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Research Article

Ikaros negatively regulates inducible nitric oxide synthase expression in macrophages: Involvement of Ikaros phosphorylation by casein kinase 2

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Abstract. Ikaros is known as a critical regulator of lymphocyte development. We examined the regulatory role of Ikaros in LPS/IFN-γ-induced inducible nitric oxide synthase (iNOS) expression by macrophages. Our results showed that IK6 (Ikaros dominant negative isoform) induction increases the iNOS expression. Ikaros DNA binding activity on the iNOS promoter was decreased, and a mutation of the Ikaros-binding site on the iNOS promoter resulted in an increase in LPS/IFN-γ-induced iNOS expression. LPS/IFN-γ increased the histone (H3) acetylation on the Ikaros DNA binding site. These results suggest that Ikaros acts as a negative regulator on iNOS expression. Treatment with a casein kinase 2 (CK2) inhibitor reversed LPS/IFN-γ-induced decrease in Ikaros DNA binding activity. Moreover, overexpression of kinase-inactive CK2 decreased iNOS expression and a significant amount of CK2α1 translocated into the nucleus in LPS/IFN-γ-treated cells. Overall, these data indicate that LPS/IFN-γ decreases the Ikaros DNA binding activity *via* the CK2 pathway, resulting in an increase of iNOS expression.

Keywords. Ikaros, inducible nitric oxide synthase, casein kinase 2, macrophage, nitric oxide.

Introduction

Ikaros plays an important role in lymphoid differentiation and has been identified as a transcription factor of lymphocyte-specific TdT and CD3δ [1 – 3]. The Ikaros gene was initially reported to contain eight (human, mouse) exons that were spliced alternately to generate at least 8 isoforms, IK1–IK8. Ikaros proteins dimerize by means of the oligomerization domain at the C terminus that contains two zinc fingers, whereas the DNA binding domain at the N terminus contains four zinc fingers [4, 5]. Alternative spliced isoforms have less than two N-terminal zinc fingers and some isoforms have a dominant negative effect [6, 7]. In particular, the IK6 isoform, which lacks all of the N-terminal zinc fingers and is known to be a strong dominant negative isoform. It is overexpressed in chronic myelogenous leukemia and acute lymphoblastic leukemia patients [8 – 14].

The Ikaros protein can act as both a positive and negative regulator of transcription. For the negative

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regulation of transcription, Ikaros combines with various chromatin-remodeling complexes, which regulate the transcription of the target gene by modulating histone acetylation [15]. In lymphocytes, most of the Ikaros exists within a stable 2-MDa complex that contains the components of the NuRD (nucleosome remodeling and deacetylation complex) complex, which includes the ATP-dependent chromatin remodeler Mi-2β and histone deacetylase (HDAC). In addition, Ikaros can recruit the SWI/SNF chromatin-remodeling complex to the appropriate lineage-specific gene targets such as CD8, and interact with the putative co-repressors Sin3 and CtBP [16–20].

Ikaros is expressed in all hematopoietic progenitors as well as in T, B, NK cells and macrophages. A decrease in Ikaros activity by the knockout or overexpression of IK6, results in the abnormal development of T cell and severe lymphoid defects, including a complete loss of T, B, NK cells and dendritic cells [21, 22]. Although the initial studies of Ikaros focused on the lymphoid defects, Ikaros mutant mice also exhibit myeloid defects and aberrant erythropoiesis. Moreover, a new isoform of Ikaros, IKx, is expressed selectively during myeloid differentiation [23]. Hence, Ikaros plays an important role in the lymphoid and myeloid lineage.

NO is a short-lived free radical that mediates various biological functions and is generated by nitric oxide synthase (NOS). There are three distinct NOS genes in mammals, neuronal NOS (nNOS), inducible NOS (iNOS), and constitutive NOS (cNOS) [24]. iNOS expression in macrophage requires stimulation by cytokines, microbial products, and various agents, and is responsible for the prolonged and profound production of NO [25]. Eukaryotic gene expression is regulated through a complex mechanism that involves a number of activators and repressors. A transcription factor may work to either stimulate or repress the transcription of a gene. NO production is regulated by a variety of cellular signaling pathways, which vary according to the types of cells and ligands. The expression of the iNOS gene in macrophages is regulated by a variety of inducible transcription factors [26–28], particularly by NF-κB in response to LPS. However, the precise role of the transcriptional factor functioning as a repressor in the LPS/IFN-γ induction of iNOS is not completely understood. Moreover, although the role of Ikaros in the differentiation of lymphoid cells is well known, the role of Ikaros in the response of macrophages to LPS/IFN-γ has not been elucidated.

This study examined the molecular mechanism of LPS/IFN-γ-induced iNOS expression in macrophages. To best of our knowledge, this is the first report to show the involvement of Ikaros in iNOS gene regulation. The results demonstrate a new repressive signaling pathway for the LPS/IFN-γ-induced expression of iNOS by Ikaros. In addition, the results show that the Ikaros transcription factor acts as a repressor on the iNOS promoter and the phosphorylation of Ikaros by casein kinase 2 (CK2) causes a loss of DNA binding ability.

Materials and methods

Cell culture. The mouse monocyte/macrophage cell line, RAW264.7, was maintained in RPMI 1640 medium (Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum (Cambrex) and 2% penicillin/streptomycin (Cambrex).

Plasmids and site-directed mutagenesis. The cDNA corresponding to open reading frame (ORF) of mouse Ikaros (pHA-IK1) was cloned by RT-PCR. The following primers containing an *Eco*RI site and HA coding sequence or a *Xho*I site were used : mouse IK1-HA-F (5′-CCTGGAATTCATGTACCCATACGATGTTCCAGATTACGCTATGGATGTCGATGAGGGTC-3′), mouse IK1-R (5′-GCTGCTCGAGTTAGCTCAGGTGGTAACGATGCTCC-3′). The cDNA was amplified by Superscript one-step RT-PCR using PLATINUM Taq kit (Invitrogen, Carlsbad, CA). The cDNA was cloned into the pcDNA3 cloning vector (Invitrogen) and verified by DNA sequencing. The clone of the Ikaros dominant negative isoform, IK6 (pEGN-IK6) was generated by cloning the relevant regions into the pEGFP-N1 vector (Clontech, JAPAN), which encodes the enhanced green fluorescence protein (EGFP). For this cloning, pHA-IK1 was used as a template for the PCR amplification of IK6. The forward mega primer containing the 3′ sequence of the Ikaros exon 2 sequence (corresponding to N-terminal amino acids 1–13) and 5′ sequence of Ikaros exon 7 was used. The following primers were used: IK6-F (5′-GGGGGAATTCATGGATGTCGATGAGGGTCAAGACATGTCCCAAGTTTCAGACAAGTGCCTGTCAGACATG-3′), IK6-R (5′-GGGGGGGCCCGGCTCAGGTGGTAACGATGCT-3′).

The CK2α1-HA cDNA (complete CDS) was also cloned into the *Eco*RI/*Xho*I sites of pcDNA3 (Invitrogen) to generate pHA-CK2α1. The kinase-inactive mutant clone (pHA-CK2α1K68M) of CK2α1 was generated from pHA-CK2α1 using a Muta-Direct™ Site Directed Mutagenesis Kit (Intron Biotech, Korea) according to the manufacturer's instructions, and the mutant construct was verified by DNA sequencing. The following primers were used for site-directed mutagenesis: CK2α1-K68M-F (5′-

AAAAAGTTGTTGTTATGATTCTCAAGCCAG-TAAAAA-3′), CK2α1-K68M-R (5′-TACTGGCTT-GAGAATCATAACAACAACTTTTT-3′).
For the luciferase reporter assay, PCR amplification of the iNOS promoter region (from −1569 to +160) was carried out with the following primers containing *Bgl*II/*Hin*dIII sites. (iNOS promoter-F : 5′-GGGAA-GATCTCCATAAGCTGTGTGTGTGTG-3′, iNOS promoter-R : 5′-CCCCAAGCTTACTAGGCTAC-TCCGTGGAG-3′) and the PCR fragment was cloned into the pGL3 vector (Promega, Madison, WI) to generate pGL3-iNOS. The clone was verified by DNA sequencing. The Ikaros binding site (from −75 to −63) mutant clone (pGL3-MT iNOS) of the iNOS promoter was generated from pGL3-iNOS using a Muta-Direct™ Site Directed Mutagenesis Kit (Intron Biotech) according to the manufacturer's instructions, and the mutant construct was verified by DNA sequencing. The following primers were used for site-directed mutagenesis: MT iNOS-F (5′-TCTC-CCTTTGATATCAGTTATGCA-3′), MT iNOS-R (5′-TGCATAACTGATATCAAAGGGAGA-3′).

