UNIVERSITY OF CALIFORNIA, SAN DIEGO

IKK epsilon Deficiency Exacerbates Inflammation in the Absence of an Interferon Response in Murine Arthritis by Delaying Neutrophil Apoptosis

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

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by

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The Thesis of Christopher Sai-Hau Chung is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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LIST OF ABBREVIATIONS

IKKε = I Kappa B Kinase epsilon

FasL = Fas ligand

TNF = tumor necrosis factor

G6PI = glucose 6-phosphate isomerase

FLS = fibroblast-like synoviocytes

IL = interleukin

IL-1ra = interleukin-1 receptor antagonist

IFNαβR = type 1 interferon α/β receptor

RA = rheumatoid arthritis

IFN = interferon

RF = rheumatoid factor

ACPA = anti-citrullinated protein antibody

HLA = human leukocyte antigen

PTPN22 = protein tyrosine phosphatase non-receptor type 22

NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells

TANK = TRAF family member-associated NF-κB activator

IRF = interferon regulatory factor

STAT1 = signal transducers and activators of transcription

GAF = gamma activated factor

ISGF3 = interferon stimulated gene factor 3

ROS = reactive oxygen species

PS = phosphatidylserine

TRAIL = TNF related apoptosis-inducing ligand

FADD = Fas associated protein with death domain

XIAP = X-linked inhibitor of apoptosis

TLR = toll-like receptor

RIG = retinoic acid-inducible gene

FCS = fetal calf serum

SDS = sodium dodecyl sulfate

PMA = phorbol 12-myristate 13-acetate

HTAB = hexadecyltrimethylammonium bromide

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ABSTRACT OF THE THESIS

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Rheumatoid arthritis (RA) is a chronic autoimmune disorder of the joints. In K/BxN arthritis, disease is mediated by autoantibodies directed toward glucose 6-phosphate isomerase (G6PI). It is currently believed that G6PI is deposited on the joint cartilage where it facilitates autoantibody-mediated attack. This model is heavily dependent on the recruitment of neutrophils in order to contribute to the inflammatory phenotype. We investigated the role of I kappa B kinase epsilon, a non-canonical activator of NF-kB, in the absence of an interferon response in the

pathogenesis of RA. Arthritis in *Ikbke*$^{-/-}$ mice is attenuated and is characterized by reduced levels of pro-inflammatory cytokines and neutrophil-recruiting chemokines in the joints. Interestingly, these mice are sensitized to therapeutic doses of interferon therapy, which to date has not shown efficacy at reasonable doses in human RA. Accordingly, *Ifnar1*$^{-/-}$ mice experience increased joint swelling in the K/BxN serum transfer model of arthritis as compared to wild-type mice. Unexpectedly, targeting of *Ikbke*$^{-/-}$ enhances the level of arthritis in the *Ifnar1*$^{-/-}$ mice.

Ifnar1$^{-/-}$ mice are neutrophilic and have expanded neutrophil-recruiting capacities. We propose that the mechanism by which IKKϵ enhances arthritis in the absence of interferon signaling is through the desensitization of neutrophils to apoptosis. IKKϵ deficiency prevents proper neutrophil clearance by prolonging neutrophil lifespan and delaying apoptosis. Therefore, arthritis in doubly deficient mice is enhanced by both the increased neutrophil recruiting capacities displayed by loss of interferon signaling and extended lifespans of these recruited neutrophils via loss of IKKϵ.

1 INTRODUCTION

1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology and is characterized by systemic inflammation of the synovial joints. The most common clinical presentations of disease are joint stiffness, swelling, and pain among others. Although the pathogenesis of this disease is still not fully understood, genetics seems to play a role as studies have shown that disease concordance among monozygotic twins is more than three times that of dizygotic twins (Lee et al. 2001). However, genetics can only account for about 50% of the predisposition to disease, leaving the rest up to environmental factors, the most common of which is smoking. The disease affects women at a rate three times higher than men, and prevalence increases with age. Our understanding of the molecular mechanisms of inflammation in RA has increased dramatically over the past several decades. The hallmark pathological changes associated with RA include the presence of autoantibodies, including the classical rheumatoid factor (RF), which recognizes the Fc portion of IgG antibodies, and the emerging anti-citrillunated peptide antibodies (ACPAs), and the increased expression of a vast repertoire of inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), and interleukin-1 (IL-1) (Scott et al. 2001).

Several genetic risk factors for RA have been elucidated. The specific human leukocyte antigen (HLA)-DRB1 locus is strongly associated with patients who are rheumatoid factor or ACPA positive (McInnes et al 2011). Additionally, a

specific mutation in protein tyrosine phosphatase N22, PTPN22, responsible proper T cell signaling in lymphocytes, has shown to be correlated with greater incidence of RA in Europe and North America (Andersson et al. 2008). Furthermore, a PTPN22 single nucleotide polymorphism 1858 was found to be greatly associated with ACPAs. Combination of the two has resulted in 100% specificity for RA diagnosis (Andersson et al. 2008).

Although the mechanism underlying loss of tolerance has yet to be elucidated, key immune regulatory factors have been implicated in development of RA. Both the adaptive and innate arms of the immune system have been shown to be necessary for pathogenesis. Much of the evidence for the involvement of the adaptive branch of the immune system is the presence of several hallmark autoantibodies found in patients with RA. These autoantibodies include rheumatoid factor as well as ACPAs. Examples of self-proteins that can become citrillunated include α-enolase, keratin, fibrinogen, fibronection, collagen, and vimentin (McInnes et al. 2011). Not all patients exhibit ACPA-positive phenotypes and presence of ACPA is generally an indicator of poorer prognosis, including more severe joint destruction (Scott et al. 2010). The synovial lining of patients with RA exhibits elevated numbers of infiltrating T cells. T cell targeted therapies in the literature have reported mixed efficacies. The role of B cells in pathogenesis is confirmed by the success of rituximab, a B cell targeted drug, in RA (McInnes et al. 2011). Additionally, plasma B cells are the main producers of autoantibodies seen in RA.

Activation of the innate immune system is crucial for the pathogenesis of RA. Synovitis, inflammation of the synovial lining of the joints, is caused and perpetuated by a number of events that involve the migration of innate immune cells, proliferation of synoviocytes, and production of inflammatory cytokines. Cartilage damage and bone erosion are the result of overproduction of metalloproteinases and osteoclast activation, respectively.

1.2 I Kappa B Kinase Epsilon

First characterized as a lipopolysaccharide inducible protein, I kappa B kinase epsilon (IKKε) is found predominantly in immune cells and activates the important immune-regulating transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Shimada et al. 1999). Further studies have also revealed that IKKε is a co-factor involved in the endogenous interferon signaling pathway and, together with TRAF family member-associated NFκB activator (TANK) binding kinase-1, has been shown to phosphorylate interferon regulatory factors (IRFs) 3 and 7, promoting IRF translocation to the nucleus and transcription of type 1 interferons (Sharma et al. 2003).

IKKε also plays a role downstream of interferon signaling by phosphorylating signal transducers and activators of transcription-1 (STAT1), which prevents the formation of gamma-activated factor (GAF) but allows the formation of interferon stimulated gene factor 3 (ISGF3). The consequence of this balance results in promotion of type I interferon signaling and greater transcription of type I interferon inducible antiviral genes (Ng et al. 2011). The ability of IKKε to modulate both the interferon response as well as NF-κB related

genes suggests its potential as a target for therapeutic enhancement of interferon-beta treatment in rheumatoid arthritis.

1.3 Neutrophil Apoptosis

Neutrophils are crucial effectors of the innate branch of the immune system and are vital for the recognition and defense against invading foreign pathogens. However, the life span of neutrophils is a tightly regulated process and dysregulation or impairment of these apoptotic pathways can have negative effects on the host. Neutrophils possess a wide arsenal of defense mechanisms to defend against infections including the production of reactive oxygen species (ROS), secretion of antimicrobial peptides, phagocytosis, and extrusion of nuclear DNA in a process known as NETosis (Mocsai 2013).

Proper neutrophil cell death and clearance, via apoptotic or necrotic mechanisms and phagocytic engulfment, respectively, are important for the resolution of inflammation after successful neutralization of bacterial infection (Luo & Loison 2007). Apoptosis and necrosis are two common forms of programmed cell death, both of which are utilized by neutrophils. Constitutive neutrophil cell death most closely resembles an apoptotic event, characterized by cell shrinkage, externalization of the lipid membrane component phosphatidylserine (PS), nuclear condensation, and mitochondrial polarization (Luo & Loison 2007).

