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# AA28–67 domain within MyD88 suppresses *c-myc* activity and expression to regulate differentiation and function of dendritic cells

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**Abstract** The mechanism by which *c-myc* expression in undifferentiated cells rapidly declines following induction of differentiation is poorly characterized. We demonstrate here that MyD88, which can activate NF-κB and MAPK, also suppresses *c-myc* activity and expression. The aa 28–67 domain, a highly conserved region within MyD88, plays a critical role in the MyD88-mediated inhibition. Indeed, deletion of the aa 28–67 domain (MyD88Δ28–67) or mutation of the highly conserved amino acid residue phenylalanine (aa 36) to aspartic acid (MyD88ΔF36D) significantly promoted *c-myc* activity and expression. Additionally, we found that MyD88Δ28–67-mediated *c-myc* activity and expression could be abrogated using PI3K inhibitor, suggesting that the PI3K/Akt signaling pathway may be involved in MyD88-mediated suppression of *c-myc*. Compared to MyD88-transduced DCs, MyD88Δ28–67- and MyD88ΔF36D-transduced DCs derived from MyD88−/− bone marrow cells had lower antigen-presenting ability. Thus, MyD88 induces the differentiation and maturation of DCs not only by activating NF-κB and MAPK but also via suppressing *c-myc* activity and expression.


## Introduction

The proto-oncogene *c-myc* encodes a helix–loop–helix transcription factor that is involved in a number of crucial cellular processes including cell proliferation, cell growth, differentiation and apoptosis [1–5]. Microarray expression analyses demonstrate that *myc* proteins control the expression of many target genes [4]. Recent studies show that *myc* also regulates chromatin structure in a global fashion [6,7]. Both the activity and expression of *c-myc* are tightly regulated by external signals, such as growth factors and extracellular matrix contacts, as well as by internal clocks, such as cell cycles. Indeed, a wide variety of signals that control promoter activity, RNA polymerase elongation, and mRNA processing can determine when and how much *myc* is synthesized [8,9].

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Myeloid differentiation factor MyD88 is a critical adaptor molecule involved in inducing the differentiation of antigen-presenting cells such as dendritic cells (DCs). MyD88 can integrate signals following activation of an array of Toll-like receptors (TLRs) and the IL-1 receptor/IL1RacP (IL1 receptor accessory protein) resulting in the activation of nuclear factor-kappaB (NF-κB) and mitogen-activated protein kinases (MAPKs) and leading to the up-regulation of proinflammatory cytokine gene expression, differentiation and maturation of antigen-presenting cells [10–14]. Although some TLRs also recruit TRIF (Toll/IL-1R domain-containing adaptor inducing IFN-β), TIRAP (TIR domain-containing adaptor protein) and TRAM (TRIF-related adapter molecule), with the exception of TLR3, most TLRs recruit MyD88 [10,12,13]. MyD88 has a modular structure composed of a death domain (DD) at the N-terminus and a Toll-IL-1 receptor (TIR) domain at the C-terminus, separated by a short linker region [15]. Three highly conserved motifs denoted Box1, Box2 and Box3 in the MyD88 TIR domain have been shown to be important for the signaling function [11,14] and for differentiation and maturation of DCs induced by Toll-like receptor ligand (TLRL). The MyD88 death domain can couple TLR-MyD88 to the activation of downstream targets such as NF-κB, MAPKp38, extracellular regulated kinase (ERK)1/2, and Janus kinases, which are associated with inflammation [16].

DCs are the most effective antigen-presenting cells, and they can be induced from monocytes to immature DCs to mature DCs under certain conditions such as the presence of GM-CSF and various TLR ligands. Since *c-myc* expression levels are high in undifferentiated cells but decline rapidly following induction of differentiation [17], the mechanism by which *c-myc* is regulated by MyD88-dependent TLR pathways is not clear. Herein, we demonstrate that MyD88 can inhibit *c-myc* activity and expression, and that the aa 28–67 domain, a highly conserved region within MyD88, plays a critical role in the MyD88-mediated inhibition.

