A Selective Small Molecule IKKβ Inhibitor Blocks NF-κB Mediated Inflammatory Responses in Human Fibroblast Like Synoviocytes, Chondrocytes and Mast Cells

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NF-κB, nuclear factor κB; IKK, IκB kinase; LPS, lipopolysaccharide; PGN, peptidoglycan; PMA, phorbol myristate acetate; TNF, tumor necrosis factor; IL, interleukin; PBMC, peripheral blood mononuclear cell; HFLS, human fibroblast like synoviocyte; MC, mast cell; M-CSF, macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated on activation, normal T cell expressed and secreted; MMP, matrix metallpproteinase; TIMP (tissue inhibitors of metalloproteinases); PGE, prostaglandin E; COX, cyclooxygenase; RA, rheumatoid arthritis; TLR, toll-like receptor; MOI, multiplicity of infection.

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ABSTRACT

IKKβ is essential for inflammatory cytokine-induced activation of NF-κB (Yamamoto and Gaynor, 2004). NF-κB plays a pivotal role in the function of major cell types that contribute to the pathophysiological process of rheumatoid arthritis (RA) (Yamamoto and Gaynor, 2001). Here we report the mechanism and the effect of the IKKβ inhibitor ML120B, a β-carboline derivative (Castro et al., 2003), on NF-κB signaling and gene activation in RA relevant cell systems. ML120B is a potent, selective, reversible and ATP competitive inhibitor of IKKβ with an IC_{50} of 60 nM when evaluated in an IκBα kinase complex assay. ML120B does not inhibit other IKK isoforms or a panel of other kinases. ML120B concentration-dependently inhibits TNFα stimulated NF-κB signaling via inhibition of IκBα phosphorylation, degradation and NF-κB translocation into the nucleus. For the first time, we have demonstrated that in human fibroblast like synoviocytes (HFLS), TNFα or IL-1β induced RANTES and MCP-1 production is IKKβ dependent. Also for the first time, we have demonstrated that LPS or peptidoglycan (PGN) induced cytokine production in human cord blood derived mast cells (MC) is IKKβ dependent. In addition, in human chondrocytes ML120B inhibits IL-1β induced matrix metalloproteinase (MMP) production with an IC_{50} of approximately 1 μM. ML120B also blocked IL-1β induced prostaglandin E_2 (PGE_2) production. In summary, ML120B blocks numerous NF-κB regulated cell responses which are involved in inflammation and destructive processes in the RA joint. Our findings support the evaluation of IKKβ inhibitors as anti-inflammatory agents for the treatment of rheumatoid arthritis (RA).

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Introduction

NF-κB is a transcription factor which activates numerous genes that regulate several immune and inflammatory processes including RA (Baeuerle and Henkel, 1994). NF-κB generally exists as a dimer in the cytosol bound to distinct inhibitory IκB subunits. NF-κB activation is primarily mediated by phosphorylation and rapid degradation of IκB, followed by translocation of NF-κB to the nucleus where it activates transcription of specific genes (Karin, 1999). Examples of genes dependent on the activation of NF-κB include cytokines, such as tumor necrosis factor (TNFα), interleukin-6 (IL-6), IL-8 and IL-1β; chemokines, such as MCP-1, RANTES; and matrix metalloproteinases (MMPs), such as MMP1, MMP3, and MMP13. NF-κB activity is tightly controlled by the IκB kinase complex, consisting of IKKα, IKKβ and IKKγ. IKKβ is essential for the inflammatory cytokine-induced activation of NF-κB (Mercurio et al., 1997; Yamamoto and Gaynor, 2004). Since IκB kinase (IKK) catalyzed phosphorylation of IκB proteins is an essential step in the signal-induced activation of NF-κB, targeting IKKβ represents an opportunity for developing novel therapeutics for inflammatory disease indications such as rheumatoid arthritis, asthma, and many others.

Rheumatoid arthritis is a chronic destructive disease of the joints, characterized by inflammation, synovial hyperplasia, and abnormal cellular and humoral immune responses. Several cell types have been implicated as major contributors in the pathophysiological process of RA (Gravallese, 2002), including fibroblast-like synoviocytes (FLS), macrophages, T cells, B cells, osteoclasts, chondrocytes, dendritic cells and mast cells. Specific cytokines, chemokines, and tissue-destructive enzymes have been identified as key players in the pathologic process of RA. Multiple lines of evidence suggest that NF-κB plays a key role in regulating the inflammatory process in these cell types. Therefore, identification of selective IKKβ inhibitors as potential therapeutics has received considerable interest.

Here we report that ML120B is a potent and selective IKKβ inhibitor. We have examined cellular mechanisms and functional effects of ML120B in RA relevant cell systems. Our results demonstrate that ML120B specifically blocks the NF-κB signaling pathway. ML120B concentration-

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dependently blocks LPS, TNFα, or IL-1β stimulated cytokine production in inflammatory cells including human peripheral blood mononuclear cell (PBMC), human fibroblast like synoviocytes (HFLS) and human mast cells (MC). ML120B also blocks IL-1β stimulated collagenases (MMP1, MMP13), stromelysin (MMP3) and prostaglandin E_2 (PGE_2) production in the human chondrocyte cell line SW1353. Taken together, these results suggest a potential use of this class of compounds as therapeutic agents in the treatment of RA.

Methods

Reagents Nonidet® P-40, Triton® X-100, Tween® 20, BSA, LPS (*E.coli* O26:B6), DMSO, Toluidine blue, PMA, Ionomycin and PGN were purchased from Sigma (St. Louis, MO). αCD3 and αCD28 were obtained from BD Biosciences (San Jose, CA). rhTNFα, rhIL-1β, IL-4, IL-6 were obtained from R&D Systems (Minneapolis, MN). Phospho-IkappaB-alpha (Ser32/36) (12C2) monoclonal antibody was purchased from Cell Signaling Technologies (Beverly, MA) and labeled with W8044 europium chelate by Perkin Elmer (Boston, MA). Streptavidin Alexa 647 and Blasticidin were purchased from Invitrogen (Carlsbad, CA). GST-IκBα (5-55) was produced in *E. Coli* as described (Chen et al., 1996; Lee et al., 1997; Lee et al., 1998) and labeled with biotin using PEO-Maleimide Activated Biotin from Pierce (Rockford, IL) as per manufacture's instructions. Titertube deep well 96-well plates were purchased from BioRad (Hercules, CA). PE-conjugated anti-human TLRs antibodies are from eBioscience (San Diego, CA).

Compound β-carboline compounds ML120B (*N*-(6-chloro-7-methoxy-9*H*-β-carbolin-8-yl)-2-methylnicotinamide) and PS 1145 (*N*-(6-chloro-9*H*-β-carbolin-8-yl) nicotinamide) were synthesized at Millennium Pharmaceuticals, Inc (Cambridge, MA). In all cell based assays, compounds were pre-incubated with cells for 30 min or 1hr before stimulation. The compound was dissolved in DMSO. The final concentration of DMSO in all the assays is 0.5%.

Cell lines The 293 NF-κB-Luc and 293 AP-1-Luc reporter stable cell lines were generated at Millennium Pharmaceuticals, Inc. (MPI, Cambridge, MA). Briefly, the construct of NF-κB-TA-Luc or AP-1- Luc engineered with a Blasticidin selection gene was used to stably transfect the 293 cell line. Positive clones were selected using Blasticidin and evaluated by their responsiveness to TNFα or PMA/Ionomycin stimulation. HFLS were obtained from Cell Applications, Inc.(San Diego, CA). Human chondrosarcoma cells SW1353 and HeLa cells were purchased from American Type Culture Collection (ATCC, Manassas, VA).

Human plasma Normal human blood was obtained through MPI's blood donation program. Normal human plasma was pooled from multiple donors, and aliquots were stored at -80^{O}C.

Preparation of IKK complex The IKK complex was purified and activated as described previously (Chen et al., 1996; Lee et al., 1997; Lee et al., 1998) with minor modifications. HeLa cell S100 lysates were precipitated in 30% ammonium sulfate and resuspended in Buffer A (50 mM Hepes pH 7.5, 100 mM NaCl, 10 mM 2-glycerophosphate, 2 mM DTT, 0.5 mM EDTA) and were passed over a Superose™ 6 gel filtration column pre-equilibrated in Buffer A. Fractions containing IKK activity were pooled and activated with 100 nM recombinant MEKK1 at 37°C for 45 minutes in Buffer A supplemented with 10 mM $MgCl_2$, 250 μM ATP, and 2 μM Microcystin-LR.

IKK Complex Enzyme Assay Phosphorylation of GST-IκBα (5-55) was measured by incubating test samples in 3% DMSO with activated IKK complex as described above; 300 nM GST-IκBα (5-55), and varying ATP concentrations in 50 mM Hepes pH 7.5, 10 mM 2-glycerophosphate, 10 mM $MgCl_2$, 5 mM DTT, and 0.1 % BSA were incubated in a reaction volume of 30 μL for 1 hour at room temperature. Reactions were terminated with the addition of 10 μL of 250 mM EDTA, followed by the addition of 40

μL of detection mixture containing 2 nM Europium labeled anti-phospho IκB and 50 nM streptavidin Alexa 647 in 50 mM Hepes pH 7.5, 0.1 % BSA, and 0.01 % Tween® 20. After 1 hour, plates were read on a Wallac Victor™ plate reader.

IKK Complex Reversibility Assay IKK complex was incubated for 1 hour with 10μM ML120B. After incubation samples were passed through a Zeba gel filtration column (Pierce, Rockford, IL) according to manufacturer's instructions. Samples were assayed as described above, but at an ATP concentration of 1mM.

IKKβ Kinase Assays Recombinant IKKβ (Upstate Biotechnology, Lake Placid, NY) was assayed under identical conditions as the IKK Complex Assay with the following modifications. 1 nM IKKβ was substituted for activated IKK complex and the ATP concentration was adjusted to 1 μM.

Kinase Selectivity Assays All kinase selectivity assays were performed against recombinant kinases at or below the Km of ATP. Assays against IKKα were performed through Upstate Kinase Profiler™ (Upstate Biotechnology, Lake Placid, NY). Assays against PKCα, PKCδ, PKCθ, CSK, Src, and Lyn were performed in a time resolved fluorescence format. All other assays were performed in Flashplate® assays.

Western Blot HeLa cells or human PBMCs were pre-incubated with ML120B or DMSO for 1 hour prior to the stimulation with TNFα (50 ng/mL) for 5 or 20 minutes. Cells were lysed using whole cell lysis buffer, and protein concentrations were determined using a BCA kit (Pierce, Rockford, IL). 20 μg of cell lysate was loaded into each well of a 4-12 % NuPAGE® Bis-Tris gradient gel. The blot was blocked in 5 % milk/PBST and incubated with polyclonal anti-phosphorylated IκB antibody or polyclonal

anti-IκB antibody (Cell Signalling, Beverly, MA), and subsequently anti-rabbit secondary antibody (Cell Signalling, Beverly, MA). ECL Plus™ (Amersham, Piscataway, NJ) was used for detection.

