKβ, but not IKKα, is required maintain a critical threshold level of this CXCL12 receptor. Time-lapse video microscopy

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Correspondence to: Prof. Kenneth B. Marcu, Biochemistry and Cell Biology Dept., Stony Brook University, Stony Brook, NY 11794-5215 USA. Phone: +1.631.632.8553; Fax; +1.631.632.9730 kenneth.marcu@stonybrook.edu.
[#]Present Addresses for DMH and MR are respectively: Cedar's Sinai Medical Center, Los Angeles, CA and Scripps Research Institute, La Jolla, CA

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CONFLICT OF INTEREST
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experiments in primary MEFs reveal that IKKα is required both for polarization of cells towards a CXCL12 gradient and to establish a basal level of velocity towards CXCL12. In addition, CXCL12 modestly up-regulates IKKα-dependent p52 nuclear translocation and IKKα-dependent expression of the CXCL12 gene. On the basis of our collective results we posit that IKKα is needed to maintain the basal expression of a critical protein co-factor required for cell migration to CXCL12.

Keywords

CXCL12; CXCR4; cell migration; NF-κB; IKKα; IKKβ

INTRODUCTION

The chemokine CXCL12, also known as stromal cell derived factor 1 (SDF-1), regulates the migration and homing of stem/progenitor cells. Along with its unique receptor, CXCR4, CXCL12 exerts a pivotal role during embryogenesis for lymphopoiesis, myelopoiesis, cardiogenesis, angiogenesis and neurogenesis (1). In postnatal life, CXCL12/CXCR4 signaling plays an important role in the homing of hematopoietic and lymphopoietic cells, and in the targeted trafficking of stem/progenitor cells during tissue repair and/or regeneration. Moreover, since CXCR4 is expressed by several types of cancer cells, CXCL12 exerts pleiotropic effects regulating processes essential to tumor cell metastasis {reviewed in (2)}. Binding of CXCL12 to CXCR4 activates multiple signaling pathways including MAPK p42/44, Jak/STAT and the PI-3K-AKT-NF-κB axes (3-7). CXCR4 expression is regulated by NF-κB and the CXCR4 promoter contains p50/p65 binding sites (6, 8), Moreover, CXCL12 stimulation of mesoangioblasts (blood vessel stem cells) triggers NF-κB p65 nuclear translocation, which is required for mesoangioblast migration in response to CXCL12 (9).

NF-κB transcription factors mediate stress-like inflammatory responses, participate in developmental programming and regulate normal and malignant cell growth and survival (10-12). In the canonical NF-κB signaling pathway, specific NF-κB subunits are released from their inhibitory IκBs by virtue of the serine-threonine kinase activities of the IKK signalosome. In response to extracellular stress signals, IκBα is phosphorylated by the IKK complex thereby targeting it for ubiquitination and subsequent proteasomal destruction. The removal of IκB results in exposure of the nuclear localization sequence of NF-κB, leading to nuclear translocation of NF-κB hetero-dimers (including p65/RelA:p50 and c-Rel:p50) and the subsequent activation of their target genes (13). The IKK complex consists of two serine-threonine kinases, IKKα and IKKβ, and NEMO/IKKγ, a regulatory or docking protein that facilitates IKK complex assembly and the regulated transmission of upstream activating signals to IKKα and IKKβ (13-15). Most extracellular stimuli cause only transient IKK activation and mechanisms to limit IKK activity are physiologically important because persistent NF-κB activity is associated with numerous pathological conditions.

IKKβ is almost always the IκBα kinase *in vivo*, although IKKα can infrequently assume this role (16, 17). In contrast to IKKβ, IKKα functions to attenuate or resolve acute

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inflammatory responses by more than one mechanism (18-20) and is the essential kinase activator of the non-canonical NF-κB pathway (21). Upon activation by the NF-κB inducing kinase (NIK), IKKα phosphorylates specific serine residues in the carboxy-terminal domain of NFκB2/p100, leading to its proteasomal processing into the mature NF-κB p52 subunit and subsequent nuclear translocation of p52-RelB hetero-dimers (22). Non-canonical NF-κB activation also requires new protein synthesis and thus is not activated as rapidly as the classical IKKβ/NEMO dependent pathway. Moreover, only a few extracellular signals have been identified that can activate the NIK-IKKα non-canonical NF-κB pathway (including CD40L, LTβ and BAFF) (11). Interestingly, extracellular stimuli resulting in cellular responses that require sustained NF-κB induction appear to activate both the IKKβ/NEMO and NIK/IKKα signaling pathways (23-26). In addition to driving nuclear translocation of RelB/p52 heterodimers, the IKKα dependent non-canonical pathway has also been reported to activate p65/p52 (25) and a subset of p50/p65 heterodimers (sequestered in the cytoplasm in p100 complexes) (26), suggesting that IKKα can also contribute to certain pro-inflammatory responses.

Although a host of cytokines and chemokines are targets of NF-κB transcription factors, only a paucity of data (and only in either immortalized or engineered cell lines *in vitro)* has suggested that canonical NF-κB activation in migrating cells may contribute to their chemotactic responses (27-29). We have previously shown that both the IKKβ-driven canonical and the IKKα-dependent p52/RelB non-canonical NF-κB pathways are simultaneously critical for cell migration to HMGB1 (30, 31). Even though it is well established that HMGB1 (32-34) and CXCL12 (6, 8, 35-38) both activate the canonical NF-κB pathway, until our recent published work, it was not known if their unique chemotactic properties require cells to express specific NF-κB target genes needed for cells to migrate towards these two chemoattractants. Here we show that IKKβ and IKKα mediated canonical and non-canonical NF-κB signaling pathways are essential for the migration of fibroblasts and macrophages in response to CXCL12. IKKβ, but not IKKα, is required to maintain a threshold level of cell surface CXCR4, which is needed to maintain CXCL12-elicited chemotaxis. In conjunction with the latter functional role of IKKβ, IKKα, (via its unique function to activate the RelB/p52 non-canonical NF-κB pathway), is critically important for the initial polarization and velocity of cell movement towards a CXCL12 gradient.

MATERIALS AND METHODS

1.1 Ethics Statement

All animal work was approved by the IACUC committee of Stony Brook University in accordance with USA NIH guidelines for the use of animals in biomedical research. These studies utilized only *in vitro* experiments with primary embryonic fibroblasts (MEFs) or bone marrow progenitors (BMPs) isolated from the femurs of adult mice and subsequently differentiated to mature macrophages in vitro. Mouse pups or adult mice were euthanized by an IACUC approved protocol prior to the isolation of MEFS or BMPs.

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1.2 Conditional and inducible IKKα KO mice

Mice with IKKα alleles flanked by LoxP recombination sites (*IKKα$^{f/f}$ mice*) that express Cre recombinase under the control of the macrophage lysozyme (MLys) promoter only in mature macrophages (MΦ) and neutrophils (*IKKα$^{f/f}$:MLysCre mice*) (30). Alternatively, other mice express a tamoxifen (4-OHT) inducible Cre gene (*IKKα$^{f/f}$:CreERT2 mice)* that have been previously described (30). All animal work was approved by Stony Brook University's IACUC committee in accordance with NIH guidelines.

