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Sulforaphane inhibits the engagement of LPS with TLR4/MD2 complex by preferential binding to Cys133 in MD2

Jung Eun Koo [a], Zee-Yong Park [b], Nam Doo Kim [c], Joo Young Lee [a,*]

[a] College of Pharmacy, The Catholic University of Korea, Bucheon 420-743, Republic of Korea
[b] School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 500-712, Republic of Korea
[c] Daegu-Gyeongbuk Medical Innovation Foundation, New Drug Development Center, Daegu 706-010, Republic of Korea

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ABSTRACT

Toll-like receptors (TLRs) are key pattern-recognition receptors that recognize invading pathogens and non-microbial endogenous molecules to induce innate and adaptive immune responses. Since activation of TLRs is deeply implicated in the pathological progress of autoimmune diseases, sepsis, metabolic diseases, and cancer, modulation of TLR activity is considered one of the most important therapeutic approaches. Lipopolysaccharide (LPS), an endotoxin of gram-negative bacteria, is a well-known agonist for TLR4 triggering inflammation and septic shock. LPS interacts with TLR4 through binding to a hydrophobic pocket in myeloid differentiation 2 (MD2), a co-receptor of TLR4. In this study, we showed that sulforaphane (SFN) interfered with the binding of LPS to MD2 as determined by *in vitro* binding assay and co-immunoprecipitation of MD2 and LPS in a cell system. The inhibitory effect of SFN on the interaction of LPS and MD2 was reversed by thiol supplementation with *N*-acetyl-L-cysteine or dithiothreitol showing that the inhibitory effect of SFN is dependent on its thiol-modifying activity. Indeed, micro LC-MS/MS analysis showed that SFN preferentially formed adducts with Cys133 in the hydrophobic pocket of MD2, but not with Cys95 and Cys105. Molecular modeling showed that SFN bound to Cys133 blocks the engagement of LPS and lipid IVa to hydrophobic pocket of MD2. Our results demonstrate that SFN interrupts LPS engagement to TLR4/MD2 complex by direct binding to Cys133 in MD2. Our data suggest a novel mechanism for the anti-inflammatory activity of SFN, and provide a novel target for the regulation of TLR4-mediated inflammatory and immune responses by phytochemicals.

1. Introduction

Toll-like receptors (TLRs) recognize specific patterns derived from invading microorganisms or damaged cells/tissues and are critical in provoking innate and adaptive immune responses. Among 13 members of the mammalian TLR family, TLR4 is responsible for the recognition of lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria that is a major inducer of sepsis. When expressed on the cellular membrane, TLR4 exists as a complex with a co-receptor, myeloid differentiation 2 (MD2) [1]. MD2 is an essential component for LPS recognition by TLR4/MD2 complex and has a hydrophobic pocket that interacts with LPS [2,3]. The five carbon chains of lipids in the lipid A component of *Escherichia coli* LPS are completely buried inside the hydrophobic pocket in MD2 whereas the sixth chain is partially exposed to the MD2 surface that forms the core hydrophobic interface for interaction with TLR4. Binding of LPS to the TLR4/MD2 complex triggers the formation of homodimers composed of two TLR4/MD2/LPS complexes [3]. Upon LPS engagement, TLR4/MD2 recruits Toll/IL-1 receptor (TIR) domain-containing adaptors such as myeloid differentiation primary response gene 88 (MyD88) and TIR-containing adaptor inducing IFNβ (TRIF) [4]. MyD88 is the first adaptor to be recruited to the TLR4/MD2 complex in response to LPS stimulation. Subsequently, TLR4/MD2 is internalized and trafficked to the endosome where it encounters TRIF. Activation of MyD88-dependent signaling pathway leads to the activation of nuclear factor kappa B (NFκB) and mitogen-activated protein kinases (MAPKs). In particular, TRIF promotes the activation of interferon regulatory factor 3 (IRF3) leading to the expression of type I interferons (IFNs) [4]. Both pathways culminate in the production of immune and inflammatory cytokines and pro-inflammatory enzymes. Since it is well established that deregulation of TLR4 activity is closely implicated in the development and aggravation of various chronic diseases, pharmacological regulation of TLR4 activation would be a beneficial strategy for preventing or treating inflammatory diseases including sepsis [5,6].