RT-PCR analysis. The total RNA was isolated from the cultured cells using TRIzol (Invitrogen) according to the manufacturer's instructions. The total RNA was amplified by SuperScript one-step RT-PCR with a PLATINUM Taq kit (Invitrogen). The PCR products were run on a 1.2% agarose gel and visualized by ethidium bromide staining. The following primers were used to amplify Ikaros and GAPDH cDNAs : Ikaros-F (5′-CGATGAGGGTCAAGACATGTCC-C-3′), Ikaros-R (5′-GTCTTCTGCCATCTCGTTGT-GG-3′), GAPDH-F (5′-CCATGGAGAAGGCTGG-GG-3′), GAPDH-R (5′-CAAAGTTGTCATGGAT-GACC-3′), IK-multi-F (5′-CGATGAGGGTCAAG-ACATGTCCC-3′), IK-7R (5′-GTCTTCTGCCAT-CTCGTTGTGG-3′).

Immunoblot analysis. The cells were harvested and homogenized by sonication in a homogenization buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 10% SDS, 0.3 mM PMSF). The protein concentration of the cell lysate was determined using Bio-Rad protein DC assay (Bio-Rad Lab, Hercules, CA) with BSA (Sigma, St. Louis, MO) as a standard. Cell lysate (20 μg protein) was separated on a 8–15% SDS-PAGE, transferred to a Hybond ECL membrane (Amersham, Arlington Heights, IL) and probed separately using the anti-iNOS antibody (BD Transduction Laboratories, San Jose, CA), anti-CK2α1 (C-18, Santa Cruz Biotech, Santa Cruz, CA) antibody. The blots were developed using a Westzol plus chemiluminescence kit (Intron). In all immunoblotting experiments, the blots were reprobed with the anti-actin antibody (Sigma) to control the protein loading.

NO measurement. The level of NO was measured as the amount of nitrite released from the macrophage [29]. Supernatants (100 μl aliquots) were combined with equal volumes of Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride, 2.5% phosphoric acid) and incubated at room temperature for 10 min. The optical density was measured at a wavelength of 540 nm using sodium nitrite as the standard.

Nuclear extract and EMSA. The cells were collected and suspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1 mM PMSF, 0.1% NP-40). The cells were incubated on ice for 15 min and centrifuged at 1500 *g* for 10 min at 4 °C. The collected nuclei were resuspended in buffer C (20 mM HEPES, pH 7.9, 0.2 M NaCl, 1.5 mM $MgCl_2$, 0.2 M EDTA, 25% glycerol, 1 mM PMSF), and kept on ice for 30 min with intermittent agitation. The samples were centrifuged at 13 000 *g* for 10 min at 4 °C. The supernatant was collected and stored at −70 °C. The protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad).

EMSA was performed using a LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Probes were generated by Biotin-end labeling. The protein-DNA complexes were resolved on a 6% polyacrylamide gel and then transferred to a nylon membrane (Millipore, Billerica, MA). The chemiluminescence of the shifted bands was detected by a Chemiluminescent Nucleic Acid Detection Module (Pierce). The following oligonucleotide was used as probe: Ikaros binding site on iNOS promoter (IK-iNOS): 5′-GT-ACTCAGCT**TTTGGGAAT**CTCCTGTCA-3′ (core recognition sequences were shown in boldface).

Stable and transient transfection. Transfection was performed using a TransIT-LT1 reagent (Mirus Bio, Madison, WI) according to the manufacturer's instructions. For transient assay, cells were treated with various reagents for 24–48 h after transfection. For stable cell generation, cells were treated with G418 (200 μg/ml for RAW264.7) for 2 weeks. The medium containing G418 was refreshed every 2–3 days until colonies were formed. The individual colonies were transferred to a new culture plate and the stable cell construction was confirmed by Western blot or RT-PCR analysis.

Ikaros siRNA transfection. The siRNA duplexes for Ikaros knockdown and control siRNA were purchased from Santa Cruz Biotech. Cells were transfected with siRNAs using Amaxa nucleofector apparatus according to the manufacturer's recommendations.

Luciferase reporter assay. The cells were transiently transfected with pGL3-iNOS or pGL3-MT iNOS using the TransIT-Lt1 reagent, as described above. After transfection, the cells were incubated for 24 h in the fresh medium, and further incubated under various conditions. The cells were harvested and incubated in a cell lysis buffer (20 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM EDTA, 2 mM DTT, 10 % glycerol, 1 % Triton X-100, 1.25 mg/ml lysozyme, 2.5 mg/ml BSA) for 5 min at room temperature. The protein extract were collected by centrifugation and the protein concentration was quantified using a Bio-Rad protein assay kit (Bio-Rad). The luciferase activity was measured using a Bright-Glo™ Luciferase Assay System (Promega) according to the manufacturer's instructions. The pCMV β vector (β-galactosidase control expression plasmid, Clontech) was also co-transfected into each cell with the pGL3 reporter vector to normalize transfection efficiency of each well. The β-galactosidase activity was measured using a β-gal Assay Kit (Stratagene, La Jolla, CA) according to the manufacturer's instruction.

Chromatin immunoprecipitation (ChIP) assay. Cells were cultured on a 10-cm dish and incubated under various conditions. The histones were cross-linked to the DNA by adding formaldehyde directly to the culture medium to a final concentration of 1 %, followed by incubation for 10 min at 37 °C. The cells were washed several times with ice-cold PBS containing the protease inhibitors (1 μM PMSF, 1 μg/ml aprotinin and 1 μg/ml pepstatin A). The cells were collected by scraping and were centrifuged at 2000 rpm at 4 °C for 5 min. The cell pellet was resuspended in SDS lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1 % SDS) and incubated for 10 min on ice. The cell lysate was sonicated to shear the DNA lengths between 200–1000 bp. Protein-DNA complex was immunoprecipitated using anti-Ikaros antibody (M-20, Santa Cruz biotech) and reverse-cross-linked at 65 °C for 4 h. DNA was purified by phenol/chloroform extraction and ethanol precipitation. PCR amplification for iNOS promoter was performed. The following primers were used for PCR analysis of the Ikaros binding site flanking region on the iNOS promoter: iNOS ChIP-F (5′-CAGGAAGAGATGGCCTTGCA-3′), iNOS ChIP-R (5′-GAGTCTCAGTCTTCAACTCC-3′).

Immunofluorescence. Cells grown in 12-well Champer slides were washed in PBS and fixed with 3.7 % paraformaldehyde solution for 15 min at room temperature. Permeabilization was performed with 0.5 % Triton X-100 in PBS for 15 min and blocked with 5 % bovine serum albumin (Santa Cruz) in PBS for 30 min, and this was followed by incubation with the anti CK2α1 antibodies (1 : 1000 dilution) overnight. Bound antibodies were detected with FITC-conjugated anti-goat antibodies (1 : 400 dilution) for 1 h at room temperature. The cells were also stained with DAPI for 5 min and examined with a fluorescent microscope (5303 Axiophot II, Oberkochen, Germany).

Statistical analysis. All of experiments were repeated at least three times. Data are reported as a mean ± SE. p values were determined by a Student's t-test. (* $p < 0.05$).

