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Cutting Edge: Monarch-1 Suppresses Non-Canonical NF-κB Activation and p52-Dependent Chemokine Expression in Monocytes[1]

John D. Lich, Kristi L. Williams, Chris B. Moore, Janelle C. Arthur, Beckley K. Davis, Debra J. Taxman, and Jenny P-Y. Ting[2]

CATERPILLER (NOD, NBD-LRR) proteins are rapidly emerging as important mediators of innate and adaptive immunity. Among these, Monarch-1 operates as a novel attenuating factor of inflammation by suppressing inflammatory responses in activated monocytes. However, the molecular mechanisms by which Monarch-1 performs this important function are not well understood. In this report, we show that Monarch-1 inhibits CD40-mediated activation of NF-κB via the non-canonical pathway in human monocytes. This inhibition stems from the ability of Monarch-1 to associate with and induce proteasome-mediated degradation of NF-κB inducing kinase. Congruently, silencing Monarch-1 with shRNA enhances the expression of p52-dependent chemokines.   The Journal of Immunology, 2007, 178: 1256–1260.

Monarch-1, also known as Pypaf7, harbors an N-terminal pyrin domain and is expressed exclusively in cells of myeloid lineage (1). We recently demonstrated that Monarch-1 suppresses proinflammatory cytokine production in monocytes stimulated with TLR ligands, TNF-α, and *Mycobacterium tuberculosis* (2). The mechanisms by which Monarch-1 performs this anti-inflammatory function are not clear; however, a role for Monarch-1 in the inhibition of NF-κB was suggested by these studies.

NF-κB activation occurs through two distinct mechanisms referred to as the canonical and non-canonical pathways. The canonical pathway proceeds very rapidly and can be activated by a number of upstream kinases that signal through the IKKαβγ complex. This results in nuclear accumulation of primarily RelA/p50 heterodimers that induce early immune response genes. In contrast, the non-canonical pathway displays slower kinetics and is dependent upon NF-κB-inducing kinase

(NIK)[3] (3). In this alternative pathway, NIK activates IKKα leading to the nuclear accumulation of p52-containing NF-κB complexes that induce a different set of inflammatory genes to support the ongoing immune response (4). The present study was initiated to elucidate the mechanisms by which Monarch-1 suppresses NF-κB in monocytes. We found that Monarch-1 suppresses activation of the non-canonical pathway by associating with NIK and inducing its proteasome-mediated degradation.

Materials and Methods

Cell lines, Abs, and reagents

HEK293T and Cos-7 cells were maintained in DMEM (Invitrogen Life Technologies) with 10% FCS, 100 mg/ml penicillin, and 100 mg/ml streptomycin. THP-1 derived cell lines stably expressing Ha-Monarch-1 or shRNA targeting Monarch-1 have been described (2). The Abs used were: anti-NIK (H-248), anti-p52 (C-5), anti-p50 (H-119), and anti-CagA (b-300; control Ab) from Santa Cruz Biotechnology; anti-HA Abs (12CA4 and 13F10) from Roche; and anti-V5 from Invitrogen Life Technologies. CD40L was obtained from Peprotech, MG132 from Calbiochem. Ha-Monarch-1 has been described (2). NIK (MGC:45335) was obtained from the American Type Culture Collection Mammalian Genome Collection. The luciferase reporter plasmids and p53 were obtained from Dr. A. Baldwin (Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC). Monarch-1 truncation mutants were PCR amplified and cloned into pcDNA3.1 V5/HIS TOPO cloning vector.

Luciferase assays

HEK293T cells were transfected with 50 ng of NF-κB or p53 reporter plasmid and 500 ng of NIK or p53. Monarch-1 was cotransfected at the indicated concentrations and pcDNA3.1 was used to equalize the plasmid concentration among samples. Luciferase assays were performed in triplicate (2).

RNA preparation and real-time PCR

Total RNA was isolated with RNeasy (Qiagen). Real-time PCR was performed using SYBR Green as described (5). Primer sequences are available upon request. Results were normalized to 18S ribosomal RNA internal controls and expressed in relative numbers.