Sulforaphane [SFN, 1-isothiocyanato-4-(methylsulfinyl)-butane] is an organosulfur compound obtained from cruciferous vegetables such as broccoli or cabbages, that exhibits anti-inflammatory

* Corresponding author. Fax: +82 2 2164 4059.
E-mail address: joolee@catholic.ac.kr (J.Y. Lee).

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and anti-cancer properties. SFN suppresses LPS-induced inflammatory responses and modulates TLR4 activation. SFN attenuates LPS-mediated induction of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and tumor necrosis factor-α (TNF-α) in macrophage cell lines [7,8]. Furthermore, oral administration of SFN reduces the increased levels of inflammatory cytokines in blood and decreases dermal inflammation and edema in mice challenged with LPS [7]. To date, several cellular targets of SFN that function in the modulation of TLR4 signaling have been investigated. NFκB translocation and IκBα degradation are suppressed by SFN in LPS-stimulated endothelial cells [9]. Suppression of LPS-induced expression of inflammatory genes such as TNF-α, IL-1β, COX-2, and iNOS by SFN is dependent on Nrf2 [10]. In addition, direct modification of cysteine residues in TLR4 has been reported to be one of the mechanisms involved in its inhibition by SFN [7].

Since binding of LPS to MD2 is the initial step leading to TLR4 activation, MD2 has been suggested as a regulatory target for the prevention of TLR4 activation by LPS. However, it has not been elucidated if MD2 would be a regulatory target of SFN. Our results demonstrate that SFN inhibits the interaction of LPS with MD2 in macrophages via direct binding to a specific amino acid, Cys133, in MD2. Our findings validate MD2 as a target of SFN in its anti-inflammatory effect on TLR4 activation and provide critical information on the mechanism by which anti-inflammatory phytochemicals can suppress TLR4 activation.

1.1. Materials and methods

1.1.1. Animals

Animal care and the study protocols were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Catholic University of Korea (permission # 2012-5-001). Mice were purchased from Orient Bio (Seoul, Korea) and acclimated under specific pathogen-free conditions in the animal facility for at least a week before the experiments. The mice were housed in a room controlled for temperature (23 ± 3 °C) and relative humidity (40–60%).

1.1.2. Reagents

Sulforaphane (SFN) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Biotinylated LPS was purchased from Invivogen (San Diego, CA, USA). Alexa Fluor 594® conjugated with LPS was obtained from Molecular Probes Inc. (Eugene, OR, USA). All other reagents were purchased from Sigma–Aldrich unless otherwise described.

1.1.3. Cell culture

Bone marrow cells were isolated from C57BL/6 mice and differentiated to macrophages as described previously [7]. Bone marrow cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal bovine serum (Life Technologies, Grand Island, NY, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM Hepes buffer, 20% L929 cell-conditioned medium, 100 units/ml penicillin, and 100 μg/ml streptomycin for 6 days, and adherent cells were used as macrophages. Ba/F3 cells, an IL-3-dependent murine pro-B cell line expressing TLR4 and Fag-MD2, were cultured in RPMI 1640 medium containing murine IL-3, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a 5% CO_2/air environment.

1.1.4. In vitro assay for LPS binding to MD2

LPS binding assay was performed as described previously [11]. Briefly, a 96-well microplate was coated with polyclonal anti-human MD2 antibody (Abnova, Taipei, Taiwan) in 50 mM Na_2CO_3 in buffer (pH 9.6). Recombinant human MD2 (0.1 μM; R&D systems) in 10 mM Tris–HCl buffer (pH 7.5) was added to the precoated wells. After washing with phosphate-buffered saline (PBS), biotinylated LPS (50 ng/ml) was added and incubated for 30 min at room temperature followed by addition of streptavidin conjugated to horseradish peroxidase and further incubation for 1.5 h at 37 °C. The activity of horseradish peroxidase was determined using Ez-WayTM TMB substrate kit (Koma Biotech, Seoul, Korea). The optical density of each well was measured at 450 nm.