HeLa cells NF-κB Nuclear Translocation Assay HeLa cells were maintained in Dulbecco's® Modified Eagle Media with 10% FBS, 2 mM glutamine and 1% Pen/Strep solution. The effect of ML120B on the nuclear translocation of p65, in response to stimulus, was examined by using high content screening technology from Universal Imaging Corp™ (UIC, Downingtown, PA). Briefly, HeLa cells were seeded into a 96-well black, clear-bottom Packard ViewPlate at 5000 cells/per well the day before the experiment. ML120B was serially diluted 1:1 in DMSO with final concentrations ranging from 20 μM to 0.04 μM. After a 1hour pre-incubation with ML120B, cells were stimulated with TNFα (10 ng/ml) for 30 min, fixed with 4% paraformaldehyde solution and permeabilized with 0.5% Triton® X-100. After being washed with 0.5% phosphate-buffered saline, cells were blocked with 0.5% blocking reagent (Roche, Indianapolis, IN). Further, cells were stained using "Cellomics Hit Kit®" (Cellomics, Pittsburgh, PA) for the detection of NF-κB Activation following the kit manual. Images were acquired with Discovery-1™ system and nuclear translocation data was analyzed by MetaMorph™ Software from UIC (Downingtown, PA)

NF-κB DELFIA Assay In order to quantitatively measure NF-κB binding activity upon activation, we developed a NF-κB DELFIA® assay based on the Trans-AM™ NF-κB Assay kit (Active Motif, Carlsbad, CA). Cells were plated in a 96 well plate the day before the experiment. After pre-incubation with serially diluted ML120B at 37^{O}C for 1 hour, cells were stimulated with TNFα (10 ng/ml) at 37^{O}C for 30 minutes. Whole cell extract was prepared from the cell pellet. 20 μg of whole cell extract was added in duplicate into a 96-Well Trans-AM™ NF-κB plate containing the immobilized NF-κB consensus site (5'-GGGACTTTCC-3'). Following a 1 hour incubation with the primary antibody, wells were washed with DELFIA® wash buffer (Perkin Elmer, Boston, MA) and incubated with Eu-N1-labeled

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anti-mouse IgG for DELFIA® readout. The NF-κB activation was quantified as Europium counts of Time-Resolved Fluorometry on a Wallac Victor™ plate reader.

NF-κB or AP-1 Luciferase Reporter Assay 293/NF-κB-Luc and 293/AP-1-Luc cells were maintained in DMEM (Invitrogen, Carlsbad, CA) with 10% FBS (Hyclone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin 2mM L-glutamine and 5 μg/mL Blasticidin (Invitrogen, Carlsbad, CA). Cells were seeded at a density of $0.05x10^6$ cells per well in black poly-D-Lysine 96-well plates (Discovery Labware Inc., Bedford, MA) the day before the assay. ML120B or PS1145 was serially diluted 1:1 in DMSO, to produce concentrations ranging from 20 μM to 0.04 μM. Media was changed to AIM-V® (Invitrogen, Carlsbad, CA) for the assay. Cells were pre-incubated with IKKβ inhibitor (ML120B or PS1145) for 1 hour at 5% CO_2 at 37^OC. For the NF-κB assay, cells were stimulated with 0.5 ng/mL TNFα (R&D Systems, Minneapolis, MN) or 10 pg/mL PMA and 2 μM ionomycin for 3 hours. For the AP-1 assay, cells were stimulated with 10 pg/mL PMA and 2 μM ionomycin for 3 hours. Media was removed and cells were lysed in 1x passive lysis buffer (Amersham Bioscience, Piscataway, NJ). 20 μL of lysate was used in accordance with luciferase assay kit (Promega, Madison, WI). The assay was run in triplicate and data was analyzed using XLfit software.

Transient Transfection Assays Using Luciferase Reporter Panel The following PathDetect *Cis*-reporter luciferase plasmids were purchased from Stratagene (La Jolla, CA): NF-κB, AP-1, CRE, SRE, ISRE and NFAT. The IL-8-luciferase reporter was obtained from the A. Casola laboratory and contains the promoter region of IL-8 (-162 to +44) upstream of the luciferase gene (Casola et al., 2000) The ELAM-luciferase reporter was generated in house and contains the promoter region of the human E-Selectin gene from -730 to +52 cloned into a modified TATA-less version of pMCS-Luc (Stratagene). Transient transfection and assay of ML120B and PS1145 on the luciferase reporter panel was carried out as following; 293T cells were plated at a density of 17,000 cells per well in 100μl culture medium

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(DMEM, 10%FBS, 1% Penicillin/Strepromycin, 1% L-glutamine) in Biocoat™ Poly-D-Lysine 96 well plates (Becton Dickinson, San Jose, CA). The following day cells were transfected with a cocktail containing 100 ng of DNA consisting of 50 ng luciferase reporter DNA, 5 ng internal control pTK-Renilla (Promega, Madison WI) and 45 ng empty vector using Fugene transfection reagent (Roche, Indianapolis, IN) following manufacturers instructions. Following overnight incubation, fresh medium containing 0.1% FBS was added to the plates and cells were incubated overnight. Cells were pre-incubated with IKKβ inhibitor compounds (ML120B or PS1145 prepared in DMSO) added at a dose range from 10 nM to 31.6 μM (3 fold serial dilution) and 30 minutes later various stimulants for the reporters were added to induce the different pathways being monitored; PMA, 50 ng/mL (Sigma, St. Louis, MO); Ionomycin, 1 μg/mL (Sigma, St. Louis, MO); Forskolin, 20 μM (Sigma, St. Louis, MO); IFNβ, 1000 U/mL (R&D Systems, Minneapolis, MN) and TNFα, 50 ng/mL (R&D Systems, Minneapolis, MN). The cells were incubated for a further 6.5 hours, after which cells were harvested and luciferase values were measured using the dual luciferase assay kit (Promega, Madison WI). Each individual assay was performed in quadruplicate per experiment and the complete transfection procedure for the reporter panel was performed a minimum of two times. The data was analyzed using XLfit software.

Human Peripheral Blood Mononuclear Cell (PBMC) Assay Heparinized human whole blood was obtained from normal donors. PBMCs were separated on a Ficoll-Paque™ Plus (Amersham, Piscataway, NJ) gradient. Compound was serially diluted 1:1 in DMSO with final concentrations ranging from 20 μM to 0.04 μM. $4x10^5$ cells per well were seeded in a 96-well plate and incubated at 5% CO_2 at 37ºC. Cells were pre-incubated with compound for 1 hour, then stimulated with either LPS (100 ng/mL) or αCD3 (0.25 μg/mL) and αCD28 (0.25 μg/mL) for 5 hours. Supernatant was collected for cytokine analysis.

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Cytokine Analysis Quantitative measurement of cytokines in culture supernantant or plasma was performed by Pierce Biotechnology, Inc.(Woburn, MA) using the SearchLight® Human Cytokine Array, a multiplexed sandwich enzyme-linked immunosorbent assay (ELISA).

Cytotoxicity LDH Release Assay Compound cytotoxicity was monitored by the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. The supernatant from human PBMC assays was tested for LDH release following the vendor's kit manual (Roche, Indianapolis, IN). 2% Triton® X-100 treated cells are used to determine the maximum releasable LDH activity in the cells. 0.5% DMSO treated cells were used as a basal level LDH activity.

Human Fibroblast-Like Synoviocytes (HFLS) Assay Cryopreserved HFLS cells were thawed and cultured in synoviocyte growth culture medium (Cell Applications, Inc., San Diego, CA). After 2-3 rounds of subculture, 1 $X10^5$ cells per well were plated in black poly-D-lysine 96-well plates (Discovery Labware, Inc., Bedford, MA) one day before the experiment. ML120B was serially diluted 1:1 with DMSO, and pre-incubated with the cells at 5% CO_2 at 37^OC for 1 hour. For cytokine studies, the cells were stimulated with 10 ng/mL human TNFα or 10 ng/mL human IL-1β and cultured for 16 hours. The supernatant was harvested and analyzed for RANTES, MCP-1, IL-6 and IL-8 production (Pierce Biotechnology, Woburn, MA). To measure the effects of ML120B on NF-κB activation, the cells were stimulated with either IL-1β (10 ng/mL) or TNFα (10 ng/mL) for 30 minutes. Whole cell extract was used to measure of NF-κB activation by the NF-κB DELFIA® assay as described above except that compound was serially diluted 1:1 in DMSO with final concentrations ranging from 50 μM to 0.1 μM.

Human Cord Blood Derived Mast Cells Assay Human PBMC's were separated by Ficoll (Amersham, Piscataway, NJ) gradient from human cord blood (Cambrex Bio Science, Walkersville, Maryland) and then cultured in mast cell culture media (RPMI 1640 containing 10% fetal bovine serum 2 mM L-

glutamine, 0.1 mM nonessential amino acid, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 μg/mL gentamycin and 0.2 μM 2-mercaptoethanol) in the presence of 100 ng/mL human stem cell factor (SCF, R&D System, Minneapolis, MN), 10 ng/mL human IL-6 and 10 ng/mL human IL-10. Non-adherent cells were transferred to a new flask containing mast cell culture media with fresh cytokines every week. To assess the maturity of the mast cells, starting from week 6, an aliquot of cells were stained with toluidine blue and the expression of c-Kit (BD Bioscience, San Jose, CA) was analyzed by FACS. When > 95% of the cells were confirmed to have differentiated into mature mast cells, the cells were processed for pharmacology studies. The cells were pre-incubated with serially diluted ML120B at 5% CO_2 at 37^{O}C for 1 hour, and then stimulated with 10 ng/mL LPS or 100 μg/mL PGN overnight. The supernatant was collected and subjected to cytokine profiling analysis (Pierce Biotechnology Inc., Woburn, MA).

Expression of Toll Like Receptors (TLRs) on Human Cord Blood Derived Mast Cells 0.5 X 10^5 mast cells were washed with cold 1X PBS with 1% BSA. Cells were stained with PE conjugated anti-human TLR 2, TLR 4, TLR 3 or TLR9 antibodies respectively. PE-conjugated mouse IgG or PE-conjugated Rat IgG2α were used as epitope controls. The expression of TLRs on mast cell surface was analyzed by FACS.

