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# E3 ubiquitin ligase NKLAM ubiquitinates STAT1 and positively regulates STAT1-mediated transcriptional activity

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## Abstract

Signal transducer and activator of transcription 1 (STAT1) is critically important for the transcription of a large number of immunologically relevant genes. In macrophages, interferon gamma (IFNγ) signal transduction occurs via the JAK/STAT pathway and ends with the transcription of a number of genes necessary for a successful host immune response. The predominant mechanism of regulation of STAT1 is phosphorylation; however, there is a growing body of evidence that demonstrates STAT1 is also regulated by ubiquitination. In this report we show that JAK1 and STAT1 in macrophages deficient in an E3 ubiquitin ligase termed Natural Killer Lytic-Associated Molecule (NKLAM) are hyperphosphorylated following IFNγ stimulation. We found NKLAM was transiently localized to the IFNγ receptor complex during stimulation with IFNγ, where it bound to and mediated K63-linked ubiquitination of STAT1. In vitro nucleofection studies demonstrated that STAT1-mediated transcription was significantly reduced in NKLAM-KO macrophages. There was no obvious defect in STAT1 nuclear translocation; however, STAT1 from NKLAM-KO macrophages had a reduced ability to bind a functional gamma activation DNA sequence. There was also less mRNA expression of STAT1-mediated genes in NKLAM-KO macrophages treated with IFNγ. Our results demonstrate for the first time that NKLAM is a positive regulator of STAT1-mediated transcriptional activity and is an important component of the innate immune response.

### Keywords

Innate immunity; Interferon gamma; Macrophage; NKLAM; STAT1; Ubiquitination

## 1. Introduction

Natural killer lytic-associated molecule (NKLAM) is a transmembrane E3 ubiquitin ligase and a member of the RING-in between RING-RING (RBR) family of proteins. Originally discovered in NK cells, NKLAM is expressed in other hematopoetic cells, including T cells, monocytes and macrophages [1,2]. NKLAM, like its closely related family member Dorfin,

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contains two centrally located transmembrane domains. The N-terminal end of the molecule contains a really interesting new gene (RING) domain followed by an in-between RING domain (IBR) and another RING domain [3]. The C-terminal portion of NKLAM is devoid of any known domains. Studies from our laboratory have shown that NKLAM colocalizes with NK cell granules and bacteria-containing phagosomes in macrophages [1,4]. NKLAM is weakly expressed in unstimulated cells and upregulated by cytokines (e.g. IFNγ) and bacterial products such as lipopolysaccharide (LPS) [4]. Studies from our laboratory have demonstrated that NK cells from NKLAM-knock out (NKLAM-KO) mice have diminished anti-tumor activity [5]. Additionally, we have shown that NKLAM plays a role in controlling tumor metastasis [6]. As a family, RBR members have been shown to play a role in neurological diseases as well as innate immunity. Mutations in the *parkin* gene (PARK2) are associated with early-onset Parkinsonism and recessive juvenile Parkinsonism [7,8]. Dorfin overexpression has been shown to lessen deleterious neurological effects in a mouse model of amylotrophic lateral sclerosis [9]. Polymorphisms in PARK2 are also associated with increased susceptibility to infection by *Salmonella typhi, Salmonella paratyphi*, and certain *Mycobacterium* strains [10–13]. Our laboratory found that NKLAM colocalized with internalized *Escherichia coli* in phagosomes and that macrophages from NKLAM-KO mice have a diminished bacteria killing response [4]. Precisely how NKLAM affects bacterial killing is currently under investigation in our laboratory. However, we have shown that NKLAM-KO macrophages have dysregulated NF-κB signaling that includes attenuated p65 phosphorylation, delayed nuclear translocation and diminished NF-κB transcriptional activation in response to LPS [4]. This leads to reduction in inducible nitric oxide synthase and less nitric oxide production. Therefore, one mechanism by which NKLAM affects innate immunity is by regulating the cellular transcriptional response.

STAT1 is a member of a family of seven signal transducing transcription factors that transduce signals from growth factors, interleukins, and cytokines [14]. Receptor ligation induces a cascade of phosphorylation events that culminate in gene transcription. In the canonical IFNγ signaling pathway, IFNγ ligation to its cognate receptor IFNGR initiates the transactivation of constitutively associated JAK1 and JAK2 tyrosine kinases. Phosphorylation of IFNGR1 on a tyrosine residue by JAK1 provides an SH2-mediated binding site for STAT1. STAT1 is then activated via phosphorylation at tyrosine residue 701 by JAK1. This tyrosine phosphorylation event stabilizes a “parallel” STAT1 dimer confirmation, enhances nuclear trafficking and promotes DNA binding [15].

Although phosphorylation is the predominant and most studied post-translational regulatory mechanism, members of the STAT family can also be regulated by ubiquitination. Kim and Maniatis were the first to demonstrate that STAT1 was ubiquitinated and degraded by the proteasome [16]. More recent studies further support ubiquitin-mediated STAT degradation. Nuclear E3 ubiquitin ligase STAT–interacting LIM protein (SLIM), ubiquitinates STAT1 and STAT4, causing their degradation in vitro and in vivo [17]. Similarly, E3 ligase Smad ubiquitination regulation factor 1 (Smurf1) was shown to bind a PY domain in STAT1 via a WW domain in Smurf1 and facilitate ST AT1 proteasomal degradation [18]. Additionally, Sendai virus C proteins monoubiquitinate STAT1 causing its degradation in order to circumvent the innate immune response [19]. There are also reports that suggest ubiquitination positively regulates transcription factor activation. Leidner et al. demonstrated

that cotransfection of plasmids encoding ubiquitin and NFκB subunit RelB into HEK 293 cells increased the transcriptional activity of RelB in an ubiquitin concentration dependent manner [20]. Similarly, transcription factor FOXO is monoubiquitinated in response to cellular oxidative stress, which promotes its nuclear localization and increased transcriptional activity [21].

These studies indicate that transcription factor ubiquitination may positively or negatively regulate transcriptional activity. It is likely that the outcome of transcription factor ubiquitination is cell type and stimulation specific. In this present study, we provide novel data that RBR family member NKLAM ubiquitinates STAT1. Our results show for the first time that E3 ubiquitin ligase NKLAM is a transient component of the IFNGR complex and serves to enhance STAT1-dependent transcription.

## 2. Material and methods

### 2.1. Macrophage culture

All experiments on mice were approved by the Institutional Animal Care and Use Committees at Saint Louis University and the VA St. Louis Health Care System. This study was carried out in strict accordance with the provisions of the USDA Animal Welfare Act Regulations and Standards, PHS policy, recommendations in the Guide for the Care and Use of Laboratory Animals and VA policy. Wild type (WT) C57BL/6 and corresponding age-matched NKLAM-KO mice were used in all studies. For isolation of bone marrow, euthanized mice were sprayed with 70% ethanol and the femurs and tibias were dissected. The bones were flushed with DMEM and the collected marrow was resuspended in BM20 media (DMEM supplemented with 20% fetal bovine serum (FBS), 20% L929-cell conditioned media, 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 1 mM sodium pyruvate). The bone marrow cells were cultured for 7 days in non-tissue culture petri dishes with a partial media change on day 3. RAW 264.7 (ATCC#TIB-71) and HEK 293 (ATCC#CRL-1573) cells were acquired from ATCC. AG129 bone marrow was a kind gift from Dr. Mark Buller (Saint Louis University).