Results

Ikaros down-regulates iNOS expression and directly binds to the iNOS promoter. The role of Ikaros transcription factor in macrophage function was examined by constructing a stably IK6 (dominant negative isoform of IK1)-expressing macrophage cell line (RAW264.7) and comparing the production of inflammatory mediators in macrophages by LPS and/or IFN-γ treatment. The stably IK6-overexpressed RAW264.7 cell was generated and the expression of IK6 was confirmed by fluorescence detection of the expressed IK6-EGFP fusion protein and RT-PCR analysis (Fig. 1A). The level of inflammatory mediators in IK6-expressing cells was then determined. The production of inflammatory cytokines (TNF-α, IL-6) in the IK6-overexpressing cells was not altered compared with the mock control (Supplementary Fig. 1). In contrast, the overexpression of IK6 significantly elevated the level of NO production when compared with the mock control treated with LPS/IFN-γ (Fig. 1B). Western blot analysis revealed that the level of LPS/IFN-γ-induced iNOS protein was higher in the IK6-overexpressed cells than in the mock-control (Fig. 1C) and densitometric analysis was performed to quantify the Western blot data (Fig. 1D). To provide additional evidence for the increase of iNOS expression by IK6 overexpression, RAW264.7 cells were transiently transfected with various doses of vector DNA carrying IK6. NO production and iNOS expression were increased by IK6 transfection in a dose-dependent manner (Supplementary Fig. 2). Moreover, specific knockdown of Ikaros by siRNA led to increase in iNOS–NO system responses to LPS/IFN-γ (Fig. 1E, F).

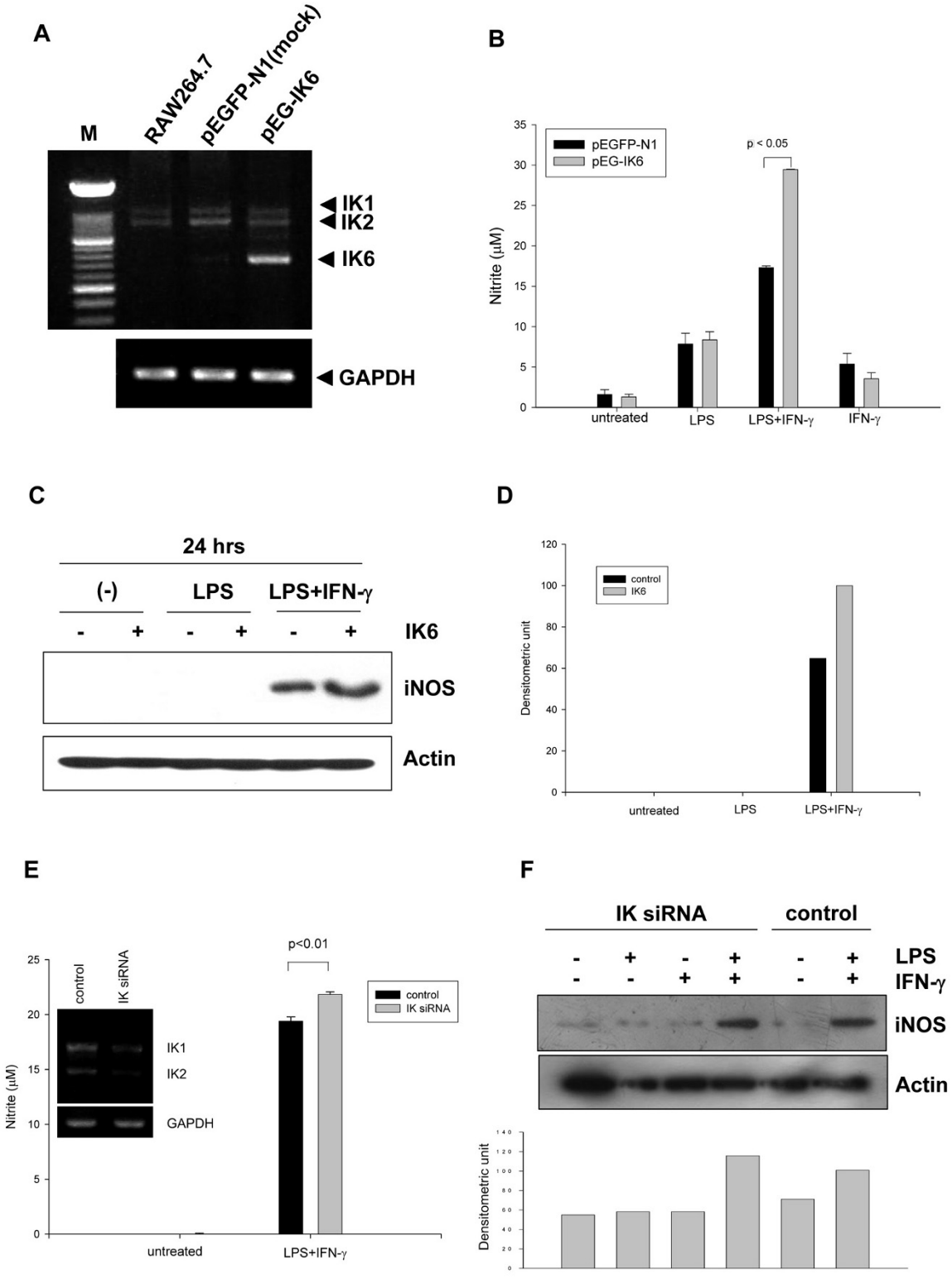

Figure 1. Nitric oxide (NO) production and inducible NO synthase (iNOS) expression in the IK6-overexpressed or Ikaros-knockdown RAW264.7 cells. (*A*) RAW264.7 cells were stably transfected with the vector carrying the IK6 isoform. The stably expressed clone was identified by RT-PCR. GAPDH served as a normalized control. IK6-transfected cells were treated with LPS (1 μg/ml) and/or IFN-γ (50 U/ml) for 24 h. (*B*) The level of NO production was determined using a Griess reagent. (*C*) iNOS expression levels were determined by ECL Western blot assay using the anti-iNOS antibody. (*D*) Densitometric analysis was performed to quantify the iNOS expression in (*C*). (*E, F*) RAW264.7 cells were transfected with siRNA (IK siRNA) specific for Ikaros and then treated with LPS/IFN-γ for 24 hours. Knockdown of Ikaros was checked using RT-PCR analysis using Ikaros-specific primers (IK-multi-F, IK-7R) (*E*). NO production and iNOS expression were determined as the methods described above. (*F*) To quantify iNOS expression, densitometric analysis was performed. All the results are representative of at least three independent experiments.