Adenovirus Transduction of IKKβ wt, IKKβ DN and IκB SR in Human Mast Cells The recombinant adenovirus constructs carrying the IKKβ wild type (IKKβ wt), IKKβ dominant-negative (IKKβ DN K44M) and IκB super repressor (IκB SR) were kindly provided by Philip A. Barker (Bhakar et al., 2002). Transduction efficiency was monitored by GFP expression. Mast cells were seeded in 24-well culture plate at 2 X 10^5 per well. Adenovirus titer MOI was optimized in a pilot experiment so that all the wells have similar transduction efficiency. After a 24 hour infection with corresponding adenoviruses, cells were stimulated with either purified LPS at 50 ng/ml (gift from Dr. Kate Fitzgerald, University of Massachusetts Medical School, Worcester, MA) or PGN at 100 μg/ml for 16 hours. The

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supernatant was collected and subjected to cytokine profiling analysis (Pierce Biotechnology Inc., Woburn, MA).

Human Chondrocytes SW1353 Assay The human chondrosarcoma cell line SW1353 was cultured in DMEM® with 2 mM Glutamax® (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (Hyclone, Logan, UT). $0.05x10^6$ cells per well were seeded into a 96-well Poly-D-Lysine plate (Discovery Labware, Inc., Bedford, MA) one day before the experiment. The culture media was changed to fresh AIM-V® media containing 2% FCS on the day of the experiment. Serially diluted ML120B in DMSO, ranging from 20 μM to 0.02 μM, was pre-incubated with cells for 1 hour at 5% CO_2 at 37^OC, and then stimulated with 10 ng/mL human IL-1β (R&D System, Minneapolis, MN) for 24 hours. The supernatant was harvested and used to measure MMP1, MMP3, MMP13 and PGE_2 production by ELISA (Amersham Bioscience, Piscataway, NJ).

Statistical Analyses The IC_{50} assays were run in triplicate and XLfit software was used to fit dose response curves to the mean values. The IC_{50} values and their 95% confidence intervals were calculated with equation #205 (Sigmoidal Does-response Model). Differences between IC_{50} estimates were assumed significant at p=0.05 if the 95% confidence intervals of the estimates did not overlap. Where appropriate, differences in means were assessed using Student's t-test in Microsoft Excel. All p-values were two-tailed, and were considered significant when $p \leq 0.01$. Results are expressed as the mean ± S.D. of a representative experiment.

Results

Identification of ML120B as a potent and specific inhibitor of IKKβ In an earlier report we described β-carboline PS1145 as an IKKβ inhibitor with a certain level of specificity and potency (Figure 1A) (Castro et al., 2003). Further chemical optimization has led to the identification of ML120B as a more potent and selective IKKβ inhibitor (Figure 1B). ML120B was determined to have an IC_{50} of 60 nM at 50 μM ATP in a time resolved fluorescence assay measuring the phosphorylation of IκBα at various inhibitor concentrations in an *in vitro* kinase assay utilizing IKK complex purified from HeLa cells and GST-IκBα as a substrate. (Figure 2A). Kinetic analysis shows ML120B to be a competitive inhibitor of ATP as indicated by the double reciprocal plots of velocity versus ATP concentration at fixed inhibitor concentrations (Figure 2B). ML120B was also shown to be a reversible inhibitor of the IKK complex (Figure 2C). To confirm that the activity of ML120B was indeed targeting IKKβ, an in vitro kinase assay was run utilizing recombinant IKKβ. ML120B was determined to have an IC_{50} of 45 nM ± 1 nM (95% C.I.) against recombinant IKKβ (Figure 2D).

To examine the selectivity of ML120B, inhibitory activities of MN120B were evaluated against a panel of 30 tyrosine and serine/threonine kinases, including IKKα. As shown in Table 1, no inhibition below 50 μM was observed across this panel of kinases, thus demonstrating the selectivity of ML120B for IKKβ. ML120B was also screened at 10 μM against a NovaScreen® (Hanover, MD) panel of 33 receptors, transporters and channels. ML120B showed >100 fold selectivity over this panel (data not shown). To further evaluate the selectivity of ML120B, both ML120B and PS1145 were screened against a panel of eight luciferase reporters that represent different well-characterized cellular pathways (Table 2). ML120B showed an inhibitory effect only on the NF-κB reporter or reporters that contain an NF-κB element in their promoter regions (IL-8 and ELAM). This data confirms that compared to the first generation β-carboline compound PS1145, ML120B is a selective IKKβ inhibitor specific to the NF-κB pathway.

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ML120B blocks NF-κB signaling pathway Upon activation by cellular stimuli, IKK phosphorylates IκBα. Phosphorylated IκBα is rapidly degraded through ubiquitin-dependent pathways, which allow released NF-κB factor to translocate to the nucleus and activate NF-κB mediated gene expression. To dissect how ML120B exerts its effect on the NF-κB signaling pathway, we examined the effect of the IKKβ inhibitor on TNFα induced IκB phosphorylation and IκBα degradation in HeLa cells. TNFα stimulation causes IκBα phosphorylation within 5 to 10 minutes and IκBα degradation within 10 to 30 minutes post stimulation. ML120B blocked TNFα stimulated IκBα phosphorylation and degradation concentration-dependently when assessed by western blot analysis (Figure 3). To examine the effects of ML120B on NF-κB translocation and DNA binding activity, we used high content image analysis to measure and quantify nuclear translocation of p65 in the absence or presence of ML120B on HeLa cells. As indicated in Figure 4A, ML120B concentration-dependently blocked TNFα induced nuclear translocation of p65 with an IC_{50} of 2.4 μM (Figure 4B). Furthermore, ML120B blocked TNFα induced NF-κB linked luciferase reporter activity in a concentration-dependent manner with an IC_{50} of 1.1 μM (95% confidence interval: 0.9, 1.3) (Figure 5A). However, ML120B showed minimal inhibition of PMA/ionomycin induced AP-1 linked luciferase activity as shown in Figure 5A. In contrast, the first generation β-carboline compound PS1145 inhibited AP-1 luciferaser reporter activity with an IC_{50} of 8.8 μM±2.9 μM (95% confidence interval). For comparison, PS1145 concentration-dependently inhibited NF-κB luciferase activity with an IC_{50} of 1.7 μM (95% confidence interval: 5.9, 11.7) (Figure 5B). Given these confidence intervals, PS1145 is significantly more potent against NF-κB than AP-1, however, ML120B is more selective and more potent than the first generation compound PS1145.

ML120B inhibits NF-κB mediated gene activation in RA relevant inflammatory cells Activated macrophages are known to contribute to synovial inflammation in RA, and IKKβ has been shown to be a key regulator for macrophage function through activation of the NF-κB signaling pathway (Guha and

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Mackman, 2001). Importantly, anti-TNFα therapy such as etanercept and infliximab have proven to be effective therapeutic agents for RA. We therefore investigated the effect of ML120B on TNFα release from human peripheral blood mononuclear cells (PBMCs). Human PBMCs were isolated from normal donors and exposed to LPS in the absence or presence of inhibitor. ML120B concentration-dependently inhibited LPS stimulated TNFα production with an IC_{50} of 3.3 μM (Figure 6A). Consistent with the mechanism of IKKβ inhibition, ML120B also concentration-dependently blocked TNFα induced IκBα phosphorylation in human PBMCs (Figure 6C). T cell activation leads to IKKβ dependent NF-κB upregulation, which contributes to inflammatory responses in RA. To evaluate the effect of ML120B on T cell signaling pathways, we evaluated αCD3/αCD28 induced IL-2 production with ML120B treatment in human PBMCs. As indicated in Figure 6B, ML120B concentration-dependently blocks αCD3/αCD28 induced IL-2 production with an IC_{50} of 3.4 μM. The cytotoxicity of ML120B was monitored by measuring LDH release in the supernatant of human PBMC. We found that ML120B did not cause LDH leakage at concentrations up to 20 μM (Figure 6D), suggesting that the effect of ML120B is not due to cellular toxicity of the inhibitor. The LDH release in ML120B treated cells is not statistically different compared to DMSO treated cells ($P>0.01$).

In addition to macrophage and T cells, human fibroblast-like synoviocytes (HFLS) also play an important role in the pathogenesis of RA. Cytokine-induced NF-κB activation in HFLS is IKKβ dependent (Aupperle et al., 2001; Hammaker et al., 2003). To evaluate the effect of an IKKβ inhibitor on HFLS, we tested the inhibitory effect of ML120B on IL-1β and TNFα induced cytokine and chemokine production. ML120B inhibits TNFα or IL-1β induced IL-6 and IL-8 production with IC_{50} values of 5.7 μM -14.5 μM. Interestingly, we found that ML120B concentration-dependently inhibits either TNFα or IL-1β induced production of chemokines (RANTES, MCP-1) and MMPs (MMP3, MMP13 and MMP1) with greater potency when compared with IL-6 and IL-8 production (Figure 7A, 7B). The IC_{50} values are summarized in Table 3. To confirm that the inhibition was NF-κB dependent, we measured NF-κB activity at a 30 minute time point with IL-1 or TNFα stimulation in the presence or absence of ML120B.

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Consistent with the reduction of cytokine/chemokine production, ML120B showed a concentration-dependent inhibition of p65 binding activity measured by NF-κB DELFIA (Figure 7C) with an IC_{50} of 3.4 μM after IL-1β stimulation and 5.7 μM after TNFα stimulation. These new findings indicate that the chemokine system may play a more direct role in the destructive phase of RA than is currently suspected, and IKKβ inhibition may lead to a greater effect in protecting joint disruption and blocking cell infiltration to the joints. We observed differential inhibitory effects when HFLS was stimulated by TNFα or IL-1β (Table 3 and Figure 7). Donor variation is very large and the mechanisms for differences in response to IL-1β and TNFα stimulations remain to be elucidated. Recent studies indicated that different inflammatory stimuli induce distinct IKK profiles (Cheong et al., 2005; Covert et al., 2005; Werner et al., 2005). Therefore, different genes may be alternately regulated by the differential usage of κB site by different promoters.

The potential role of mast cells (MCs) in the pathophysiological process of RA has been demonstrated previously (Woolley and Tetlow, 2000; Woolley, 2003). To further investigate the function of IKKβ in toll-like receptor (TLR) signaling in human MC, we investigated the effect of ML120B on mast cell function with human cord blood PBMC derived mast cells, characterized by positive toludine blue staining and positive c-Kit expression by FACS (data not shown). In addition, we evaluated the expression pattern of TLRs in human cord blood derived mast cells by FACS analysis. Human MCs express both TLR2 (receptor for PGN) and TLR4 (receptor for LPS) but have minimal expression of TLR3 and TLR9 (Figure 8). Purified LPS (gift from Dr. Kate Fitzgerald) was used to avoid potential cross signaling through other TLRs from contamination. We used adenoviral-mediated over-expression of a dominant-negative version of IKKβ (IKKβ DN) or IκB super repressor (IκB SR) to elucidate the functional role of IKKβ and NF-κB in TLRs signaling in human MCs. Both IKKβ DN and IκB SR can prevent LPS or PGN induced NF-κB dependent cytokine production (IL-1β, TNFα and IL-6). (Figure 9A, 9B). Consistent with our findings with the adenoviral-mediated over-expression system, ML120B showed concentration-dependent inhibition of PGN or LPS induced IL-1β, TNFα and IL-6

production with IC_{50} values ranging from 2.2 to 5.6 μM (Figure 9C, 9D). P values from t-test were used to analyze the statistical differences.