1.3 Reagents

Recombinant murine CXCL12/SDF-1 was obtained from PeproTech (Rocky Hill, NJ). Human recombinant PDGF and human recombinant complement C5a were purchased from R&D Systems (Minneapolis, MN); purified fibronectin was obtained from Roche (Indianapolis, IN). Tamoxifen (4-hydroxytamoxifen, 4-OHT) was obtained from Sigma-Aldrich (St. Louis, MO); Alexafluor 647-conjugated anti-mouse CXCR4 antibody was purchased from Biolegend (San Diego, CA). All materials for the in vitro cell migration assays were obtained from Neuroprobe (Cabin John, MD) and included 48 well microchemotaxis chamber and 8 μm pore size cellulose nitrate filters (for macrophages) and 8 μm pore size PVP-free polycarbonate filters (for fibroblasts).

1.4 Cells and tissue culture

Immortalized WT, IKKα KO, p52 KO and RelB KO MEFs were maintained as previously described in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Bone marrow progenitors from the femurs of IKKα WT (*IKKα$^{f/f}$*), *IKKα$^{f/f}$:MLysCre* and *IKKβ$^{f/f}$:MLysCre* adult mice were differentiated to MΦ in M-CSF conditioned DMEM/10%FBS for 7 days as previously described (30); and the loss of IKKα or IKKβ in myeloid cell progenitors does not affect the efficiency of their differentiation to mature macrophages or neutrophils (30). Primary MEFs were isolated from 5-6 day old *IKKα$^{f/f}$:CreERT2* mouse embryos also as described in prior reports (30, 31).

1.5 Retroviral transduction

IKKβ and IKKα KO MEFs were stably transduced with a Moloney murine retroviral vector containing a murine CXCR4 cDNA expressed as part of a bi-cistronic IRES-Puromycin expression cassette (39). Murine CXCR4 cDNA was subcloned upstream of an IRES-puromycin cassette in the BIP murine Moloney retroviral vector (40, 41). The generation of amphotyped viruses, infection of cells and selection of stable puromycin resistant cell populations have been previously described (30, 40, 41).

1.6 In vitro chemotaxis assays

Chemotaxis assays with MEFs and MΦ were performed as previously described (30, 31). MEF and MΦ migration assays were performed with 5×10^4 and 1×10^5 cells respectively per well of a 48 well microchemotaxis (Boyden-type) chamber. Chemotaxis of MEFs utilized 8 μm pore size PVP-free polycarbonate filters pre-coated with fibronectin (50 μg/ml) and chambers were incubated for 3 hours at 37°C. MEFs were quantified as the

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number of migrated cells per high power field (400×), and the background control (serum-free media) was subtracted so data are expressed as net migrated cells. Macrophage chemotaxis employed cellulose nitrate filters (8 μm pore size) in chambers incubated for 3 hours at 37°C. Distances of macrophages migrated into the nitrocellular filters were measured by the leading front method as previously described (42). Macrophage migration data is presented as net cell movement per 3 hours. In our experience and prior work (30, 31), nitrocellulose filters, which measure the leading front distance of migrating cells, is a superior and more sensitive technique for myeloid cells (including macrophages, monocytes and neutrophils) than counting cells per high power field with polycarbonate filters. Mesenchymal cells such as fibroblasts do not migrate into the nitrocellulose filters, even when employing very large pore size (12 μm) filters. For fibroblast migration assays we used thin (10 μm depth) polycarbonate filters with straight 8 μm holes to count cells adhering to the underside of the filter.

For IBIDI time-lapse chemotaxis experiments, cells of different genotypes were distinguished from each other by staining with 500 nM Green CMFDA or 500 nM Orange CMRA CellTracker™(Invitrogen) for 15 min in a humidified tissue culture incubator. Labeled cells were washed, resuspended at 4×10^5 cells/ml in DMEM (phenol red free) +0.1% BSA, mixed and placed (300 μl) in a μ-Slide (IBIDI/Integrated BioDiagnostics Inc.) pre-coated with 50 μg/ml fibronectin. After cells had firmly attached to the substratum, slides were laid on a 37°C humidified stage of an UltraVIEW ERS Spinning Disk confocal microscope (Perkin Elmer). A 0-30 μg/ml CXCL12 gradient, mixed with fluorescent beads (Molecular Probes) for microscope viewing, was allowed to form in the chamber's channel as described (43). Pictures of cells at the edge of the gradient were captured every 2 minutes for up to 3 h (microscope objective Zeiss, 5X magnification, numerical aperture 0.15; EM-CCD Hamamatsu C9100 Camera; UltraVIEW ERS acquisition software). Directional tracks are defined as those with ending points closer to the CXCL12 source compared to their starting points, whereas non-directional tracks are the opposite. Indeterminate tracks starting and ending at the same distance from the CXCL12 gradient (moving laterally) are also considered non-directional tracks. Cell tracks were analyzed with ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997-2008).

1.7 Flow cytometry

Primary mature macrophages were detached from cell culture dishes by incubation in DPBS containing 10% EDTA for 10 minutes at 4°C. Cells were resuspended at 5×10^6 cells per ml in flow cytometry staining buffer (DPBS + 1% BSA + 0.02% NaN_3); and 5×10^5 cells were blocked with 2 μg of mouse IgG for 15 minutes on ice and subsequently stained with 1 μg of Alexafluor 647-conjugated anti-mouse CXCR4 antibody (Biolegend) for an additional 15 minutes on ice. Cells were washed twice with flow cytometry staining buffer and then fixed in 2 % paraformaldehyde prior to analysis. For intracellular staining, macrophages were permeabilized by washing twice with 1× BD perm/wash buffer (BD Biosciences, San Jose, CA). Next, cells were resuspended in 100 μl of perm/wash buffer and blocked with 2 μg of mouse IgG for 30 minutes on ice. Cells were stained with 1 μg of fluorophore conjugated anti-CXCR4 antibody for 30 minutes on ice and then washed twice with perm/wash buffer

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and fixed with 2% paraformaldehyde. Flow cytometric data were collected using a BD FACS Calibur II flow cytometer and the data were analyzed using FlowJo (Tree Star Inc., Ashland, OR). As a negative control for the specificity of the anti-CXCR4 antibody, cells were stained with an isotype control antibody conjugated with the same fluorophore.

1.8 Immunoblotting and cell fractionation

Immunoblotting and the preparation of nuclear and cytoplasmic fractions were performed as previously described (30, 41). HRP conjugated secondary antibody signals were detected with an enhanced chemiluminescent detection kit (GE Healthcare, Piscataway, NJ). Nuclear p52 levels were normalized with respect to nuclear lamin B1. Film images were acquired with a Fluor-S Multimager (BioRad). Scanned protein bands were quantified with Quantity One 4.5.0 software (BioRad) and processed with Adobe Creative Suite.