1.1.5. Immunoprecipitation and immunoblotting

To determine LPS binding to MD2, biotinylated LPS was incubated with Ba/F3 cells expressing TLR4 and Flag-MD2. Protein extracts were immunoprecipitated with anti-flag antibody (Sigma–Aldrich) and protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C on a rocker. Immune complexes were washed three times and solubilized with Laemmli sample buffer. The solubilized proteins were resolved on SDS–PAGE and electrotransferred to polyvinylidene difluoride membranes. The membranes were probed with primary antibody as indicated, and then with secondary antibody conjugated to horseradish peroxidase. Streptavidin conjugated to horseradish peroxidase (Zymed, Carlsbad, CA, USA) was used to detect biotin-LPS. Reactive bands were visualized using the ECL system (Amersham Bioscience, Piscataway, NJ, USA).

1.1.6. Immunostaining and confocal imaging

Bone marrow-derived primary macrophages were grown on glass cover slips (18 mm diameter; Marienfeld Laboratory Glassware, Lauda-Konigshofen, Germany). After 1 h pre-treatment with SFN, the cells were incubated with Alexa Fluor 594® conjugated with LPS (Molecular Probes Inc.). The cells were fixed with paraformaldehyde, washed three times with PBS, and blocked with 1% bovine serum albumin for 50 min. The coverslips were incubated with anti-MD2 antibody in blocking buffer overnight at 4 °C, followed by incubation with FITC-conjugated anti-rabbit IgG secondary antibody (Sigma) and mounting with anti-fade solution (Molecular Probes Inc.). The slides were examined with an LSM710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with 40× objective. Images were obtained with ZEN2011 software (Carl Zeiss).

1.1.7. In-solution digestion and micro liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Purified mouse MD2 was obtained from Dr. J.O. Lee (KAIST, Korea) [3] and 1 μg of protein was incubated with SFN (20 μM) for 1 h at 37 °C. In-solution digestion and micro LC-MS/MS analysis were carried out as described in the previous study [7].

1.1.8. Molecular modeling of covalent binding of SFN to cysteine 133 in MD2

For prediction of a covalent binding model of SFN with MD2, we used the crystal structure of human TLR4-human MD2-LPS complex (PDB code 3FXI) and human MD2 in complex with lipid IVa (PDB code 2E59). The protein structure was minimized using the Protein Preparation Wizard in the Schrodinger software graphical user interface Maestro (version 9.3). SFN was docked into a minimized crystal structure of Cys133 in the human MD-2 crystal structure using the covalent docking routine in Prime (Prime, version 3.1, Schrödinger, LLC, New York, NY, 2012). The covalent docking was performed using default settings, keeping all residues fixed except for the connecting Cys133. The molecular graphics for the refined covalent docking model of the SFN were generated using PyMol package (http://www.pymol.org).

1.1.9. Statistical analysis

Data are expressed as mean ± SEM. Comparisons of data between groups were analyzed by one-way ANOVA and Tukey's multiple range test. Values of $p<0.05$ were considered significant.

2. Results

To investigate whether sulforaphane (SFN) inhibited the interaction of LPS with MD2, we performed an *in vitro* assay to determine the level of LPS bound to recombinant MD2. SFN reduced the association of MD2 with biotinylated LPS (Fig. 1A), showing the inhibitory effect of SFN on the interaction of LPS with MD2.

To confirm whether this inhibitory effect of SFN was observed in cellular systems, Ba/F3 cells expressing TLR4 and Flag-tagged MD2 were treated with biotinylated LPS in the absence or presence of SFN and the amount of co-immunoprecipitated LPS and MD2 was determined by immunoblot analysis. SFN attenuated the association of LPS with MD2 in a dose-dependent manner (Fig. 1B),