Finally, chondrocyte activation is a known contributor to joint inflammation and destruction in RA and it has been shown that IL-1 mediated chondrocyte activation leads to production of MMPs and other mediators (Fernandes et al., 2002; Abramson, 2004). We used ML120B to evaluate the role of IKKβ in IL-1β mediated signaling in the human chondrocyte cell line SW1353. ML120B concentration-dependently inhibited IL-1β induced MMP13 and MMP1 production with the IC_{50} of 0.9 μM and 1.2 μM, respectively (Figure. 10A). In addition, IL-1β stimulated PGE2 production was also blocked by ML120B with an IC_{50} of 1.7 μM (Figure 10B).

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Discussion

IKKβ has been identified as the primary kinase responsible for NF-κB activation in cytokine stimulated cells and thus identification of selective IKKβ inhibitors as potential therapeutics for chronic inflammatory diseases has received considerable interest. In this report we describe the characterization of a potent and selective IKKβ inhibitor, ML120B. We demonstrate that ML120B is highly selective and specifically inhibits IκBα phosphorylation, NF-κB nuclear translocation, and transcription in a concentration-dependent manner. In order to evaluate the pharmacological effects of ML120B, we tested several cell types relevant to RA including human fibroblast-like synovioctes (HFLS), mast cells, and chondrocytes for inhibition of cytokine, chemokine and metalloprotease expression. In all cases we found that ML120B displays inhibitory effects in a concentration-dependent manner.

A chemical screening effort using the endogenous IKK complex allowed us to identify β-carboline derivatives as novel IKKβ inhibitors (Castro et al., 2003). Lead optimization led to the identification of ML120B, a potent ATP competitive inhibitor of IKKβ. ML120B selectively inhibits only IKKβ kinase activity when screened against a panel of 30 other kinases. In addition, ML120B does not inhibit the kinase activity of the related proteins IKKα or IKKε. Furthermore, in a panel of cellular pathway selectivity reporter assays, ML120B only showed an inhibitory effect on the NF-κB reporter or reporters that contains an NF-κB element in their promoter region (IL-8 and ELAM). Compared to the first generation β-carboline compound PS1145, ML120B has been shown to be a selective inhibitor specific to the NF-κB pathway. Thus, ML120B is a useful tool to evaluate the effects of IKKβ inhibition on the activation of the NF-κB pathway in a variety of cell-based systems relevant to RA.

To determine whether IKKβ activation can be blocked by ML120B in cells, we tested the inhibitory effect of ML120B on IκBα phosphorylation, on nuclear translocation of NF-κB, and on transcriptional regulation. ML120B inhibited TNFα induced IκB phosphorylation and degradation in

HeLa cells and inhibited nuclear translocation of NF-κB with an IC_{50} of 2.4 μM. ML120B also concentration-dependently blocked TNFα induced NF-κB DNA binding activity.

The cellular activity and selectivity of ML120B was further evaluated at the transcription level using NF-κB and AP-1 luciferase reporter assays. ML120B selectively inhibits NF-κB activity but does not inhibit AP-1 activity. It has been shown that cytokine activated IKKβ is essential for NF-κB activation (Mercurio et al., 1997). LPS induced cytokine production in human monocytes and T-cell receptor (TCR) mediated IL-2 production in T cells are both IKKβ dependent (O'Connell et al., 1998). As expected, ML120B inhibited both LPS stimulated TNFα production and αCD3/αCD28 co-stimulated IL-2 production in human PBMCs with similar IC_{50} values around 3 μM. Overall the data demonstrated that ML120B is a selective IKKβ inhibitor that blocks IκBα phosphorylation, nuclear translocation and NF-κB mediated transcription.

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovitis and bone destruction (Gravallese, 2002). Multiple lines of evidence suggest that several cell types play critical roles in the pathophysiological process of RA. We evaluated HFLS, mast cells and chondrocytes for ML120B mediated inhibition of NF-κB dependent responses. HFLS play a critical role in the process of cartilage and bone erosion in RA, presumably via the synthesis and secretion of inflammatory mediators including IL-6 and IL-8 (Georganas et al., 2000). *In vitro* IKKβ blockade with a dominant negative adenoviral construct inhibited IL-1 or TNFα stimulated IL-6, IL-8 and ICAM-1 production (Aupperle et al., 2001). In our study, ML120B inhibited TNFα or IL-1β induced IL-6 or IL-8 production in HFLS with IC_{50} values ranging from 5.7-14.5 μM (Table 3). Several lines of evidence indicate that NF-κB and MAP kinase /AP-1 pathways are both involved in IL-6 and IL-8 expression in HFLS (Neff et al., 2001), and that cross-talk exists between IL-1 and IL-6 signaling (Deon et al., 2001). Therefore, blocking NF-κB with a selective IKKβ inhibitor may only partially inhibit IL-6 or IL-8 production induced by TNFα or IL-1β in HFLS.

Several studies have examined the pathogenic role of chemokine and chemokine receptor interactions in RA (Nanki et al., 2001). We report here for the first time that in HFLS, both RANTES and MCP-1 production induced by TNFα or IL-1β are IKKβ/NF-κB dependent. The selective IKKβ inhibitor ML120B concentration-dependently blocked TNFα or IL-1β induced RANTES and MCP-1 production with IC_{50} values ranging from 0.7 μM to 1.8 μM (Table 3). We found ML120B is more potent in blocking the RANTES and MCP-1 production than its effect on blocking IL-6 and IL-8 production (Table 3) Pharmacia Corp's IKKβ inhibitor SC514 inhibits IL-1β stimulated IL-6 and IL-8 production in HFLS with an IC_{50} of 20 μM (Kishore et al., 2003). Compared to the first generation β-carboline IKKβ inhibitor PS1145, and other classes of IKKβ inhibitors, ML120B is a highly selective, potent inhibitor. In a separate report we demonstrated that ML120B was efficacious in an experimental model of RA. The oral administration of ML120B dose dependently inhibited paw swelling, offered significant protection from arthritis-induced body weight loss and cartilage and bone erosion (unpublished data). Considering the pivotal role of HFLS in the pathophysiology of RA, the inhibitory effect of ML120B on RANTES, MCP-1, IL-6 and IL-8 production may directly contribute to the *in vivo* efficacy of the compound.

Active arthritis is associated with increased cell infiltration, as well as expression of pro-inflammatory cytokines and MMPs (Smeets et al., 2003). ML120B concentration-dependently blocks TNFα or IL-1β induced MMP13, MMP3 and MMP1 production. Results from 2-3 donors are summarized in Table 3. These data suggest that IKKβ inhibition may play a dominant role in protecting joint disruption and blockade of inflammatory cell migration in RA joints.

The mast cell is a tissue-based inflammatory cell of bone marrow origin. Mast cells respond to signals of innate and acquired immunity with the immediate and delayed release of inflammatory mediators (Taylor and Metcalfe, 2001). The mast cell is now considered to play a pivotal role not only in allergic reactions but also in a number of other inflammatory disorders (Stevens and Austen, 1989; Boyce, 2003; Boyce, 2004). A potential role for mast cells in RA has also been highlighted recently (Benoist and

Mathis, 2002; Lee et al., 2002; Woolley, 2003) and it has been suggested that mast cells provide a critical cellular link between soluble factors and the synovial eruption through activation of cytokine production upon activation. Additionally, cytokine production by mast cells has been linked to NF-κB mediated events (Boyce, 2004). We therefore evaluated ML120B for inhibition of mast cell cytokine production in response to inflammatory initiators. The toll-like receptor (TLR) family is the essential recognition and signaling component of mammalian host defense (Akira and Sato, 2003). TLRs are responsible for the production of inflammatory cytokines in bone marrow-derived mast cells (BMMCs) (Stassen et al., 2001). Human cord blood PBMCs can be differentiated *in vitro* into mast cells (Hsieh et al., 2001). We studied the expression of TLR in this cell type by Flow Actuated Cell Sorting (FACS). Human cord blood derived mast cells express both TLR 2 and TLR 4 but have minimal expression of TLR 3 and TLR 9. We used adenoviral over-expression of a dominant negative form of IKKβ (IKKβ DN) and IκB super repressor (IκB SR) to study the function of IKKβ/NF-κB in TLR signaling. Either LPS or PGN induced IL-1β, TNFα and IL-6 production was abolished by IKKβ DN or IκB SR. In line with this finding, the selective IKKβ inhibitor ML120B has similar effects on human MCs. ML120B concentration-dependently inhibited LPS or PGN induced IL-1β, TNFα and IL-6 production with IC_{50} values of 2.2 μM to 5.6 μM. Our results indicate that inhibition of IKKβ may have a beneficial effect on attenuating local inflammation through the direct blockade of mast cell activation. Interestingly, in the same experiment we found that IL-4 and IL-13 production in MC is not affected by ML120B (data not shown), indicating that these two cytokines may use an NF-κB independent regulatory mechanism in human MCs.

Activated chondrocytes are also thought to play an important role in joint destruction in RA. Matrix Metalloproteases (MMPs), a family of zinc-dependent enzymes, play a prominent role in matrix degradation. Collagenases (MMP1, MMP13) and stromelysins (MMP3) are increased in RA synovium/cartilage (Close, 2001). IL-1 stimulates the release of degrading enzymes by FLS at the cartilage-pannus interface. At the same time, IL-1 may also activate chondrocytes to release these enzymes, which contributes to the cartilage destruction at sites distant to the pannus. ML120B blocked

IL-1β induced MMP13 and MMP1 production in SW1353 cells with IC_{50} values of 0.9 μM and 1.2 μM respectively, suggesting that chondrocytes may be a suitable cell target for IKKβ inhibition in RA. Recent evidence suggests that cyclooxygenase (COX-2), a NF-κB mediated gene product, is a mediator of angiogenesis, and COX-2 activity is known to be upregulated in the rheumatoid arthritis synovium (Woods et al., 2003). Activation of COX-2 leads to the production of the pro-inflammatory mediator PGE_2. We investigated the effects of ML120B in IL-1β stimulated PGE_2 production in human SW1353 cells and found that ML120B inhibited IL-1β induced PGE_2 with an IC_{50} of 1.7 μM. These data suggest a potent inhibitory effect of ML120B on chondrocyte activation and cartilage degradation.