### 2.2. Plasmid constructs and transfection

STAT1 alpha Flag pRc/CMV was a gift from Jim Darnell (Addgene plasmid # 8691). The pGAS-luc plasmid was obtained from Agilent Technologies. Human NKLAM and myc-tagged NKLAM plasmids have been described elsewhere [3]. For transient transfection of plasmids into HEK 293 cells Lipofectamine 3000 was used according to the manufacturer's instructions.

### 2.3. Immunoblotting

Whole cell protein lysates were separated using SDS-PAGE then transferred to PVDF membrane. Membranes were blocked with 1% (wt/vol) BSA in Tris-buffered saline plus 0.1% Tween-20 (TBS-T) then incubated in primary antibody with rocking overnight at 4 °C. The antibodies for STAT1, pSTAT1 (Tyr701), JAK1, ubiquitin K63 linkage, and pJAK1 were purchased from Cell Signaling. The anti-flag tag (M2) and beta actin antibodies were from Sigma-Aldrich. The anti-NKLAM antibody has been described previously [1] and the anti-

IFNGR1 was purchased from Leinco. After three washes in TBS-T, the blots were probed with HRP-conjugated secondary antibodies and the proteins were visualized with BioRad Immun-Star Western C chemiluminescence kit. Images were captured and analyzed using a BioRad Chemidoc XRS+ imager (BioRad).

### 2.4. Flow cytometry with biotinylated IFNγ

Murine IFNγ (25 μg) was biotinylated as described by Brooks et al. [22]. Briefly, 3 μL of biotin-X-NHS (10 mg/mL in DMSO) was added to 25 μg of murine IFNγ for 4 h at room temperature. The biotinylated IFNγ was then purified from reaction components using Zeba spin columns (Life Technologies). The biotinylated IFNγ retained its full biologic activity as determined by phosphorylation of macrophage STAT1 (data not shown). Biotinylated IFNγ was then used to assess the surface expression of IFNGR1. Briefly, WT and NKLAM-KO ($2 \times 10^5$) bone marrow-derived macrophages (BMDM) were stimulated with 100 U/mL biotinylated IFNγ for 30 or 60 min at 37 °C. An aliquot of cells ($2 \times 10^5$) was also treated with biotinylated IFNγ and kept on ice (60 min) to prohibit internalization of the receptor during stimulation. The treated cells were washed once in cold PBS and streptavidin-PE was used to detect the bound IFNγ by flow cytometry.

### 2.5. Cell surface IFNGR1 immunoprecipitation

BMDM were grown to confluence on 100 mm petri dishes and then stimulated with 100 U/mL IFNγ for 30 or 60 min. The monolayers were washed 2 times with ice cold PBS then collected with the addition of 1.7 mM EDTA. After pelleting, the cells were resuspended in 100 μL PBS/3% BSA and 2 μg of anti-IFNGR1 antibody was added. After incubation on ice for 1 h, the cells were washed twice in 5 mL of ice cold PBS to remove any unbound anti-IFNGR1 then lysed in 500 μL of lysis buffer (1% Triton X-100, 65 mM Tris; pH 7.5, 137 mM NaCl, 10% glycerol). Total protein concentration was determined by BCA. Immunocomplexes were isolated using protein G magnetic beads (Bio-Rad). Additionally, IFNGR complexes were isolated using bound biotinylated-IFNγ. Briefly, WT or NKLAM-KO macrophages were incubated with 100 U/mL biotinylated IFNγ for 30 or 60 min. The cells were washed with cold PBS then lysed in lysis buffer. Streptavidin-agarose beads were then added to equivalent amounts of protein and incubated for 3 h. The isolated immune complexes were washed three times with lysis buffer and then solubilized in sample buffer. Western blots were then performed for the desired proteins.

### 2.6. Determination of IFNGR1-associated phosphatase activity

BMDM were grown to confluence on 100 mm petri dishes and then stimulated with 100 U/mL IFNγ for 30 or 60 min. The monolayers were washed 2 times with ice cold PBS then lysed in 500 μL of lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, pH 7.5). Total protein concentration was determined by BCA. Anti-IFNGR1 antibody was added for 1 h at 4 °C. Immunocomplexes were isolated with magnetic beads following a two hour incubation at 4 °C. Isolated complexes were washed 3 times in cold lysis buffer then resuspended in 1 × phosphatase reaction buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM DTT, 13.5 mM paranitrophenyl phosphate; pNPP). The reactions (120 μL total volume) were incubated at 37 °C for a total of 16 h. Small aliquots (3 μL) were taken at 1, 3, 6, and 16 h to measure the absorbance at 405 nm using a Nanodrop spectrophotometer.

### 2.7. Oligonucleotide pull down

The 5′-biotinylated 20-mer oligonucleotide used in this study corresponded to a functional gamma interferon activation sequence (GAS) within the iNOS promoter [23]. The forward strand was as follows: 5′-TCCTTTTCCCCTAACACTGT-3′. A mutated duplex was used as a control: 5′-TCCTTTGCCCCTACCACTGT-3′ (mutated bases are underlined). Equal amounts of the forward and corresponding reverse strands were heated at 100° for 60 min then cooled to room temperature to form duplexes. Adherent BMDM cultures were treated with 100 U/mL IFNγ for 30 or 60 min. Cellular nuclear and cytoplasmic fractions were isolated using the Pierce NE-PER kit. An aliquot of nuclear extract (50 μg) was incubated with 2 μg annealed duplex in binding buffer (1% NP-40, 140 mM NaCl, 50 mM HEPES-KOH, pH 7.5) at room temperature for 2 h. Biotinylated complexes were isolated with Streptavidinagarose beads that had been blocked in 2% BSA in binding buffer. Complexes were solubilized and subjected to SDS-PAGE and Western blot analysis for the desired protein.

### 2.8. Macrophage nucleofection and luciferase activity measurement

WT or NKLAM-KO macrophages ($4 \times 10^6$) were mixed with 3 μg of the luciferase reporter plasmid pGAS-luc and suspended in nucleofection solution T (Lonza). Cells were nucleofected using program T-20 with the nucleofector I device (Amaxa Biosystems). The nucleofected macrophages were resuspended in DMEM plus 10% FBS, transferred to 12-well plates and stimulated with 100 U/mL IFNγ for 6 h. At the desired time, the cells were collected, lysed in 1 × reporter lysis buffer and snap frozen at −80 °C to aid in cell disruption. The total firefly luciferase activity was determined using the Promega Luciferase Assay System (Promega). The total protein amount was determined using the BCA protein assay kit. Transfection efficiency was assessed by flow cytometry after nucleofection of cells with the green fluorescence protein reporter plasmid pmaxGFP (Amaxa) and was found to be similar between genotypes (data not shown).

### 2.9. Real time PCR

WT and NKLAM-KO were treated with 100 U/mL IFNγ for 3 or 6 h. Total RNA was extracted isolated using a Qiagen RNeasy kit. Then 100 ng of RNA was used to synthesize cDNA using the Taqman reverse transcription kit (Applied Biosystems). Real-time PCR was performed in 96-well plates with iTaq Universal SYBR Green Supermix (Bio-Rad). 18S served as an internal standard for normalization. For graphical presentation of quantified RT-PCR results, the mean ΔΔCT values for each gene was calculated by subtracting the mean ΔCT value of the reference gene (18S) from the mean ΔCT value of the treated gene. Data were graphed as $2^{\Delta\Delta CT}$.