Extrinsic neutrophil apoptosis is triggered by the ligation of so-called "death receptors" by their respective ligands. The two most well characterized death receptor-ligand pairs are Fas-Fas ligand and TNF receptor-TRAIL (TNF

related apoptosis inducing ligand). Ligation of these death receptors leads to the formation of intracellular death complexes. Fas signaling results in recruitment of Fas associated death domain, FADD, which can subsequently activate caspase-8 into its active cleaved form. Although activated caspase-8 is in itself enough to trigger the other effector caspases, most notably capase-3, it can also cleave Bid, resulting in the formation of truncated Bid, a small cytotoxic protein capable of disrupting the mitochondrial membrane, thus initiating a mitochondrial-mediated form of apoptosis. However, this step is not necessary for the execution of extrinsic apoptosis (Galluzi et al. 2012).

In contrast, intrinsic neutrophil apoptosis is initiated independent of signaling occurring as a result of receptor ligation on the neutrophil surface. These include irradiation, genotoxic stress, and withdrawal of growth factors (Leitch et al. 2008). These stress signals will lead to the formation of the apoptosome, which includes but is not limited to proteins such as Apaf1 and cytochrome c released as a result of mitochondrial permeabilization. The apoptosome proceeds to activate caspse-9 via cleavage into its active form, which in turn actives caspase-3 through proteolytic cleavage. Intrinsic neutrophil apoptosis is regulated by the Bcl-2 family of proteins. The pro-apoptotic members of this family, including Bax, Bak, Bad, and Bid are responsible for liberating cytochrome c from the mitochondria (Leitch et al. 2008). These proteins are regulated by their pro-survival counterparts, including A1, Bcl-x_L, and Mcl-1.

Cross talk between the two pathways is also evident in caspase-8's ability to cleave Bid into its cytotoxic truncated form, tBid. Localization of tBid to the

mitochondria promotes its permeabilization, thus triggering the intrinsic apoptotic mechanisms. There is an additional family of proteins that prevents apoptosis known as IAPs, or inhibitors of apoptosis. These include cellular IAP-1 and 2 (cIAPs) as well as X-linked IAP (XIAP). These proteins sequester caspases in the cytosol to prevent them from carrying out their apoptotic effector functions. In non-inflammatory conditions, high levels of the pro-apoptotic Bcl-2 proteins are constitutively expressed. Mcl-1, along with its other pro-survival family members, is expressed at low levels under these same conditions. (Simon 2003). Overall, these mechanisms are vital toward the resolution of inflammation.

1.4 Type I Interferons

Interferons are cytokines that play a major role in antiviral immunity and modulate the immune system in order to fight viral infections. They can be ubiquitously expressed by nearly every major cell type and are essential for the clearance of many pathogens. There are three main classes of interferons: type I, type II, and type III. Type I is the largest family and the two most well characterized interferons belonging to this group are interferon-alpha (IFN-α) and interferon-beta (IFNβ). Stimulation of interferon production begins with surface or intracellular binding of receptors that activate transcription factors that are able to initiate transcription of interferons. Interferons are pleiotropic, with its effects exerted through the production of interferon-stimulated genes (ISGs).

The induction of type I interferons is dependent on the specific stimuli and receptors that are engaged. One of the most common mechanisms of interferon production begins with the engagement of pattern recognition receptors known

as Toll-like receptors (TLRs), which are able to recognize and bind a host of foreign viral and bacteria antigens. TLRs that have been shown to signal downstream production of interferon include TLR3, TLR4, TLR7, and TLR9 (Gonzalez-Navajas et al. 2012). Other TLR-independent mechanisms have also been shown to induce the transcription of type I interferons. These include the cytosolic RNA sensor RIG-I, cytosolic DNA sensor STING, as well as the DNA-dependent activator of IRF (Gonzalez-Navajas et al 2012).

In rheumatoid arthritis, patients have been reported to show elevated levels of IFN-β in their synovial tissue. The exact role of IFN in RA has not fully been elucidated. IFN-β has been reported to have anti-inflammatory effects via the down regulation of TNF-α and IL-1β, which promotes the induction of IL-10 and IL-1ra (Holten et al. 2002). Several different studies have shown efficacy of IFN-β therapy in mouse models of arthritis. However, human clinical trials have been mixed and tolerance to treatment continues to be problematic (Tak 2004).

1.5 The K/BxN Mouse Model of RA

First described in 1996, the K/BxN mouse model was developed by Drs. Benoist and Mathis when their groups fortuitously crossed a KRN mouse, which contains a transgenic T cell receptor that recognizes a bovine pancreas RNase, to a non-obese diabetic (NOD) mouse. The transgene positive offspring developed spontaneous arthritis, characterized by swelling and redness in all the distal joints (Kouskoff et al. 1996). Clinically, histologically, and immunologically, the model is strikingly similar to RA in humans. Genetic analyses revealed that the I-A^{g7} molecule of class II MHC on the NOD background is responsible for the

development of disease in KRNxNOD offspring. Absence of either $CD4^+$ T cells or mature B cells completely eliminates arthritis both histologically or by clinical assessment of ankle thickness.

Autoreactive T cells in the K/BxN mice recognize a peptide presented on the $I\text{-}A^{g7}$ molecule of MHC class II presenting cells in the NOD background. Subsequent interaction with B cells results in the production of autoantibodies, which have been identified as autoreactive toward the ubiquitously expressed glucose-6-phosphate isomerase (G6PI). G6PI electrostatically arrays on the surface of cartilage. Hence, anti-G6PI causes preferential swelling of the distal joints, similar to human RA. In humans however, anti-G6PI autoantibodies are not specific for RA.

2 METHODS

2.1 Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

2.2 Mice

KRN T cell receptor (TCR) transgenic mice were a kind gift from Drs. D. Mathis and C. Benoist (Harvard Medical School, Boston, MA), and Institut de Génétique et de Biologie Moléculaire et Cellulaire (Strasbourg, France), and were maintained on a C57Bl/6 background (K/B). Arthritic mice were obtained by crossing K/B mice with NOD/Lt (N) mice, producing K/BxN mice. Progeny bearing the Vβ6 transgenic TCR were identified by quantitative PCR using custom designed primers to detect the transgenic receptor and anti-Vβ6 FITC (BD-PharMingen, San Diego, CA) labeled antibodies. These were tenth generation backcrossed onto C57Bl/6. C57Bl/6 and NOD/Lt mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice were bred and maintained under standard conditions in the University of California, San Diego Animal Facility that is accredited by the American Association for Accreditation of Laboratory Animal Care. All animal procedures were approved by the UCSD Institutional Animal Care and Use Committee (IACUC).

2.3 Serum Transfer and arthritis scoring

Blood from 6-8 week old adult K/BxN mice were collected and spun down at 14,000 x g for 15 minutes on a Beckmann table top centrifuge. Sera containing

autoantibodies were pooled. Recipient mice received a 150 μl injection intraperitoneally on day 0. Clinical assessment of arthritis was attained using three different scoring methods. Ankle thickness was measured with a Mitutoyo 700-118-20 Quick Mini Digital Thickness Gage. Data are reported as change in ankle thickness relative to baseline measurements made prior to injection.

2.4 RNA Isolation and quantitative real-time PCR

Mice were sacrificed on day 4 post serum injection and ankles and wrists were collected, pooled, and snap frozen in liquid nitrogen. RNA was subsequently isolated using a PerfectPure RNA Fibrous Tissue Kit (5PRIME, Gaithersburg, MD) following the manufacturer's instructions. Synthesis of cDNA from 500 ng total RNA was performed using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). Quantitative real-time PCR was performed with a BioRad iCycler (BioRad, Hercules, CA) using TaqMan® Gene Expression Assays (Applied Biosystems, Carlsbad, CA). Fold induction was expressed by the $2^{-\Delta\Delta Ct}$ method normalized to the geometric mean of three housekeeping genes: β-actin, Ywhaz, and 18S.

2.5 Isolation of Neutrophils

Donor mice were injection i.p. with 1 ml of 4% thioglycollate (Difco, Franklin Lakes, NJ). For functional, non-recruitment studies, 2 ml of 2% Bio-gel in 1X PBS were injected i.p in order to elicit a more pure neutrophil response. After 4 hours, the mice were sacrificed and cells were harvested by peritoneal lavage using 10 mL of sterile PBS and 5mM EDTA. Red blood cells were removed by

hypotonic lysis using 5 mL of ACK buffer (0.15 M NH_4Cl, 10 μM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4). Neutrophils were purified using a discontinuous sucrose gradient centrifugation protocol. Neutrophils were layered over Histopaque 1077, which was on a 1.119 g/ml Percoll layer. Cells were centrifuged at 4000 x g and the neutrophils were collected at the sharp interface. Neutrophils were recovered at a purity of 90-95% as determined by Wright-Giemsa staining. Neutrophils were resuspended at in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum (GIBCO® by Invitrogen, Carlsbad, CA), 1% penicillin/streptomycin, 1% L-glutamine, and β-mercaptoethanol at a concentration of 1 x 10^6 cells/ml for further assays.