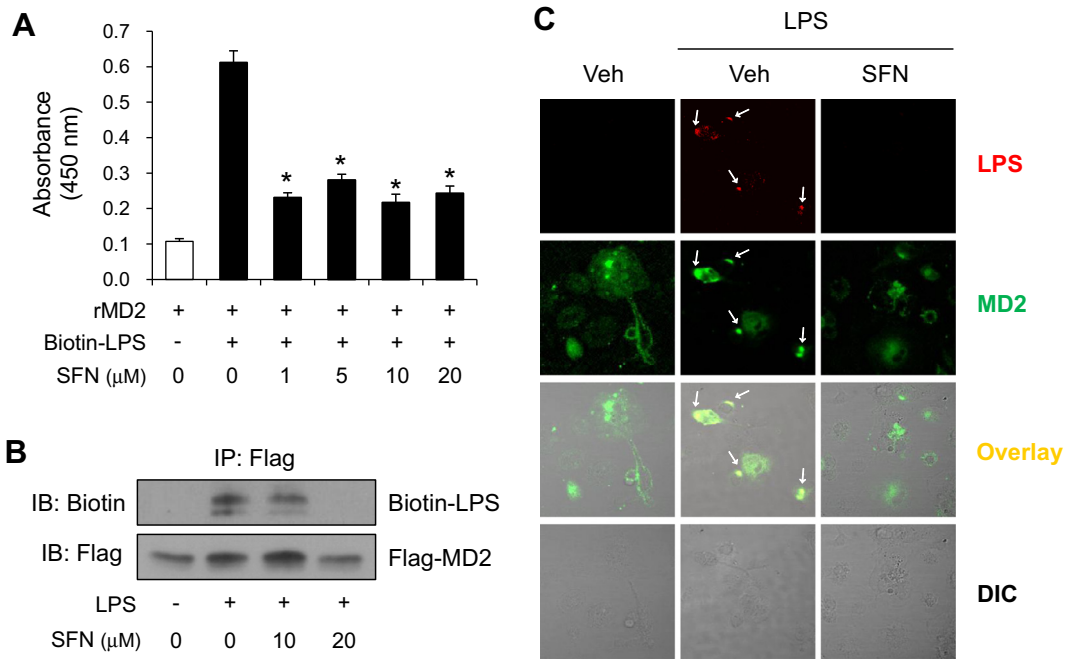

Fig. 1. Sulforaphane inhibits LPS binding to MD2. (A) *In vitro* binding assay using recombinant MD2 (rMD2) and biotinylated LPS to analyze LPS binding to rMD2. rMD2 was pre-incubated with SFN for 1 h at 37 °C and added to wells coated with anti-MD2 antibody, followed by addition of biotinylated LPS (50 ng/ml). Values are means ± SEM ($n = 3$). $^{*}p<0.05$. (B) Ba/F3 cells expressing TLR4 and Flag-tagged MD2 were pre-treated with SFN for 1 h and then stimulated with biotinylated LPS (1 μg/group) for 20 min. Cell lysates were immunoprecipitated with anti-Flag antibody and immunoblotted to detect biotinylated LPS and MD2. (C) Bone marrow-derived primary macrophages were pre-treated with SFN (20 μM) for 1 h and then treated with Alexa Fluor 594 conjugated LPS (1.5 μg/group) for 30 min. Cells were stained with anti-MD2 antibody and FITC-conjugated anti-rabbit IgG secondary antibody. A set of representative pictures from two independent experiments is presented.