1.9 Sybr green real time RT-PCR assays

Total cell RNAs were prepared and Sybr green real time RT-PCR was performed and quantified as previously described (30) Forward (F) and reverse (R) PCR primer sequences were as follows:

Gapdh F (GCTCACTGGCATGGCCTTC),

Gapdh R (CCTTCTTGATGTCATCATACTTGGC);

Cxcl12 F (GCACGGCTGAAGAACAACAAC),

Cxcl12 R (TTCCTCGGGCGTCTGACTC).

1.10 Statistical analysis

P values were determined with either Prism V4.0 Software or InStat (both obtained from GraphPad Inc., San Diego, CA) to 4 significant figures by two tailed Student's t test, one way or two way ANOVA with Tukey's multiple comparisons post test as indicated in the figure legends of specific experiments.

RESULTS

2.1 The IKKα and IKKβ driven NF-κB pathways are both required for cell migration in response to CXCL12

We have previously shown that the NF-κB canonical pathway is required for cell migration to HMGB1 and CXCL12 but not to other cytokines (9), and that the IKKβ and IKKα-driven canonical and non-canonical NF-κB pathways are both essential for cell migration to HMGB1 (30, 31). Akin to our findings with HMGB1-mediated cell migration, we find that IKKβ and IKKα are essential for the migration of immortalized MEFs (Figure 1A) but not to a PDGF positive control. To determine if this was also a property of other cell types lacking IKKα or IKKβ, we performed analogous experiments with mature primary macrophages differentiated from bone marrow progenitors of *IKKβ*$^{f/f}$*;MLysCre* and *IKKα*$^{f/f}$*;MLysCre* mice, in which IKKβ or IKKα respectively are absent in mature myeloid cells. Immunoblots demonstrating that IKKβ and IKKα proteins are not expressed by mature myeloid cells

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derived from these mice were reported previously (30) and other representative examples are shown in Suppl. Figure 1A. Indeed, akin to IKKβ and IKKα KO MEFs, mature macrophages derived from these mice also are unable to migrate to CXCL12 as compared to mature macrophages from WT mice. In contrast, macrophages from each of these 3 mouse strains migrate equally well to the positive control, complement activation fragment C5a (Figure 1B). The essential IKKα requirement for chemotaxis in response to CXCL12 is functionally linked to the non-canonical NF-κB pathway, because MEFs lacking either the non-canonical NF-κB subunits p52 or RelB also failed to migrate towards CXCL12, while their ability to respond to PDGF was similar to that of WT MEFs (Figure 1C).

2.2 IKKβ is required to maintain sufficient levels of CXCR4

Since the CXCL12 receptor CXCR4 is a direct target of the canonical NF-κB pathway (6, 8), we performed experiments to investigate if the essential requirement for IKKβ was linked to significant differences of CXCR4 expression levels in WT and IKKβ KO cells. To this end, we analyzed the relative expression levels of total cellular and cell surface CXCR4 protein in WT and conditional IKKβ KO *IKKβ$^{f/f}$;MLysCre* mature macrophages by quantitative flow cytometry. We performed this analysis by flow cytometry, and not by immunobloting nor by immunofluoresence staining of cells, because only flow cytometry provides a quantitative assessment of CXCR4 expression on a single cell basis. This analysis of total cellular and cell surface levels of CXCR4 in multiple batches of WT and IKKβ KO macrophages revealed that the expression levels of CXCR4 were significantly reduced in IKKβ KO cells relative to their WT counterparts (Figure 2A). Since cell surface CXCR4 is internalized upon binding to its ligand CXCL12 (44, 45), we observed a significantly greater reduction of cell surface CXCR4 with IKKβ KO Macs compared to WT Macs after 2 hr of exposure to CXCL12 (see results shown as fold change in MFI with an without CXCL12 treatment for total and cell surface levels of CXCR4 in Figure 2A&B respectively). Similar to our observations with primary mature macrophages, we also noted reduced cell surface CXCR4 levels in IKKβ KO MEFs compared to WT or NF-κB p52 KO MEFs when cells were treated with CXCL12 (Supplementary Figure 2). Representative quantitative flow cytometry experiments comparing cell surface and total CXCR4 expression in un-stimulated versus CXCL12-treated cells are shown in Supplementary Figure 3. In addition, these quantitative experiments demonstrate that total levels of CXCR4 in unstimulated IKKβ KO primary mature macrophages are significantly reduced compared to their WT counterparts (Supplementary Figure 3C). To investigate if the IKKβ-dependent maintenance of a critical, threshold level of cell surface CXCR4 is functionally linked to IKKβ's essential requirement for cell migration to CXCL12, we enforced the expression of murine CXCR4 in both IKKβ and IKKα KO MEFs by stable retroviral transduction and evaluated their migration in response to CXCL12 by quantitative flow cytometry analysis. The enhanced cell surface expression of CXCR4 in these cells, as well as the specificity of the anti-CXCR4 antibody (31), was verified as shown in Supplementary Figure 4. Indeed, the CXCL12-induced migration of IKKβ KO MEFs was rescued by the enforced expression of exogenous murine CXCR4; in contrast, IKKα KO MEFs expressing similar levels of exogenously introduced CXCR4 still failed to migrate towards CXCL12 (Figure 3).

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2.3 IKKα is required at the earliest phase of CXCL12-mediated cell migration

To begin to explore the mechanism of action of IKKα in CXCL12 induced cell migration, we dissected the migration parameters of WT vs. induced IKKα KO primary MEFs in time-lapse microscopy experiments employing IBIDI cell migration slides (30, 43). In vitro migration assays were performed with primary *IKKα$^{f/f}$;CreERT2* MEFs sham-treated (WT control) or treated with 4-OHT (IKKα induced deletion) in IBIDI μ slides as previously described (30). Immunoblotting confirmed the ablation of IKKα protein expression in *IKKα$^{f/f}$;CreERT2* MEFs exposed to 4-OHT (30) (Supplementary Figure 1B). As shown in Figure 4A, IKKα is required in primary MEFs for migration towards CXCL12, as it is in immortalized MEFs (shown above in Figure 1). The functional impact of IKKα for directional chemotaxis is best determined at the earliest phase of the migration response, which defines cellular polarization/orientation towards the CXCL12 gradient. In this analysis, we defined directional tracks as those with ending points closer to the higher CXCL12 concentration in the gradient, compared to their starting points, whilst non-directional tracks are the opposite; and indeterminate tracks starting and ending at the same distance from the CXCL12 gradient (moving laterally) are also considered non-directional tracks {see detailed description of how the IBIDI experiments were performed in Materials and Methods} (30, 43). These time lapse time video microscopy experiments revealed that IKKα is critical for determining initial cellular orientation/polarization towards a CXCL12 gradient (compare directions of migration tracks in WT vs, induced IKKα KO cells in Figure 4B and 4C respectively and see statistically analyzed bar graph results summary in Figure 4C). The Euclidean distance (e.g. the straight line distance between starting and arriving points) and velocity of all tracks were compared to each other to ensure that all cells in the population were analyzed equally for these two migration parameters independent of their directionality to avoid biasing the results solely on the cells migrating directly towards the CXCL12 gradient. Importantly, Figures 4D and 4E reveal that IKKα is also required for optimal Euclidean migration distance and for the degree of cellular velocity within the initial 60 minutes of the chemotactic response to CXCL12.

