Cutting Edge: Monarch-1: A Pyrin/Nucleotide-Binding Domain/Leucine-Rich Repeat Protein That Controls Classical and Nonclassical MHC Class I Genes

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Cutting Edge: Monarch-1: A Pyrin/Nucleotide-Binding Domain/Leucine-Rich Repeat Protein That Controls Classical and Nonclassical MHC Class I Genes


Proteins containing a limited number of N-terminal motifs followed by nucleotide-binding domain and leucine-rich repeat regions are emerging as important regulators for immunity. A search of human genome scaffold databases has identified a large family of known and unknown genes, which we have recently called the CATERPILLER (caspase recruitment domain, transcription enhancer, r(purine)-binding, pyrin, lots of leucine repeats) gene family. This work describes the characterization of a new member, Monarch-1. Monarch-1 has four different splice forms due to the differential splicing of leucine-rich repeat motifs. It is expressed in cells of myeloid-monocytic origin. Affymetrix microarrays and small interfering RNA were used to elucidate the downstream effects of Monarch-1 expression in cells including those of myeloid-monocytic origin. These analyses show that Monarch-1 enhances nonclassical and classical MHC class I expression at the level of the promoter, RNA, and protein expression. The Journal of Immunology, 2003, 170: 5354–5358.

A recently discovered family of genes containing a nucleotide-binding domain (NBD) and a leucine-rich repeat (LRR) motif is critical for apoptosis and immune and inflammatory disorders. In humans, we described at least 22 known and novel family members by searching genomic databases and called these the CATERPILLER (caspase recruitment domain, transcription enhancer, r(purine)-binding, pyrin, lots of leucine repeats) gene family (1). Some of these genes were previously called NBD-LRR/NACHT/PYPAF genes (2–4). Among the known mammalian NBD/LRR proteins, three are genetically linked to immunologic disorders. In humans, we described at least 22 known and novel family members by searching genomic databases and called these the CATERPILLER (caspase recruitment domain, transcription enhancer, r(purine)-binding, pyrin, lots of leucine repeats) gene family (1). Some of these genes were previously called NBD-LRR/NACHT/PYPAF genes (2–4).

Monarch-1 RT-PCR

To clone the N-terminal region the following primers were used: Monarch-1 N-terminal forward (F) 5′-GGGTTACCCTAGAACCAGGCAAGGCAAGGAC-3′ and reverse (R) 5′-CAGCTGTGCTGTTCTGTGTGCTGTG-3′. To clone the suspected C-terminal region and identify LRR splice forms, the following primers were used: Monarch-1 C-terminal forward (F) 5′-CAGAGGACTCATCTGGAAG-3′ and reverse (R) 5′-CTCATAGAGAGTGAGAG-3′. The One-Step RT-PCR kit (Qiagen, Valencia, CA) was used following the manufacturer's protocol. For expression analysis, the primers were Monarch-1 prNBD F 5′-TTGACCCGGATAACAGGAAGGAC-3′ and Monarch-1 prNBD R 5′-ATCTCCCCTGACGTGTAGAAAG-3′.

5′ RACE

5′ RACE was performed using two gene-specific primers following the manufacturer's protocol (Roche, Indianapolis, IN). The gene-specific primers were: SP-1 5′-GGTCTGGTCTCAGAAAGACAGACG-3′ and SP-2 5′-CTGCGACATAGTCCCTGAGTGTTC-3′. The longest clone was chosen as the 5′ start of the Monarch-1 mRNA.

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3 Abbreviations used in this paper: NBD, nucleotide-binding domain; LRR, leucine-rich repeat; CATERPILLER, caspase recruitment domain, transcription enhancer, r(purine)-binding, pyrin, lots of leucine repeats; MHC-II, MHC class II; DET-A-NO. [Z]-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-um-1,2-diolate (DETA-NO), an inducer of nitric oxide, was used at 125 μmol/L (Alexis Biochemicals, San Diego, CA). IFN-γ was used at 1000 U/ml, TGF-β at 1 ng/ml, TNF-α at 20 ng/ml (Peprotech, Rocky Hill, NJ), and PMA (Sigma-Aldrich, St. Louis, MO) at 10 ng/ml.