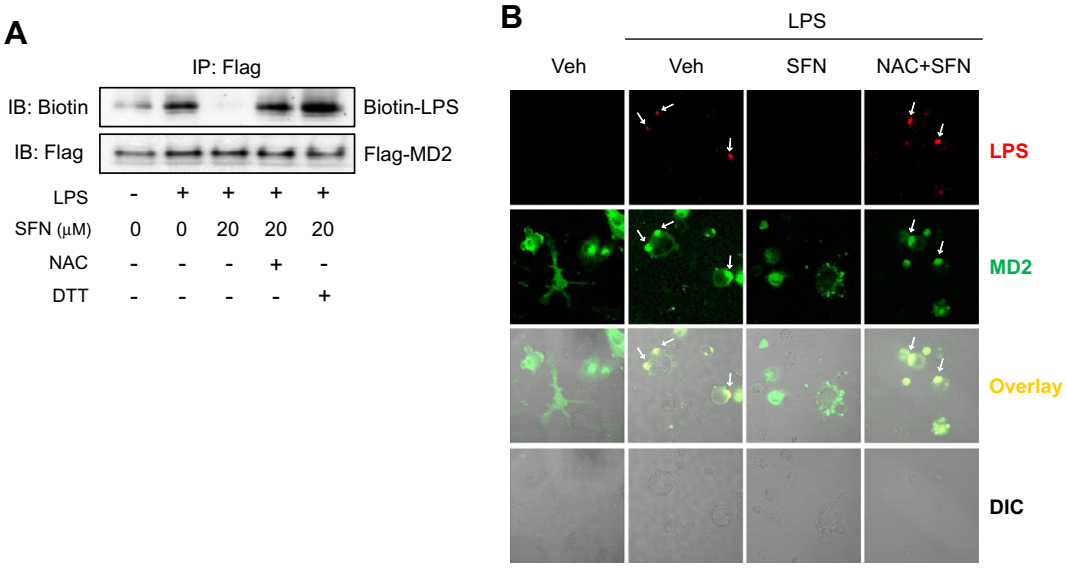

Fig. 2. Thiol supplementation reverses the inhibitory effect of sulforaphane on the interaction of LPS and MD2. (A) Ba/F3 cells expressing TLR4 and Flag-tagged MD2 were pre-treated with sulforaphane (SFN) in the absence or presence of *N*-acetyl-L-cysteine (NAC; 2 mM) or dithiothreitol (DTT; 300 μM) for 1 h and then stimulated with biotinylated LPS (1 μg/group) for 20 min. Cell lysates were immunoprecipitated with anti-Flag antibody and immunoblotted to detect biotinylated LPS and MD2. (B) Bone marrow-derived primary macrophages were pre-treated with SFN (20 μM) for 1 h in the absence or presence of NAC (2 mM) and then treated with Alexa Fluor 594® conjugated LPS (1.5 μg/group) for 30 min. Cells were stained with anti-MD2 antibody and FITC-conjugated anti-rabbit IgG secondary antibody. A set of representative pictures from two independent experiments is presented.

although the inhibitory potency of SFN was lower in the cellular system, possibly due to protein binding and metabolism of SFN in the media.

To visualize the localization of LPS and MD2, bone marrow-derived primary macrophages (BMDMs) were treated with fluorescent LPS conjugated with Alexa Fluor 594® and analyzed by confocal microscopy. SFN significantly blocked the co-localization of fluorescent LPS with MD2 in BMDMs (Fig. 1C). These results indicate that interaction of LPS with MD2 was interrupted by SFN.

Since SFN has the ability to react with sulfhydryl moieties in proteins, we investigated whether the inhibitory effect of SFN on LPS binding to MD2 was related to its reactivity with sulfhydryl groups. Association of LPS with MD2 was determined by immunoprecipitation and immunoblotting study using Ba/F3 cells stably expressing TLR4 and Flag-tagged MD2 after treatment with SFN in the absence or presence of the thiol donors *N*-acetyl-L-cysteine (NAC) or dithiothreitol (DTT). The suppressive effect of SFN on LPS binding to MD2 was reversed by supplementation with NAC and DTT (Fig. 2A). Furthermore, NAC blocked the SFN-mediated inhibition of co-localization of LPS with MD2 (Fig. 2B). These findings show that the inhibitory effect of SFN on the binding of LPS to MD2 was mediated through its reactivity with sulfhydryl groups.

To investigate whether SFN directly reacted with cysteine residues in MD2, we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis after incubation of recombinant MD2 with SFN. LC-MS/MS analysis showed that SFN covalently bound to Cys133, but not to Cys95 and Cys105, in MD2 (Fig. 3).