2.4 CXCL12 up-regulates the NF-κB non-canonical pathway

Because the IKKα dependent NF-κB non-canonical pathway is required for the earliest phase of migration in response to CXCL12 (polarization and initial velocity), these results suggest that the IKKα /p52/RelB axis could be necessary to maintain a sufficient basal level of a critical protein co-factor. In conjunction with such a mechanism, CXCL12 may also stimulate IKKα dependent p52 nuclear translocation to up-regulate the expression of a subset of IKKα/RelB/p52-dependent genes. To explore the latter possibility, MEFs were exposed to CXCL12 for up to 8 hours and cytoplasmic and nuclear cell fractions were scored for their levels of p100 (p52's precursor) and the processed p52 NF-κB subunit. Representative immunoblots are shown in Figure 5A-C with lamin-B1 and β-tubulin serving as normalization/reference controls for nuclear and cytoplasmic proteins respectively. Cells treated for a similar duration with anti-murine lymphotoxin β receptor (mLTβR) antibody, an agonist which is known to robustly activate IKKα-dependent non-canonical NF-κB signaling (46), served as a positive control. Quantitative densitometric analysis of multiple immunoblots of nuclear and cytoplasmic extracts of WT vs. *IKKα$^{-/-}$* cells indicate that

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CXCL12, as we previously had shown for HMGB1 (30), modestly induces p52 nuclear translocation in WT but not in $IKK\alpha^{-/-}$ MEFs (Figure 5E). In contrast, and as expected, LTβR stimulation induced robust p100>p52 processing in WT MEFs, while no effect was seen in IKKα null cells (Figure 5E). The reduced levels of cytoplasmic and nuclear p52 (in comparison to its p100 precursor) observed in IKKα null MEFs in Figure 5 are in agreement with prior reports showing that IKKα is required to maintain a constitutive, basal level of cytoplasmic and nuclear p52 (46, 47). Indeed, quantitative densitometric analysis of immunoblots from multiple independent experiments show that unstimulated IKKα KO MEFs present a reduced degree of p100 > p52 processing (revealed by their relative p52/p100 ratios) in comparison to WT MEFs (Figure 5D). In contrast to IKKα KO MEFs, similar independent experiments show that unstimulated IKKβ KO MEFs present p52/p100 ratios of 85±12.5%, which are comparable to WT MEFs

In light of the above biochemical observations, we next investigated if IKKα was also required for the induction of RelB/p52 target gene expression in response to CXCL12. Because the CXCL12 gene is well known direct target of RelB/p52 NF-κB heterodimers, we analyzed CXCL12 mRNA expression levels by qRT-PCR in WT and IKKα KO MEFs in response to CXCL12 with LTβ receptor activation serving as a positive control. Indeed as shown in Figure 6, CXCL12 is a modest IKKα-dependent inducer of its own expression.

DISCUSSION

Cell migration is a sophisticated, multi-step process critical for animal development, innate and adaptive immunity and the recognition and repair of damaged tissue. Inappropriate and/or dysregulated cell migration contributes to the progression of cancers, poor wound healing, inflammatory-induced tissue injury and other maladies {reviewed in (48, 49)}.

3.1 Cell migration responses towards CXCL12 are mechanistically related to chemotactic responses to HMGB1

CXCL12 (SDF-1), along with HMGB1, belong to a small group of chemoattractants that are essential for stem cell trafficking (50-54). Consequently, these molecules are critically important for the recognition and repair of damaged tissue *in vivo* (50, 53, 54). Extracellular HMGB1 engages the cell surface Receptor for Advanced Glycation End-products (RAGE) and thereby elicits a range of inflammatory reactions by acting as a cytokine and chemoattractant (33, 34, 55, 56). The pertussis toxin sensitivity of HMGB1 chemotactic responses suggested early on that at least one G-protein coupled receptor is also required for HMGB1-mediated cell migration responses (57); and recent work by our lab and others has shown that CXCR4 functions as this essential receptor for HMGB1-mediated cell migration responses (31, 58). HMGB1 forms functional complexes with a variety of endogenous and exogenous effectors (including CpG-ODNS, LPS, IL-1β and nucleosomes) to dramatically enhance inflammatory responses (59-62); and HMGB1-partner complexes signal through the specific partner molecule's receptor, independent of HMGB1 receptors, to dramatically enhance the production of cytokines associated with inflammatory responses (63). Recently, we reported that the HMGB1-mediated cell migration responses require extracellular CXCL12, which is secreted by cells migrating towards HMGB1 (31, 64). HMGB1 forms

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functional complexes with CXCL12 (64); and the mechanism of cell migration towards HMGB1 involves HMGB1-CXCL12 complexes signaling via CXCR4 (31, 58), which is believed to be sufficient to drive cell migration towards HMGB1 in the absence of other HMGB1 receptors (58). Interestingly, CXCL12 ligation of CXCR4 activates some of the same pathways as HMGB1 including canonical NF-κB signaling, moreover, CXCR4 (6, 8) and its ligand CXCL12 (46, 65), are direct targets of the IKKβ-dependent canonical and IKKα-dependent non-canonical NF-κB pathways, respectively. Importantly and unlike other cell migratory responses, we have previously shown that chemotaxis to HMGB1 or CXCL12 *in vitro* and *in vivo* uniquely requires activating of the canonical NF-κB pathway (9, 57). Furthermore, we have also previously shown that the IKKβ/canonical and IKKα/non-canonical NF-κB signaling pathways are simultaneously required for HMGB1 chemotactic responses *in vitro* and *in vivo* (30, 31), with IKKβ signaling necessary to maintain sufficient levels of RAGE (30); and IKKα signaling essential for maintaining a sufficient expression of its direct target CXCL12 for cells to migrate towards HMGB1/CXCL12 complexes (31, 58). In this new study we present evidence that the IKKβ and IKKα-driven NF-κB signaling pathways are also both essential for cell migration to CXCL12 alone, independent of HMGB1, thereby linking the regulation of cell migration to the two unique chemoattractants mediating cell recruitment to damaged tissues.

3.2 IKKβ and IKKα-dependent NF-κB signaling are simultaneously essential for directed cell movement towards CXCL12/SDF-1

Our data collectively points to constitutive IKKβ-dependent canonical NF-κB signaling as essential to maintain a specific threshold level of cell surface CXCR4, the sole receptor that positively mediates directed cell migration to CXCL12. Thus, on the basis of the new data presented here and our prior work (30), IKKβ-dependent canonical NF-κB signaling works by a common mechanism of action to regulate chemotactic responses to HMGB1 and CXCL12 by maintaining sufficient expression levels of the receptors for these two cytokines.