## 3. Results

### 3.1. STAT1 and JAK1 are hyperphosphorylated in NKLAM-KO BMDM

The binding of IFNγ to the surface-expressed IFNGR sets off a cascade of phosphorylation events. To examine the effects of NKLAM on the regulation of the JAK/STAT signaling pathway we treated adherent monolayers of WT and NKLAM-KO macrophages with IFNγ

and examined the phosphorylation state of JAK1 and STAT1. We found that both JAK1 and STAT1 in NKLAM-KO macrophages were hyperphosphorylated in response to IFNγ. The largest difference in JAK1 phosphorylation between WT and NKLAM-KO macrophages was observed early in the time course but equalized by 60 min (Fig. 1A and B). In NKLAM-KO macrophages STAT1 hyperphosphorylation remained elevated out to two hours of stimulation (Fig. 1C and D). There were no differences in the expression of total JAK1 or STAT1 between WT and NKLAM-KO BMDM.

JAK1 and JAK2 are constitutively associated with IFNGR1 and IFNGR2, respectively. Following binding of IFNγ to the IFNGR, JAK1 and JAK2 are brought in close physical proximity and are phosphorylated via transactivation without the need for an additional initiating kinase. Interferon gamma signaling is negatively regulated by number of phosphatases. CD45, a cell surface-expressed receptor tyrosine phosphatase, is capable of dephosphorylating all murine JAK family members [24]. Additionally, SHP-1, a non-receptor tyrosine phosphatase expressed in hematopoetic cells, also dephosphorylates JAK1 [25]. Thus, diminished phosphatase activity in NKLAM-KO macrophages is an attractive explanation for the observed hyperphosphorylation response. We therefore examined the phosphatase activity within the IFNGR complex itself. In WT macrophages, the phosphatase activity in unstimulated IFNGR complexes and that from macrophages treated with IFNγ for 30 min were similar. However, the phosphatase activity isolated from cells that were stimulated with IFNγ for 60 min was dramatically elevated (Fig. 2A). This increase in intra-IFNGR1 complex phosphatase activity may serve to attenuate IFNγ signaling and temporally corresponds to the decrease in pSTAT1 (Tyr701) at 2 h of IFNγ stimulation (Fig. 1C). In contrast, the phosphatase activity in IFNGR1 complexes from 60 min IFNγ-stimulated NKLAM-KO macrophages was significantly reduced (Fig. 2B) and corresponds to the persistent pSTAT1 (Tyr701) observed after 2 h of IFNγ stimulation (Fig. 1C).

### 3.2. NKLAM is a transient component of the IFNGR complex

Our observation that phosphatase activity was significantly diminished in IFNGR complexes isolated from NKLAM-KO macrophages, prompted us to examine the presence of NKLAM within the IFNGR complex. The anti-IFNGR1 antibody we used recognizes an extracellular domain of IFNGR. Thus, adding anti-IFNGR1 antibody prior to gently lysing the cells, allows the isolation of only cell surface expressed-IFNGR1. Interestingly, by using this method of isolating only surface IFNGR1, unphosphorylated STAT1 is constitutively associated with the IFNGR complex in unstimulated WT and NKLAM-KO macrophages (Fig. 3A, time 0). In WT macrophages, STAT1 becomes phosphorylated on tyrosine 701 after 30 min of IFNγ stimulation followed by a reduction in STAT1 phosphorylation by 60 min. In contrast, STAT1 phosphorylation persisted in NKLAM-KO macrophages stimulated with IFNγ for 60 min. We also found that NKLAM was transiently associated with the IFNGR complex after 30 min of IFNγ stimulation.

To further strengthen our observations, we also isolated surface expressed IFNGR using biotinylated IFNγ. Adherent WT and NKLAM-KO BMDM were stimulated with biotinylated-IFNγ for 30 or 60 min at 37 °C. After lysis, IFNGR complexes were isolated with streptavidin agarose beads. To isolate IFNGR without initiating IFNγ-mediated signal

transduction, adherent WT and NKLAM-KO were cooled to 4 °C prior to the addition of biotinylated IFNγ. This technique did not inhibit IFNγ binding to the receptor and did not stimulate IFNγ-mediated STAT1 phosphorylation (Fig. 3B, time 0). We found that using biotinylated IFNγ to isolate IFNGR complexes produced identical results as the experiments performed using anti-IFNGR1 antibody to immunoprecipitate IFNGR1. Again, maximal levels of NKLAM were associated with the IFNGR complex after 30 min of IFNγ stimulation.

Both methods used for isolating the IFNGR complex were successful in isolating associated STAT1. We did find that STAT1 phosphorylation in NKLAM-KO macrophages was slightly higher after 60 min of IFNγ stimulation compared to WT macrophages. However, the levels of phosphorylation were not as dramatic as the whole cell lysates. One possible explanation is that IFNGR1-associated STAT1 constitutes a very small, highly compartmentalized portion of total cellular STAT1 and is likely subject to only kinases and phosphatases within the IFNGR1 complex. Once STAT1 is phosphorylated and leaves the IFNGR1 receptor complex, STAT1 could become a target for other cellular phosphatases (e.g. nuclear localized phosphatases). Unexpectedly, we did find that NKLAM-KO macrophages had a slightly higher phosphatase activity at baseline and after 30 min of IFNγ (Fig. 2B). Higher phosphatase activity at baseline would likely maintain the dephosphorylated state of proteins associated with the IFNGR1 complex. After 30 min of IFNγ stimulation, the IFNGR1-associated phosphatase activity in NKLAM-KO macrophages remains elevated in comparison to WT macrophages and results in less STAT1 phosphorylation (Fig. 3A and B, NKLAM-KO). After 60 min of IFNγ stimulation, the IFNGR1-associated phosphatase activity in NKLAM-KO macrophages decreases resulting in a concomitant increase in STAT1 phosphorylation (Fig. 3A and B, NKLAM-KO).

To rule out differential IFNGR1 expression between WT and NKLAM-KO BMDM, we immunoblotted non-reduced whole cell lysates for IFNGR1 and found no difference in cell expression of IFNGR1 (Fig. 3C). Whole cell lysates from AG129 mice that do not express IFNα/β or γ receptors [26] do not produce an IFNGR1 band. Additionally, to rule out the possibility of differential surface expression or receptor internalization we treated WT and NKLAM-KO macrophages with biotinylated IFNγ on ice, and used streptavidin-PE to visualize the presence of the IFNGR1 receptor. As shown in Fig. 3D, WT and NKLAM-KO macrophages express similar numbers of cell surface IFNGR1 receptors at all timepoints tested.

### 3.3. NKLAM associates with STAT1

STAT1 plays a critical role in IFNγ signal transduction and is a target for ubiquitin ligases [16–18]. Therefore, we next examined the possibility that NKLAM and STAT1 associate physically. RAW 264.7 cells were treated with IFNγ to physiologically elevate the intracellular levels of NKLAM. The cells were then stimulated with LPS for 30 min. NKLAM was immunoprecipitated from cytosolic and nuclear lysates and the subsequent immunoblots were probed for STAT1. As shown in Fig. 4A, NKLAM and STAT1 are associated in an IFNγ-dependent manner; this association was further strengthened by treating the cells with LPS. There was no significant STAT1 signal in untreated cells (unt).