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Cell lines
HeLa cells were transfected with 1 μg of pcDNA3-hemagglutinin (HA) vector or HA-tagged Monarch-1 via FUGENE (Roche) and selected with 500 μg/ml G418. U937 small interference RNA (siRNA) clones were selected with 500 μg/ml puromycin.

Cell preparation and purification
PBMC were isolated from buffy coats (American Red Cross, Durham, NC) using lymphocyte separation medium (ICN Pharmaceuticals, Costa Mesa, CA). T cells, B cells, monocytes, and CD15+ granulocytes were individually selected by a MACS column (Miltenyi Biotec, Auburn, CA). Monocyte-derived dendritic cells were generated by differentiating PBMCs with GM-CSF and IL-4 for 8 days.

RNA preparation and real-time PCR
Total RNA was isolated using the SV40 Total RNA System (Promega, Madison, WI) with a DNase I digestion step. Real-time PCR was performed with the TaqMan sequence detection system (Applied Biosystems, Foster City, CA). Monarch-1 F 5'-AGAGGACCTGGTAGGAGATC-3', R 5'-CTTCAGACGAGGCTAC-3', probe 5'-CCCGTCCTCTTGCAGGACAC-3', probe 5'-CGCGTGCCGTTCATCTGAC-3'; B 5'-TCACCTGAGGGGTCTGAC-3', probe 5'-ACCAGAAGGGAGGAGGC-3'; HLA-B A 5'-GGGACGGGCCAGACCATGAT-3', R 5'-GGCCCGTCTTCTGCTGAAG-3', probe 5'-CAAGCAACACACACAG-3'; LMP2 F 5'-GCCGAGCGGAGAAGGCTAC-3', probe 5'-CATATTGACAACGCCTCCAGAA-3'; C 5'-ACCTCAACTACATGGTTTAC-3'.

Affymetrix analysis
Total RNA from pcDNA-HA and HA-Monarch-1 HeLa stable clones was prepared using RNeasy Mini columns (Qiagen). Ten micrograms of RNA were reverse-transcribed using Superscript II (Stratagene, La Jolla, CA), labeled using Superscript II (Stratagene, La Jolla, CA), and analyzed on HG U133A chips at the University of North Carolina Genomics Facility (Chapel Hill, NC) according to the Affymetrix technical manual (http://www.affymetrix.com). Sample quality was assessed by Affymetrix analysis of the on-line version of this article contains supplemental material.

Cytometric fluorometric analysis of HLA
Flow cytometry was performed as previously described (13). FITC-conjugated human pan-reactive HLA Ab (Caltag Laboratories, Burlingame, CA) and control FITC mouse IgG2a κ isotype Ab (BD PharMingen, San Diego, CA) were used.

siRNA construction and transfection
Wild-type and mutant human Monarch-1 short hairpin RNAs were stably expressed in the human U937 monocyte cell line by transfection of plasmids containing short hairpin RNA transcription cassettes followed by clonal selection in puromycin as described. The targeted sequence is GTCCATGCTACACAG.

Results and Discussion
Identification of the human Monarch-1 cDNA
Previously, we identified genes for novel NBD/LRR proteins with structural similarities to CIITA via searches of the published Celera and the National Center for Biotechnology Information human genome databases (1). One of the predicted new genes, Monarch-1, was cloned by RT-PCR using primer pairs specific for both the suspected N- and C-terminal regions of the gene. The 5′ end of the longest clone was isolated using RACE-PCR of cDNA from U937 cells. The full-length cDNA is 3731-bp long with a 220-bp 5′ UTR, a 323-bp 3′ UTR and a 3189-bp open reading frame (accession number AY116204, supplemental Fig. 1A). Monarch-1 is located on human chromosome 19q13.4. Comparison with known mRNAs in the database revealed the 3′ one-third of this gene was previously identified as RNO2 (14). During the preparation of this manuscript, the sequence for a new gene PYPAF7, has been published and is identical to isofrom I of Monarch-1 except for an arginine deletion at aa 692 in PYPAF7 (3). Analysis of additional clones led to the isolation of a form missing aa 692 (PYPAF7). The Monarch-1 cDNA contained in 10 exons, encodes a predicted protein of 1063 aa as with a predicted molecular mass of 118 kDa (supplemental Fig. 1B). To investigate whether multiple Monarch-1 splice forms exist, PBMC total RNA was subjected to RT-PCR with primers spanning the end of the NBD through the C-terminal LRR region of Monarch-1 (not shown). At least four splice forms of the Monarch-1 LRR region are evident. Sequence analysis of the four prominent bands shows that these novel splice forms correspond to differential splicing of the LRR (accession numbers AY116205, AY116206, and AY116207) (supplemental Fig. 1, C, E, and G). The full-length Monarch-1 contains 10 exons (isofrom I) while the shorter forms lack exon 9 (isofrom II) (supplemental Fig. 1D), exons 7 and 8, (isofrom III) (supplemental Fig. 1F), and exons 7 through 9 (isofrom IV) (supplemental Fig. 1H), respectively. Analysis of Monarch-1 using RT-PCR with primers specific for the N-terminal region suggests that alternative N-terminal splice forms do not exist (not shown).