In contrast, the critical need for IKKα-dependent non-canonical NF-κB p52/RelB activation for cell migration towards CXCL12 is not linked to CXCR4 expression levels. Although the precise downstream target of the non-canonical NF-κB IKKα/p52/RelB signaling axis needed for cell migration to CXCL2 remains to be identified, our time lapse video microscopy experiments reveal that IKKα is required for the initial cell orientation/polarization and velocity towards a CXCL12 gradient. Taken collectively, along with our cell fractionation experiments showing that IKKα up-regulates the basal level of p100>p52 processing, our results herein provide evidence that cells migrating towards CXCL12 require the IKKα/p52/RelB pathway to maintain a sufficient level of a critical effector/adaptor of CXCR4-mediated CXCL12 signaling that is specifically needed for cell migration to CXCL12 alone. In contrast, cell migration towards HMGB1/CXCL12 complexes are believed to signal via an alternate conformation of the CXCR4 receptor (58).

In summary, we posit that the IKKα/p52/RelB pathway functions in a constitutive fashion to maintain basal levels of this putative CXCR4 signaling effector/co-factor; and our future work will in part be directed to identify this potentially novel regulatory protein factor.

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CONCLUSIONS

Our experiments show that IKKβ and IKKα are each essential for cell migration towards CXCL12 for different reasons. The results demonstrate that IKKβ is needed to maintain a threshold level of cell surface CXCR4, the CXCL12 receptor, while the dual requirement for IKKα is not linked to CXCR4 expression. Time lapse video microscopy analysis performed with inducible IKKα KO primary fibroblasts reveal that the essential need for IKKα becomes apparent within the initial 30-60 minutes of a CXCL12 chemotactic response, as IKKα appears to be critical for both initial cell polarization and velocity towards CXCL12. We also present evidence that CXCL12 is a modest iIKKα-dependent inducer of NF-κB p52 nuclear translocation and also up-regulates its own IKKα/RelB/p52-dependent expression. Because IKKα up-regulates the basal level of p100>p52 processing and is essential at the earliest phase of CXCL12-mediated cell migration, taken together our data suggests that IKKα is required to maintain the basal expression of a critical NF-κB p52/RelB target gene, which is required for CXCL12-mediated cell migration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations

C5a	complement component 5a
HMGB1	High Mobility Group Box 1
HPF	high power field
IKK	Inhibitor of NF-κB kinase
MEFs	Mouse Embryo Fibroblasts
MΦ	mature macrophages
PDGF	platelet derived growth factor
RAGE	receptor for advanced glycation end products
SDF-1/CXCL12	Stromal Cell Derived Factor 1/C-X-C motif ligand 12
SF	serum free media
WT	wild type

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1. Cell migration to CXCL12 requires IKKβ- and IKKα-dependent NF-κB signaling
2. IKKβ is required to maintain a sufficient threshold level of CXCR4
3. IKKα is required for cell polarization and velocity towards CXCL12
4. IKKα is needed to maintain a sufficient level of active NF-κB p52/RelB heterodimers
5. CXCL12 modestly induces IKKα-dependent p52 nuclear translocation.

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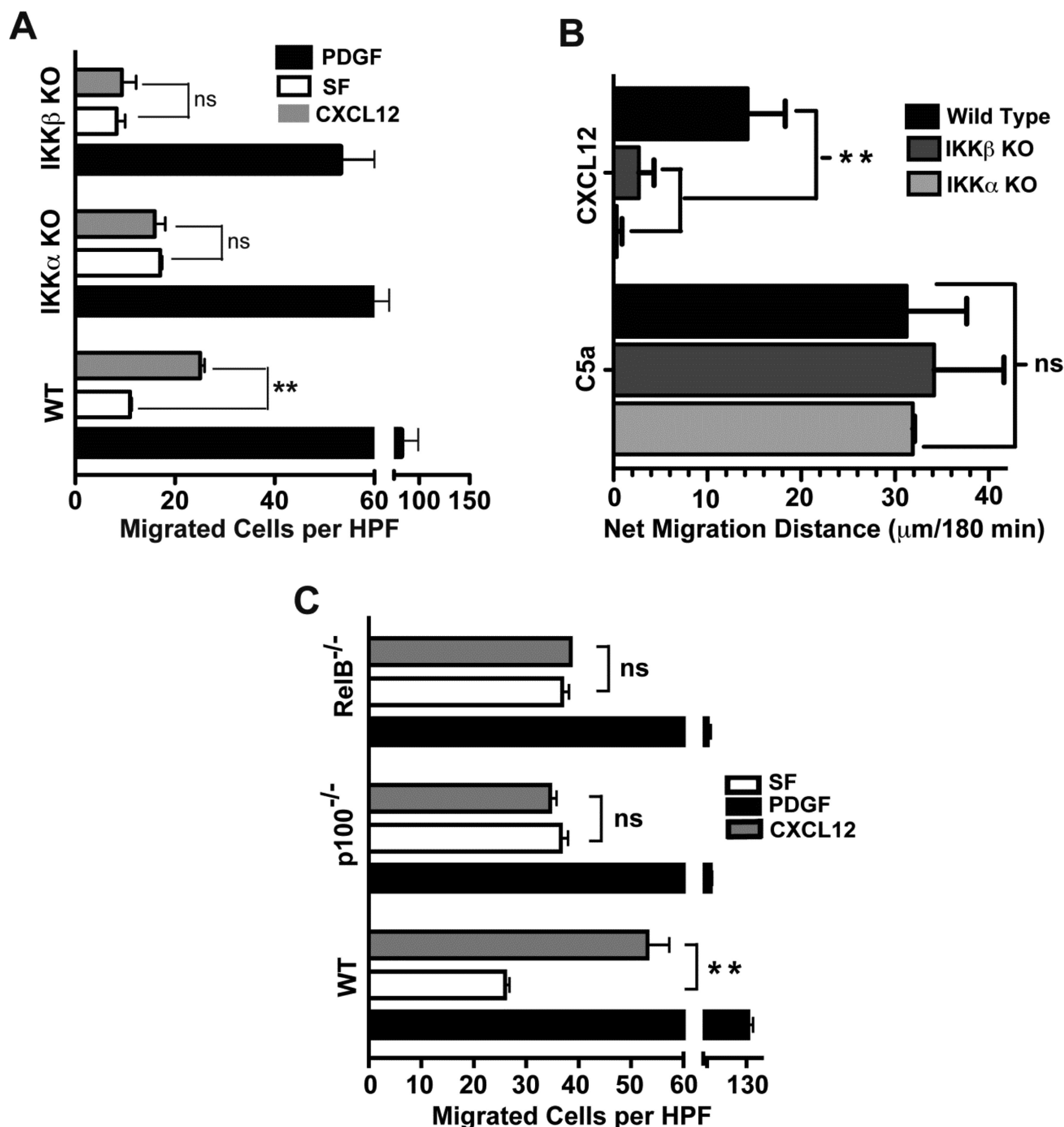

Figure 1. IKKα, IKKβ and NF-κB p52 and RelB are each required for cell migration in response to CXCL12