This is likely due to the fact that NKLAM is expressed at very low levels in unstimulated cells. The control immunoprecipitation was negative.

To further test the association of STAT1 and NKLAM, flag-tagged STAT1 and myc-tagged NKLAM were co-transfected into HEK 293 cells and the subsequent lysates were immunoprecipitated with anti-STAT1, -flag tag, -myc tag, or –NKLAM antibodies. Western blots for STAT1 revealed a band at the correct molecular weight for STAT1 in all immunoprecipitates (Fig. 4B, top panel). The membranes were stripped and reprobed for NKLAM (Fig. 4B, middle and bottom panels). The bottom panel is an overexposure of the middle panel. The presence of NKLAM in the STAT1 and flag-tagged immunoprecipitates provides strong evidence that NKLAM and STAT1 are physically associated.

### 3.4. NKLAM positively regulates STAT1 K63-linked ubiquitination

We next examined the ubiquitination state of STAT1 in WT and NKLAM-KO BMDM. In order to elevate the cellular levels of NKLAM in a physiological manner, the monolayers were primed with IFNγ for 18 h then stimulated with 100 ng/mL LPS for the times indicated. STAT1 was immunoprecipitated and the immunocomplexes were immunoblotted for various proteins (Fig. 5A). STAT1 ubiquitination was significantly elevated in WT as compared to NKLAM-KO macrophages (Fig. 5, top panel). There was evidence of ubiquitination in NKLAM-KO BMDM; however, it was not LPS-dependent. Interestingly, STAT1 was transiently, K63-linked ubiquitinated in WT BMDM after 30 min of LPS stimulation. The increase in K63-linked STAT1 ubiquitination observed in NKLAM-KO BMDM was minor. Coincidentally, NKLAM was associated with STAT1 after 30 min LPS stimulation (Fig. 5A, bottom panel). In whole cell lysates, NKLAM was ubiquitinated in an LPS-dependent manner (Fig. 5B). STAT1 expression was slightly increased with IFNγ treatment but the overall expression pattern of STAT1 was similar between genotypes.

### 3.5. NKLAM positively affects STAT1 transcriptional activity and DNA binding ability

STAT1 regulates the expression of a large number of immunologically important genes. Our observation that NKLAM bound to STAT1 and mediated its K63-linked ubiquitination prompted us to determine what effect NKLAM may have on STAT1 DNA binding and transcriptional activity. To this end, we tested STAT1 transcriptional activity using a STAT1 responsive luciferase reporter plasmid containing 4 tandem canonical GAS elements. WT and NKLAM-KO BMDM were nucleofected with pGAS-luc reporter plasmid and stimulated with IFNγ for 6 h. We found that the luciferase activity in NKLAM-KO macrophages was significantly less than in WT macrophages, suggesting that NKLAM plays a positive role in regulating STAT1 transcriptional activity (Fig. 6A).

Our experiments with the pGAS-luc plasmid suggest that NKLAM plays a role in STAT1-mediated gene transcription. To further strengthen this observation, we assessed changes in mRNA expression of iNOS, a STAT1-regulated gene that contains a known GAS element. As show in Fig. 6B, treatment with IFNγ stimulated WT iNOS mRNA expression that was significantly elevated over NKLAM-KO. These results are consistent with our data that showed NKLAM-KO BMDM express less iNOS in response to LPS [27]. The effect of NKLAM on STAT1-regulated genes was not limited to genes with GAS elements. We also

found that NKLAM-KO BMDM expressed less CCL5/RANTES mRNA in comparison to WT BMDM when stimulated with IFNγ (Fig. 6C). STAT1 is necessary for IFNγ-induced CCL5 expression [28], but the CCL5 promoter does not contain a GAS element. However, the expression of transcription factor named interferon regulatory factor 1 (IRF-1) is mediated by STAT1 via a GAS element in the IRF-1 promoter [29]. IRF-1 subsequently regulates CCL5 expression by binding an interferon stimulated response element (ISRE) within the CCL5 promoter [30].

Current research shows that STAT1 ubiquitination results in proteolytic degradation via the proteasome. Our data suggest that in addition to degradation, STAT1 ubiquitination may positively regulate STAT1 transcriptional activity; therefore, we next examined STAT1 nuclear protein expression and translocation. There were no significant differences in cytosolic or nuclear STAT1 expression between genotypes, nor was there any difference in translocation of STAT1 into the nucleus (Fig. 7A, top panel). Subsequent Western blotting for K63-linked ubiquitin did show an increase in both the cytosolic and nuclear fractions from WT but not NKLAM-KO BMDM (Fig. 7A, third panel). These observations prompted us to examine STAT1 DNA binding ability. Nuclear lysates from IFNγ-stimulated macrophages were used in an oligonucleotide pull down assay in which the oligonucleotide contained a working GAS element from the iNOS promoter region (Fig. 7B). The control (Cnt) oligonucleotide contained mutated bases that prohibit STAT1 binding and did not pull down any STAT1 [23]. STAT1 from unstimulated WT or NKLAM-KO macrophages minimally bound the oligonucleotide. However, there was significant STAT1 recovery using nuclear lysates from WT macrophages stimulated with IFNγ. Interestingly, the lack of NKLAM expression negatively altered the ability of STAT1 to bind to the oligonucleotide, indicating that NKLAM promotes STAT1 DNA binding.

## 4. Discussion

RING in between RING ubiquitin ligases are a small family of ubiquitin ligases with diverse functions. Previous research has demonstrated that RBR ligases play roles in the progression of neurological diseases. There is now a growing body of evidence that certain RBR ligases are involved in host susceptibility to infectious agents. In support of this concept, work from our laboratory has demonstrated similar findings in that NKLAM-deficient macrophages lack the bactericidal activity of wild type macrophages. Full elucidation of the mechanism of action of NKLAM as a component of the innate immune system remains to be determined.

Interferon gamma initiates signal transduction via the JAK/STAT pathway, a pathway driven by protein phosphorylation reactions. The phosphorylation state of any given phosphoprotein is determined by the activity of competing kinases and phosphatases. RBR ubiquitin ligases have no known kinase or phosphatase activity; however, E3 ubiquitin ligase SLIM impairs the tyrosine phosphorylation of STAT4 independent of proteasomal degradation [17]. The authors suggest that SLIM acts as an adaptor protein for a phosphatase or alters the conformation of STAT4 to allow interaction with a phosphatase. The hyperphosphorylation we observed following IFNγ stimulation of NKLAM-KO macrophages (Fig. 1) could be the result of over active kinase activity or decreased phosphatase activity. Liu et al. found that patients with autosomal dominant chronic

mucocutaneous candidiasis have a hyperphosphorylated STAT1 phenotype that is due to impaired STAT1 dephosphorylation [31]. There are a number of kinases that phosphorylate JAK1, such as PKC zeta and Tyk2 [32,33]; however, these kinases are not known to phosphorylate JAK1 in response to IFNγ. A more attractive explanation for the observed JAK/STAT1 hyperphosphorylation phenotype may be diminished phosphatase activity or total lack of phosphatase protein, rather than enhanced kinase activity.