2.6 Myeloperoxidase Activity Assay

Day 5 arthritic mice were sacrificed according to IACUC approved protocols. Knee and ankle synovial aspirates were collected and pooled per mouse on the same day. Biological samples were lysed in 150 μl of a buffer containing 0.5 M sodium acetate, 1% hexadecyltrimethylammonium bromide (HTAB), and 1% NP-40. Samples were pelleted and supernatant was collected and assayed immediately according to manufacturer's instructions for the Myeloperoxidase Chlorination Fluorometric Assay Kit (product #10006438) provided by Cayman Chemicals.

2.7 Quantification of Apoptosis

Bone marrow cells were harvested from donor tibias and femurs and cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10%

fetal calf serum (GIBCO® by Invitrogen, Carlsbad, CA), 1% penicillin/streptomycin, 1% L-glutamine, and β-mercaptoethanol. Cultures were incubated at 37° C with 5% CO_2 prior to further assays. Apoptosis was induced by 60 second exposure to UV irradiation, 5 μg of anti-Fas antibody (Jo-2 clone, BD Biosciences), 100 ng of LPS, or 0.2 μM staurosporine. Apoptosis was assessed by flow cytometry.

2.8 Flow Cytometry

Neutrophils (5×10^5) were washed and resuspended in 1X Annexin Binding Buffer (eBiosciences). Cells were incubated with 1 μg of Fc block (BD Pharmingen) at 4° C for 15 minutes. Without washing, cells were stained with anti-Gr-1 (eBiosciences), anti-CD11b (eBiosciences) and Annexin V (eBiosciences) and incubated on ice protected from light for 20 minutes. Cells were washed twice in 1X Annexin V binding buffer and resuspended at a final volume of 200 μl. Cells were subsequently stained with propidium iodide or 7-aminoactinomycin D immediately prior to analysis on a BD Accuri C6 flow cytometer. Data was analyzed using FlowJo software version 10.0.6 (FlowJo, LLC, Ashland, Oregon).

2.9 Western Blotting

Neutrophils were plated at 5×10^6 cells/ml and either incubated in 5 μg/ml anti-Fas antibody (Jo2 clone; BD Biosciences) or received a 60 second exposure to UV radiation. Cells were washed with 1X PBS. Neutrophil lysates were collected in either 50 or 100 μl of a cocktail containing Phosphosafe™ Extraction Reagent (BD Millipore) and Complete mini protease inhibitor (Roche). Lysate protein

concentrations were quantified using a Bradford Assay (Pierce). Total protein was quantified using a Coomassie (Bradford) Protein Assay (Thermo Scientific). Whole protein lysates (25 μg) were fractioned on Bis-Tris buffered 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to Immobilon-Psq polyvinylidene diflouride membranes (EMD Millipore).

2.10 Bone Marrow Derived Neutrophils

Mice were anaesthetized and sacrificed in accordance with approved IACUC protocols. Femur and tibia were isolated from the mice and excess muscle tissue and tendons were removed. Bones were placed in 20mM HEPES-HBSS supplemented with 0.5% FCS at pH 7.4. Distal ends of the bones were removed and using a 25-gauge needle and 10cc syringe, the marrow was thoroughly flushed from the bones with the supplemented HBSS. Marrow extracts were drawn through an 18-gauge needle to disaggregate the cells and then passed through a 70 μm cell strainer (Falcon). The cells were then lysed in 0.2% NaCl for 45 seconds and equilibrated with 1.2% NaCl. Cells were spun down and resuspended in 1 ml of HBSS solution. Cells were layered over 5 ml of 62% Percoll and spun for 30' at 1000 x g. Neutrophils were collected at the interface and purity was verified at >90% by flow cytometry. A majority of contaminating cells were B lymphocytes. Neutrophils were then resuspended in RPMI-1640 supplement with 10% fetal calf serum and antibiotics for further assays.

2.11 Quantification of NETosis

Neutrophils were plated at a concentration of 1 x 10^5 cells per well in a volume of 50 μl in a 96-well black clear bottom plate. Stimulants were made in a solution of 2.5 μM Sytox Orange (Invitrogen). PMA and DNase were at a final concentration of 100 nM and 60U per well, respectively. Fluorescence was detected in a Tecan GENios plate reader detecting every 2 minutes for 5 hours. Images were taken at 5 hours with a fluorescence microscope.

2.12 Histology

Whole knee joints and hind paws were fixed in 10% formalin, decalcified, trimmed, and embedded. Sections were prepared from the tissue blocks and stained with hematoxylin and eosin (H&E) and Toluidine blue (HistoTox, Boulder, Colorado, USA). Histopathological scoring was performed as previously described on a scale of 0–4 for inflammation, bone erosion and cartilage damage (Guma et al. 2009).

2.13 Statistics

Data are expressed as means ± SEM. Significance was assessed by analysis of variance (ANOVA) for arthritis studies. Bonferroni post hoc tests were used for multiple pairwise comparisons. For single comparisons, one-way ANOVA was performed at 95% confidence. All statistical analyses were performed using Prism software version 6.0b (GraphPad Software, La Jolla, CA).

3 RESULTS

3.1 *Ikbke*$^{-/-}$ mice have attenuated arthritis

Groups of C57BL/6 and *Ikbke*$^{-/-}$ mice were injected intraperitoneally with 150 μl of K/BxN serum on day 0. The *Ikbke*$^{-/-}$ mice displayed a reduction in swelling throughout the course of the 10-day experiment (Fig. 1A, 1B). Histological examinations were performed on the knee joints of the mice in order to investigate the extent of bone and cartilage damage. *Ikbke*$^{-/-}$ mice showed improved histological integrity of the joint, including less infiltration of immune cells, better retention of cartilage and less bone erosion (Fig. 1C).

3.2 IFNαβR and IL-1ra deficiency exacerbates arthritis

Previous reports have demonstrated that *Ikbke*$^{-/-}$ mice were more sensitive to therapeutic doses of IFNβ (Corr et al 2009). Additionally, *Ikbke*$^{-/-}$ fibroblasts treated with IFNβ produced greater levels of the IL-1 receptor antagonist, IL1ra (Corr 2011). Therefore, we decided to investigate the effects of targeting the interferon or interleukin-1 pathways to determine their roles in inflammation in this model of arthritis. Both the *Ifnar1*$^{-/-}$ and *Il1rn*$^{-/-}$ mice suffered from enhanced paw swelling in the 10-day K/BxN serum transfer time course experiments (Fig. 1A, 1B). The K/BxN serum transfer model of arthritis is dependent on IL-1, so it was not surprising that ablation of the IL-1 receptor antagonist results in worsened and less controlled swelling. Histological examination shows an increased severity of bone erosion as well as cartilage degradation in both *Ifnar1*$^{-/-}$ and *Il1rn*$^{-/-}$ mice (Fig. 1C).

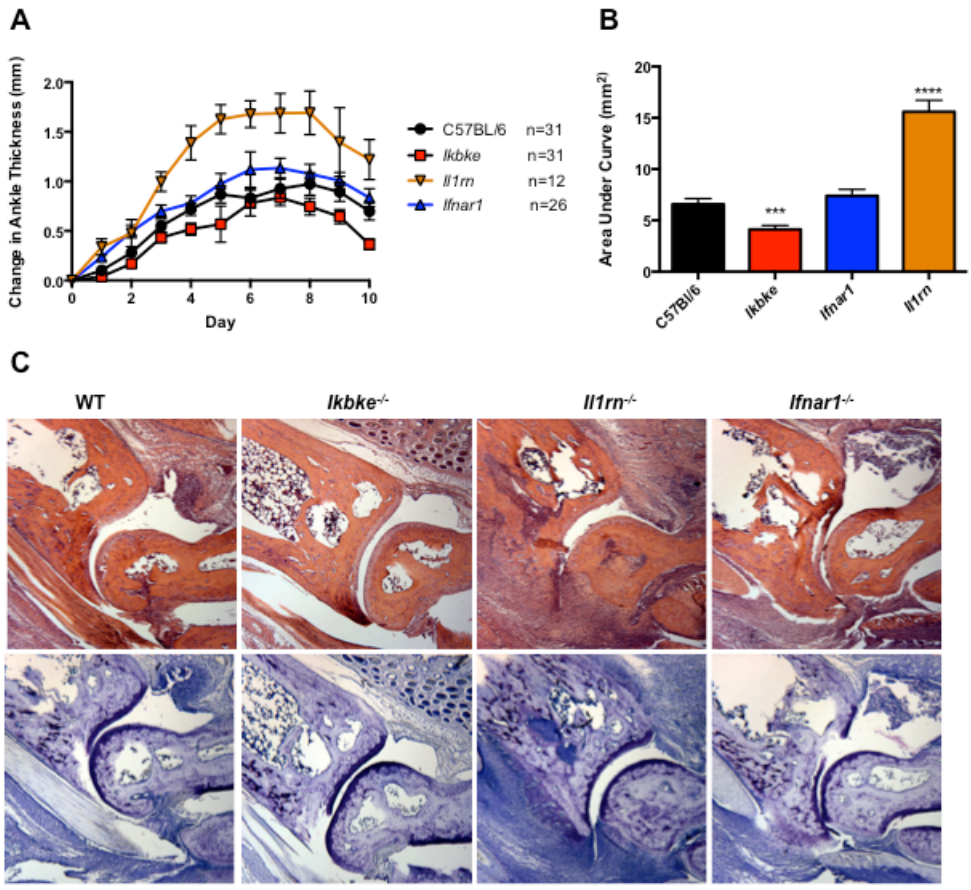

Figure 1. Ikbke$^{-/-}$ mice have attenuated arthritis.
Wild-type (n=31), Ikbke$^{-/-}$ (n=31), *Il1rn*$^{-/-}$ (n=12), and *Ifnar1*$^{-/-}$ (n=26) mice were injected intraperitoneally with 150 µl of K/BxN serum on day 0. Disease was monitored over a 10 day time course. Mice were sacrificed on day 10 and legs were harvested and stored in formalin for histology. (A) and (B) Time course experiments of mice injected with K/BxN serum. Shown is baseline corrected change in ankle thickness. (C) Histology of knee joints from day 10 arthritic mice. H&E (top) and Toluidine Blue (bottom). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA.