(A) Chemotaxis of immortalized WT, IKKβ KO and IKKα KO MEFs (5×10^4 cells) was evaluated using a 48 well microchemotaxis chambers as previously described (30). Cells were allowed to respond to CXCL12 (50 ng/ml), serum-free media (SF) as the negative control and PDGF (10 ng/ml) as the positive control for robust cell migration. The number of cells that traversed an 8 μm pore-size polycarbonate filter after a 3 hour incubation at 37°C were counted using a microscope at 400× magnification. Numbers represent mean ± SEM of cells per high power field (HPF), n = 4, ** indicates $p<0.01$, ns = not significant. PDGF served as a positive migration control for all 3 cell backgrounds but only to illustrate that each cell type migrate towards PDGF but in contrast to WT MEFs, IKKα KO and IKKβ KO cells do not migrate towards CXCL12. The absolute degrees of migration of WT, IKKα

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KO and IKKβ KO MEFs towards PDF are somewhat differ from each other, which likely reflects intrinsic properties of these different cell lines. Thus, statistical analyses of the results in Figure 1A were done for WT MEFs exposed to media vs. CXCL12: IKKα KO MEFs exposed to media vs. CXCL12; and IKKβ KO MEFs exposed to media vs. CXCL12.

(B) Primary mature WT and conditional IKKα and IKKβ KO macrophages (10^5 cells) were exposed to CXCL12 (50 ng/ml) or C5a (2 nM) as a positive control in 48 well microchemotaxis chambers for 3 hrs as previously described (30). Data is presented as net migration distance per 400× field after subtracting basal migration in serum free media (n = 3-4). Mean value of WT macrophage basal migration towards serum free media control was 35 ± 2.6 μm. Statistical significance is indicated, ns = not significant.

(C) Chemotaxis of WT, NF-κB p100/p52$^{-/-}$and NF-κB RelB$^{-/-}$ MEFs (5×10^4 cells) was evaluated using a 48 well microchemotaxis chambers as previously described (30). Cells were allowed to respond to CXCL12 (50 ng/ml), serum-free media (SF) as the negative control and PDGF (10 ng/ml) as the positive control for robust cell migration. The number of cells that traversed an 8 μm pore-size polycarbonate filter after a 3 hour incubation at 37°C were counted using a microscope at 400× magnification. Numbers represent mean ± SEM of cells per high power field (HPF), n = 4, ** indicates p<0.01, ns = not significant.

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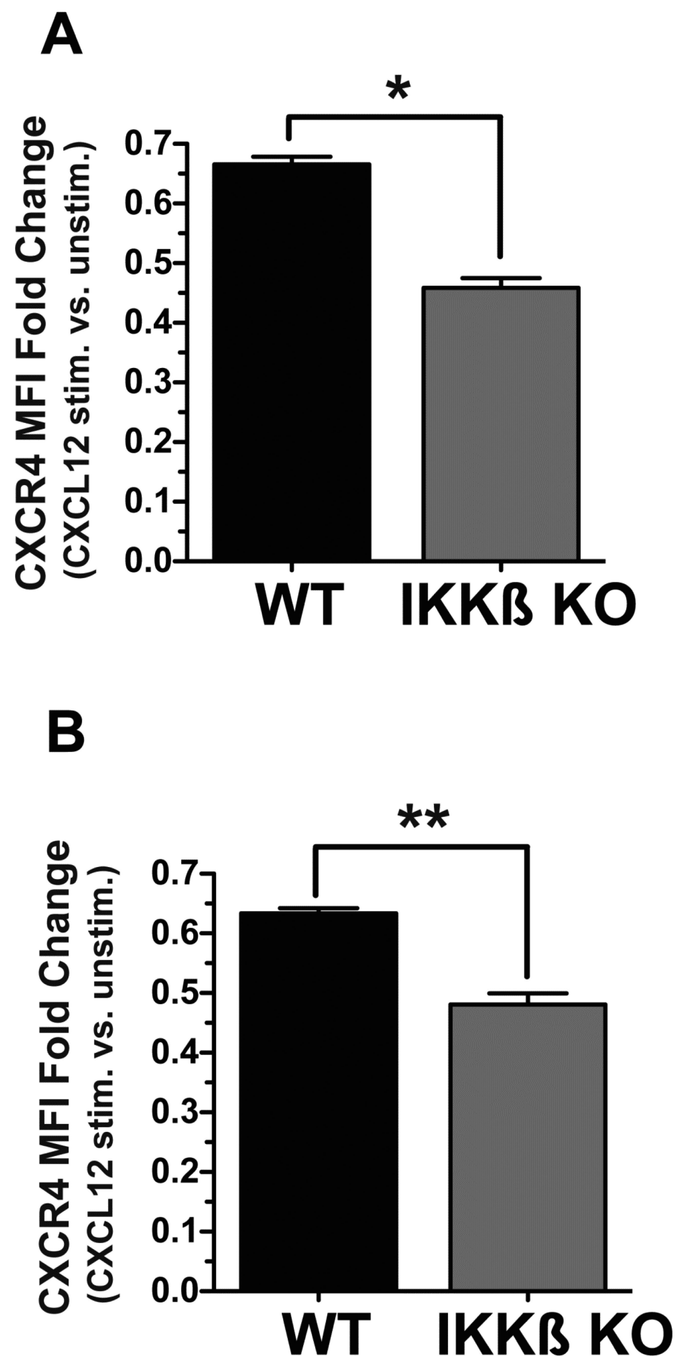

Figure 2. CXCR4 protein expression levels in Wt versus IKKβ conditional KO macrophages

Mature macrophages derived from WT and myeloid cell conditional *IKKβ*$^{f/f}$*:MLysCre* mice were stained with anti-CXCR4 antibody after cell permeabilization to reveal total cellular CXCR4 protein expression **(A),** or without permeabilization to quantity cell surface CXCR4 levels **(B)** and then analyzed by flow cytometry. Data are shown as mean fluorescence intensities (MFI) from 4 independent experiments and are expressed as fold change of cells stimulated with CXLC12 (50 ng/ml) for 2 hr vs. unstimulated cells. *p<0.05 and **p<0.01 by one way ANOVA with Tukey's multiple comparisons post test.