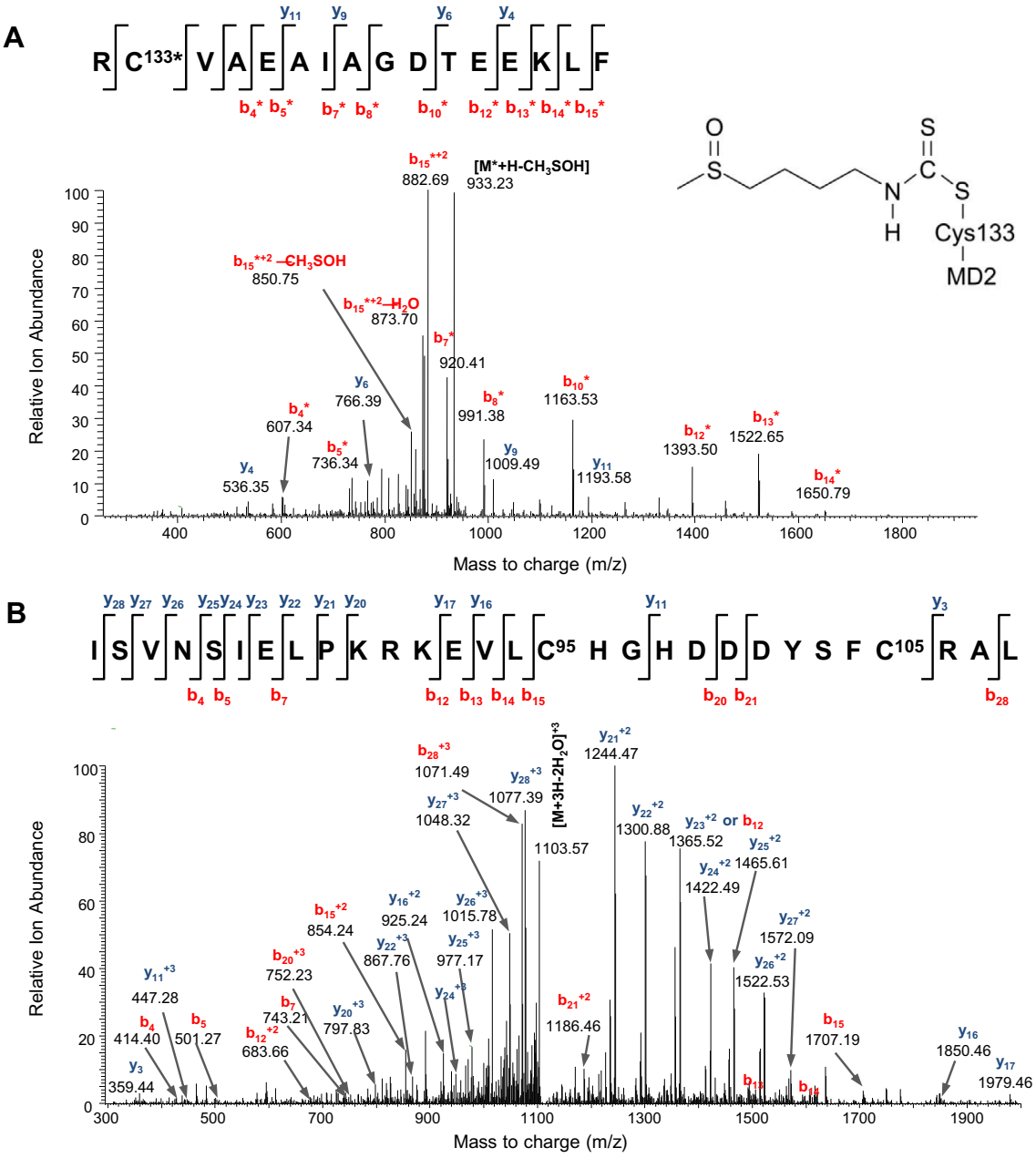

Fig. 3. Sulforaphane preferentially binds to Cys133 in MD2. Recombinant MD2 was incubated with sulforaphane (SFN; 20 μM) for 1 h at 37 °C and in-solution digestion and micro liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed. A MS/MS spectrum of the SFN-Cys133 adduct was acquired using a LTQ linear ion trap mass spectrometer. MS/MS spectra of mouse MD2 sequences (A, Arg132-Phe147, RC[133]VAEAIAGDTEEKLF; B, Ile80-Leu108, ISVNSIELPKRKEVLC[95]HGHDDDYSFC[105]RAL) were selected and the fragment ions were assigned as b (red) or y (blue) series ions. Mass accuracy of ±0.5 Da was used in the assignments. * denotes fragment ions with one SFN. The proposed structure of the SFN-Cys133 MD2 adduct is presented in A. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Cys133 in MD2 is a free cysteine residue whereas Cys95 and Cys105 participate in the formation of intramolecular disulfide bonds. Therefore, it seems that free cysteine is primarily accessible to SFN whereas cysteines in disulfide bonds are less favorable for SFN interaction. The results demonstrate that SFN preferentially modifies Cys133 in MD2 via direct covalent interaction.

Next, we performed docking simulation analysis to generate a molecular model explaining how covalent binding of SFN to Cys133 interferes with LPS engagement to MD2. The SFN-Cys133 adduct inside the hydrophobic pocket of MD2 is in close proximity with Ile46, Phe76, Phe147, Phe151, Val135, and Leu149 (Fig. 4A). A schematic diagram