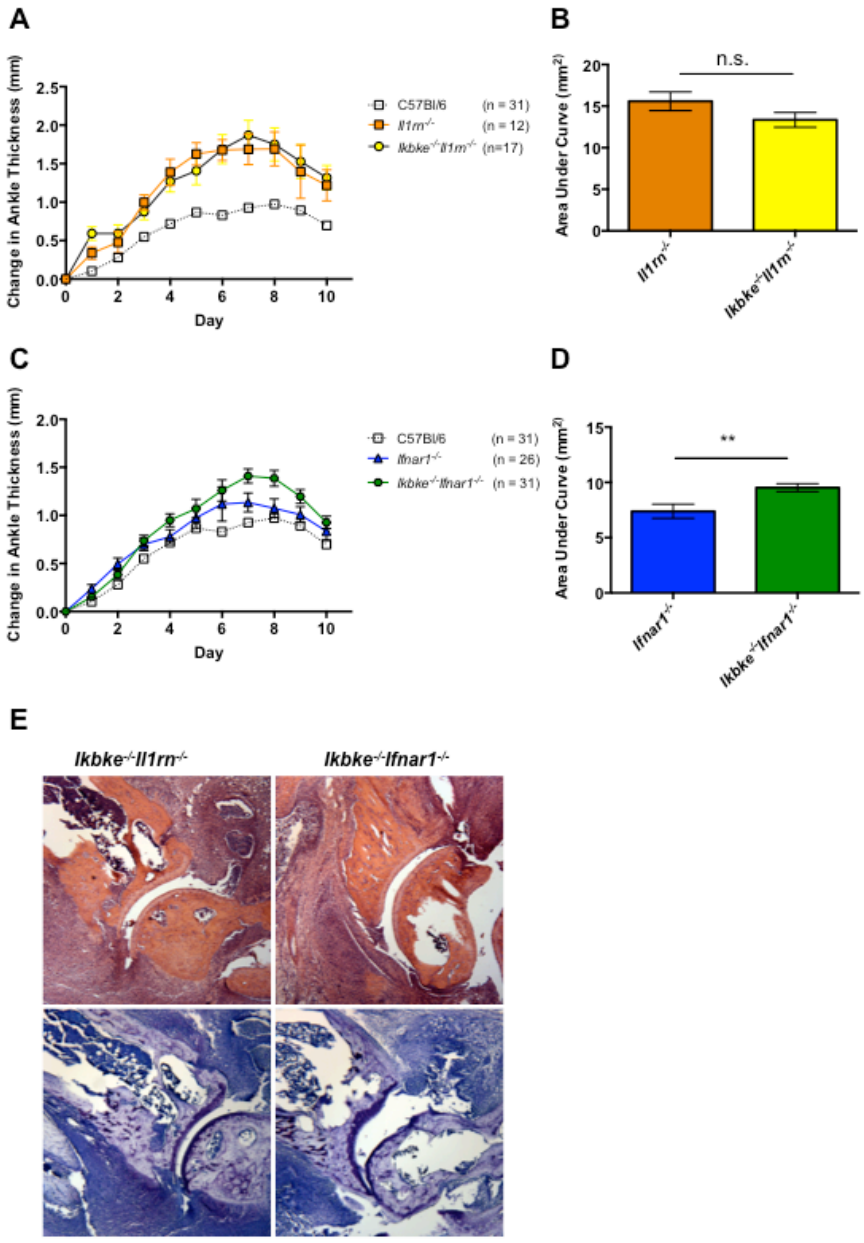

Figure 2. Mice are not rescued by loss of *Ikbke* and inflammation is further prolonged in *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ mice.
Ikbke$^{-/-}$*Il1rn*$^{-/-}$ (n=17) and *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ (n=31) mice were injected i.p. with 150 µl of K/BxN serum on day 0. Disease was monitored over a 10-day time course. Mice were sacrificed on day 10 and legs were harvested and stored in formalin for histology. The wild-type mice are shown as a dotted line for comparison in each panel. (A) and (B) Time course experiments of mice injected with K/BxN serum. Shown is baseline corrected change in ankle thickness. (C) Histology of knee joints from day 10 arthritic mice. H&E (top), Toluidine Blue (bottom). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA.

3.3 Targeting *Ikbke* in *Ifnar1*$^{-/-}$ mice paradoxically enhances swelling

To investigate the role of IKKϵ in the interferon and IL-1 signaling pathways in this model of arthritis, double knockout mice were generated. Serum transfer experiments with these mice demonstrated unique results. In the *Ikbke*$^{-/-}$*Il1rn*$^{-/-}$ mice, disruption of IKKϵ did not significantly affect the outcome of arthritis (Fig. 2A, 2B). This result suggests that the mechanism of IKKϵ depends critically on the presence of IL-1Ra in order to curb the proinflammatory effects of IL-1 and exert its beneficial effects in reducing arthritis. Surprisingly, *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ double knockout mice experienced a greater course of disease and increased paw swelling than seen in the *Ifnar1* knockouts alone (Fig. 2C, 2D). Through an unknown mechanism, IKKϵ is able to further exacerbate the increased swelling seen in the absence of interferon signaling. Histology reveals elevated levels of bone erosion in *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ joints (Fig 2E) and evidence of cartilage degradation.

3.4 IKKϵ deficient mice have a reduced chemokine profile whereas interferon receptor deficient mice have higher levels of chemokine expression

In order to elucidate a mechanism for inflammation in these mice, wrists from day 4 arthritis mice were harvested for cytokine and chemokine analysis via quantitative PCR. Day 4 was chosen as the time point to investigate mRNA levels because it immediately precedes the peak of swelling in serum-transfer mice. Although the pro-inflammatory cytokine, IL-1β was reduced in IKKϵ-deficient mice, which is expected due to its role in activating NF-κB dependent genes,

there was little difference in its expression between *Ifnar1*$^{-/-}$ and *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ mice (Fig. 3). The immunomodulatory cytokine, IL-10, which is widely known for its reported anti-inflammatory effects, was not affected by either IKKϵ or IFN receptor deficiency. Interestingly, neutrophil-attracting chemokines, KC, MIP-2, and GCP-2, which are reduced in IKKϵ deficient mice, are significantly up-regulated in IFN receptor deficient mice (Fig. 3). Reduction of KC and MIP-2 is observed in IKKϵ/IFN receptor double knockouts; however, GCP-2 is significantly overexpressed at similar levels to the IFN receptor knockout mice alone (Fig. 3).