We examined the phosphatase activity associated with the IFNGR complex and found phosphatase activity within WT IFNGR1 complex increased after 60 min of continual IFNγ stimulation. This observation is in line with studies that suggest phosphatases provide tight negative control by dephosphorylating JAK and STAT proteins [34]. Interestingly, we found the phosphatase activity in the IFNGR complex from NKLAM-KO macrophages decreased after 60 min IFNγ stimulation. Presumably this would enhance the phosphorylation state of certain IFNGR complex target proteins (e.g. JAK and STAT). Indeed, we do observe JAK1 and STAT1 hyperphosphorylation in NKLAM-KO BMDM. STAT1 phosphorylation is increased at each time point tested and phosphorylation is prolonged out to two hours of IFNγ stimulation in NKLAM-KO macrophages (Fig. 1C). Possible explanations are that NKLAM recruits a phosphatase to the IFNGR complex or induces the activation of a constitutively associated phosphatase. The latter concept is not well established but there is precedent for phosphatase activation via ubiquitination [35,36]. SHP-2 is constitutively associated with IFNGR1 [37] but studies demonstrating ubiquitin-dependent activation are lacking. CD45 is also aplausible candidate and is a proven JAK1 phosphatase [24]. Localization to the plasma membrane would allow CD45 access to the IFNGR complex. Further research into potential phosphatases is warranted.

We isolated the IFNGR complex from IFNγ stimulated macrophages by IFNGR1 immunoprecipitation or by specifically isolating bound biotinylated IFNγ with streptavidin. Both techniques demonstrated for the first time that NKLAM is transiently associated with the receptor in an IFNγ-dependent manner. The IFNGR1 has been shown to be ubiquitinated by the K3 and K5 proteins of the Kaposi's sarcoma-associated herpesvirus, which induced its degradation [38]. The IFNGR is not a likely target for NKLAM, as we saw no decrease in total IFNGR1 protein or change in IFNGR1 surface expression between NKLAM-KO and WT macrophages (Fig. 3C and D). Unexpectedly, we found that STAT1 is constitutively associated with the IFNGR1 complex as evidenced by the presence of STAT1 in IFNGR complexes isolated from unstimulated cells (Fig. 3A and B). Though present, STAT1 was only phosphorylated on tyrosine 701 following IFNγ stimulation. Our observation is in contrast to current IFNγ signaling dogma that states STAT1 is recruited to the IFNGR complex in a SH2-dependent manner [15]; however, there is evidence of STAT proteins being constitutively associated with ErbB1 and IL-22 receptors [39,40].

Recent studies have shown that STAT1 ubiquitination results in its degradation via the proteasome and negatively regulates STAT1-mediated gene expression [18]. Currently, STAT proteins have not been defined as targets for any of the known RBR ubiquitin ligases. The localization of NKLAM within the IFNGR complex puts NKLAM in close proximity to several IFNγ signaling proteins and suggests a novel role for NKLAM as a component of the IFNγ signaling pathway. Results from STAT1 co-immunoprecipitations show that

STAT1 is transiently K63-linked ubiquitinated when NKLAM is present within the IFNGR complex. We cannot rule out the possibility that NKLAM-mediated STAT1 degradation plays a role in IFNγ signaling in macrophages. It is possible that NKLAM-mediate STAT1 degradation may occur at specific cellular locations such as the IFNGR complex and is not evident upon examination of total cellular STAT1.

Importantly, we did observe that the lack of NKLAM expression negatively affected STAT1 binding and GAS-mediated transcription. This suggests that NKLAM has an overall positive effect on STAT1 mediated transcriptional activity. There is a growing body of evidence to suggest that ubiquitination, independent of proteasomal degradation, can positively regulate transcriptional activity. Leidner et al. demonstrated that polyubiquitination of NF-κB subunit RelB enhanced transcriptional activity without affecting nuclear localization or DNA binding, while treatment with PMA/ionomycin treatment led to RelB proteasomal degradation [20]. The authors suggest that polyubiquitination may affect the interaction of RelB with co-activators. This notion is supported in a study by Adhikary et al. that showed HectH9 ubiquitination of Myc was required for recruitment of co-activator p300 and transactivation of multiple genes [41]. Similarly, transcription factor FOXO4 is monoubiquitinated in response to hydrogen peroxide treatment, resulting in nuclear localization and increased transcriptional activation.

Recent STAT1 mutational studies have defined a key lysine residue that is critical for DNA binding. Huntelmann et al. demonstrated that mutation of STAT1 lysine 567 to an alanine completely abrogated the ability of STAT1 to bind DNA [42]. The authors also show that STAT1 lysine 567 has direct access to bound DNA. It is unknown at this time as to whether lysine 567 is a viable target for ubiquitin ligases. The possibility that ubiquitin ligases could potentially control transcription factor function via ubiquitination of key lysine residues is an attractive hypothesis that deserves further investigation.

We did find that NKLAM is present within the nuclear fraction of IFNγ-stimulated WT BMDM (Fig. 4A); however, we do not have conclusive evidence that NKLAM is a true nuclear localized protein. Studies from Ahmed et al. have demonstrated that IFNGR1 and STAT1 alpha are recruited to the GAS elements within the promoters of IRF-1 and IFNγ-inducible indoleamine 2,3 dioxygenase [43], thus it is entirely possible that NKLAM translocates to the nucleus along with the IFNGR1 macromolecular complex (Fig. 8).

In conclusion, we provide novel data that expands the current knowledge base of RBR E3 ligase NKLAM. Our data suggest that NKLAM is a key component of IFNγ signal transduction and regulates STAT1-mediated transcriptional activity. Further research is required to not only expand the known list of NKLAM substrates, but to fully elucidate the precise mechanism(s) of action of NKLAM in the context of innate immunity.

## 5. Statistical analysis

Statistical differences were assessed using a two-tailed, unpaired Student's *t*-test or one-way ANOVA with Microsoft Excel software or GraphPad software. A p value of 0.05 or lower was considered statistically significant.

## Acknowledgments

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## Abbreviations

| | |
|---|---|
| **NKLAM** | natural killer lytic-associated molecule |
| **STAT1** | signal transducer and activator of transcription 1 |
| **RBR** | RING in between RING |
| **JAK1** | Janus kinase 1 |
| **IFNγ** | interferon gamma |
| **IFNGR** | interferon gamma receptor |
| **GAS** | gamma interferon activation sequence |
| **BMDM** | bone marrow-derived macrophage |
| **LPS** | lipopolysaccharide |
| **Ub** | ubiquitin |
| **WT** | wild type |
| **KO** | knock out |
| **iNOS** | inducible nitric oxide synthase |

| | |
|---|---|
| **ISRE** | interferon stimulated response element |
| **IRF-1** | interferon regulatory factor 1 |