Immunoprecipitations and Western blot analysis

HEK293T cells were transfected using FuGene 6 (Roche). The cells were lysed as described (2). Nuclear and cytoplasmic fractions were generated using the NE-PER kit (Pierce). Protein concentrations were determined by Bradford assay (Bio-Rad) and equilibrated samples were immunoprecipitated with 2 μg of the indicated Ab for 18 h with rotation. Ab complexes were captured with protein A/G agarose beads (Pierce). The beads were washed, eluted into sample buffer, boiled, and separated by SDS-PAGE (2). Unless indicated, all plasmids were used at equal concentrations.

Pulse-chase analysis

Cos-7 cells were transfected with 3 μg of the indicated plasmids using FuGene 6 (Roche). The cells were incubated for 18 h, starved for 30 min in methionine/cysteine free DMEM with 5% FBS, pulsed with 0.4 mCi/ml [^{35}S]methionine for 30 min, washed with warm PBS, and incubated in methionine fortified DMEM containing 10% FBS. At the indicated time points, cells were washed in ice-cold PBS then lysed in 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, 50 mM Tris (pH 8), 50 mM NaF, and 2 mM EDTA supplemented with protease inhibitors (Roche). NIK was immunoprecipitated and eluted into reducing sample buffer. Proteins were fractionated by SDS-PAGE and gels were dried. Control samples consisting of protein A/G beads alone confirmed the specificity of protein bands visualized in autoradiographs. Autoradiographs were scanned and analyzed by densitometry.

Results and Discussion

Monarch-1 inhibits non-canonical NF-κB in monocytes.

Previously, we reported that Monarch-1 inhibits TLR-induced NF-κB driven luciferase. However, this reporter assay could not determine which NF-κB pathway was inhibited (2). Activation of canonical NF-κB requires the degradation of IκBα to release RelA/p50 heterodimers. RelA is then phosphorylated allowing the expression of NF-κB responsive genes such as *IκBα* and *NF-κB2/p100*. To determine the role of Monarch-1 in canonical NF-κB activation, THP-1 monocytes stably expressing Ha-Monarch-1 (THP-Ha-Mon1) or an empty vector control (THP-EV) were stimulated with the TLR2 ligand Pam3Cys for the indicated times (Fig. 1A). Activation was monitored by Western blot analysis of IκBα degradation, RelA phosphorylation, and the induction of NF-κB responsive genes. No difference in IκBα degradation or RelA phosphorylation was detected between THP-EV and THP-Ha-Mon1 cells at the time points assayed. In addition, the expression of *NF-κB2/p100* and *IκBα* was equally up-regulated in both cells. Although a more detailed kinetic analysis indicated that Monarch-1 did decrease RelA phosphorylation at 60 min (Fig. 1B), these results indicate that initial activation of canonical NF-κB is not affected by Monarch-1.