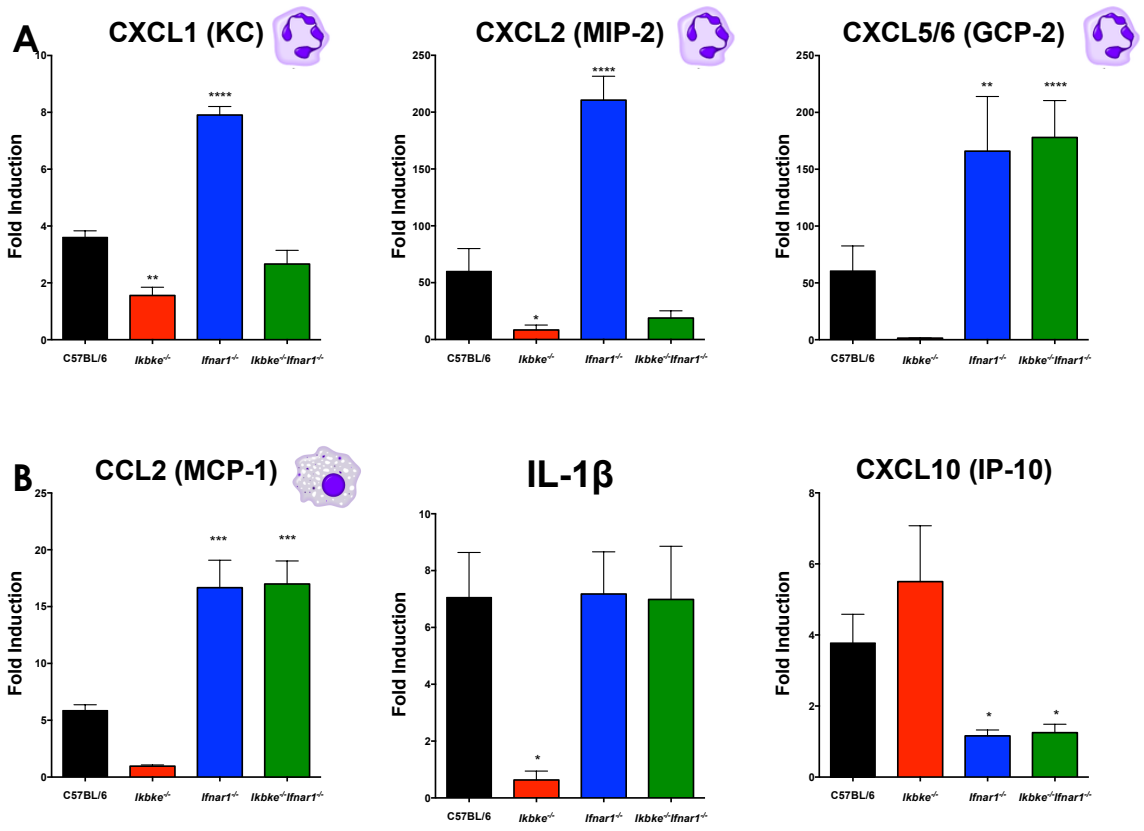

Figure 3. Ikbke mice express less cytokine and chemokine mRNA than wild-type mice; neutrophil recruiting chemokine mRNA is elevated in *Ifnar1*$^{-/-}$ and *Ikbke*$^{-/-}$ *Ifnar1*$^{-/-}$ mice.
Wrists from day 0 and 4 wild-type, *Ikbke*$^{-/-}$, *Ifnar1*$^{-/-}$, and *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ mice were harvested and snap frozen in liquid nitrogen. 500 ng of total RNA was used to synthesize cDNA and qPCR was performed. (A) Neutrophil attracting chemokines and (B) Monocyte attracting chemokine (CCL2), NF-κB controlled proinflammatory cytokine IL-1β, and Interferon stimulated immunomodulatory cytokine IP-10. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA.

3.5 *Ikbke*$^{-/-}$ mice have reduced neutrophil recruitment

In order to assess the effects of diminished chemokine levels in *Ikbke*$^{-/-}$ mice and elevated levels of the same chemokines in Ifnar1 mice, we looked at the neutrophil recruiting capabilities of these mice *in vivo*. Mice were given 1 ml injections of thioglycollate intraperitoneally and lavaged using phosphate-buffered saline. After purification of neutrophils, total neutrophil recruitment was

determined through flow cytometry. Previous reports have demonstrated that an early deficiency in interferon signaling in the bone marrow can results in neutrophilia during acute viral infection (Seo et al. 2011). In line with those observations, we found that by as early 2 hours, *Ifnar1*$^{-/-}$ mice posses greater neutrophil recruitment capabilities than wild-type (Fig. 4). This effect persists through 12 hours and numbers begin to stabilize similarly to wild-type by 18 hours post injection. The rapid decline seen in the *Ifnar1*$^{-/-}$ mice between 12 and 18 hours may suggest an accelerated program of neutrophil apoptosis in these mice.

Alternatively, *Ikbke*$^{-/-}$ mice show modest reduction in neutrophil numbers by 18 hours. In the doubly-deficient mice, recruitment is elevated compared to wild-type through 12 hours post injection (Fig. 4). However, they do not experience the rapid decline in numbers at 18 hours like the *Ifnar1*$^{-/-}$ mice, which suggests that the apoptotic or cell death programming is altered in these neutrophils, presumably by the absence of Ikbke. This finding prompted us to investigate whether neutrophil apoptosis is impaired in the absence of IKKϵ.

Myeloperoxidase activity was measured in the synovial fluid of day 5 arthritic knee and ankle joints. The data reflect the findings in the peritonitis experiments, with *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ mice showing the greatest levels of MPO activity in the synovial joints (Fig. 5). Therefore, neutrophil numbers are indeed elevated in the joints of *Ifnar1*$^{-/-}$ and *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ arthritic mice, further supporting the role of neutrophils in triggering the inflammation and paw swelling observed in these mice.

3.6 Fas Ligand expression on neutrophil surface is unaffected by IKKϵ deficiency

Fas ligand is a surface molecule involved in extrinsic neutrophil apoptosis. Secretion of Fas ligand activates Fas receptors on the neutrophil surface, which initiates the extrinsic apoptosis pathway.

In our experiments, the absence of IKKϵ did not significantly alter the expression of FasL on the surface of neutrophils (Fig. 6). Hence, the potential effect that IKKϵ could have on FOXO3a is not impairing the expression of FasL.

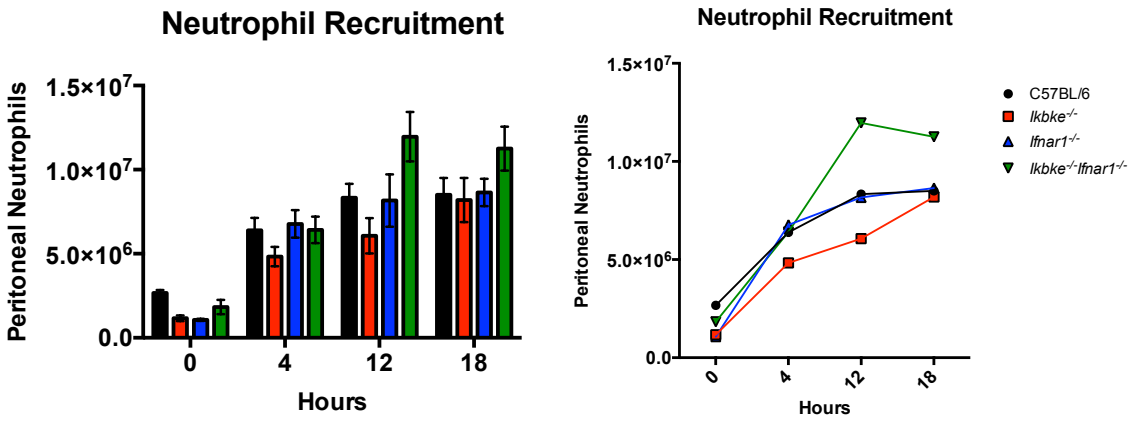

Figure 4. Disparate recruitment profiles between Ikbke$^{-/-}$ and Ifnar1$^{-/-}$ mice correlate with chemokine production
C57BL/6, *Ikbke*$^{-/-}$, *Ifnar1*$^{-/-}$, and *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ mice were injected with 1 ml of thioglycollate intraperitoneally. At the indicated time points, peritoneal lavages were performed and peritoneal neutrophils were isolated and quantified.

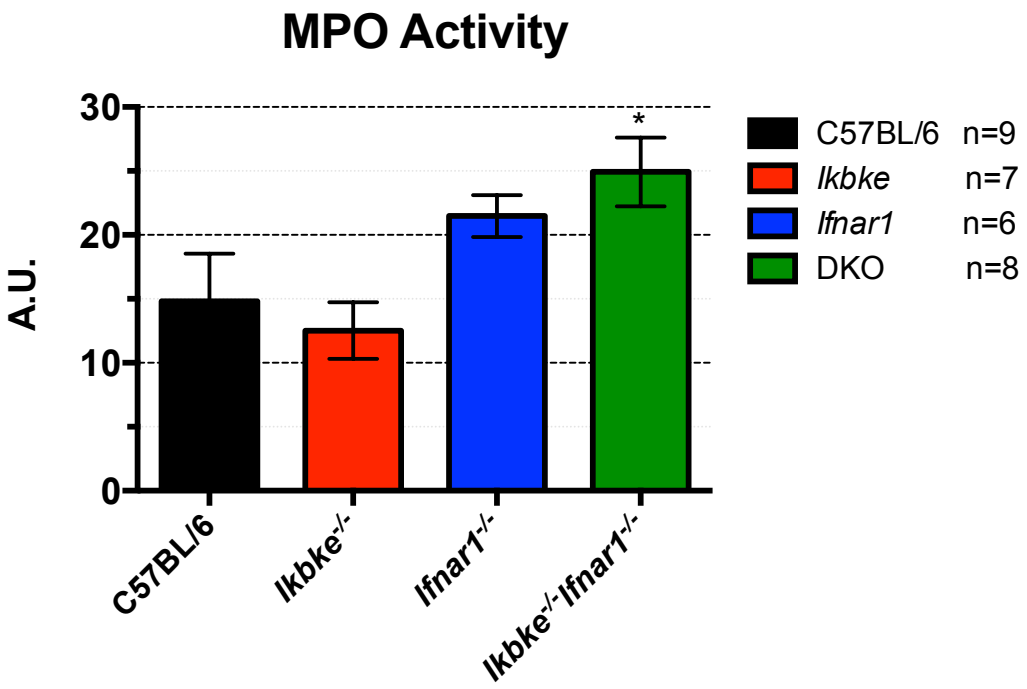

Figure 5. MPO activity in the joints of day 5 arthritic mice.
C57BL/6, *Ikbke*$^{-/-}$, *Ifnar1*$^{-/-}$, and *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ mice were injected with 150 μl of K/BxN serum intraperitoneally. On day 5, synovial aspirates and tissue were collected from the knees and ankles and pooled. MPO activity assay was performed to the manufacturer's recommendations (Cayman Chemicals) *, $P < 0.05$ by 1-way ANOVA with Bonferroni post-hoc test.