RA is a chronic progressive inflammatory disease of the joint, involving several cell types thought to play key roles in the abnormal immune response, synovial hyperplasia and joint destruction observed in the course of the disease. These cellular processes are controlled through production of several molecular mediators including cytokines and chemokines and it is likely that NF-κB is a key transcription factor regulating this inflammatory response. Based on the critical role that IKKβ plays in cytokine mediated NF-κB activation, targeting IKKβ represents a novel approach for the treatment of RA. We have developed ML120B, a potent and selective inhibitor of IKKβ that specific to the NF-κB pathway. ML120B has shown consistent inhibitory effects towards key cell types and inflammatory mediators that are involved in the pathology and progression of RA. The results of these studies suggest a potential use of this class of IKKβ inhibitors as new therapeutic agents in the treatment of RA**.**

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Footnotes

This work was presented as a poster in ***Keystone Symposia NF-κB: Biology and Pathology. Utah, USA 2004:*** Wen D, Gangurde P, Morgan JG, Nong Y, Savinainen A, Kujawa J, Schopf L, Hepperle M, Harriman G, Jaffee B, Ocain T, Gutierrez-Ramos JC and Xu Y: NF-κB Regulation by IKKβ in Rheumatoid Arthritis.

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Legends for Figures

FIG.1. **Chemical Structures of β-carboline compounds. A.** PS1145: *N*-(6-chloro-9*H*-β-carbolin-8-yl) nicotinamide. **B. ML120B:** *N*-(6-chloro-7-methoxy-9*H*-β-carbolin-8-yl)-2-methylnicotinamide

FIG.2. **ML120B is a potent, reversible and ATP competitive inhibitor of IKKβ**. **A.**. Dose dependent inhibition of IKK complex by ML120B. Inhibition of activated IKK complex by ML120B at 50 μM ATP using biotinylated-GST tagged IκBα (5-55) as a substrate. The data represent the average of triplicate measurements with error bars indicating standard deviations. See "Experimental Procedures" for details. **B.** ML120B is an ATP competitive inhibitor of the IKK complex. Phosphorylation of biotinylated-GST tagged IκBα (5-55) was measured at fixed inhibitor concentrations of 167 μM (closed squares), 111 μM (closed triangles), 75 μM (closed diamonds), 50 μM (closed circles), 33μM (open squares), 22 μM (open triangles), and 0 μM (open diamonds). **C.** ML120B is a reversible inhibitor of the IKK complex. IKK enzyme alone or pre-incubated with 10μM ML120B was passed through a desalting column and assayed for activity. 100% activity was recovered from the pre-incubated sample as compared to IKK with 10 μM ML120B added after desalting. **D**. ML120B specifically inhibits recombinant IKKβ. The data represent the average of triplicate measurements with error bars indicating standard deviations. See "Experimental Procedures" for details.

FIG.3. **ML120B inhibits kinase activity in cells.** HeLa cells were pre-incubated with ML120B for 1hr and subjected to TNFα (30 ng/mL) stimulation. Total cell lysates were collected for Western Blot. **A**. TNFα can induce IκB phosphorylation and degradation in 5-20 minutes. **B**. ML120B concentration-dependently blocked TNFα induced IκB phosphorylation in HeLa cells.

C. ML120B concentration-dependently blocked TNFα induced IκB degradation in HeLa cells. **D**. In HeLa cells, ML120B block TNFα induced IκB phosphorylation at 5 and 10 minutes. **E**. In HeLa cells, ML120B block TNFα induced IκB degradation at 10 and 20 minutes.

FIG.4. **ML120B inhibits TNFα induced NF-κB p65 cytoplasm to nuclear translocation in HeLa cells. A**. Image data of NF-κB p65 localization by Discovery-1. HeLa cells were seeded in a 96-well plate the day before the experiment. The cells were pre-incubated with serial diluted ML120B (range from 20 μM to 0.04 μM) prior to the simulation with TNFα (10 ng/mL). Cells were fixed with 4% paraformaldehyde and permiabilized with 0.5% Triton X-100 post 30 minutes TNFα stimulation. Cells were stained for both FITC labeled p65 and Hoechst following the Cellomics Hit Kit Manuel. Images were acquired with Discovery-1 system (Universal Imaging Corp. UIC). **B.** IC_{50} graph of high content image analysis by MetMorph software. Image data acquired with Discovery-1 system were analyzed by MetaMorph software form UIC. Quantitative data of NF-κB p65 localization in the cells was calculated for IC_{50} with XLfit.

FIG.5. **ML120B dose dependently inhibits NF-κB dependent transcriptional activity in 293/NF-κB luciferase reporter assay but has minimal effect on 293/AP-1 dependent transcriptional activity.** 293 / NF-κB-Luc or 293 / AP-1-Luc cells were pre-incubated with 1:1 serial diluted ML120B (20 μM to 0.04 μM) in a 96-well plates for 1 hr. Cells were stimulated with TNFα (0.5 ng/mL) in Fig.5A or PMA (10 pg/mL) / ionomycin (2 μM) in Fig 5B for 3 hours respectively. Luciferase activity was measured using cell lysates.

FIG.6. **ML120B cellular activity in Human PBMC Assay. A**. ML120B concentration-dependently blocks LPS stimulated TNFα production. Fresh isolated human PBMCs were seeded in a 96-well plate at 4 X 10^5 per well. Cells were pre-incubated with ML120B (20 μM to

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0.04 μM) for 1 hour and stimulated with LPS (100 ng/mL) for 5 hours. Supernatant was collected for TNFα ELISA. **B.** ML120B concentration-dependently blocks αCD3/αCD28 crosslink induced IL-2 production. Same as above described, PBMCs were co-stimulated with αCD3 (0.25 μg/mL) and αCD28 (0.25 μg/mL) for 5 hours. Supernatant was collected for IL-2 ELISA. **C**. ML120B concentration-dependently blocks TNFα induced IκB phosporylation. 8 X 10^6 fresh isolated human PBMCs were seeded in 60 mm dishes. Cells were pre-incubated with serial diluted ML120B (20 μM, 5 μM and 1.25 μM) at 5% CO_2 37^{O}C for 1 hour and stimulated with TNFα (50 ng/mL) for 5 minutes or 10 minutes. Total cell lysates were used to determine the level of IκB phosphorylation by Western blot. Same blot was probed by house keeping gene Actin to verify the equal loading. **D**. Cytotoxicity (LDH) assay. ML120B cytotoxicity was monitored by LDH release in human PBMC assay. 2% Triton treated cells was used as maximal LDH release control. Compound solvent DMSO was used as minimal basal LDH release control.

FIG.7. **Effect of ML120B on Human Fibroblast Like Synoviocyte (HFLS).** **A**. ML120B concentration-dependently blocks TNFα induced MCP-1, RANTES and IL-6 production in HFLS. 1 X 10^5 HFLS were seeded in a 96 well plate. Cells were pre-incubated with 1:1 serial diluted ML120B (20 μM to 0.04 μM) for 1 hour and stimulated with TNFα (30 ng/mL) for 16 hours. The supernatant was harvested and analyzed for RANTES, MCP-1 and IL-6 production (PerBio, Pierce). **B**. ML120B concentration-dependently blocks IL-1β induced MCP-1, RANTES and IL-6 production. Same as above described in Fig. 6A, except that the cells were stimulated with IL-1β (10 ng/mL) for 16 hours. **C**. ML120B blocks both IL-1β and TNFα induced NF-κB p65 binding activity. HFLS were pre-incubated with serial diluted ML120B (20 μM to 0.04 μM) for 1 hour. Cells were stimulated with either IL-1β (10 ng/mL) or TNFα (30 ng/mL) for 30 minutes. Nuclear extracts were prepared. NF-κB p65 binding activity was quantitatively measured by NF-κB DELFIA assay as described.

FIG 8. **TLR Expression in Human Cord Blood Derived Mast Cells**. PBMCs were freshly isolated from human cord blood (Cambrex) and *in vitro* differentiated into mature mast cells as described in Experimental Procedure. The maturity of mast cells was monitored by toluidine blue staining and c-Kit expression on cell surface by FACS (data not shown). The expression of Toll-Like Receptor (TLR) was tested by FACS analysis with specific antibody to TLR 2, TLR 4, TLR 3 and TLR 9. Human cord blood derived mast cells express both TLR4 (Receptor for LPS) and TLR2 (Receptor for PGN), but has minimum expression of TLR 3 and TLR9.

FIG.9. **Role of IKKβ in Human Mast Cell Function.** **A.** 4 X 10^5 matured mast cells were infected with optimized adenoviruse titer (MOI). The similar infection rate for vector alone (GFP), wild type IKKβ (IKKβ wt), dominant negative IKKβ (IKKβ DN) and IκB super repressor (IκB SR) was confirmed with GFP expression. After 24hours infection, cells were then stimulated with LPS (10 ng/mL) for 16 hours. The supernatant was harvested and analyzed for IL-1β, TNFα and IL-6 production (PerBio Pierce). P values were used to analysis the statistical differences. **** $p<0.001$, *** $p<0.005$, ** $p<0.01$, * $p<0.05$. **B.** Same as A, after 24 hours infection, cell were stimulated with PGN (100 μg/mL) for 16 hours. The supernatant was harvested and analyzed for IL-1β, TNFα and IL-6 production (PerBio Pierce). P values definition is same as Fig 8A. **C**. Human MC were seeded in a 96-well plate and pre-incubated with 1:1 serial diluted ML120B (20 μM - 0.04 μM) for 1hr. Cells were stimulated with LPS (10 ng/mL) or PGN (100 μg/mL) overnight. The supernatant was collected for cytokine profiling analysis (PerBio, Pierce). The data was expressed as percentage inhibition of cytokine production. The IC_{50} values were calculated with Excel XLFit program. **D**. Summary of LPS or PGN stimulated IL-1β, TNFα and IL-6 production in human cord blood derived mast cells. IC_{50} results are expressed as the mean ± S.D. of 3 different experiments.

FIG.10. **Effect of ML120B on Human Chondrocyte Activities.** **A**. ML120B concentration-dependently blocks IL-1β stimulated MMP13 and MMP1 production in SW1353. Cells were pre-incubated with 1:1 serial diluted ML120B (20 μM to 0.04 μM) for 1hr and stimulated with IL-1β (10 ng/mL) for 24 hours. The supernatant was harvested for MMP1, and MMP13 ELISA. **B.** ML120B concentration-dependently blocks IL-1β induced PGE_2 production in SW1353. Same as above described in Fig. 10 A, the supernatant was also collected for PGE_2 ELISA.

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Table 1. Selectivity of ML120B against a Panel of Kinases. Among the 30 kinases examined, ML120B exhibited <50% inhibition against all 30 kinases at the corresponding highest concentration tested (50, 84 or 100 μM). ML120B was also screened against other isoforms of IKK. ML120B selectively blocks IKKβ kinase activity but has minimal effect on IKKα or IKKε.