## Materials and methods

### Dendritic cells and cell lines

Monocyte/macrophage RAW264.7 cells (ATCC, Manassas, VA) were grown in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. MyD88−/− bone marrow cells (BMCs) were kindly provided by Shizuo Akira's Lab (Research Institute for Microbial Disease, Osak University, Osaka, Japan). Murine bone marrow-derived DCs (BMDCs) were prepared as described previously [18]. Briefly, BMCs were collected by removing the femur bones of mice, removing each end and flushing out the bone marrow with RPMI 1640 medium using a syringe. The pooled cells were harvested by centrifugation at 600*g* for 10 min, resuspended in 2 ml ACK buffer for 5 min at room temperature to lyse red blood cells, and then cultured in media containing 1000 U/ml GM-CSF for 7 days.

### Reagents, antibodies and flow cytometry analysis

CpG ODNs, R848, peptidoglycan (PGN), lipopolysaccharide (LPS) from *Escherichia coli* and polyinosinic-polycytidylic acid (polyI:C) were purchased from Invivogen (San Diego, USA) and used to stimulate TLR9, TLR7/8, TLR2, TLR4 and TLR3. The concentrations used in all experiments unless stated otherwise were as follows: 5 μM CpG ODNs, 0.1 μg/ml R848, 5 μg/ml PGN, 1 μg/ml LPS and 10 μg/ml polyI:C.

FITC-conjugated anti-CD11c (N418), anti-mouse CD86 (GL1), anti-mouse CD80 (16-10A1), anti-mouse CD40 (3/23) and PE-labeled anti-mouse CD11b (M1/70), CD4 (L3T4) and CD8α (Ly-2) were purchased from PharMingen. To assess surface marker phenotypes of BMDCs, the cells were collected in ice cold PBS, incubated with the indicated antibodies, washed twice, resuspended in PBS with 1% paraformaldehyde and 1% FCS and kept at 4 °C prior to flow cytometric analysis (FAScan, Becton Dickson). For each analysis, an isotype-matched control mAb was used as a negative control.

### RNAi knock-down in vitro

To generate a MyD88 knockdown in RAW264.7 cells, a MyD88-targeted siRNA structure was designed and generated according to our previous method [18]. After hybridization, the oligonucleotides were inserted into the pSUPER vector at the *Bgl*II and *Hin*dIII sites and then transfected into RAW264.7 cells. A stably transfected cell line was established in the presence of 1000 μg/ml G418. A non-targeting control structure was also included to evaluate non-specific effects of siRNA upon the cells. Silencing of the target molecule was confirmed using RT-PCR and western blotting.

### Preparation of MyD88 domain-negative forms and amino acid residue mutants

Various MyD88 domain-negative forms and amino acid residue mutants were prepared by PCR using appropriate primers listed online in the supplementary Table S1. To generate MyD88 domain-negative forms, four primers, including two oligonucleotides containing two-sided reverse complementary sequences on the indicated domain and 5′ and 3′ end primers, 5′-atgcccctcaacgtgaacttcacc-3′ and 5′-ttatgcaccagagtttcgaagc-3′, were added to the PCR mixture. To generate mutant amino acid residues, four primers including 5′ and 3′ end primers, and two oligonucleotides containing two reverse complementary sequences with mutant nucleotides were added to the PCR mixture. The deletions and mutants were confirmed by sequence assay. The fragments were cloned into pcDNA3.1/V5-His Topo TA-expressing vector according to the protocol (Invitrogen).

### Preparation of lentiviruses containing MyD88, MyD88 domain-negative forms, MyD88 with mutant residues and c-myc

The PLenti6/V5/D-Topo kit was used to clone MyD88, MyD88 domain-negative forms, MyD88 with mutant residues and *c-myc* according to the recommended protocol (Invitrogen). Lentiviruses were generated by co-transfecting 293 T cells with packaging vectors (CMVΔ8.9 and VSVg) based on the manufacturer's instructions (Invitrogen). Forward primer