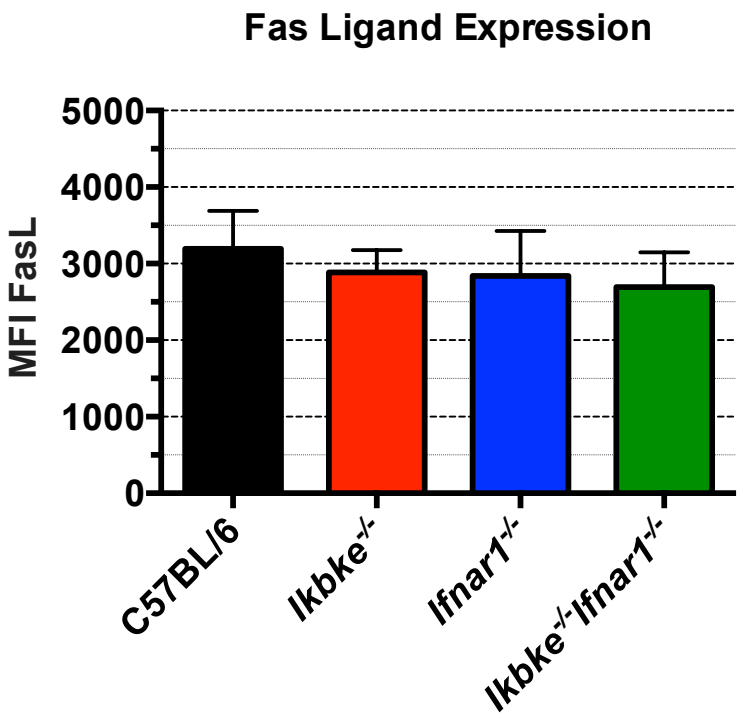

Figure 6. FasL surface expression on neutrophils is not defective in *Ikbke*$^{-/-}$ mice C57BL/6, *Ikbke*$^{-/-}$, *Ifnar1*$^{-/-}$, and *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ mice were injected with 1 ml of thioglycollate intraperitoneally. At four hours, peritoneal lavages were performed and peritoneal neutrophils were isolated. Cells were stained with FasL (eBioscience) and analyzed by flow cytometry. Shown is median fluorescence intensity (MFI).

3.7 Apoptosis is diminished in *Ikbke*$^{-/-}$ neutrophils

Here, we investigated the role of IKKϵ in neutrophil apoptosis. Peritoneal neutrophils were isolated from mice after intraperitoneal injections with 1 ml of 4% thioglycollate. Four hours after injection, peritoneal lavage was performed to harvest PMNs. Neutrophils were subjected to the apoptotic stimuli of UV irradiation, anti-Fas, Staurosporin, or LPS. Gating of neutrophil population was carried out using Gr1 and CD11b surface markers (Fig. 7). Apoptosis was analyzed by Annexin V and propidium iodide counter staining (Fig. 7). Flow cytometry revealed that the *Ikbke*$^{-/-}$ cells showed reduced levels of apoptosis in

response to UV irradiation by 6 hours (Fig. 8), which has been reported to activate the intrinsic apoptotic pathway (Fulda & Debatin 2006). Another activator of the intrinsic apoptosis pathway, staursporine, also showed that the *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ neutrophils have significantly reduced apoptosis relative to the *Ifnar1*$^{-/-}$ (Fig.9) Fas stimulation did not significantly affect apoptosis by 6 hours (Fig. 9), suggesting that the extrinsic pathway of apoptosis is not greatly affected by absence of IKKϵ.

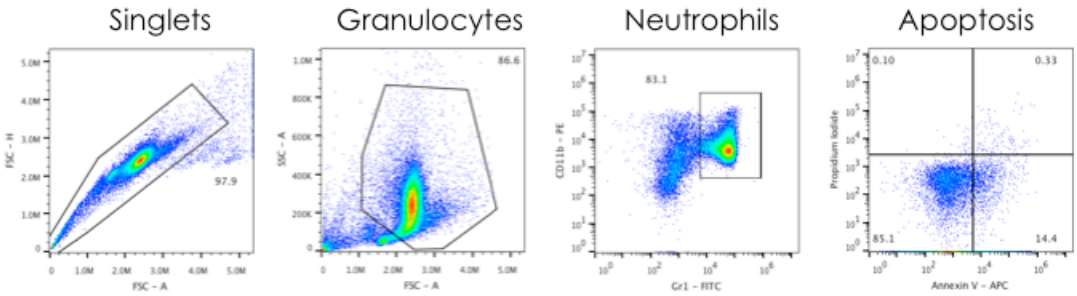

Figure 7. Representative gating of neutrophil population for analysis of apoptosis. Peritoneal neutrophils were isolated in 10ml of 1X PBS containing 5 mM ETDA from mice 4 hours after i.p. injection of 1ml 4% thioglycollate. Shown from left to right is chronological order of gating. Neutrophils were identified as Gr1^{+} and CD11b^{+} cells. Apoptosis was characterized by Annexin V^{+} and propidium iodide^{-} staining.

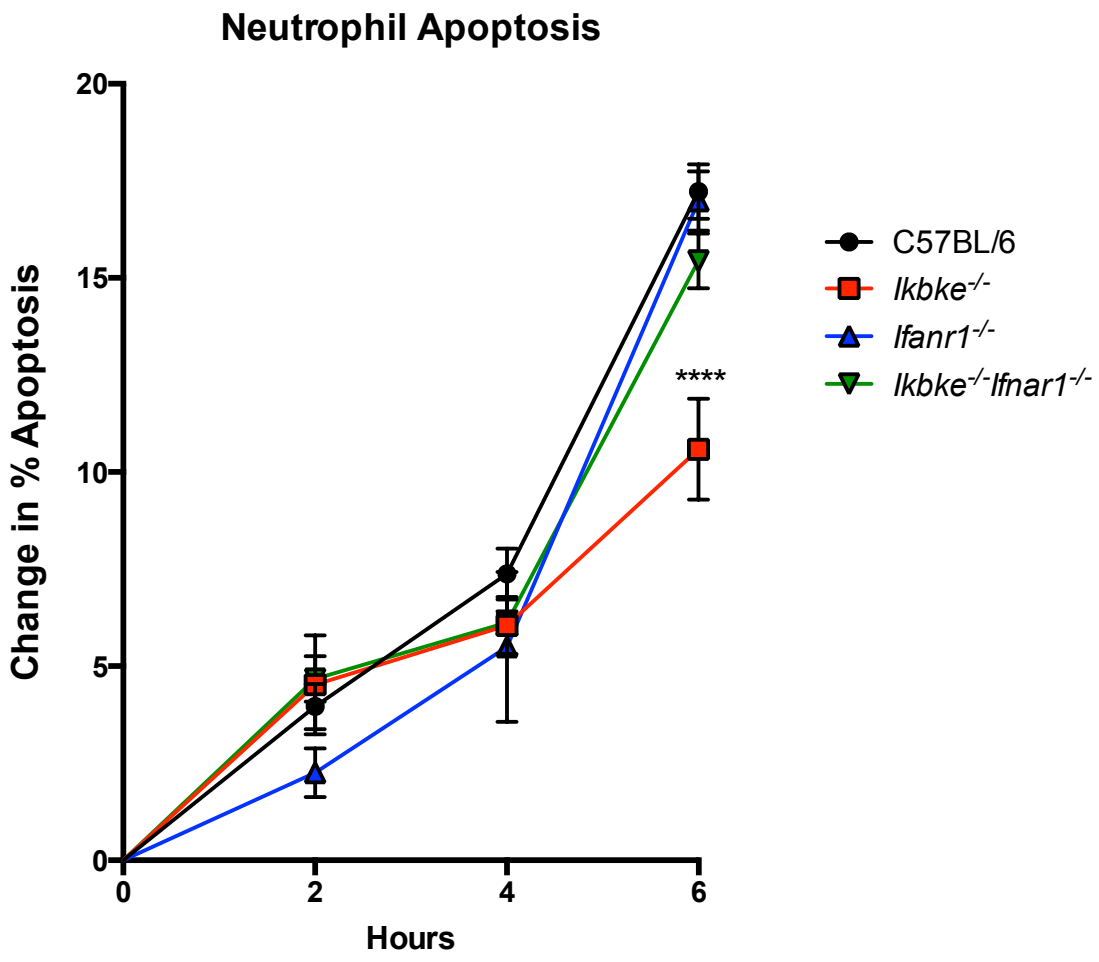

Figure 8. *Ikbke*$^{-/-}$ neutrophils are refractory to apoptosis
C57BL/6, *Ikbke*$^{-/-}$, *Ifnar1*$^{-/-}$, and *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ mice were injected with 1 ml of thioglycollate intraperitoneally. At 4 hours, peritoneal lavages were performed and peritoneal neutrophils were isolated. Neutrophils were exposed to UV radiation for 60 seconds and apoptosis was measured by Annexin V staining and quantified by flow cytometry. **, $P < 0.01$; ***, $P < 0.001$ by 2-way ANOVA with Bonferroni post-hoc test.

