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E3 ubiquitin ligase NKLAM positively regulates macrophage inducible nitric oxide synthase expression

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ABSTRACT

Stimulated macrophages generate potent anti-microbial reactive oxygen and nitrogen species within their phagosomes. Previous studies have shown that the E3 ubiquitin ligase natural killer lytic-associated molecule (NKLAM) is a macrophage phagosomal protein that plays a role in macrophage anti-bacterial activity. *In vivo*, NKLAM-knockout (KO) mice produce less nitric oxide (NO) upon exposure to lipopolysaccharide (LPS) than wild type (WT) mice. *In vitro*, we found that NO production and inducible nitric oxide synthase (iNOS) protein were diminished in LPS-stimulated NKLAM-KO bone marrow-derived and splenic macrophages. Additionally, LPS-stimulated NKLAM-KO macrophages displayed defects in STAT1 tyrosine phosphorylation and production of interferon beta (IFNβ). The JAK/STAT pathway is critical for the production of IFNβ, which augments iNOS protein expression in mice. iNOS protein expression is also regulated by the transcription factor NFκB, thus we investigated whether NKLAM influences NFκB function. LPS-stimulated NKLAM-KO macrophages showed evidence of delayed nuclear translocation of the NFκB subunit p65. This was associated with a reduction in p65/DNA colocalization. The defect in p65 translocation was independent of IKBα degradation. NKLAM-KO macrophages also expressed less p65 and showed evidence of defective p65 phosphorylation at serine 536. Importantly, LPS-stimulated NKLAM-KO macrophages have diminished NFκB transcriptional activity as assessed by transfection of a luciferase reporter plasmid. Collectively, our data implicate NKLAM as a novel modulator of macrophage iNOS expression.

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Introduction

Macrophages are a first line of defense against pathogens and serve as a critical bridge between the innate and adaptive arms of the immune system. Macrophages employ several bactericidal mechanisms to eradicate foreign pathogens. One such mechanism is the generation of nitric oxide (NO) from L-arginine *via* the expression and activation of inducible nitric oxide synthase (iNOS; NOS2). iNOS is a dimeric protein that is expressed in several immune cells including macrophages, dendritic cells, and neutrophils (Cruz et al., 2001; Ratajczak-Wrona et al., 2013). The production of NO by iNOS in response to infection is a critical defense mechanism. Studies have shown that iNOS-knockout mice are unable to mount an efficient immune response (Alayan et al., 2006). Stimulation with microbial products such as lipopolysaccharide (LPS), lipoteichoic acid or pro-inflammatory cytokines induces the transcriptional upregulation of iNOS protein (Zhang et al., 2013). Previous studies have shown that treatment with exogenous IFNβ enhances iNOS protein expression and NO production (Jacobs and Ignarro, 2001; Yao et al., 2001). Once activated, iNOS is capable to producing large amounts of NO. Nitric oxide can combine with the simultaneously produced super oxide anion resulting in peroxynitrite, a potent bactericidal free radical that induces oxidative damage to proteins, lipids, and nucleic acids (Habib and Ali, 2011; Tecder-Unal et al., 2008). The mouse iNOS promoter contains several transcription factor binding sites such as interferon-stimulated response elements (ISRE), IFNγ activation sites, Oct-1 and NFκB binding sites (Korhonen et al., 2005; Xie et al., 1993).

The regulation of iNOS transcription by transcription factor NFκB has been well studied. There are two NFκB consensus binding

Abbreviations: BMDM, bone marrow-derived macrophage; IFN, interferon; iNOS, inducible nitric oxide synthase; KO, NKLAM-knockout; LPS, lipopolysaccharide; NK, natural killer cell; NKLAM, natural killer lytic-associated molecule; NO, nitric oxide; RBR, RING in between RING; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor; WT, wild type; GFP, green fluorescent protein.

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sites in the iNOS promoter that are critical for the regulation of iNOS transcription (Kim et al., 1997). In the canonical NFκB pathway, NFκB is sequestered in the cytoplasm by its inhibitor IKBα. Upon stimulation with bacterial products such as LPS, IKBα is rapidly phosphorylated, ubiquitinated and degraded in the 26S proteasome. The binding of IKBα to NFκB subunit p65 masks a nuclear localization signal in p65. Once IKBα is degraded, NFκB is freed and translocates to the nucleus where it transcriptionally regulates a large number of immunologically important genes (Zerfaoui et al., 2008). NFκB p65 is subject to several forms of post-translational modifications including phosphorylation, acetylation, and ubiquitination (Hoesel and Schmid, 2013). These modifications affect the nuclear translocation, DNA-binding and transcriptional activity of NFκB (Perkins, 2006). According to the NFκB barcode hypothesis, it is the unique combination of these modifications that direct transcription in a gene-specific pattern (Moreno et al., 2010).