Table 1

Enzyme	IC50 (μM)	Enzyme	IC50 (μM)	Enzyme	IC50 (μM)
IKKα	**>100**	PKCα	>100	ERK2	>100
IKKε	**>100**	PKCδ	50	p38a	>100
PKA	>100	PKCθ	50	MKK6	>83
CaM KII	>100	CSK	>50	MEK1	>83
CDK2/Cyclin E	>100	Src	>50	cRaf-1	>83
CKII	>100	Lyn	>50	cABL1	>50
LCK	>100	MK2	>50	p70 S6K	>100
CHK1	>100	KDR	>50	ROKA	>100
MAPKAPPK2	>100	JNK1	84	SGK1	>100
PLK	>100	PRPK	>100	PRAK	>100

Table 2 ML120B and PS1145 Cellular Selectivity Assay ML120B and PS1145 were screened against a panel of eight cellular pathway luciferase reporter assays. Results clearly show that ML120B specifically inhibits the NF-κB pathway, as measured by the NF-κB reporter, or reporters that contain an NF-κB element(s) in their promoter region (IL-8 and ELAM). ML120B has minimal inhibitory effects on other pathways tested in cellular pathway selectivity assays. ML120B also shows greater selectivity than the earlier generation β-carboline inhibitor, PS1145.

Cellular Pathway Selectivity				
Reporter	**Stimulator**	**Pathway**	**ML120B IC_{50} (μM)**	**PS1145 IC_{50} (μM)**
NF-κB	PMA	IKK / inflamm / apoptosis	2.8	1.7
AP-1	PMA	JNK / stress / growth	>30	8.8
NFAT	PMA+Ionomycin	PKC & $Ca2^{+}$ / calcineurin	>30	6.6
SRE	PMA	MAPK / ERK / growth	>30	>30
CRE	Forskolin	PKA / stress	>30	>30
ISRE	IFNβ	JAK / STAT / inflamm	>30	>30
IL 8	TNFα	inflammation marker (cytokine)	6.3	3.4
ELAM	TNFα	inflammation marker (selectin)	0.5	0.9

Table 3. IC_{50} of ML120B on IL-1β or TNFα Stimulated Cytokines/Chemokines and MMPs Production. ML120B cellular potency in HFLS was tested on 2-6 donors. Average of IC_{50} values are summarized in Table 3.

	IL-1 sti IC_{50} (μM)	SD	Donor tested	TNFα sti IC_{50} (μM)	SD	Donor tested
IL-6	**5.7**	2.2	5	**14.5**	12.1	6
IL-8	**7.3**	5.0	4	**8.9**	4.2	3
MCP-1	**1.8**	1.5	6	**1.6**	1.7	6
RANTES	**0.8**	0.6	6	**0.7**	0.5	6
MMP-1	**9.7**	1.2	2	**2.8**	0.9	3
MMP-3	**1.9**	2.0	3	**2.0**	0.6	3
MMP-13	**1.9**	1.6	3	**0.9**	0.3	3

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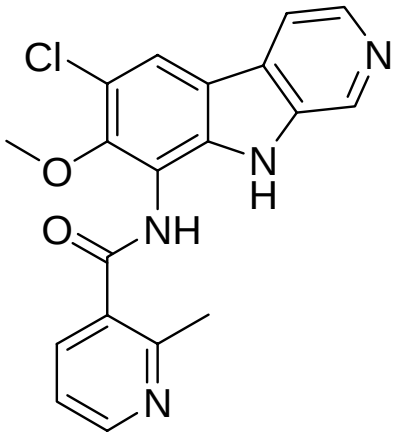
Figure 1