of SFN-MD2 interactions based on the proposed covalent docking model is presented in Fig. 4B. The proposed covalent binding model shows that SFN bound to Cys133 overlaps the position of the R3″ lipid chain of LPS and XA2 lipid chain of lipid IVa, thereby preventing LPS and lipid IVa from engaging with the hydrophobic pocket of MD2 (Fig. 4C and D). This suggests that the binding of SFN to Cys133 forms a bulky hindrance that blocks the interaction of LPS with MD2.

3. Discussion

MD2 is a crucial factor in the reaction of TLR4 with its ligand. MD2-deficient mice do not respond to *E. coli* LPS and are therefore resistant to endotoxic shock [12]. MD2-null mice are also insensitive to pulmonary inflammation following nasal instillation of LPS [13]. MD2 contains 143 amino acid residues that adopt a β cup fold with two antiparallel β sheets and create a hydrophobic pocket. The MD2 pocket is narrow and deep, with hydrophobic residues on the internal surface and positively charged residues located near the opening rim of the cavity [1,14]. A free cysteine residue at position 133 inside the binding pocket of MD2 has been shown to play a crucial role in modulating the interaction between MD2 and LPS [15]. The results of our LS-MS/MS analysis showed that SFN directly binds to Cys133 in MD2. Recently, it was reported that caffeic acid phenethyl ester, an active phenolic compound found in natural products such as propolis, prevents the engagement of LPS to TLR4/MD2 complex through the formation of an adduct with