Expression of Monarch-1 is predominantly in myeloid-monocytic cells
RT-PCR shows expression in U937 and HL-60 cells but not T/B or nonhemopoietic cell lines (not shown). To assess Monarch-1 expression in PBMC subpopulations RT-PCR was performed, and showed expression in dendritic cells, monocytes, and granulocytes (Fig. 1A). A faint band was detected in the lymphocyte preparation, however this may be due to contamination as these same preparations show a faint band for the myeloid genes, CD14 and CD15. To more definitively compare Monarch-1 expression among the myeloid-monocytic cells, real-time PCR analysis was used (Fig. 1B). High levels of Monarch-1 were detected in granulocytes, with lower expression observed in monocytes. An increase in Monarch-1 expression is observed in monocytes in response to DETA-NO (an activator of nitric oxide) consistent with previous findings of nitric oxide induction of RNO2 mRNA expression (Fig. 1C) (14). In contrast, TNF-α, IFN-γ, or a combination of the two decreased Monarch-1 expression in a time-dependent fashion.

Identification of Monarch-1-regulated genes by DNA microarray
To determine the downstream effects of increased Monarch-1, an Affymetrix DNA array analysis was performed to compare gene profiles in the presence or absence of Monarch-1. Stable
clones expressing Monarch-1 were made in the HeLa cell line because these cells do not express Monarch-1 (Fig. 2A). Two sets of stable-expressing Monarch-1 clones were independently produced on different days by transfection of HeLa cells with either the empty vector control, pcDNA, or with a pcDNA-HA-tagged Monarch-1 expression vector and selected for neomycin resistance. The first experiment resulted in two Monarch-1-containing clones, clone A with lower Monarch-1 expression and clone B with higher expression. The second experiment resulted in one clone, C, with intermediate expression. Analysis of the Monarch-1 expression level in different RNA preparations of these clones relative to total primary human PBMCs indicates that the clones express lower levels of Monarch-1 than PBMCs. Thus changes detected in Monarch-1-expressing lines are likely to be relevant, and not due to the overexpression of Monarch-1. Clones with a higher Monarch-1 level were not obtained perhaps due to toxicity.

DNA microarray analysis was performed for control and Monarch-1-expressing stable clones using Affymetrix chips comprising ~22,000 gene sequences. The most prominent change is a cluster of nine MHC-I-related sequences, including HLA-B (three sequences), HLA-C (two sequences), HLA-F (one sequence), HLA-G (two sequences), and the proteosomal subunit LMP7 required for processing of class I peptides (samples with “x”, Fig. 2B). The appearance of HLA-B, C, and G each multiple times further attests to the validity of these findings. Regulation of MHC-II genes by Monarch-1 was not observed (not shown). To assess whether additional MHC-I genes may be modulated by Monarch-1 but not included due to the stringent cutoff standards used for filtering, we examined the expression patterns of all HLA genes. All classical MHC-I (HLA-A, B, and C) and nonclassical MHC-I genes (HLA-E, F, and G) were up-regulated by Monarch-1.

To quantify the changes, real-time PCR was performed using total RNA isolated from A, B, and C stable clones. The levels of HLA-B, HLA-G, and LMP7 mRNA are enhanced in the Monarch-1 stable clones compared with controls (Fig. 3A). FACS analysis further confirmed up-regulation of MHC-I Ag (Fig. 3B). To discern the involvement of tran-
scriptional or posttranscriptional mechanisms, we transiently cotransfected a Monarch-1 expression plasmid (or a control plasmid) with a luciferase reporter driven by 220 bp of the HLA-B promoter (15) in HeLa cells (Fig. 3C). Monarch-1 enhanced the HLA-B promoter > 25 times. This enhanced activity over that seen for mRNA and protein levels may be due to transient transfection resulting in higher than physiological levels of Monarch-1.