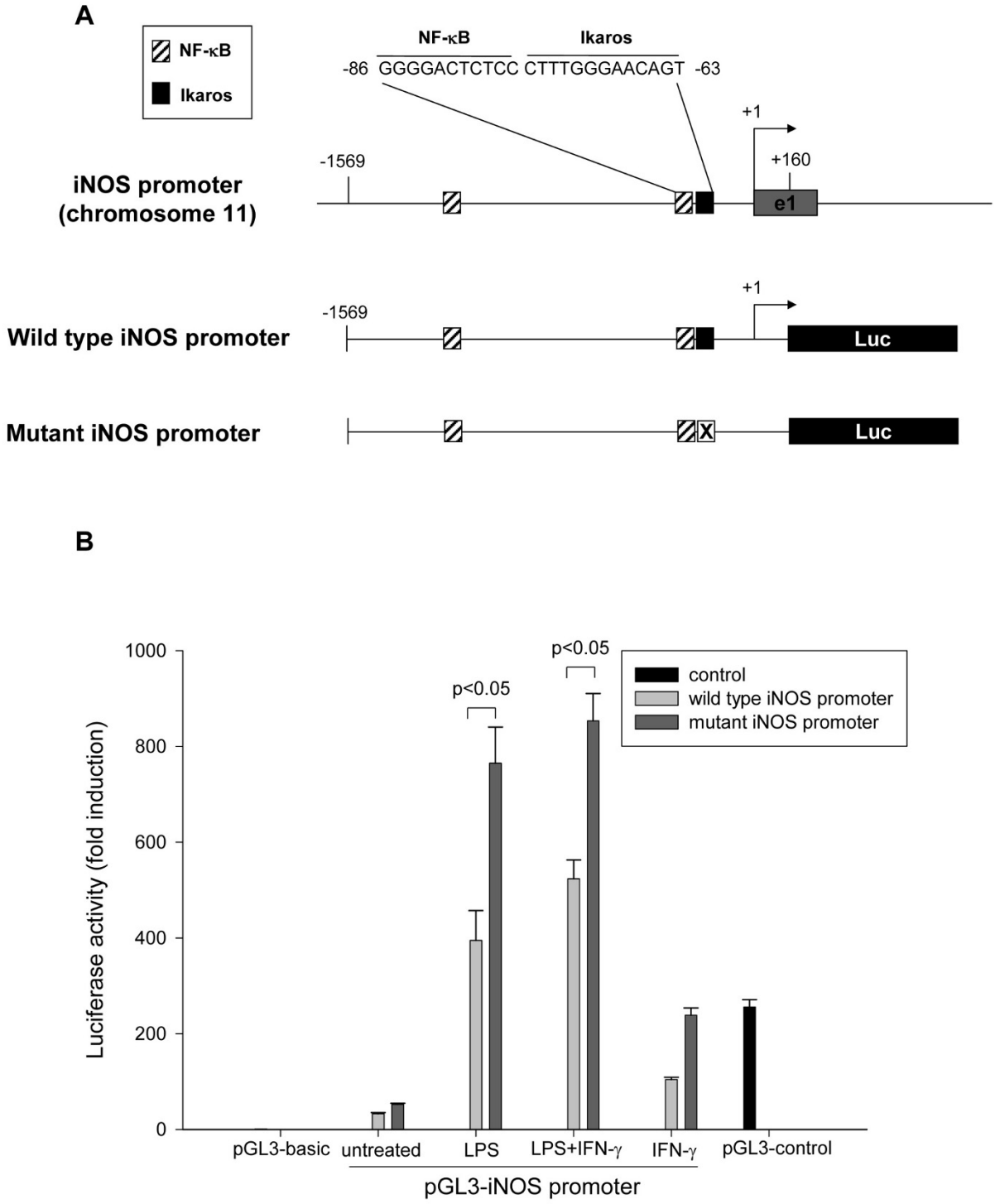

Figure 2. Luciferase reporter analysis of the iNOS promoter. (*A*) pGL3 luciferase reporter vectors carrying the wild-type or mutant iNOS promoter were constructed, as described in Materials and methods. (*B*) RAW264.7 cells were co-transfected with the constructs or pCMVβ. The luciferase and β-galactosidase activities were measured after 24 h stimulation with LPS (1 μg/ml) and/or IFN-γ (50 U/ml). The luciferase activity was normalized to the β-galactosidase activity for each transfection and determined using a Bright-Glo™ Luciferase assay system according to the manufacturer's recommendations. The experiment was performed in triplicate with identical results.

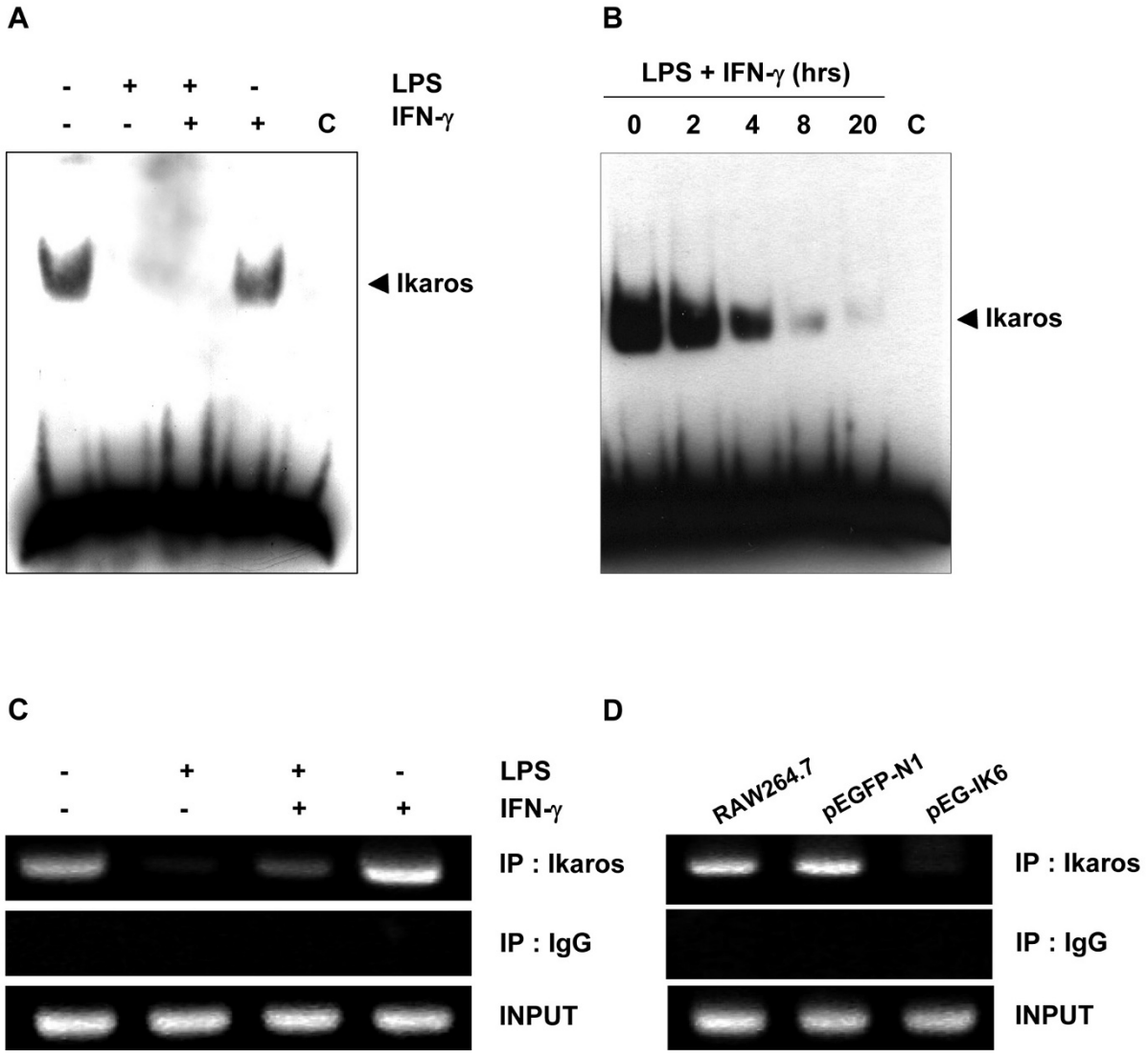

Figure 3. Effect of LPS and/or IFN-γ on the Ikaros DNA binding activity. (*A, B*) RAW264.7 cells were treated with LPS (1 μg/ml) and/or IFN- γ (50 U/ml) for 18 h or the indicated times. After treatment, EMSA was performed using IK-iNOS biotin-labeled oligonucleotide probes, respectively. C: Unlabeled IK-iNOS probe as a competitor. (*C*) Cells were treated with LPS (1 μg/ml) and/or IFN-γ (50 U/ml) for 18 h. ChIP was performed using anti-Ikaros antibody and then the DNA binding activity on iNOS promoter was determined by PCR/agarose electrophoresis using its specific primers. (*D*) DNA binding activity of Ikaros in IK6-overexpressed cell was also determined by ChIP assay. Similar observations were obtained in three independent experiments.