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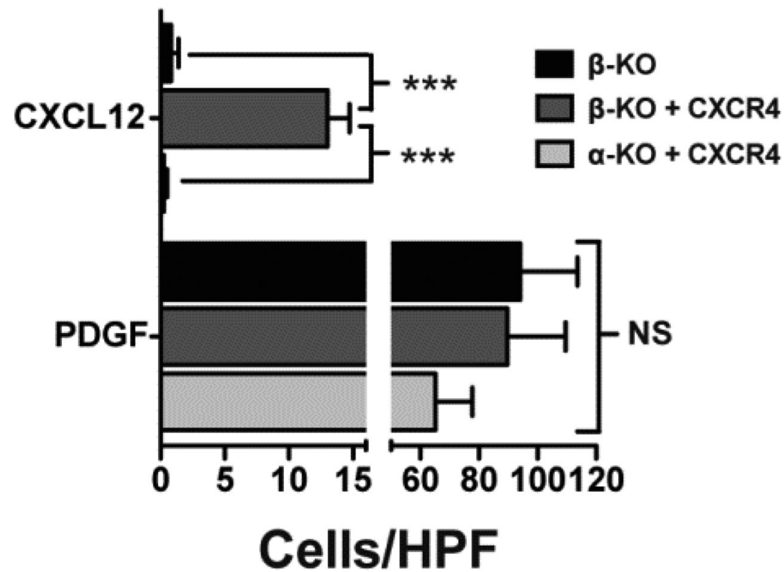

Figure 3. Enforcing CXCR4 expression rescues IKKβ but not IKKα cell migration towards CXCL12

IKKβ and IKKα KO MEFs were stably transduced with a Moloney murine retroviral vector harboring a murine CXCR4 cDNA in a bi-cistronic IRES-Puromycin expression cassette (MuCXCR4-BIP). Cell surface CXCR4 expression was verified by flow cytometry. IKKα and IKKβ KO MEFs stably transduced with MuCXCR4-BIP and IKKβ KO MEFs as a negative control were exposed for 3 hrs to CXCL12 (50 ng/ml) in 48 well microchemotaxis chamber. PDGF (10 ng/ml) served as the positive control for cell migration. Numbers represent mean ± SEM of cells per high power field (n = 5), *** = $p<0.001$, NS = not significant.

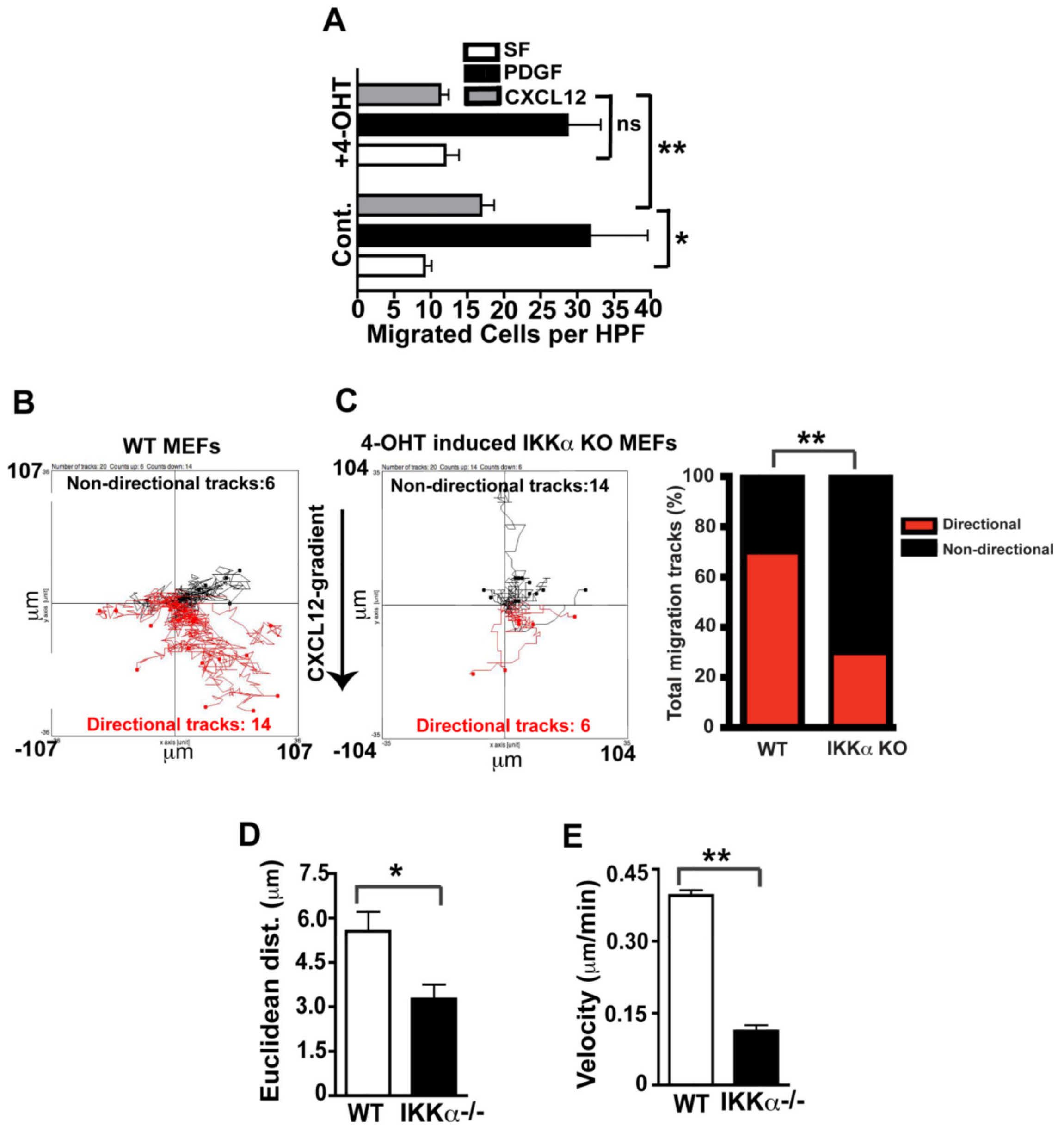

Figure 4. Functional consequences of IKKα ablation on specific CXCL12 migration parameters

(A): IKKα$^{f/f}$:CreERT2 MEFs were incubated with or without 4-OHT (100 nM) for 36 hours, and migration assays were performed using serum free medium (SF), or in response to the same media containing CXCL12 (30 ng/ml) or the positive control PDGF (10 ng/ml). Numbers represent mean ± SEM of cells per high power field (n = 3), ** = p<0.01, * = p <0.05, NS = not significant. (**B and C**): Cells were exposed CXCL12 gradients (0-30 ng/ml) in µ slides for 1 hour, and their movement was recorded by time-lapse microscopy. Red lines are tracks of cells moving in the direction of the CXCL12 gradient, and black lines are cell tracks moving in other directions. The difference in the number of directional and non-directional tracks between WT and the IKKα ablated MEFs is statistically significant (χ^2 test **p<0.01); and bar graphs summarizing these results are shown to the right of panel C. **(D and E):** WT and IKKα KO (4-OHT treated) cell tracks in Panels B & C were analyzed

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for their relative Euclidean distances and velocities, $*p<0.05$ and $**p<0.01$ by two-tailed Student's t tests.