Although the level of expression in the stable clones was less than that in primary blood cells and hence does not represent an overexpression system, the physiologic relevance of HeLa cells could be questioned. PYPAF7/Monarch-1 can interact with apoptotic speck protein (3), which is not expressed in HeLa cells but is expressed in monocytic lines. To address the regulation of MHC genes in a more relevant system, siRNA technology was used to reduce endogenous Monarch-1 expression levels in U937 cells, which express Monarch-1 and apoptotic speck protein (16). A vector containing a siRNA specific for Monarch-1 or a mutant siRNA with two mutated nucleotides was introduced into U937 cells. The bulk culture which should have a mixture of cells containing or lacking Monarch-1-specific siRNA showed a decrease of overall Monarch-1 expression compared with cells with control siRNA (Fig. 4A). Clones were then isolated under selectable conditions. Monarch-1 expression was significantly decreased in Monarch siRNA clones, but not in the controls (Fig. 4B, top panel). The levels of Monarch-1, HLA-B, and HLA-G mRNA were correspondingly decreased in the presence of Monarch-1-specific siRNAs but not siRNA controls (Fig. 4B, middle and bottom panels). These data strongly indicate that Monarch-1 controls both classical and nonclassical MHC-I genes in a physiologically relevant cell type.

This work describes a novel pyrin/NBD/LRR protein, Monarch-1, which is expressed primarily by myeloid-monocytic cells. A prominent downstream effect of Monarch-1 is induction of both classical and nonclassical MHC-I genes and LMP7. IFN-γ and TNF-α, known inducers of class I molecules, decreased Monarch-1 expression. However, no significant alteration of MHC class I HLA-G gene expression was observed in Fig. 1C at these time points (not shown). At later time points,
HLA-G expression was enhanced by these two cytokines (not shown). This indicates that Monarch-1 does not play a major role in the induction of MHC-I by TNF-α and IFN-γ. We suggest that the transcription factors induced by these two cytokines, including NF-κB and STAT-1, provide sufficiently strong signals to induce MHC-I despite the down-regulation of Monarch-1. In addition it suggests that Monarch-1 represents a new pathway for MHC-I induction separate from the TNF-α and IFN-γ pathway.

Induction of both classical and nonclassical molecules by Monarch-1 indicates that it is a novel global inducer of MHC-I. More impressively, the reduction of Monarch-1 by siRNA caused a corresponding and almost equal decrease of both classical and nonclassical MHC-I in the monocytic line, U937. This indicates that although Monarch-1 may not be required for MHC-I expression in all cells because cells without this gene still express MHC-I, it is clearly an important regulator of MHC-I in myelomonocytic cells.

The classical MHC-I molecules, HLA-A/B/C are expressed on almost all nucleated cells while the nonclassical MHC-I, HLA-E, -F and -G have a more diverse distribution. HLA-G is expressed by activated macrophages (17) and classically by trophoblasts (15), HLA-F by B cells (18), and HLA-E is ubiquitous in expression (19). A series of conserved upstream DNA sequences in the classical MHC-I promoter include the AP-1 site (20), the NF-κB site, the IFN regulatory factor (IRF) binding site, and the S-X-Y module shared with MHC-II promoters (15). In contrast, nonclassical MHC gene upstream elements exhibit nucleotide sequence variations, mutations, and deletions in these regions (21). It will be of significant interest to identify the Monarch-1 responsive regulatory DNA. The up-regulation of classical and nonclassical MHC-I genes may have broad immunologic implications for the development of both CTLs and NK cells (22). Future studies in gene deletion mice will provide further insight concerning the physiologic role of Monarch-1 in modulating CTL or NK responses in vivo.

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References
G.

ATTGGTGAGTGGGGCAGGGCAGGAGGGAACTGAAGAGTGAGAAAGCATTATTTCAGCAAAAGGTCTTTCCTCCCTTGCTCACTCCTCCAA

CCACTGGCTCAGCCTCTCCGCCCGCTGCCTGTGAATGATGCAATGGAAGGTGTGCTGGGGTCGCCCTGTGTCCCGTGCATAGGAGCATCT

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H.

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