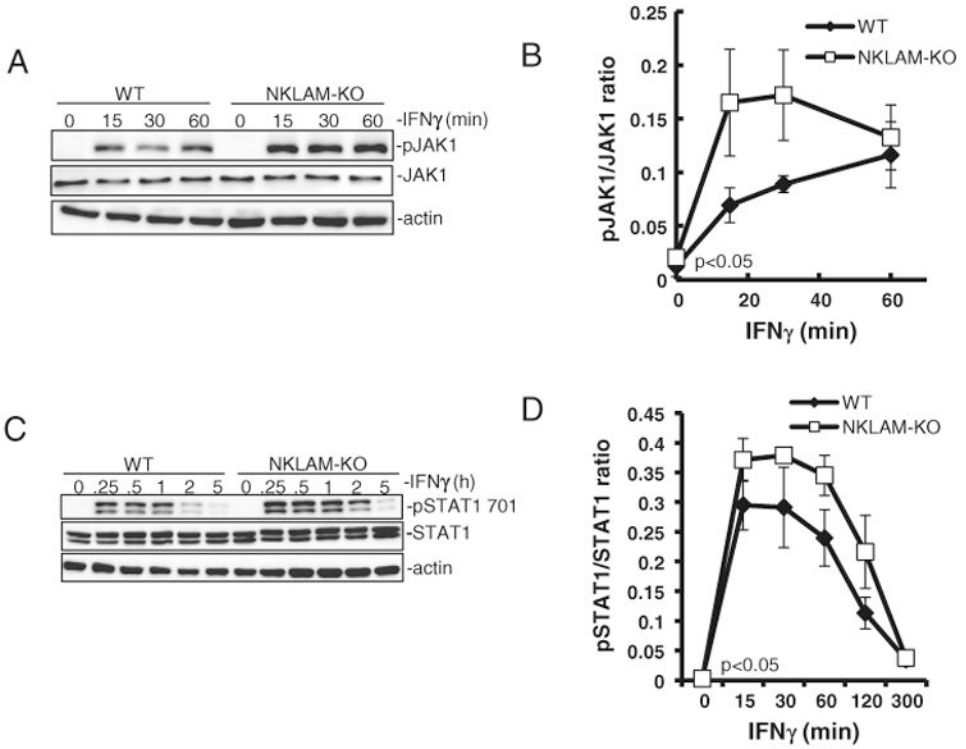


**Fig. 1.**

JAK/STAT proteins are hyperphosphorylated in NKLAM-KO BMDM upon stimulation with IFNγ. WT and NKLAM-KO BMDM were treated with 100 U/mL IFNγ for the times indicated. Equal amounts of whole cell lysate were immunoblotted for JAK1 and pJAK1 (A) or STAT1 and pSTAT1 (Tyr 701) (C). The ratio of the band density of the phosphoprotein divided by the band density of the total protein is depicted in graphical form (B, D). Actin was used as a loading control. Data are presented as mean ± SEM of at least 3 independent experiments. $p < 0.05$, by ANOVA.

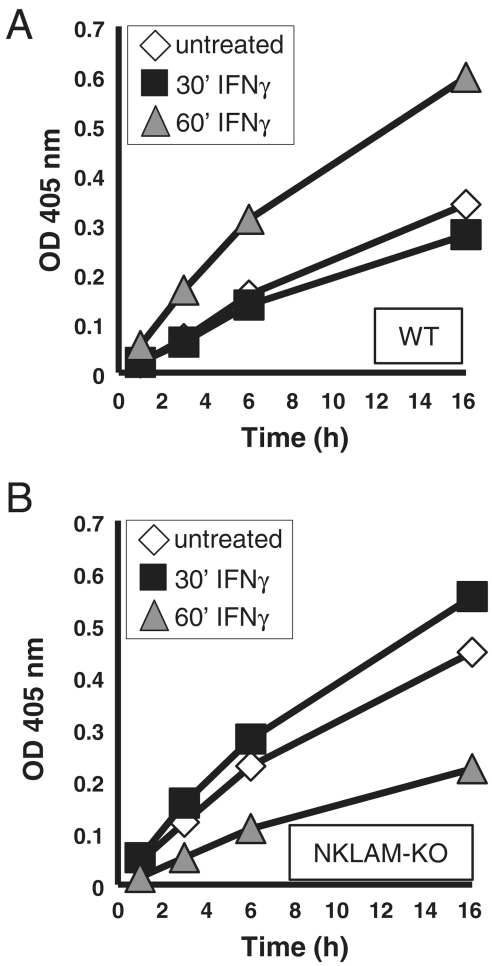


**Fig. 2.**

NKLAM-KO BMDM have diminished phosphatase activity within the IFNGR1 complex. WT (A) and NKLAM-KO (B) BMDM were treated with 100 U/mL IFNγ for 30 min (closed square), 60 min (gray triangle), or left untreated (open diamond). The IFNGR was immunoprecipitated and the intrinsic phosphatase activity was assayed using pNPP as a substrate. Graphs represent 1 of 2 independent experiments with similar results.

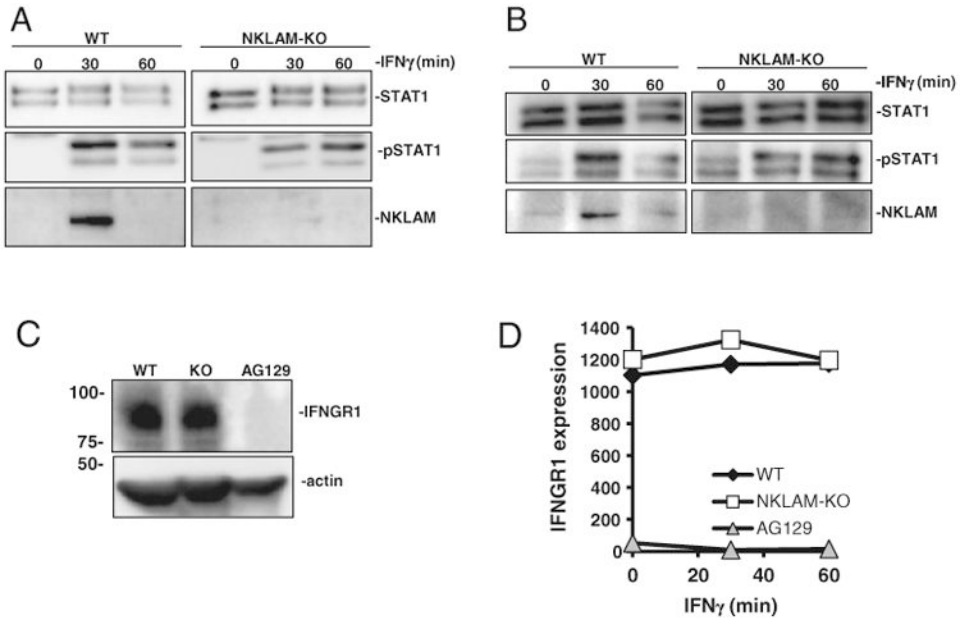


**Fig. 3.**

NKLAM transiently localizes to the IFNGR membrane complex following IFNγ stimulation. WT and NKLAM-KO BMDM were stimulated with 100 U/mL IFNγ for times indicated and cell surface IFNGR1 was isolated by immunoprecipitation using anti-IFNGR1 antibody (A) or biotinylated IFNγ (B). Western blots were performed for STAT1, pSTAT1 (Tyr701) and NKLAM. Non-reduced whole lysates were used to assess the total cellular expression of IFNGR1 in WT, NKLAM-KO and IFNGR1-KO AG129 macrophages (C). Flow cytometric analysis of surface expressed IFNGR1 was performed on WT, NKLAM-KO and AG129 macrophages. Graph represents the geometric mean of IFNGR1 expression (D). Data are representative of at least 3 independent experiments.