Since our results showed that dominant negative isoform of Ikaros increased iNOS expression, it is conceivable that Ikaros binds to its cognate elements in the iNOS promoter to inhibit the production of NO, and acts as a transcriptional repressor. This hypothesis was examined by cloning the iNOS promoter (from −1569 to +160) and identifying the various transcription factor binding sites. The Ikaros binding element was located (from −75 to −63) at the iNOS promoter near the transcription start site. The luciferase reporter vector carrying the iNOS promoter with a mutated Ikaros DNA binding site was next subcloned (Fig. 2A). The luciferase reporter vectors carrying the wild-type and mutated iNOS promoter were transiently transfected into the RAW264.7 cells. The cells were then treated with LPS and/or IFN-γ for 24 h, and the luciferase activity was measured. The iNOS promoter activity induced by LPS or LPS/IFN-γ was 1.6–2 times higher in the cells transfected with the mutated iNOS promoter than in the cells containing the wild-type iNOS promoter (Fig. 2B). Moreover, LPS- and/or IFN-γ-induced iNOS promoter activity was strongly increased when the Ikaros binding site was mutated. Therefore, we determined if Ikaros can bind a specific element within the iNOS promoter, and if its binding activity is altered by LPS and/or the IFN-γ treatment. The EMSA assays showed that using the

potent Ikaros binding element (IK-iNOS), the LPS and LPS/IFN-γ treatment decreased the level of formation of the DNA-protein complex (Fig. 3A), and its formation was decreased in a time-dependent manner after the LPS/IFN-γ treatment (Fig. 3B). To further demonstrate the specific DNA binding of Ikaros to iNOS promoter, we performed a ChIP assay using anti-Ikaros antibody. Our data show that Ikaros was able to bind with iNOS promoter strongly, but the binding activity was decreased by LPS or LPS/IFN-γ treatment (Fig. 3C). In addition, the DNA binding activity of Ikaros to iNOS promoter was abolished in IK6-overexpressed cell line (Fig. 3D).

CK2 is involved in LPS/IFN-γ-induced iNOS expression and the phosphorylation of Ikaros. It was reported that the DNA binding activity of Ikaros could be regulated by its phosphorylation, and that CK2 is responsible for Ikaros phosphorylation [30]. Therefore, this study examined whether CK2 was involved in LPS/IFN-γ-induced iNOS expression and the DNA binding activity of Ikaros. Cells were treated with LPS and/or IFN-γ for 24 h in the presence or absence of a CK2 inhibitor, 25 μM 5,6-dichloro-1-beta-ribofuranosyl benzimidazole (DRB), and the level of NO production was measured. Treatment with DRB partially prevented the LPS/IFN-γ-induced production of NO (Fig. 4A–D). Similarly, a decrease in iNOS expression was observed (Fig. 4E). Cells were also treated with DRB in the presence of LPS/IFN-γ to determine if CK2 plays a role in the Ikaros DNA binding activity. As shown in Figure 4F, DRB restored DNA binding activity of Ikaros, which was decreased by LPS or LPS/IFN-γ. To gain more insight into the role of CK2 in iNOS expression, cells were transfected with a kinase-inactive mutant of CK2α1 (CK2K68M) (Fig. 5) [31, 32]. CK2K68M was able to decrease the NO production and iNOS expression. The data demonstrate that CK2α is involved in iNOS-NO system activity by LPS/IFN-γ.

LPS/IFN-γ treatment results in translocation of CK2α to the nucleus. We have shown that CK2 is involved in the production of NO. In addition, the translocation of CK2 to the nucleus has been implicated in the response of cells to various stimulants. CK2 subunits also contain nuclear localization domains [33]. Therefore, to determine the activation pattern of CK2 in LPS/IFN-γ-treated macrophages, the expression and subcellular localization of the CK2 subunits were examined by RT-PCR and immunofluorescence, respectively. The mRNA levels of the CK2α1 and CK2β subunits were not changed by the LPS/IFN-γ treatment (data not shown). However, it was found that a large amount of the CK2α subunit translocated to the nucleus in the LPS/IFN-γ-treated cells (Fig. 6).

LPS and/or IFN-γ alter histone acetylation of the flanking region of the Ikaros binding site on the iNOS promoter. Previous studies reported that Ikaros has an association with the components of NuRD and that histone acetylation plays an important role in NOS expression [5, 34]. Therefore, this study examined the status of histone acetylation of Ikaros binding site on the iNOS promoter using a ChIP assay. The primers for this experiment were designed to detect the flanking region of the Ikaros binding site (Fig. 7A). As shown in Figure 7B, the level of histone acetylation of the H3 subunit was increased by the LPS and/or IFN-γ treatment. However, the level of acetylation of the H4 subunit was unchanged.

Discussion

In this study, our data demonstrate a novel repressive signaling pathway for iNOS expression by Ikaros in LPS/IFN-γ-stimulated macrophage, which suggests a physiological role of Ikaros in macrophages.

Activated macrophages are the source of NO that is largely produced by iNOS in response to cytokines and microbial products such as LPS, TNF-α and IFN-γ [35, 36] NO production by macrophages is regulated through various signal transduction pathways. iNOS expression depends on the activation of mitogen-activated protein kinases or transcriptional activators such as NF-κB and AP-1. However, the involvement of a transcriptional repressor in iNOS expression has not been elucidated. Since IK6 expression has been found in various types of leukemia and IKx, a new myeloid-specific isoform with various isoforms, has been found in myeloid differentiation [13, 23, 37, 38], these data suggest that Ikaros is an important gene in the myeloid cell and lymphoid cell lineages. Nevertheless, the functional role of Ikaros in macrophages remains to be determined. To examine the role of Ikaros in the function of macrophages, IK6-over-expressing macrophage cells were established. We found that normal isoforms of Ikaros such as IK1 and IK2 were mainly expressed in macrophage cells (supplementary Fig. 4A). Data also showed that the level of TNF-α and IL-6 production was not changed in the IK6-overexpressing cell compared with the control cells (supplementary Fig. 1), but the level of iNOS expression and NO production was higher. In addition, Ikaros knockdown increased iNOS–NO system activity, which is consistent with the results from IK6-overexpressed macrophage. It was also