We next analyzed the role of Monarch-1 in non-canonical NF-κB activation. This alternative pathway can be induced by TNF receptor family members such as CD40 and requires the processing of NF-κB2/p100 to its active form p52, which then rapidly translocates to the nucleus (6). THP-Ha-Mon1 cells or empty vector controls were pretreated with Pam3Cys to induce p100 expression and then stimulated with CD40L to promote p100 processing (Fig. 1C). Such prior activation of the canonical pathway followed by the subsequent activation of the non-canonical pathway has been documented (7). Cytoplasmic and nuclear extracts were prepared and non-canonical NF-κB activation was determined by monitoring p100 processing to p52 by Western blot. Pam3Cys treatment induced p100 expression in both THP-Ha-Mon1 and THP-EV cells, confirming that the canonical pathway remained intact (Fig. 1C). Subsequent CD40L treatment of THP-EV cells resulted in the accumulation of nuclear p52 within 1 h of treatment. The level of p52 peaked by 3 h and was maintained in the nucleus throughout the 6 h time course. In contrast, p52 was significantly reduced in THP-Ha-Mon1 cells. A weak p52 band was detected within 1 h of treatment; however, this effect was only transient as p52 was not detected at later time points. Importantly, no differ-
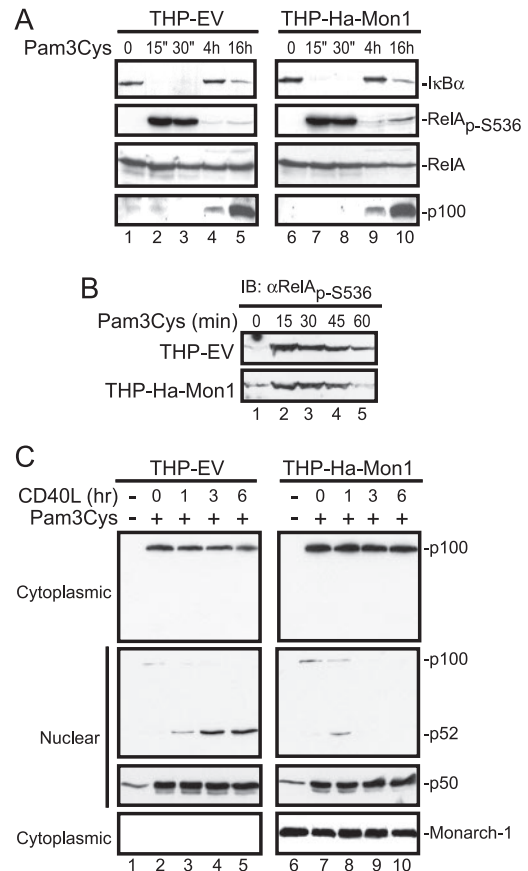
FIGURE 1. Monarch-1 suppresses non-canonical NF-κB activation. *A* and *B*, THP-EV and THP-Ha-Mon1 cells were treated with 200 ng/ml Pam3Cys for the indicated times. Western blots were probed with Ab to detect IκBα, RelA, or p100. *C*, THP-EV and THP-Ha-Mon1 cells were stimulated with 200 ng/ml Pam3Cys to induce p100 expression and then treated with 250 ng/ml CD40L to activate non-canonical NF-κB. Nuclear and cytoplasmic fractions were analyzed by Western blot.

ence in nuclear p50 levels was detected between the two cell lines, indicating that nuclear translocation of NF-κB proteins was not globally inhibited by the presence of Monarch-1. These results indicate that Monarch-1 suppresses non-canonical NF-κB activation.

Monarch-1 associates with NIK

Although many kinases can stimulate the canonical pathway, the non-canonical pathway is uniquely dependent upon NIK (8). To determine whether Monarch-1 intersects the non-canonical pathway by associating with this kinase, we performed coimmunoprecipitation experiments. HEK293T cells were transfected with Ha-Monarch-1 and NIK, and NIK complexes were immunopurified and analyzed by Western blot. Monarch-1 coprecipitated with NIK but not a control isotype Ig (Fig. 2A). As additional controls, no association was found between Monarch-1 and IKKα when both proteins were overexpressed, nor did two other CATERPILLER (CLR) proteins, CIITA and NOD2, interact with NIK (data not shown).

Monarch-1 failed to coprecipitate with endogenous NIK when expressed alone in unstimulated cells (Fig. 2A, *lane 2*). Instead, these complexes only formed when both proteins were coexpressed. It is known that ectopically expressed NIK displays a high level of functional activity, while endogenous NIK does not (9). Thus an explanation for this result is that Monarch-1 preferentially
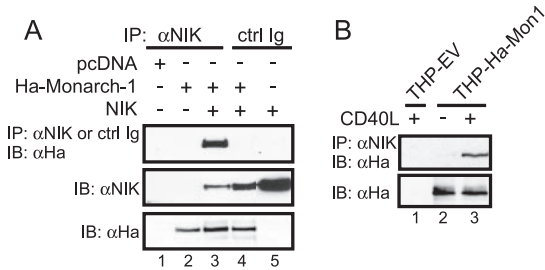
FIGURE 2. Monarch-1 associates with NIK. *A*, HEK293T cells were co-transfected with Ha-Monarch-1 and NIK or pcDNA. Cell lysates were immunoprecipitated with anti-NIK and fractionated by SDS-PAGE. Western blots were probed with anti-Ha to detect Monarch-1. Control samples (*lanes 4* and *5*) were immunoprecipitated with a control polyclonal Ab to monitor specificity. Lysate controls show the presence of NIK and Monarch-1 in the expected lanes. *B*, THP-EV or THP-Ha-Mon1 cells were treated with 250 ng/ml CD40L. Cells were lysed and immunoprecipitated with anti-NIK. Western analysis of the precipitates was performed using anti-Ha to detect Monarch-1.