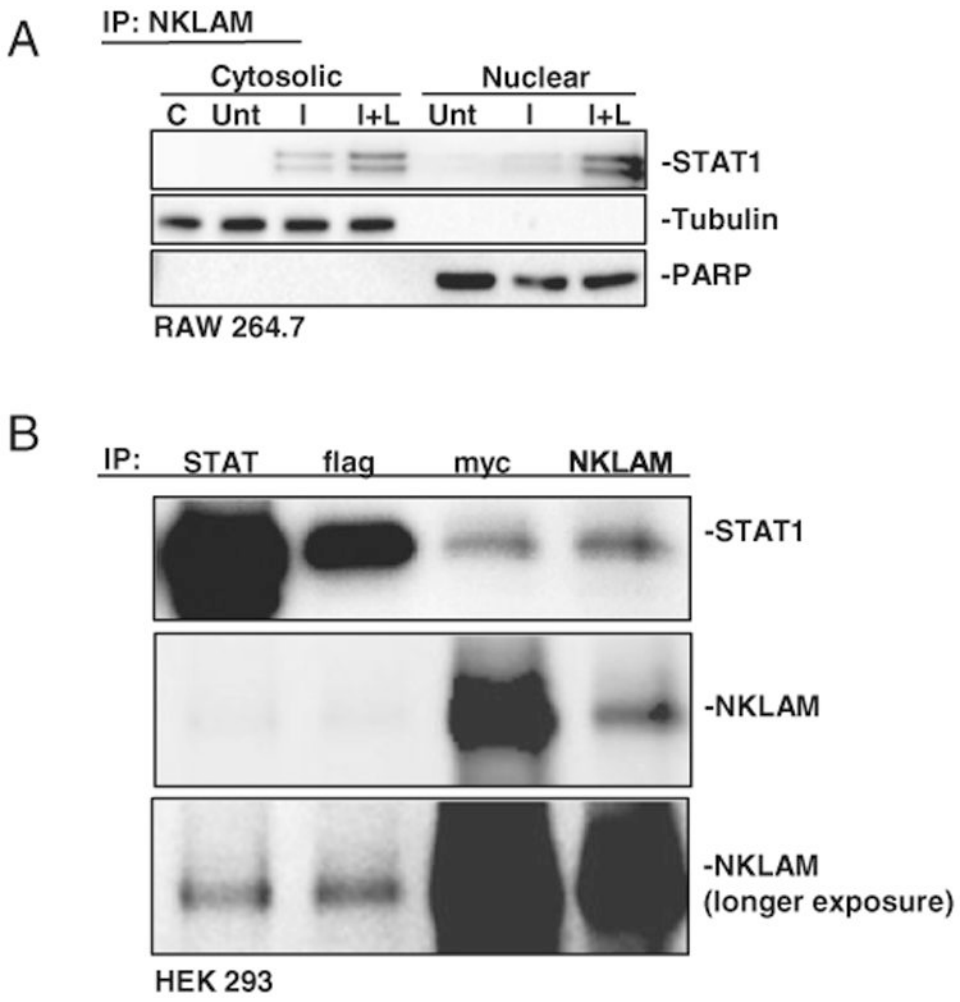


**Fig. 4.**

NKLAM is physically associated with STAT1. A) RAW 264.7 were stimulated with 100 U/mL IFNγ for 18 h (I) then treated with LPS (100 ng/mL) (I + L) for 30 min. After isolating cytosolic and nuclear fractions, NKLAM was immunoprecipitated and the subsequent immunoblots were probed for STAT1. Tubulin and PARP were used to demonstrate fraction purity. C, control (isotype control antibody), Unt, untreated. B) HEK 293 cells were cotransfected with STAT1α-flag and NKLAM-myc. Lysates were immunoprecipitated for STAT1, flag-tagged STAT1, myc-tagged NKLAM, or NKLAM then immunoblotted for STAT1 or NKLAM. Bottom panel is a longer exposure of the middle panel to show faint NKLAM bands. Data shown represents 3 experiments.

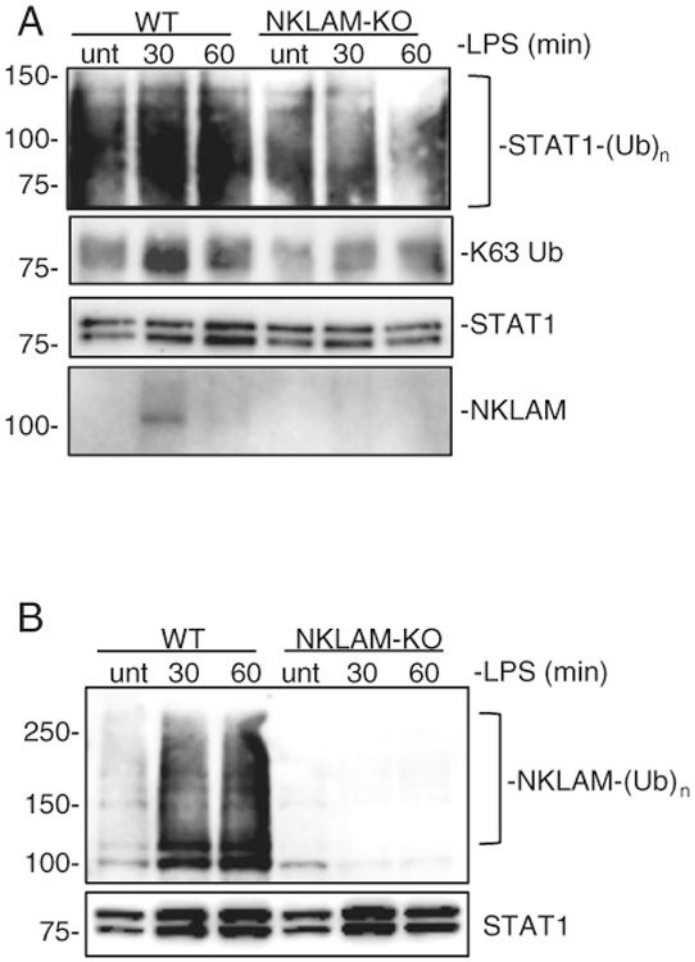


**Fig. 5.**

NKLAM positively regulates STAT1 ubiquitination. A) WT and NKLAM-KO BMDM were stimulated with 100 U/mL IFNγ for 18 h followed by short term stimulation with 100 ng/mL LPS for 30 or 60 min. STAT1 was immunoprecipitated and the resulting immunocomplexes were blotted for total ubiquitin, K63-linked ubiquitin (Ub), STAT1, and NKLAM. B) Whole cell lysates were probed for NKLAM and STAT1 expression. Data represent at least 3 independent experiments.

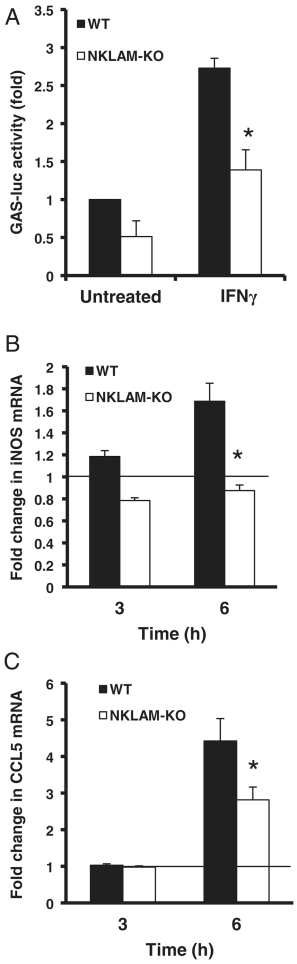


**Fig. 6.**

NKLAM positively affects STAT1-mediated transcriptional activity. A) WT (black column) and NKLAM-KO (white column) BMDM were nucleofected with pGAS-luc plasmid then stimulated with 100 U/mL IFNγ for 6 h and assayed for luciferase activity; *$p < 0.05$; $n = 5$. B–C) WT (black column) and NKLAM-KO (white column) BMDM were treated with 100 U/mL IFNγ for 3 or 6 h. The expression of iNOS and CCL5 was determined by quantitative RT-PCR. The fold change in mRNA levels (mean ± SEM) is expressed relative to untreated cultures (solid line set at 1). Graph represents at least 3 independent experiments. *$p < 0.05$; $n = 3$.