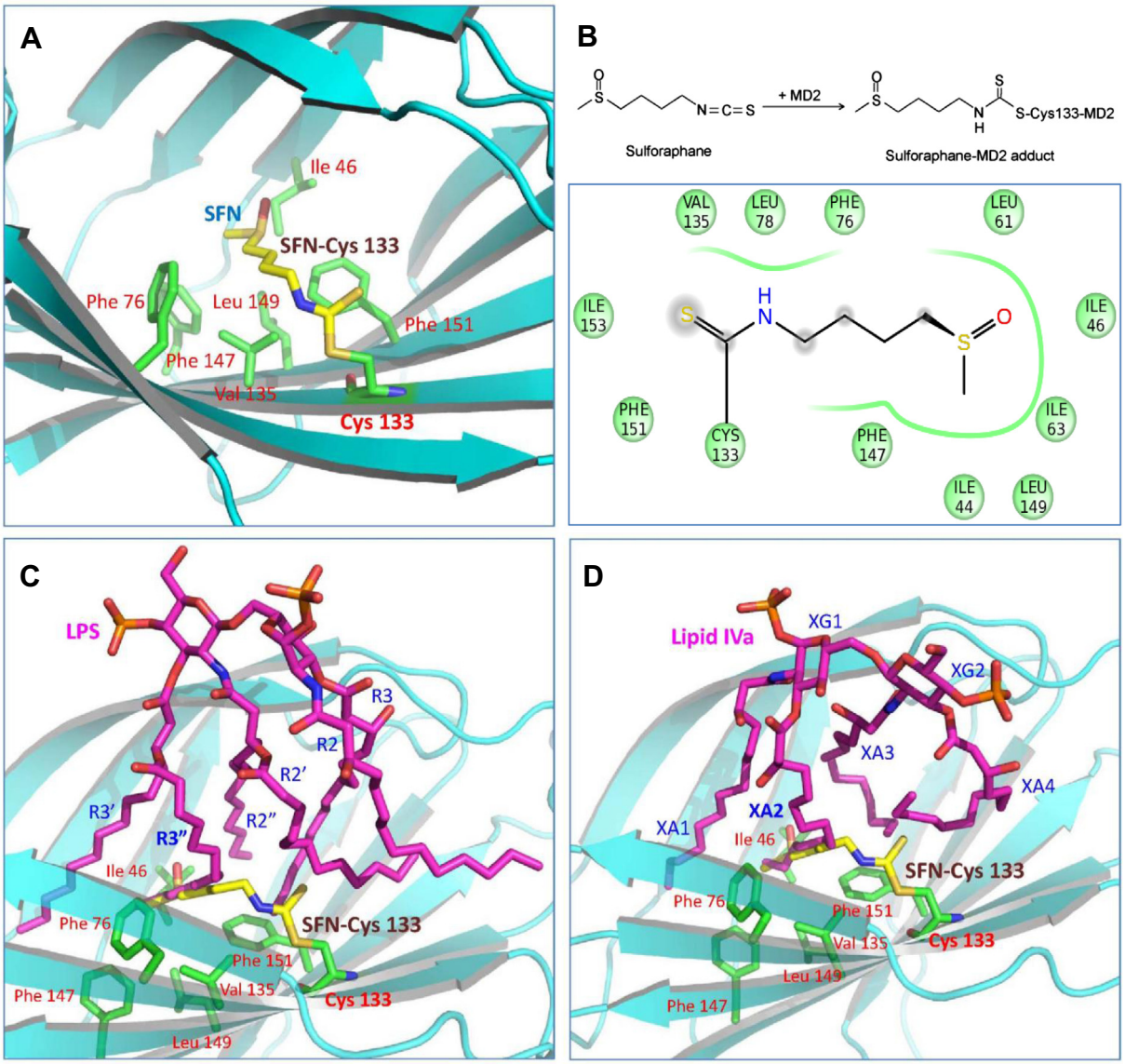

Fig. 4. Molecular model of sulforaphane binding to Cys133 in MD2. (A) Covalent docking of sulforaphane (SFN) to the crystal structure of human MD2 was modeled with Prime and PyMol package. SFN is represented as a yellow branch connected to Cys133. (B) Schematic diagram of SFN–MD2 interaction based on the proposed covalent docking model. (C, D) Molecular modeling of the SFN-Cys133 adduct superimposed onto the LPS-binding site (C) and LPS antagonist lipid IVa-binding site (D) of MD2. Fatty acids in LPS and lipid IVa are represented by the pink color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Cys133 in a hydrophobic pocket of MD2, thereby impairing downstream signal activation [11]. It should be noted that not all thiol-reactive compounds can interact with Cys133 in MD2. Curcumin is well known for its anti-inflammatory properties against LPS treatment [16]; however, it does not make the Michael addition-type reaction with the Cys133 residue even though it is buried in the deep interior of the MD2 pocket through a hydrogen bond with the Glu122 residue [16]. In addition to Cys133, the Cys95 and Cys105 residues of MD2 are critical for conferring LPS responsiveness on TLR4 [17,18]. However, SFN did not interact with Cys95 and Cys105, possibly because Cys95 and Cys105 form disulfide bonds and are therefore less accessible than free cysteine Cys133. Although it was previously suggested that SFN may bind to certain cysteine residues of NFκB subunits due to its thiol-reactivity [8], our MS/MS studies of MD2 are the first demonstration of the covalent interaction of SFN with cysteine residues of a protein.

Collectively, our results demonstrate that SFN interferes with LPS engagement to MD2 via direct modification of Cys133 in MD2. Furthermore, these findings provide information that might be useful in the development of MD2-targeting TLR4 inhibitors.

Acknowledgments

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