associates with active forms of the kinase. To explore this possibility in monocytic cells, THP-Ha-Mon1 cells were stimulated with CD40L to activate endogenous NIK (Fig. 2*B*) (6). Monarch-1 coprecipitated with endogenous NIK only in stimulated cells, thus supporting our hypothesis that complex formation depends on the activation status of NIK.

Structural domains of Monarch-1 required for NIK binding.

Monarch-1 possesses a tripartite domain architecture conserved in most CLR proteins (10). To determine which structural elements of Monarch-1 are required for NIK binding, truncation mutants were constructed and tested for the ability to bind NIK in immunoprecipitation assays. The N-terminal pyrin domain of Monarch-1 failed to coprecipitate with NIK (Fig. 3). However, the pyrin-NBD truncation mutant did coprecipitate with NIK, indicating a role for the NBD in NIK binding. NIK also coprecipitated with truncation mutants comprised of the NBD-LRR and the LRR alone. Thus, both the NBD and LRR domain of Monarch-1 encode elements that mediate NIK binding. In contrast, the pyrin domain is not required for this interaction.
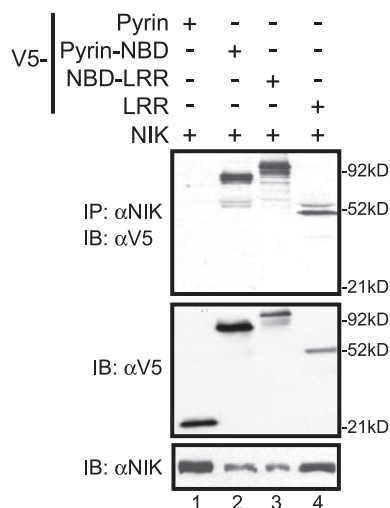

FIGURE 3. The NBD and LRR domains of Monarch-1 mediate NIK binding. HEK293T cells were transfected with NIK and the indicated Monarch-1 truncation mutant. Cell lysates were immunoprecipitated with anti-NIK and Western blots probed with anti-V5 to detect Monarch-1. The *bottom panels* show the presence of Monarch-1 and NIK in lysates.

Monarch-1 inhibits NIK-induced NF-κB activation.

To directly test the effect of Monarch-1 on NIK-induced NF-κB activation, luciferase reporter assays were performed. As expected, ectopic expression of NIK led to strong activation of an NF-κB reporter plasmid (Fig. 4*A*). Coexpression of Monarch-1 resulted in a dose dependent inhibition of NIK-induced NF-κB activity, confirming a negative regulatory role for Monarch-1. In contrast, Monarch-1 did not inhibit activation of a p53-inducible reporter plasmid indicating specificity of its function.