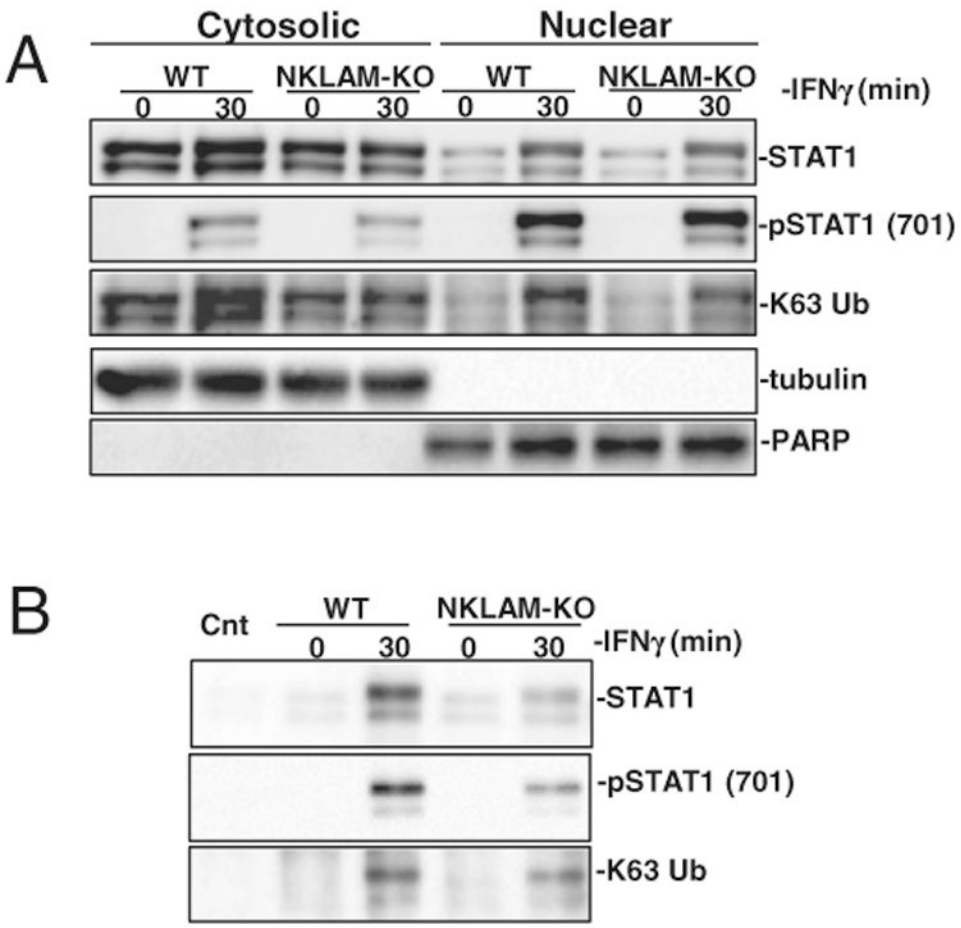


**Fig. 7.**

NKLAM affects STAT1 DNA binding. WT and NKLAM-KO BMDM were untreated or treated with IFNγ (100 U/mL) for 30 min. Isolated cytosolic and nuclear fractions were immunoblotted for STAT1, pSTAT1 (Tyr701), and K63-linked ubiquitin. Tubulin and PARP were used to demonstrate the purity of each fraction. B) The nuclear fractions from (A) were incubated with a biotinylated control oligonucleotide (Cnt) or an oligonucleotide containing a functional GAS sequence. Protein/DNA complexes were isolated and immunoblotted for STAT1 and pSTAT1 (Tyr701), and K63-linked ubiquitin. Data represent 1 of at least 3 experiments.

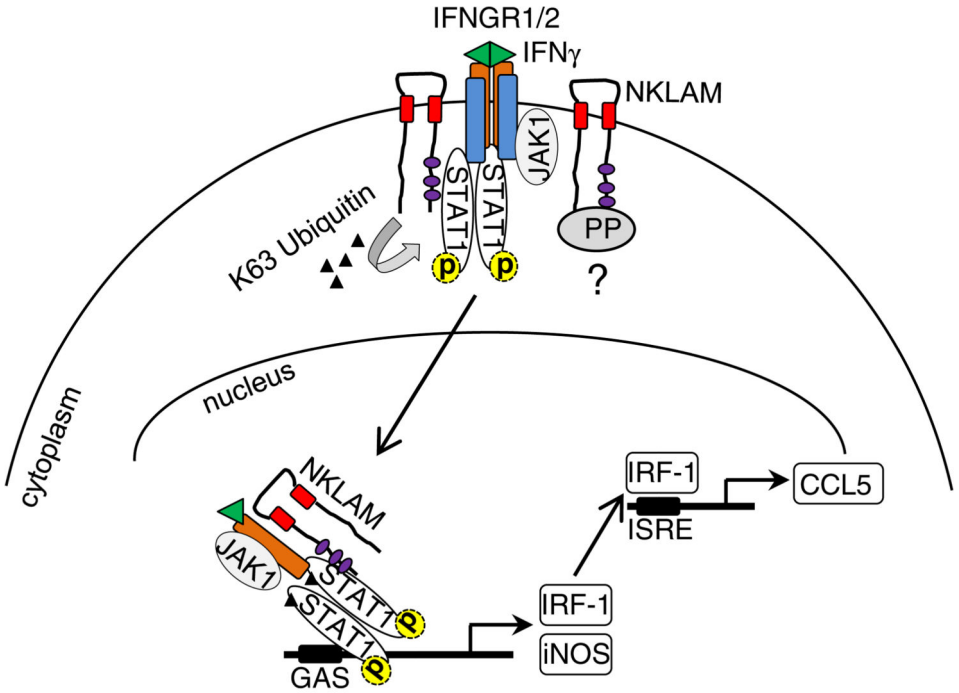


**Fig. 8.**

Proposed model of STAT1 signal transduction regulation by NKLAM. Localization to the plasma membrane would allow NKLAM to transiently associate with the IFNGR following IFNγ stimulation. Within the complex, NKLAM could serve to recruit and stabilize the presence of a phosphatase (PP), resulting in dephosphorylation of key proteins within the IFNGR complex (e.g. JAK and STAT). NKLAM localization to the IFNGR complex would also allow access to STAT1 where the subsequent K63-linked polyubiquitination (closed triangle) facilitates binding to GAS within the promoters of interferon-stimulated genes such iNOS and interferon regulatory factor 1(IRF-1). IRF-1 regulates CCL5/RANTES transcription via binding of the interferon stimulated response element (ISRE) in the CCL5 promoter.