Natural killer lytic-associated molecule (NKLAM) is a membrane-bound E3 ubiquitin ligase and a member of the RING1-in between RING-RING2 (RBR) family of proteins. Three cysteine-rich regions in the N-terminus are critical for the ubiquitin ligase activity of NKLAM (Fortier and Kornbluth, 2006). NKLAM was originally discovered in our laboratory as a protein that co-localized with granzyme B in natural killer (NK) cytolytic granule membranes (Kozlowski et al., 1999). In addition to NK cells, NKLAM is expressed in other mononuclear cells such as monocytes and macrophages (Kozlowski et al., 1999; Portis et al., 2000). NKLAM is weakly expressed under resting conditions but is upregulated by cytokines (*e.g.* IFNγ) and bacterial products such as LPS (Lawrence and Kornbluth, 2012). NK cells from NKLAM-deficient (KO) mice have diminished anti-tumor cytolytic activity (Hoover et al., 2009). *In vivo*, NKLAM plays a role in controlling tumor metastasis (Hoover et al., 2012). Recent studies from our laboratory have also demonstrated a role for NKLAM in macrophage bacterial killing. We found that macrophages (bone marrow-derived and peritoneal) from NKLAM-KO mice are significantly defective in killing *Escherichia coli*. Despite its localization to phagosomes, NKLAM does not regulate bacteria uptake or phagosome acidification (Lawrence and Kornbluth, 2012). Thus, NKLAM is likely involved in regulating some other macrophage killing mechanism within the phagosome. One such mechanism is iNOS protein expression and NO production. Results presented here indicate that NKLAM is a positive regulator of iNOS protein expression, and does so, at least in part, by modulating the activity of the transcription factor NFκB.

Materials and methods

Macrophage cultures

All experiments on mice were approved by the Institutional Animal Care and Use Committees at Saint Louis University and the VA. 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. For splenic macrophage isolation, spleens were harvested from healthy mice and the red blood cells were removed by Lympholyte-M (Cedarlane) density gradient centrifugation. The mononuclear cell layer was washed, resuspended in RPMI 1640 supplemented with 10% FBS and plated in tissue culture plastic dishes for 7 days. Cells obtained using this method are predominantly mature F4/80$^+$, CD11b$^+$ macrophages (Van Ginderachter et al., 2006).

Nitric oxide measurement

Adherent bone marrow-derived macrophages (BMDM) were incubated with various concentrations of LPS for times indicated. Culture supernatants were assayed for nitrite using the Greiss reaction. For the *in vivo* studies, mice were intraperitoneally injected with sterile PBS or 25 μg of LPS and blood was obtained at 1, 2 and 6 h post-injection. The blood was centrifuged at 4000 × *g* for 4 min and the total concentration of nitrite present in the plasma was determined using the Griess reaction following the plasma preparation protocol of Moshage et al. (Moshage et al., 1995). Briefly, plasma samples were diluted and deproteinized with the addition of 1/20th volume of zinc sulfate for a final concentration of 15 g/L. The samples were centrifuged and the resulting supernatants were assayed for total nitrite using a commercial kit according the manufacturer's protocol (Cayman Chemical Company).

Macrophage transfection and luciferase activity measurement

Adherent BMDM were harvested from dishes with 1.5 mM EDTA, washed once with cold PBS, and then resuspended in DMEM plus 10% FBS. Wild type and NKLAM-KO macrophages (4×10^6) were mixed with 3 μg of the luciferase reporter plasmid pNFκB-luc (Clontech) and suspended in nucleofection solution T. Cells were nucleofected using program T-20 with the nucleofector I (Amaxa Biosystems). The nucleofected macrophages were resuspended in DMEM plus 10% FBS, transferred to 12-well plates and stimulated with 100 ng/ml LPS for the times indicated. At the desired time, the cells were collected, lysed in 1X luciferase reporter lysis buffer and snap frozen at −80 °C to aid in cell disruption. The total firefly luciferase activity was measured using the Promega Luciferase Assay System (Promega). Protein concentrations were determined using bicinchoninic acid (BCA) protein assay reagents (Pierce). Transfection efficiency was assessed by flow cytometry after nucleofection of cells with the green fluorescence protein (GFP) reporter plasmid pmaxGFP (Amaxa).

Immunoblotting

Whole cell protein lysates were separated using SDS-PAGE and then transferred to PVDF membrane. Membranes were blocked with 1% (wt/vol) BSA in Tris-buffered saline plus 0.1% Tween-20 (TBS-T) and then incubated in primary antibody with rocking overnight at 4 °C. The antibodies for iNOS (BD Transduction Laboratories), NFκB p65 (Santa Cruz Biotechnologies), IKBα (Cell Signaling), phospho-p65 Ser536 (Cell Signaling), STAT1 (Cell Signaling), poly ADP ribose polymerase (PARP; Cell Signaling), and phospho-STAT1 (701) (Cell Signaling) were used at 1:1000. Anti β-actin antibody (Sigma–Aldrich) was used at 1:4000. After three washes in TBS-T, the membranes were probed with HRP-conjugated secondary antibodies and the proteins were visualized with Bio-Rad Immun-Star Western C chemiluminescence kit. Images were captured and analyzed using a Bio-Rad Chemidoc XRS+ imager (BioRad).

Preparation of cytosolic and nuclear fractions

WT and NKLAM-KO BMDM were treated with 100 ng/ml LPS for 15, 30 or 60 min. Cytoplasmic and nuclear protein fractions were isolated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific). Equal protein amounts were immunoblotted for NFκB p65. The membranes with nuclear and cytoplasmic cell