We next sought to determine the biologic consequences of Monarch-1-mediated suppression of NIK in monocytes using RNA silencing. THP-Ha-Mon1 cells or THP-1 cells in which Monarch-1 expression was silenced by shRNA (THP-shMon1) were stimulated with Pam3Cys and CD40L to induce activation of non-canonical NF-κB. Next, the expression of the p52-dependent genes *CXCR4*, *CXCL12*, and *CXCL13* was analyzed and compared with control THP-EV cells (11, 12). All three genes were strongly upregulated in THP-shMon1 cells, demonstrating enhanced p52 activity in these cells in the absence of Monarch-1. In contrast, gene expression was inhibited in THP-Ha-Mon1 cells, indicating reduced activity of non-canonical NF-κB in the presence of Monarch-1. No difference was detected in the expression of *CXCL8* or *CXCL9*, which are not known to be p52 dependent (data not shown). Together, these results suggest a mechanism whereby
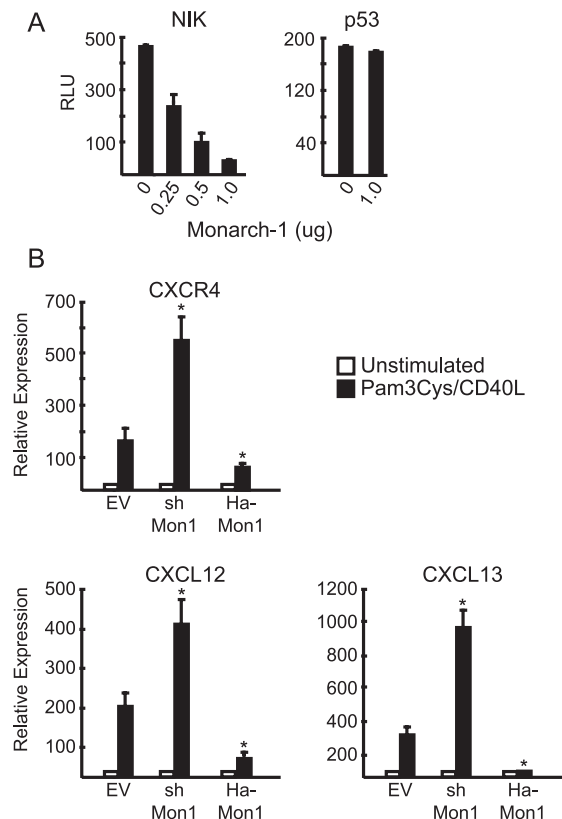
FIGURE 4. Monarch-1 suppresses NIK-induced NF-κB activation. *A*, HEK293T cells were transfected with NF-κB or p53 luciferase reporter plasmids in the presence of NIK or p53. Monarch-1 was transfected at the indicated concentrations and luciferase activity was assessed. *B*, THP-EV, THP-shMon1, and THP-Ha-Mon1 cells were treated as described in Fig. 1*B*. The expression of the indicated genes was measured by real-time PCR. The values presented are the average of three experiments measured in triplicate. The Student *t* test was used to determine statistical significance in gene expression compared with control THP-EV cells, $p < 0.05$.

Monarch-1 associates with NIK and suppresses its ability to activate non-canonical NF-κB in monocytic cells.

Monarch-1 induces NIK degradation through a proteasome-dependent pathway.

Throughout the course of this study we consistently noticed reduced levels of NIK in cells coexpressing NIK and Monarch-1, compared with cells in which NIK was expressed alone (Fig. 2*A*). Furthermore, a significant reduction in endogenous NIK was also observed upon stimulation of THP-Ha-Mon1 cells compared with THP-EV cells (Fig. 5*A*). These observations led us to question whether Monarch-1 suppresses NIK activity by regulating the stability of the kinase. To test this hypothesis, Cos-7 cells were transfected with NIK in the presence or absence of Monarch-1 and pulse-chase assays were performed. NIK protein levels declined sharply in the presence of Monarch-1 and densitometry quantified an approximate 75% decrease over the course of the experiment

FIGURE 5. Monarch-1 induces proteasome-mediated degradation of NIK. *A*, THP-EV or THP-Ha-Mon1 cells were stimulated as indicated and NIK levels were assessed by Western blot. *B*, Cos-7 cells were transfected with NIK in the presence or absence of Monarch-1 and pulse-chase assays were performed. Gels were visualized by autoradiography and NIK bands were analyzed by densitometry. The percentage of NIK remaining, as compared with the zero time point, is shown below each panel. *C*, HEK293T cells were transfected with NIK plus full-length or truncated Monarch-1. NIK levels were determined by Western blot. HSP70 was monitored to ensure equal loading. Immunoblots showing Monarch-1 expression were cropped for space considerations. *D*, HEK293T cells were transfected with NIK in the presence or absence of Monarch-1. The indicated samples were treated with increasing concentrations of MG132 6 h posttransfection. Western blots were probed with anti-NIK. Actin levels were assessed to ensure equivalent protein loading.

(Fig. 5*B*). In contrast, in the absence of Monarch-1 NIK remained stable throughout the 6 h chase period.