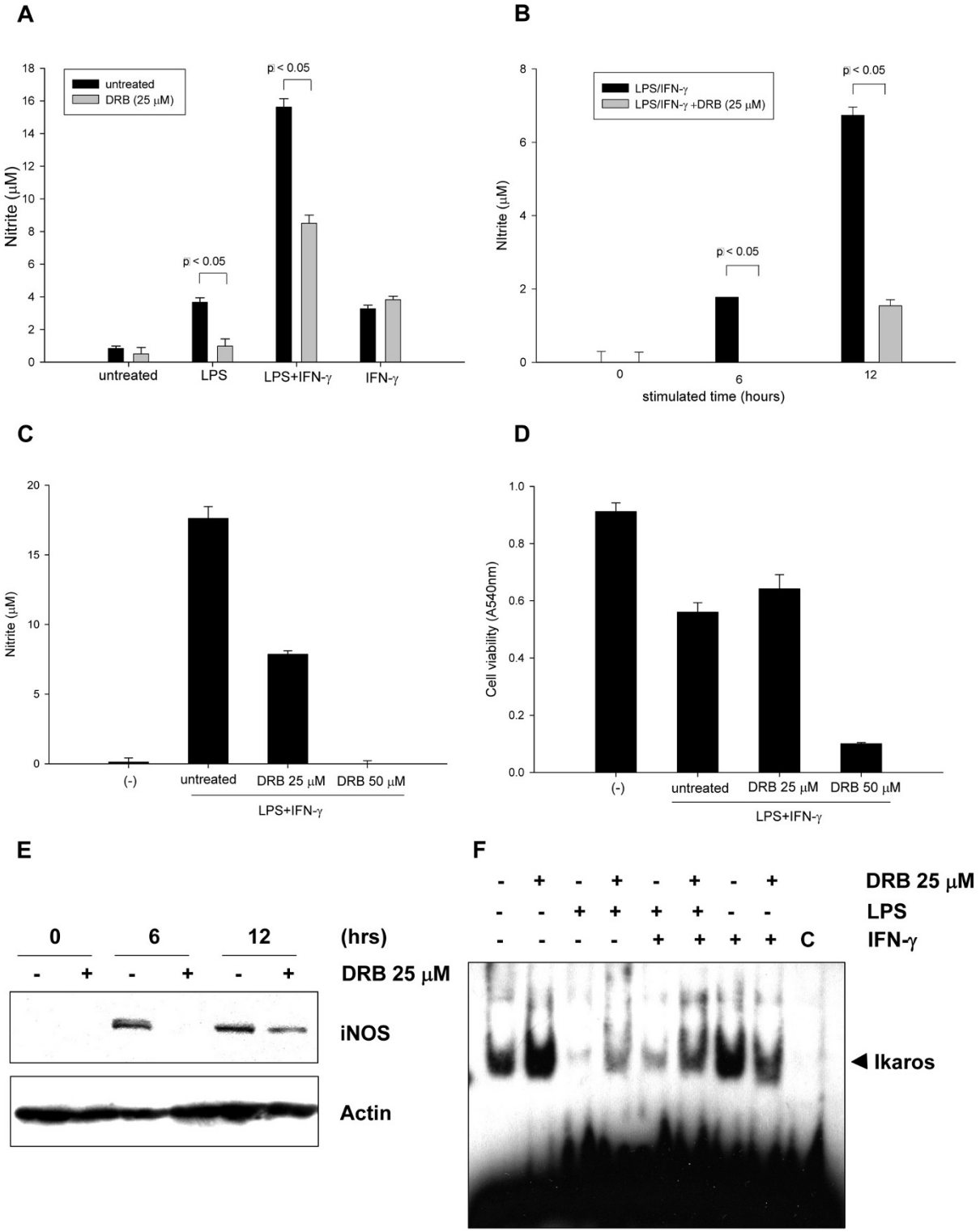

Figure 4. Effect of 5,6-dichloro-1-beta-ribofuranosyl benzimidazole (DRB) on the level of NO production, iNOS expression and Ikaros DNA binding activity. (*A*) RAW264.7 cells were treated with LPS (1 μg/ml) and/or IFN-γ (50 U/ml) in the presence or absence of DRB (25 μM) for 24 h. The level of NO production was determined using a Griess reagent. (*B*) The RAW264.7 cells were treated for the indicated times with LPS (1 μg/ml)/IFN-γ (50 U/ml) in the presence or absence of DRB (25 μM). The level of NO production was determined using the Griess reagent. (*C, D*) To examine the dose response of DRB treatment, cells were treated with LPS (1 μg/ml) and/or IFN-γ (50 U/ml) in the presence or absence of indicated concentrations of DRB. NO production and cell viability were determined. (*E*) The iNOS expression levels were determined by ECL Western blot assay using the anti-iNOS antibody. (*F*) RAW264.7 cells were treated with LPS (1 μg/ml) and/or IFN-γ (50 U/ml) for 18 h in the presence or absence of DRB (25 μM). EMSA was performed using IK-iNOS biotin-labeled oligonucleotide probe. C: Unlabeled IK-iNOS probe as a competitor. All the results are representative of at least three independent experiments.

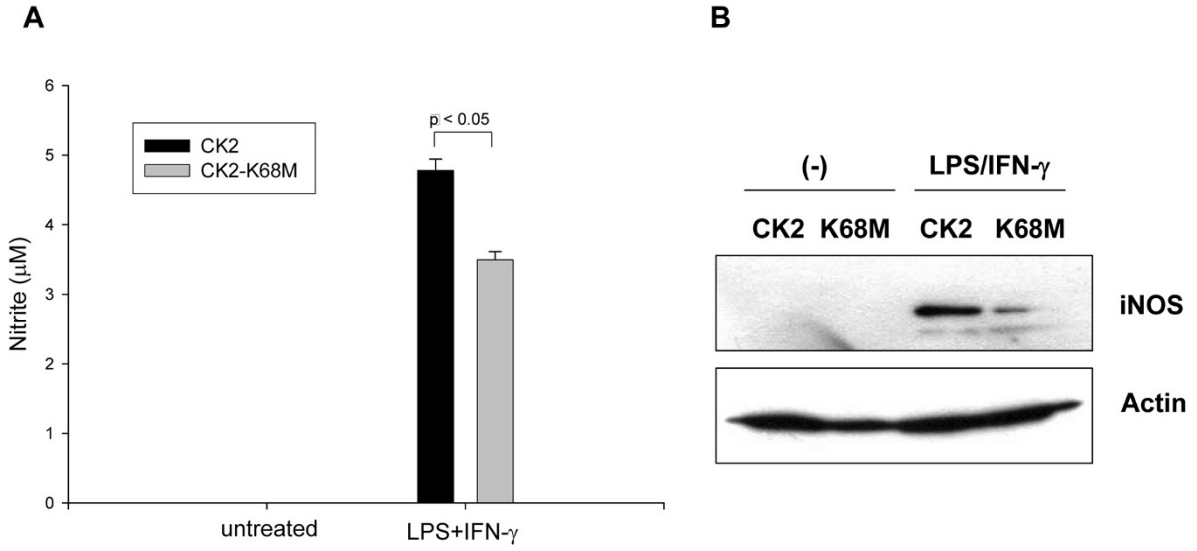

Figure 5. Effect of kinase-inactive casein kinase 2 (CK2) α1 on the level of NO production and iNOS expression. The cells was transiently transfected with the vector carrying wild-type CK2 α1 or kinase-inactive CK2α1 (K68M) and then treated with LPS (1 μg/ml) and/or IFN-γ (50 U/ml) for 6 h. (*A*) The level of NO production was determined using a Griess reagent. (*B*) iNOS expression was determined by an ECL immunoblot assay using the anti-iNOS antibody. CK2: pHA-CK2α1, K68M: pHA-inactive CK2α1. A representative experiment is shown that was reproducible at least three times.