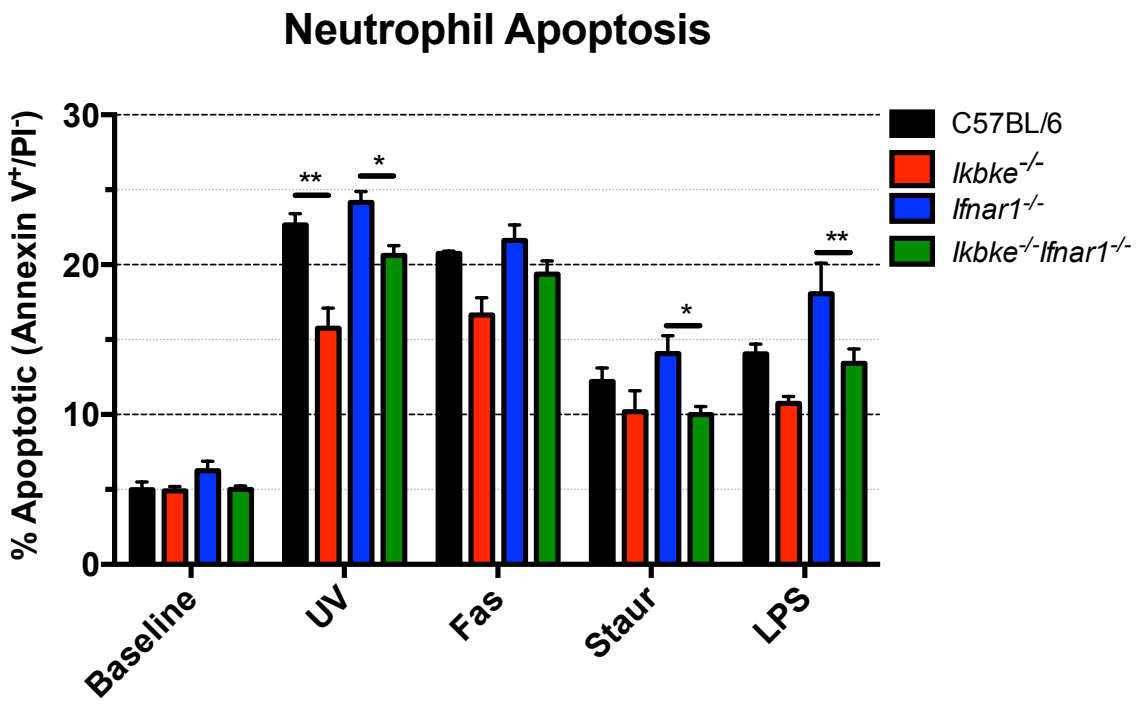

Figure 9. In response to apoptotic stimuli, Ikbke deficiency makes neutrophils more refractory to apoptosis by 6 hours.
Peritoneal neutrophils were harvested via lavage and purified using a modified discontinuous sucrose gradient separation. Neutrophils were resuspended in 1640 RPMI supplemented with 10% FCS, penicillin, streptomycin, and β-ME. Cells were then stimulated for 6 hours with anti-Fas (5 μg/ml), Staurosporin, or LPS. UV irradiation was administered for 1 min. At 6 hours post-treatment, neutrophils were collected and apoptosis was measured by Annexin V staining and analyzed by flow cytometry. ** $P < 0.05$, **, $P < 0.01$ by 2-way ANOVA with Bonferroni post-hoc test.

3.8 Delayed apoptosis in IKKε deficient neutrophils is mediated through caspase-3

The two main apoptotic pathways, intrinsic and extrinsic, require distinct sets of proteins to carry out their processes. We stimulated neutrophils via both extrinsic and intrinsic manners for different lengths of time and collected whole cell lysates. Western blot analysis revealed that *Ikbke*$^{-/-}$ *and Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ neutrophils express reduced levels of the activated cleaved caspase-3 (Fig. 10).

These results confirm that the reduced neutrophil apoptosis observed by Annexin V staining is mediated through an intrinsic pathway upstream of caspase-3. In contrast, caspase-8, which is an effector of the death receptor mediated extrinsic cell death pathway, is activated by Fas, and shows slight activation in the un-stimulated cells, but shows no differential activation in the absence of either IKKϵ or IFN receptor (Fig. 10). Based on these results, IKKϵ deficiency is not functioning through the Fas-FasL pathway to delay neutrophil apoptosis, but rather an alternate, possibly intrinsic mechanism.

3.9 *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ neutrophils undergo NETosis more readily in response to PMA

Because IKKϵ deficient neutrophils undergo less apoptosis, we wanted to explore other mechanisms of cell death in order to determine if IKKϵ plays a role in those alternate mechanisms as well. We focused on NETosis, which is an orchestrated form of cell death in neutrophils that involves the condensation and expulsion of nuclear DNA into the extracellular space, forming webs of nucleic acids that contain antimicrobial peptides and other proteases that aid in the defense against bacterial infections. In response to phorbol 12-myristate 13-acetate (PMA), IKKϵ knockout neutrophils showed a reduced level of NETosis, whereas the doubly deficient neutrophils are more sensitive to the stimuli and NET at a rate of roughly 80% after 5 hours of stimulation (Fig. 11A & B). The level of constitutive NETosis, as seen in the unstimulated panels (Fig 11A) show that *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ neutrophils have greater levels of NETosis even in the absence of stimuli. The rates of NETosis in these neutrophils correlate directly to the severity of arthritis

seen in these phenotypes in the passive serum transfer model. These results suggest that NETs may act in an inflammatory fashion when present in sterile inflammation, similar to diseases such as lupus and vasculitis.

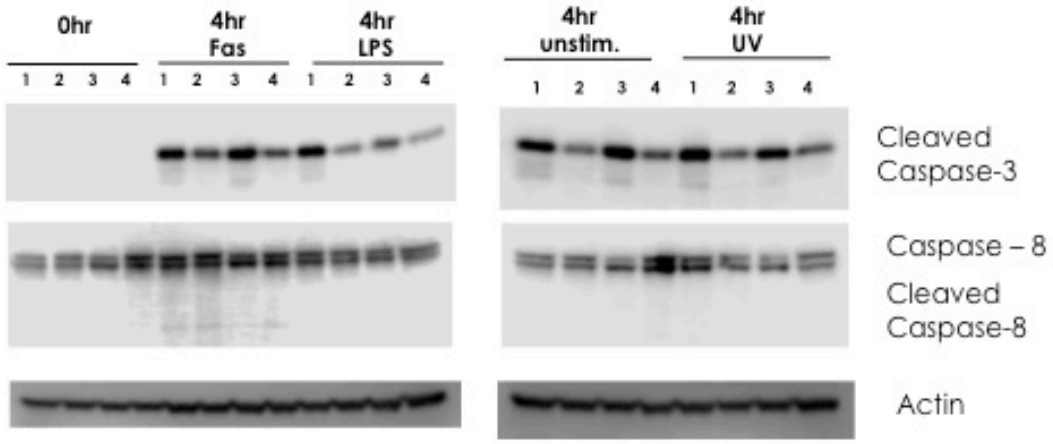

Figure 10. Caspase expression in apoptotic neutrophils.
Stimulated cells were collected (2.0 x 10^6 cells) and lysed in 50 µl of a Phosphosafe (Millipore) and Protease Inhibitor cocktail (Roche). Lysates were run on a 12% Bis-Tris gel (Life Technologies) and transferred to PVDF membranes (EMD Millipore). Membranes were probed with caspase-3 and caspase-8 antibodies as previously described. Lanes are as follows: (1) WT, (2) *Ikbke*$^{-/-}$, (3) *Ifnar1*$^{-/-}$, (4) *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$.