To determine which domains of Monarch-1 regulate NIK stability, truncated forms of Monarch-1 were coexpressed with NIK, and NIK levels were determined by Western blot. These experiments revealed that the NBD is required to reduce NIK stability (Fig. 5*C*). Interestingly, although the LRR domain associated with NIK, it had only a subtle effect on NIK stability (Fig. 5*C*, *lane 5*). The LRR domains of the CLR proteins NOD1 and NOD2 sense breakdown products of peptidoglycan to trigger downstream signaling pathways, although there is no evidence that NOD1/2 directly bind to these products (13). A specific ligand for Monarch-1 also has not been identified; however, we predict that in the presence of ligand, the LRR domain would function to regulate Monarch-1 activity. Nevertheless, our results indicate that the NBD is required for reducing NIK stability. The pyrin domain, in contrast, stabilizes NIK and may play an autoinhibitory role in Monarch-1 function (Fig. 5*C*, *lane 2*).

The stability of many cellular proteins is regulated by the proteasome. To determine whether Monarch-1 regulates NIK stability through a proteasome dependent mechanism, cells were transfected with NIK in the presence or absence of Monarch-1 (Fig. 5*D*). Proteasome inhibitor was added at increasing concentrations and NIK levels were determined by Western blot analysis. As expected, coexpression of NIK and Monarch-1 resulted in greatly reduced levels of NIK protein. This reduction was blocked in a dose dependent manner upon treatment of cells with proteasome inhibitor, demonstrating a role for the proteasome in Monarch-1-mediated NIK degradation.

This report reveals a second mechanism whereby Monarch-1 associates with and negatively regulates a signaling molecule in activated monocytes. In a previous report, we demonstrated that Monarch-1 associates with IRAK-1 following TLR stimulation and blocks its hyperphosphorylation (2). Since this correlated with decreased production of proinflammatory cytokines, we were initially surprised to find that immediate early activation of canonical NF-κB occurred normally in THP-Ha-Mon1 cells. However, it has been shown that IRAK-1 can activate downstream signaling pathways in the absence of phosphorylation (14). Therefore, it is likely that Monarch-1 regulates other functions that are associated with IRAK-1 phosphorylation such as the ability to interact with other signaling molecules (15). This may result in Monarch-1-mediated suppression of canonical NF-κB activity at later time points. Indeed, we did observe decreased RelA phosphorylation 60 min after TLR2 stimulation in the presence of Monarch-1.

It is not clear whether the suppression of IRAK-1 phosphorylation and NIK degradation occur through a common pathway. Nevertheless, the results presented here demonstrate that Monarch-1 operates at multiple points to attenuate inflammatory signaling. Given the preponderance of Monarch-1 expression in monocytes, neutrophils and eosinophils, our results suggest Monarch-1 may be critical for controlling inflammatory responses such as those that occur during allergy, asthma and infection.

Disclosures

The authors have no financial conflict of interest.

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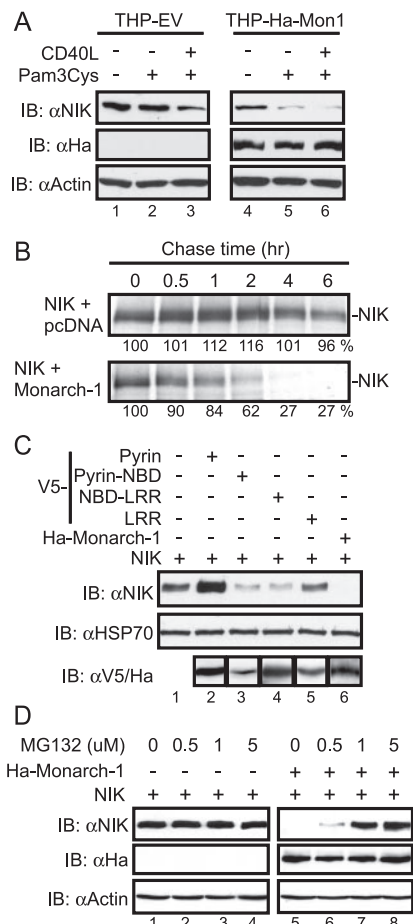
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