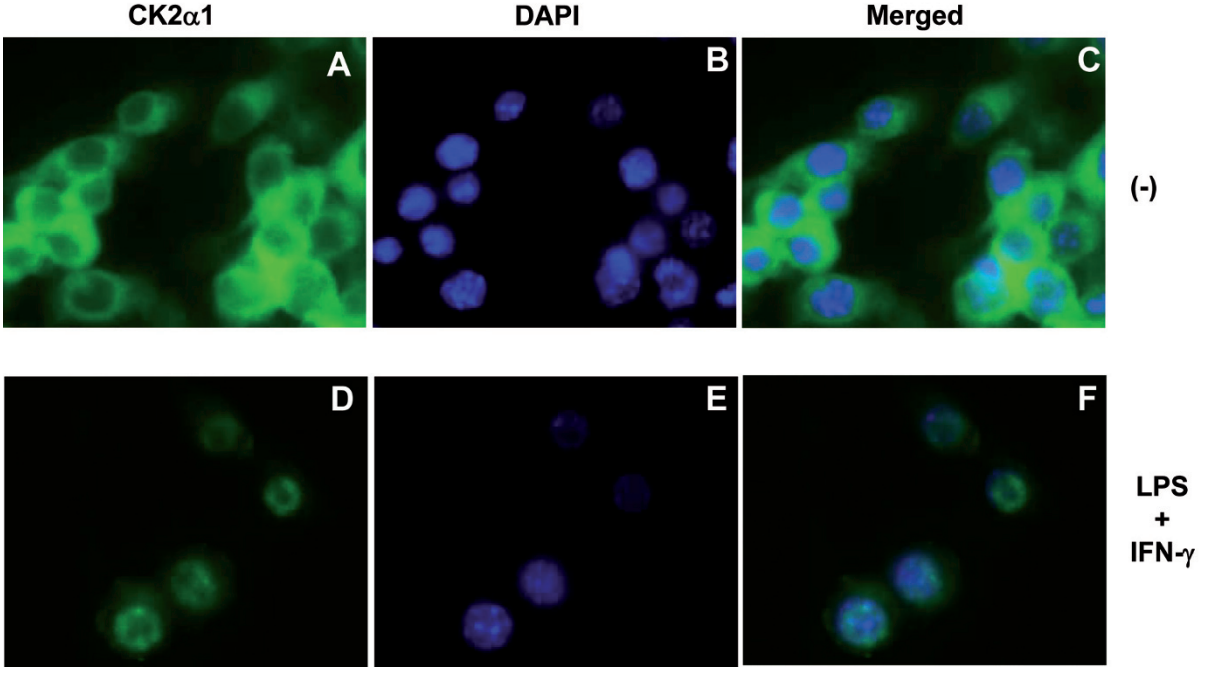

Figure 6. Nuclear localization of CK2α1 by the LPS/IFN-γ treatment. The cells were treated with LPS (1 μg/ml) and IFN-γ (50 U/ml) for 4 h, and fixed and permeabilized as described in Materials and methods. CK2α1 protein was detected using FITC-conjugated anti-CK2α1 antibody. The cells were counterstained with DAPI to visualize the nuclei and observed using a fluorescence microscope (viewed at magnification of 400×). (*A*–*C*) Untreated cells; (*D*–*F*) LPS (1 μg/ml)/IFN-γ (50 U/ml). Photographs were taken with identical exposure times. Similar observations were obtained in three independent experiments.

found that the Ikaros binding site was located on the iNOS promoter, and the binding site was involved in iNOS expression. Sequence analysis revealed that the promoters of various genes (IP-10, I-A, I-E; MHC class II) induced by the LPS/IFN-γ treatment in the macrophages contains at least one Ikaros binding site that may be involved in their regulation (unpublished observation). Recently, *in vivo* evidence that some of

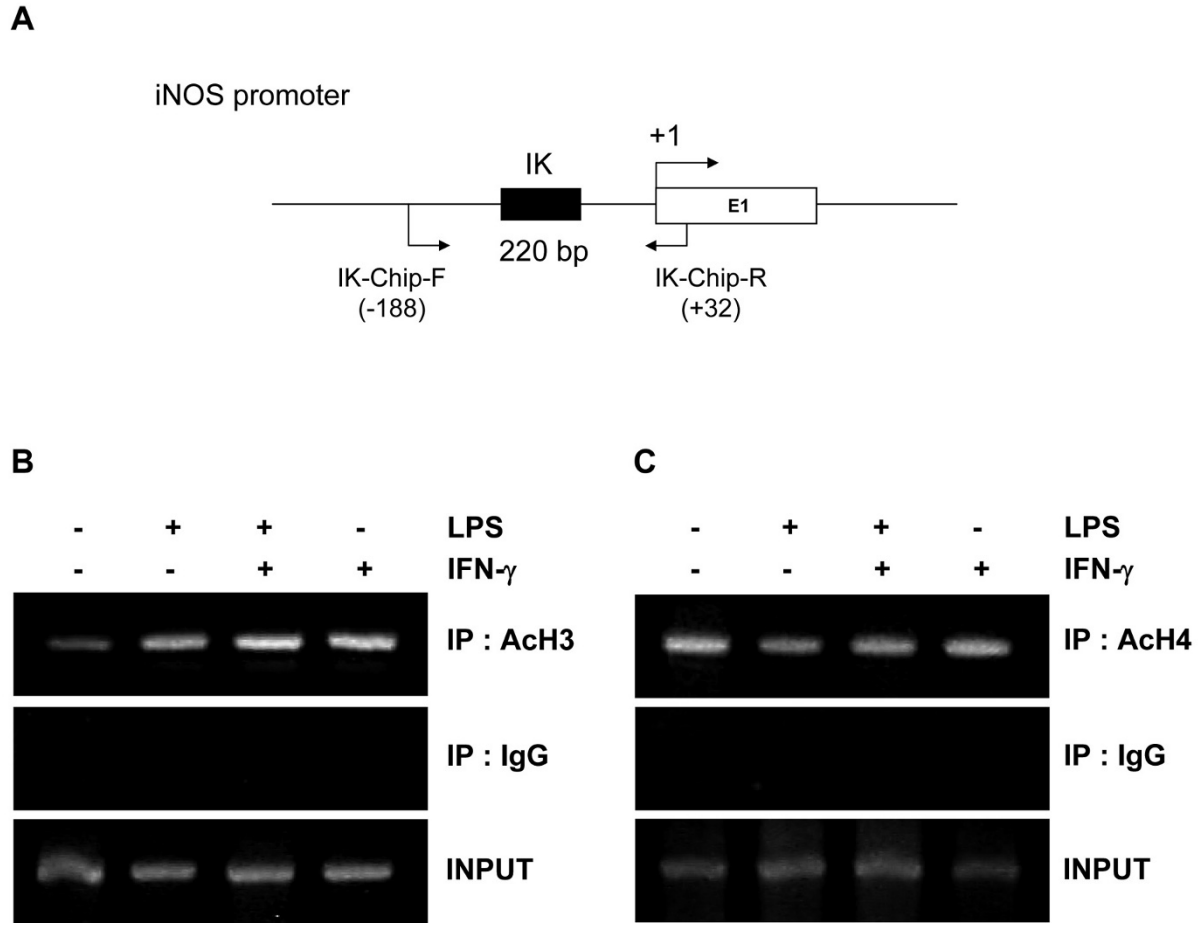

Figure 7. Effect of LPS/IFN-γ on histone acetylation in the iNOS promoter region. (*A*) The RAW264.7 cells were treated with LPS (1 μg/ml) and/or IFN-γ (50 U/ml) for 18 h. Schematic presentation of the iNOS promoter with the location of the Ikaros binding site (IK) and PCR primers used for ChIP assay. (*B, C*) The histone ChIP assay was performed using anti-acetyl-H3 (*B*) and anti-acetyl-H4 (*C*) antibodies, respectively. The experiment was performed in triplicate with identical results.

sites including Ikaros binding site are important in promoter function was demonstrated by *in vivo* footprinting and DNase I hypersensitivity analyses in activated T cells [39]. Based on these observations, it is likely that high-affinity Ikaros binding sites would be evolutionally conserved and functionally important.

From our current data, it is possible to explain the mechanism by which Ikaros controls the expression of iNOS. Down-regulated Ikaros as a result of the LPS/IFN-γ treatment would influence iNOS expression. In support of this hypothesis, we found that Ikaros expression during the treatment of LPS/IFN-γ was down-regulated in macrophage cell line RAW264.7 cells, whereas iNOS expression was not changed by LPS/IFN-γ when the down-regulated Ikaros was restored by transfection with IK1 (Supplementary Fig. 4B, C). This indicates that there may be macrophage-specific regulatory mechanisms for the change in Ikaros activity by LPS/IFN-γ. In addition, we hypothesize that after Ikaros is phosphorylated by the LPS/IFN-γ-activated kinase, the phosphorylated Ikaros is detached from the iNOS promoter. It was recently reported that Ikaros has three potent phosphorylation sites (P1, P2, P3) and these sites are phosphorylated in S, G2 and M phases of the cell cycle with the exception of the G1 phase [30]. Since our data showed that the LPS/IFN-γ treatment significantly decreased the Ikaros DNA binding activity, resulting in the increase of NO production, it is plausible that Ikaros acts as a transcriptional repressor. Based on these findings, it is also suggested that Ikaros needs to be phosphorylated for decrease in Ikaros DNA binding activity. Previous reports have shown that CK2 is the most potent kinase in Ikaros phosphorylation [30, 40]. CK2 is a serine/threonine kinase that can exist as a tetramer form containing two catalytic subunits (α1, α2) and two regulatory subunits (β) [41]. Each subunit can also regulate various cellular signaling pathways as monomer. While many different stimuli regulate the activity of CK2 in a different manner, the change