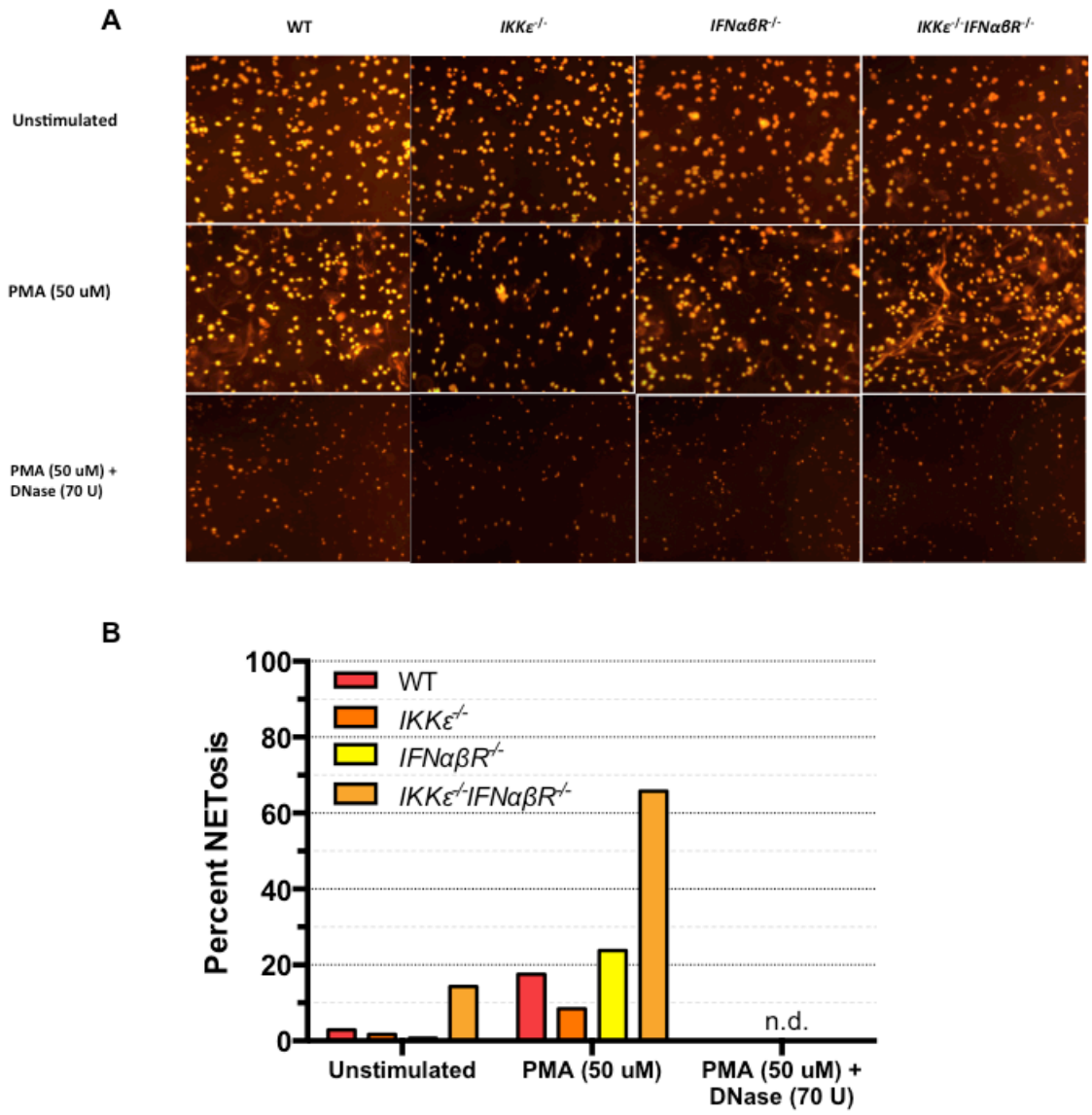

Figure 11. *Ikbke*$^{-/-}$*Ifnar1*$^{-/-}$ mice more readily undergo NETosis in response to PMA. Freshly isolated peritoneal neutrophils were resuspended at 2 x 10^6 cells/ml. 1 x 10^5 cells were plated in a 96 well plate and stimulated with the indicated agents in a cocktail of Sytox Orange as described previously. (A) Images were taken at 5 hours and (B) Quantification was determined using multiple fields covering >300 cells in total per group.

4 DISCUSSION

In the K/BxN mouse model of arthritis, autoantibodies directed towards G6PI direct the generation of an RA-like disease. Pathogenesis is dependent upon the presence and function of a wide range of immune cell types. In our serum-transfer model, innate immune cells such as macrophages, neutrophils, and FLS mediate the damage observed in the bone and cartilage of articular joints. In this paper, we examined closely the relationship between the regulatory function of neutrophils and the severity of arthritis by observing the effects in knockout mice. Our data supports a model in which IKKϵ is required for proper execution of neutrophil apoptosis in a model of sterile inflammation.

In the absence of an interferon response, mice were shown to express elevated levels of neutrophil recruiting chemokines, including GCP-2, KC, and MIP-2. These data are supported by the work of Seo et al., in which *Ifnar1*$^{-/-}$ mice recruit greater numbers of neutrophils to the lung in an acute viral infection model. This is presumably due to defects in the bone marrow, in which *Ifnar1* deficiency results in the generation of Ly6C^{int} monocytes. These monocytes will preferentially express KC, whereas their Ly6C^{hi} counterparts produce MCP-1. This altered differentiation leads to enhanced recruitment of neutrophils to sites of inflammation in the *Ifnar1*$^{-/-}$ mice (Seo et al. 2011). Our data is consistent with these reported results in the literature. Implications of these results include the support for the protective role IFN-β plays in the pathogenesis of the K/BxN serum transfer model of arthritis.

One of the recently identified functions of IKKϵ is its ability to regulate signaling molecules other than NF-κB and IRFs. Importantly, it has been reported that IKKϵ is a negative regulator of the transcription factor FOXO3 (Luron et al. 2012) in the production of endogenous IFN-β in myeloid derived dendritic cells. Phosphorylation of FOXO3 by IKKϵ results in nuclear exclusion, decreased protein stability, and increased degradation. In vitro experiments showed that FOXO3 antagonization by IKKϵ is required for IFN-β production, presumably due to the fact that that FOXO3 will inhibit NF-κB activity in the nucleus.

We are interested in IKKϵ's interaction with FOXO3 because inflammatory arthritis requires the activity of FOXO3a in order to prevent neutrophil apoptosis induced via Fas-Fas ligand interactions (Jonsson et al. 2005). In these sets of experiments, *Foxo3a*$^{-/-}$ mice were administered K/BxN serum and it was found that they did not develop clinical arthritis and had reduced paw swelling. FOXO3a deficient mice expressed elevated levels of Fas ligand on their neutrophil surfaces, suggesting that enhanced neutrophil apoptosis was responsible for the inability of these mice to develop arthritis. However, in our model, neutrophils lacking IKKϵ do not seem to portray a phenotype of reduced FasL expression, perhaps due to FOXO3a regulation by other regulatory factors. This result suggests that the mechanism by which IKKϵ delays apoptosis is independent of the extrinsic Fas-FasL signaling pathway. This is further supported by the data demonstrating that cleaved caspase-8 signal is similar among both knockout strains and the double knockout strains in response to anti-Fas treatment. Our results have indicated that although FOXO3a plays a major role

in the pathogenesis of K/BxN serum transfer arthritis, this pathway is not greatly affected in the IKKϵ knockout mice.

The role of IKKϵ in cellular apoptosis is inconsistent in the literature. *Ikbke* has been reported as an oncogene, and has been shown to be upregulated in glioma cells, contributing to the resistance of apoptosis in these cells through constitutive NF-κB activation (Guan et al. 2010). Additionally, IKKϵ has been characterized as an oncogene in various cancers, including breast and ovarian cancer (Verhelst et al. 2012). Contrastingly, other reports have attributed a pro-apoptotic role for IKKϵ. It has been shown that IKKϵ directly targets the X-linked inhibitor of apoptosis (XIAP), a negative caspase regulator, leading to its degradation and sensitizing cells to virus-induced apoptosis (Nakhaei et al. 2011). Based on the results of this study, we propose that IKKϵ acts as a promoter of neutrophil apoptosis under inflammatory conditions in order to facilitate proper neutrophil clearance. Absence of IKKϵ results in a delay of this mechanism, although the exact pathway that is affected still needs to be further elucidated.

Importantly, as shown by Corr et al. in 2009, targeting IKKϵ enhances the efficacy of systemic IFN beta treatment in arthritic mice that received K/BxN serum. What this study adds to the previous findings is that apoptosis may be affected in the neutrophil populations of IKKϵ deficient mice and that a specific apoptosis inducing agent or small molecule, such as a caspase-3 activator, may further enhance the beneficial effects of blocking IKKϵ. Further studies to elucidate the specific pathway involved will lend credence to a more defined target, one with possibly fewer side effects than the broad-scope caspases.

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