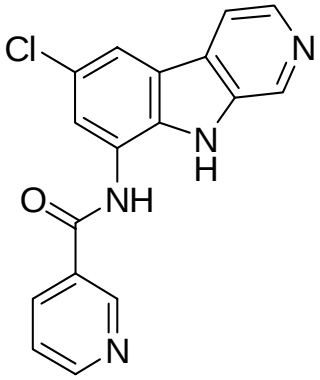
A. ML120B



Molecular Weight =367
Molecular Formula = $C_{19}H_{15}ClN_4O_2$

N-(6-chloro-7-methoxy-9*H*-β-carbolin-8-yl)-2-methylnicotinamide

B PS-1145



Molecular Formula: $C_{17}H_{11}ClN_4O$
Molecular Weight : 322.75

Chemical name: *N*-(6-chloro-9*H*-β-carbolin-8-yl)nicotinamide

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Figure 2

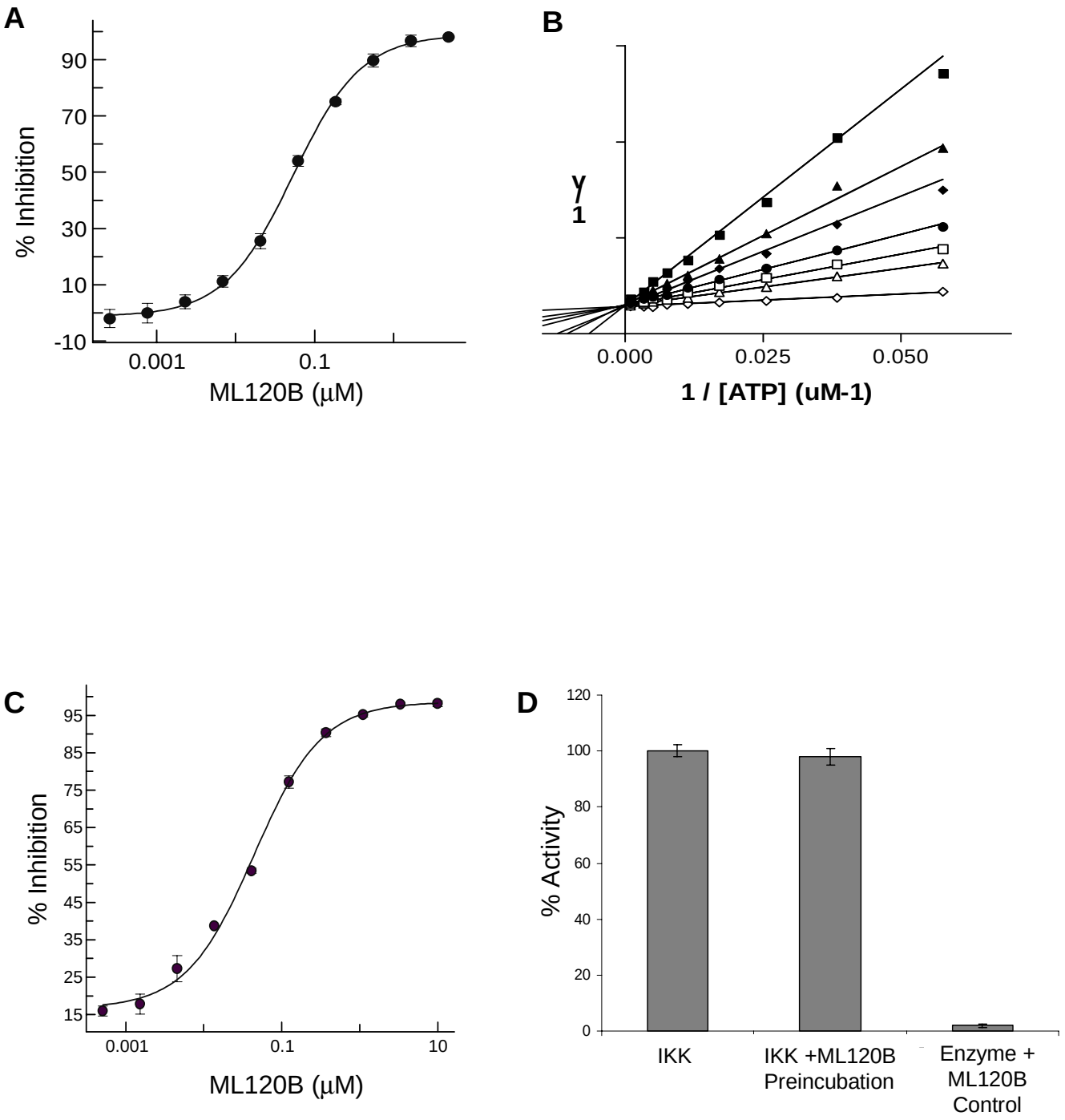

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Figure 3

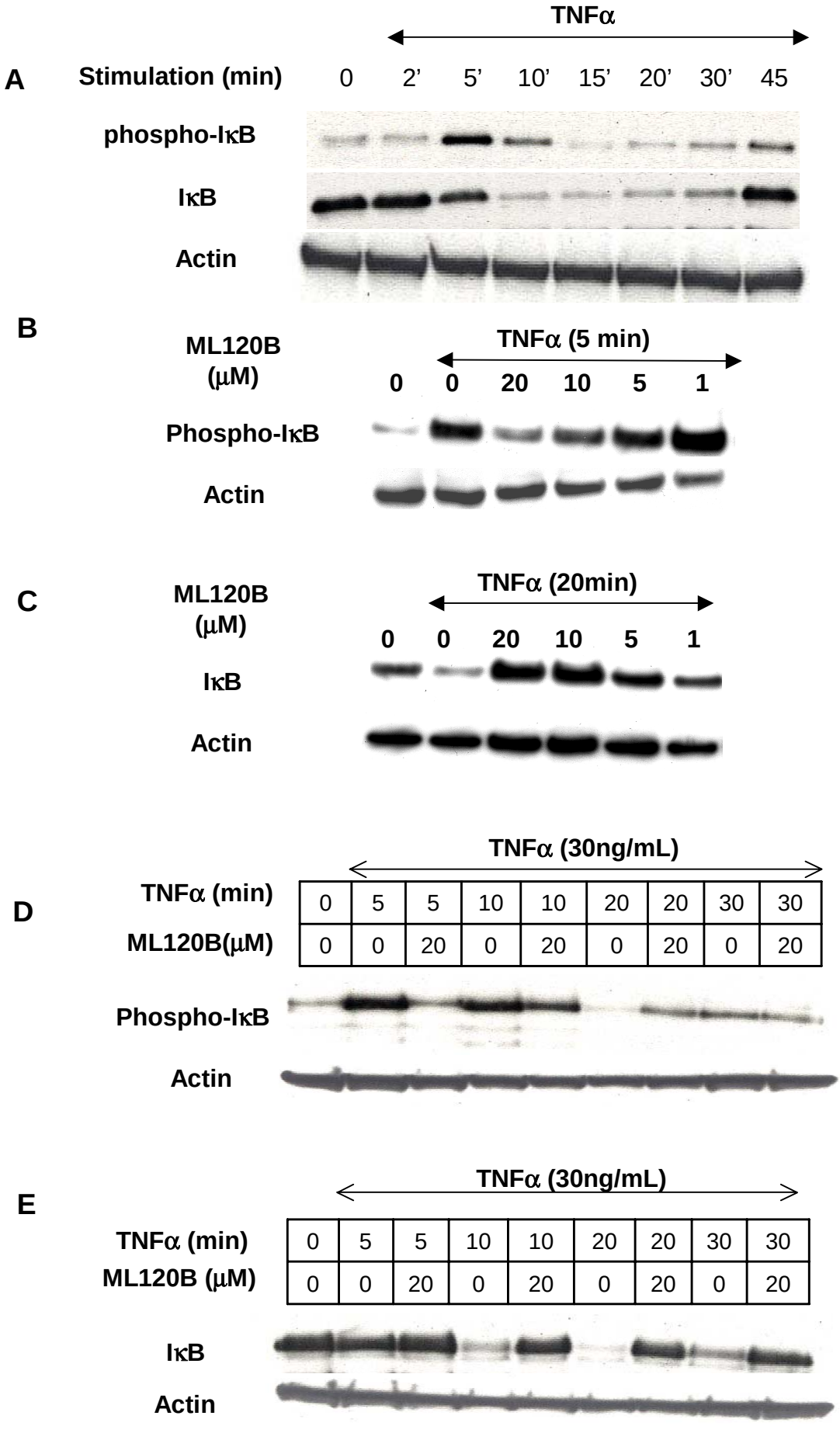

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Figure 4

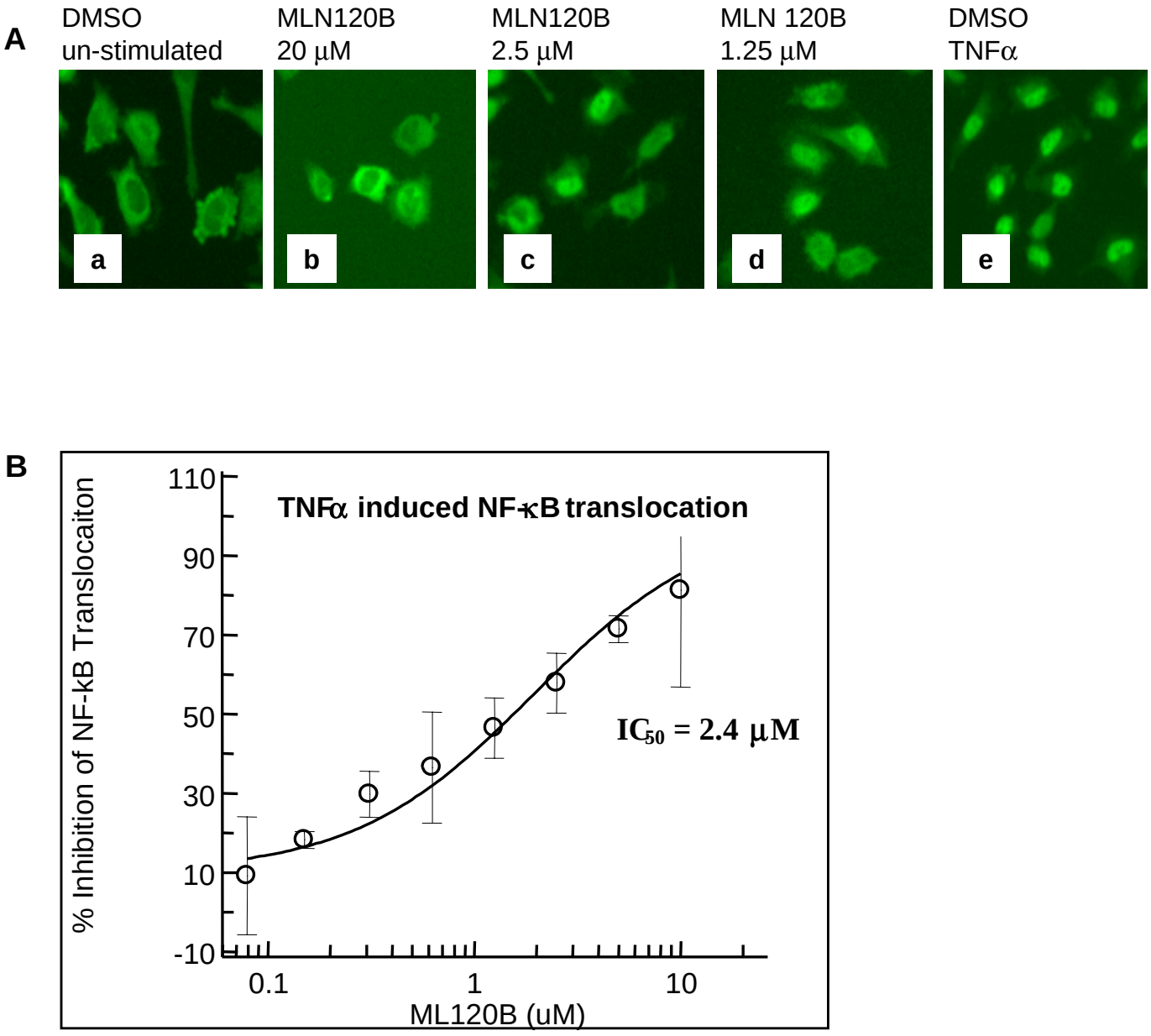

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Figure 5