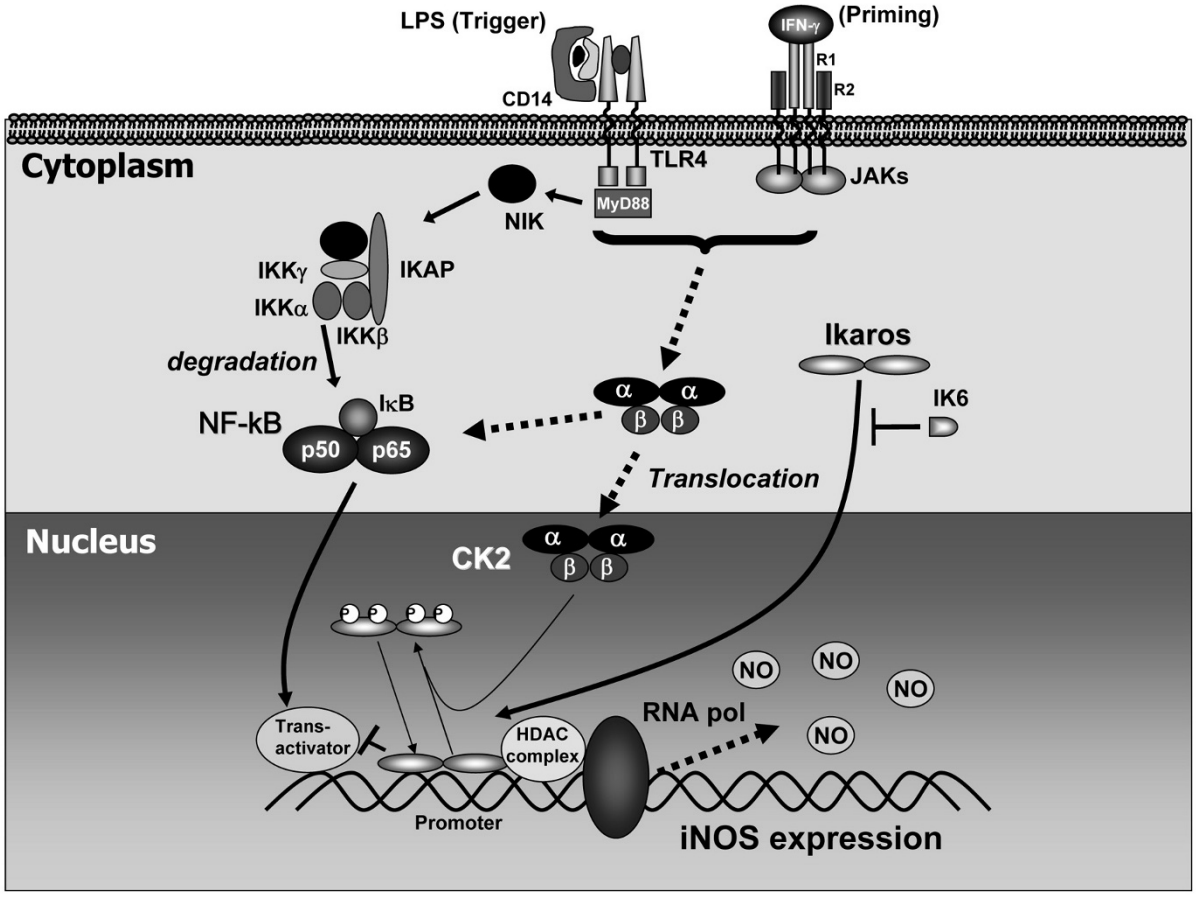

Figure 8. Schematic diagram showing the CK2/Ikaros-regulated expression of iNOS in macrophages.

in CK2 activity can influence several signal transduction pathways through a variety of mechanisms. To the best of our knowledge, there are no reports of a relationship between CK2/Ikaros and iNOS expression. Therefore, we determined whether the CK2 is involved in iNOS expression and Ikaros phosphorylation induced by LPS/IFN-γ.

The present results demonstrate that CK2 inhibitor, DRB, inhibited iNOS expression and restored the reduced DNA binding activity of Ikaros induced by the LPS/IFN-γ treatment. Our previous data also demonstrated that siRNA-mediated depletion of the CK2α completely restored the Ikaros DNA binding activity [42]. In addition, the overexpression of the CK2α kinase-deficient mutant induced a marked inhibition of NO production and iNOS expression. These results suggest that CK2 regulates iNOS expression through Ikaros phosphorylation. However, the data does not totally rule out the possibility that other transcription factors play a role in iNOS expression. It has been suggest that CK2 can regulate iNOS expression in mouse breast epithelial cell through the activation of NF-κB (EMT-6) [43]. Other studies also have found that the activation of NF-κB by IL-1 is accompanied by the CK2-mediated phosphorylation of the p65 subunit in fibroblasts and hepatoma cells [44]. Those data demonstrate that CK2 might be able to regulate the iNOS expression though Ikaros regulation as well as the NF-κB pathway.

A subpopulation of each CK2 substrate could be altered by different upstream signals and each substrate could regulate the other substrates independently. It is known that each subunit exists as different amounts of protein in different sites of the cell, because the target substrates for CK2 differ according to the location in the cell [41]. It was also reported that various kinases, including MAPK, can differentially phosphorylate the catalytic subunits of CK2 in various amino acid residues, and the altered kinase activity of the phosphorylated CK2 affects the cellular process [45–49]. In the light of these findings, it is possible that for CK2 activation, LPS/IFN-γ can both directly and indirectly deliver divergent activation signals and trigger the synergistic signal transduction pathways. In the present study, it was found that the α1 and β

subunits of CK2 were strongly expressed, but the α2 subunit was not expressed in the macrophage cell line RAW264.7 (supplementary Fig. 3). Moreover, the expression of these subunits was not altered by the LPS/IFN-γ treatment, suggesting that LPS/IFN-γ does not control the CK2 activity through an increase in CK2 expression. In addition, our results demonstrate that a large amount of the α1 subunit was located to the nucleus after the LPS/IFN-γ treatment. It has been also reported that most of Ikaros is located in the nucleus [5]. Based on these findings, it appears that the nucleus-localized CK2α1 subunit is involved in Ikaros phosphorylation in the nucleus.

Histone deacetylation by HDACs leads to a 'close' state of the histone, which represses transcription [15]. Ikaros recruits Mi-2β, which is ATP-dependent chromatin remodeler (Chd4), and the HDACs (HDAC1 and HDAC2) [15–18]. These proteins, as the components of the NuRD complex, can form a complex with Ikaros and negatively regulate the transcription of the target gene. In addition, it was reported that Ikaros regulates the acetylation and deacetylation of the histone H3 subunit, which results in the transcriptional activation of prolactin (PRL) and the repression of the pituitary growth hormone (GH), Bcl-XL [50, 51]. Our results also showed that Ikaros was able to bind to iNOS promoter, and the level of acetylhistone H3 on Ikaros binding region was increased by LPS/IFN-γ. This suggests that Ikaros controls histone deacetylation, which leads to transcriptional repression of the iNOS promoter.

Many studies have reported that transcriptional activators play a role in the regulation of iNOS expression, but there are no reports of the transcription factor functioning as a repressor. We propose a new repressive transcriptional model for iNOS expression by Ikaros (Fig. 8). LPS/IFN-γ might activate CK2α1 through various signaling pathways in macrophages. The activated CK2α1 is translocated to the nucleus and affects the Ikaros transcription factor. In addition, since IK1 (an isoform containing the whole ORF) is located mainly in the nucleus and CK2 can phosphorylate the Ikaros protein, the translocated CK2α1 might phosphorylate the Ikaros protein on the iNOS promoter. Subsequently, the phosphorylated Ikaros combined with the HDAC complex is detached from the iNOS promoter and causes the acetylation of the histone H3 on the iNOS promoter. Overall, these results are expected to lead to an understanding of the regulatory mechanisms of stimuli-induced NO production in macrophages. However, further studies on the signal transduction cascades that participate in the regulation of Ikaros activity will be needed to help discover new drugs that can control the immune responses.

Electronic supplementary material. Supplementary material is available in the online version of this article at springerlink.com (DOI 10.1007/s00018-008-8332-7) and is accessible for authorized users.

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