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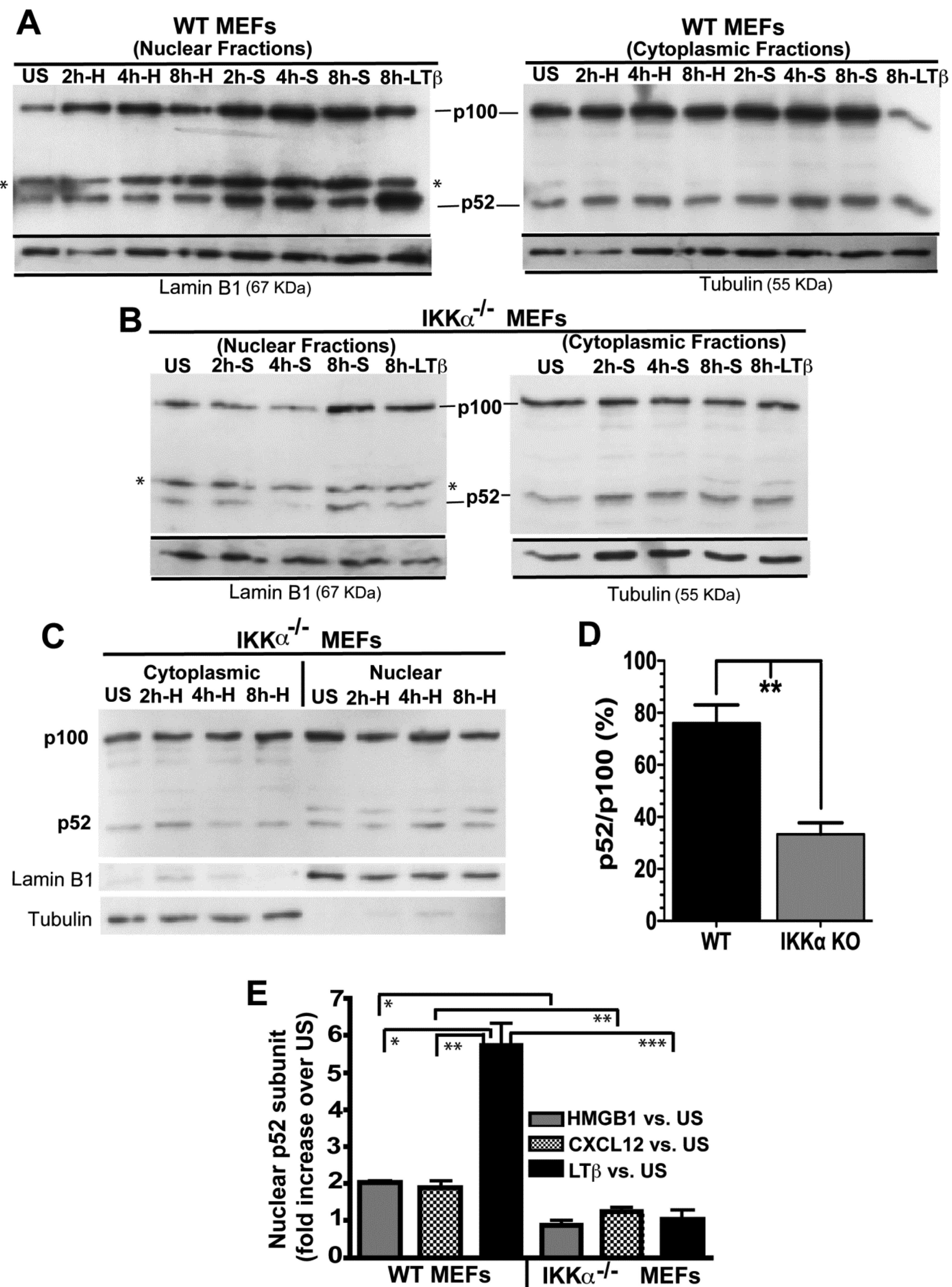

Figure 5. CXCL12 is a modest, IKKα-dependent inducer of NF-kB p52 nuclear translocation

(A) Immortalized WT or (**B and C**) IKKα$^{-/-}$ MEFs were stimulated with either 50 ng/ml CXCL12, 100 ng/ml HMGB1 or 10 μg/ml of an antagonistic anti-LTβR antibody for the indicated times. S refers to CXCL12/SDF-1 and H denotes HMGB1. Nuclear and cytoplasmic protein fractions were analyzed by western blotting as shown with p100 and p52 identified by an anti-p100/p52 antibody. The asterisk in the immunoblot denotes a cross-reactive artifact band in the anti-p52 nuclear extracts. Immunoblots were stripped and re-probed with antibodies against Lamin B1 or β-Tubulin as protein loading controls for

nuclear and cytoplasmic cell fractions, respectively. (**D**) Comparative analysis of p52:p100 levels in un-stimulated WT vs. unstimulated IKKα KO MEFs from 3 independent experiments (one of which is shown in panels A-C). **E)** Densitometric quantification of p52 nuclear import in WT and IKKα$^{-/-}$ cells in response to HMGB1, CXCL12/SDF-1 or LTβR stimulations at the 8 hr time point. Numbers represent 3 independent experiments. p52 signals were all normalized to lamin B1 as a nuclear protein reference control. Error bars are standard error of the mean. *P<0.05; **P<0.01; ***P<0.001 by two-tailed Student's t tests.

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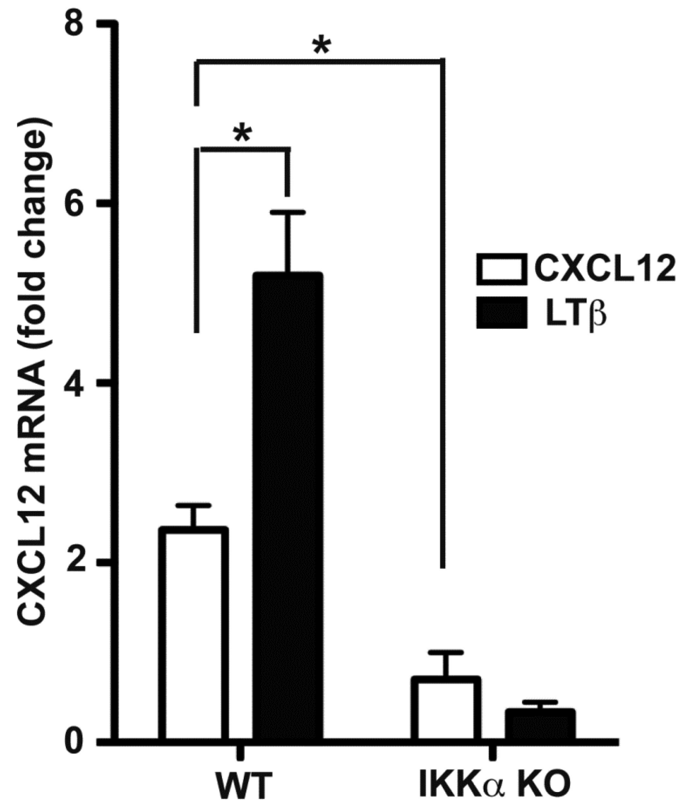

Figure 6. CXCL12 up-regulated the level of its mRNA with a dependency on IKKα

Real time RT-PCR analysis of CXCL12 mRNA expression in WT and IKKα$^{-/-}$ MEFs in response to CXCL12 or LTβR stimulation. Data were normalized to GAPDH mRNA qRT-PCRs as a reference control. $^{+}P<0.05$.