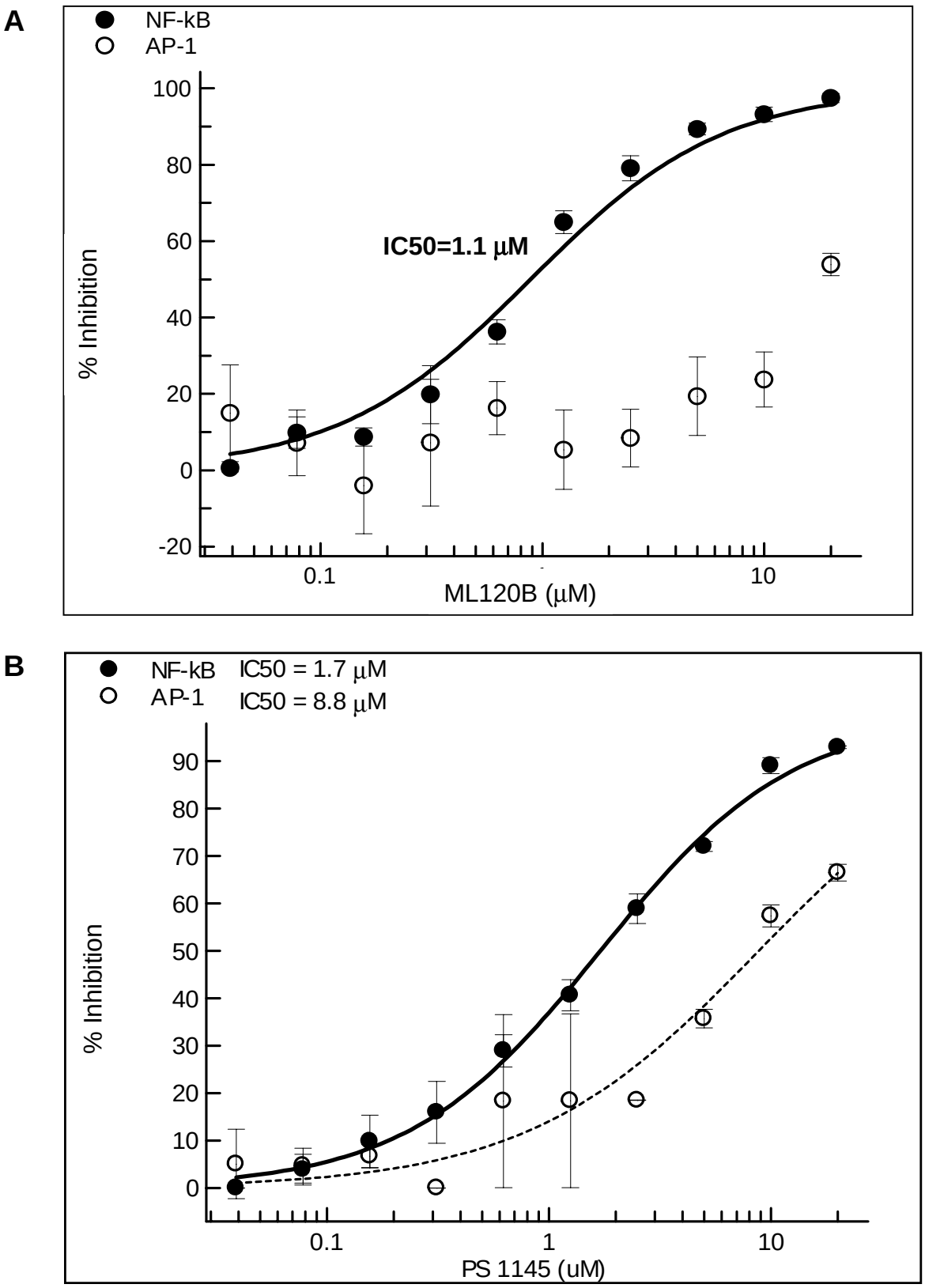

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Figure 6

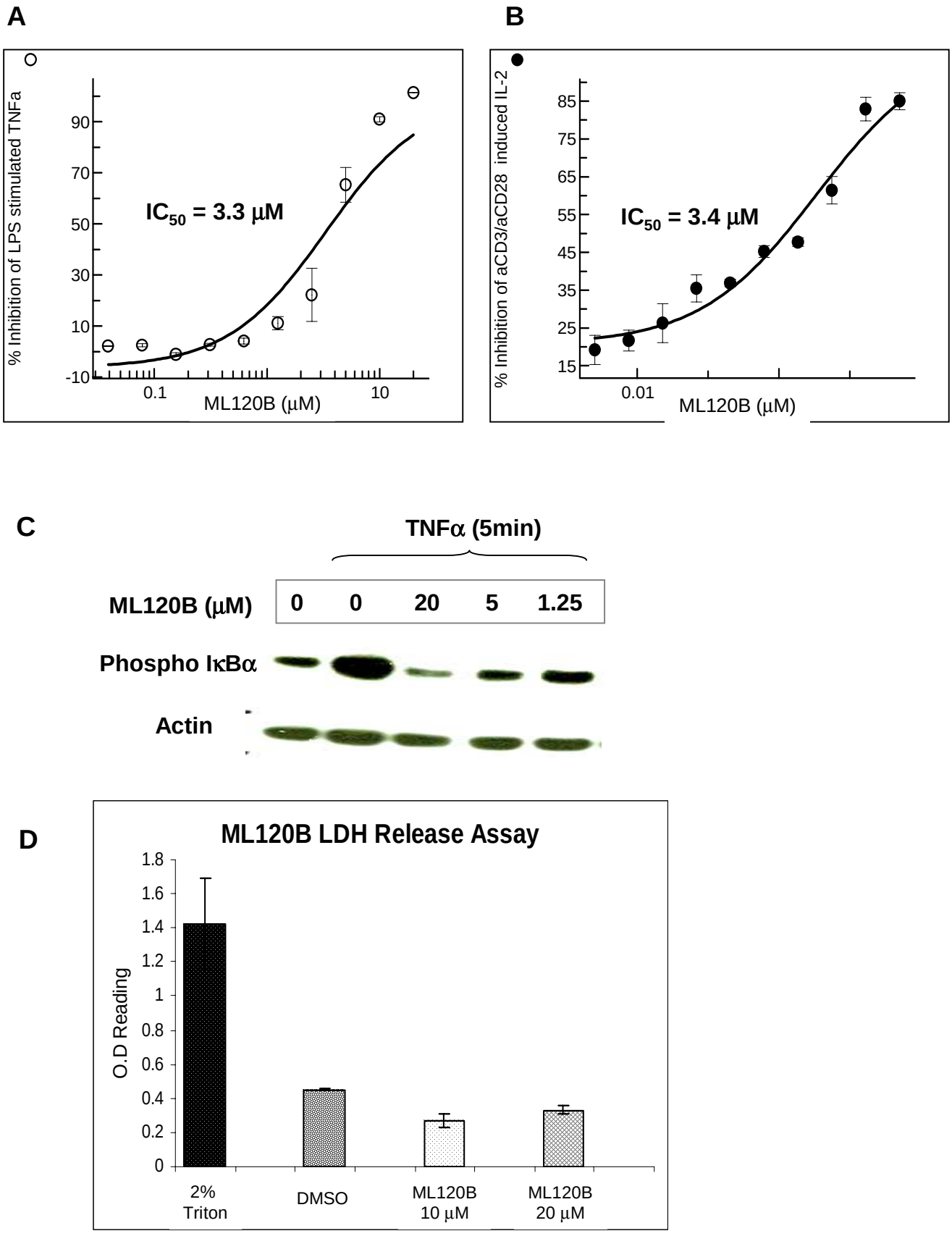

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Figure 7

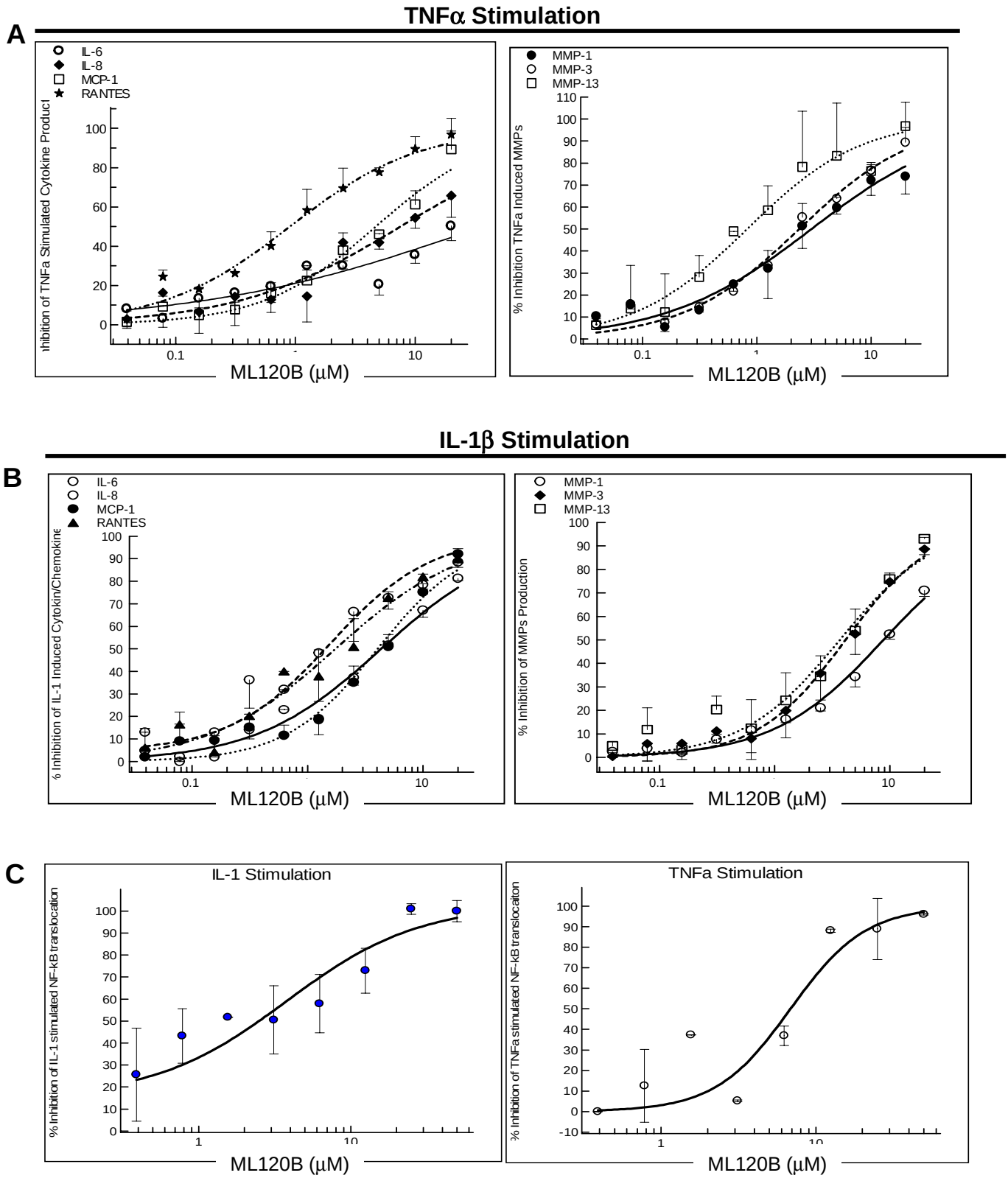

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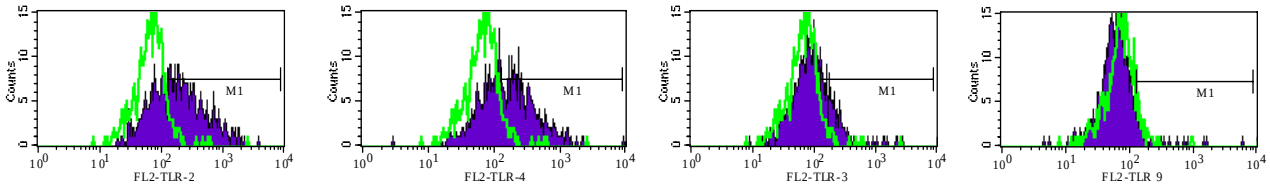
Figure 8



TLR 2 **TLR 4** **TLR 3** **TLR9**

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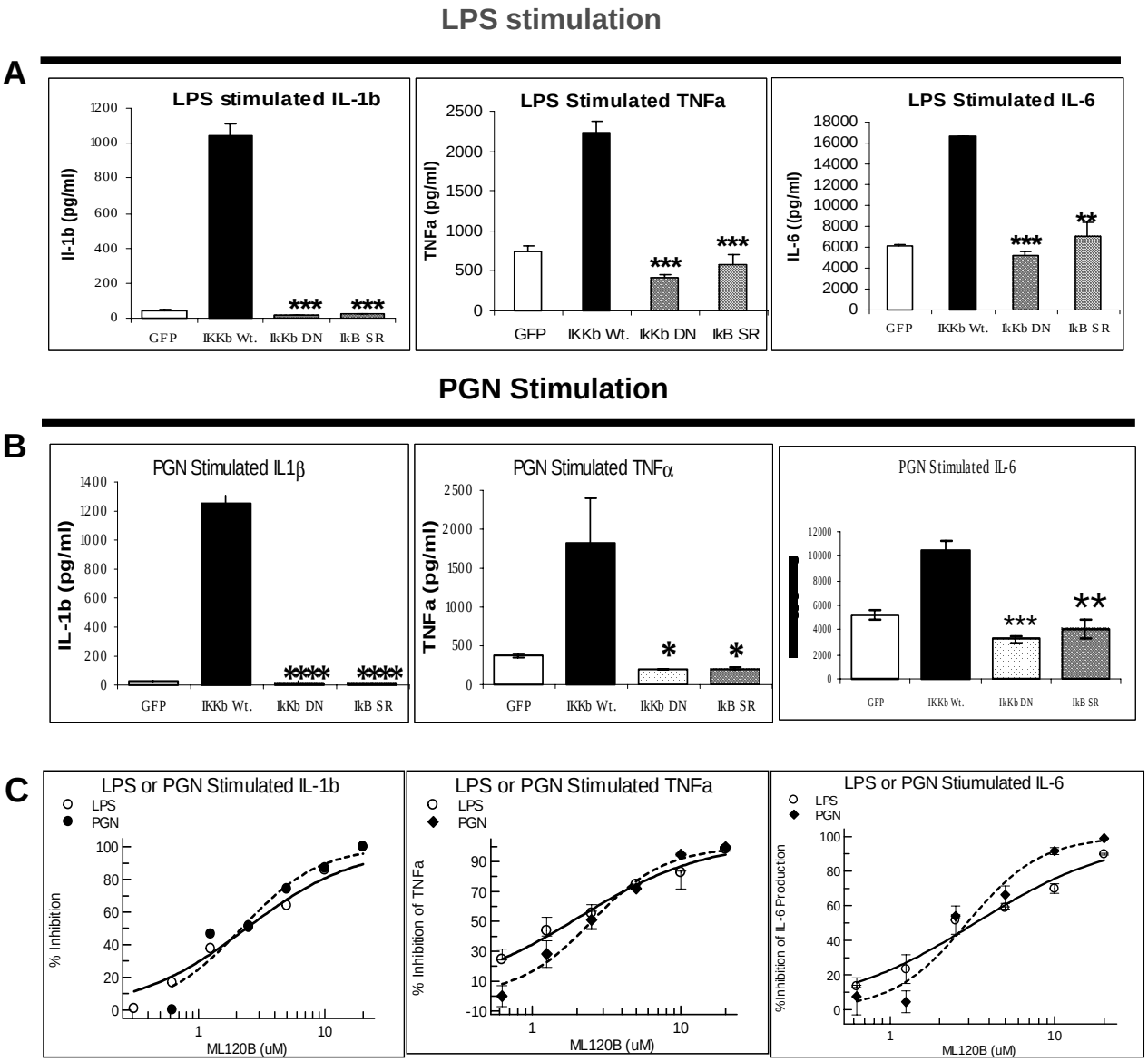
Figure 9



D

ML120B IC50 (μM)	IL-1β	TNFα	IL-6
LPS Stimulation	2.4	3.0 ± 1.3	5.6 ±3.2
PGN Stimulation	2.2	2.4 ± 0.1	2.7 ± 0.4

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Figure 10