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Alternatively spliced myeloid differentiation protein-2 (MD-2s) protein inhibits TLR4-mediated lung inflammation

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Abstract

We previously identified a novel alternatively spliced isoform of human myeloid differentiation protein-2 (MD-2s) that competitively inhibits binding of MD-2 to TLR4 *in vitro*. Here we investigated the protective role of MD-2s in LPS-induced acute lung injury by delivering intracheally (i.t.) an adenovirus construct that expressed MD-2s (Ad-MD-2s). After adenovirus-mediated gene transfer, MD-2s was strongly expressed in lung epithelial cells and readily detected in bronchoalveolar lavage fluid (BALF). Compared to Ad-EV control mice, Ad-MD-2s delivery resulted in significantly less LPS-induced inflammation in the lungs, including less protein leakage, cell recruitment, and expression of proinflammatory cytokines and chemokines, such as IL-6, KC, and MIP-2. BALF from Ad-MD-2s mice transferred into lungs of naive mice before i.t. LPS challenge diminished pro-inflammatory cytokine levels. As house dust mite (HDM) sensitization is dependent on TLR4 and HDM Der p 2, a structural homolog of MD-2, we also investigated the effect of MD-2s on house dust mite (HDM)-induced allergic airway inflammation. Ad-MD-2s given before HDM sensitization significantly inhibited subsequent allergic airway inflammation after HDM challenge, including reductions in eosinophils, goblet cell hyperplasia, and IL-5 levels. Our study indicates that the alternatively spliced short isoform of human MD-2 could be a potential therapeutic candidate to treat human diseases induced or exacerbated by TLR4 signaling, such as Gram-negative bacterial endotoxin-induced lung injury and house dust mite-triggered allergic lung inflammation.

Keywords

MD-2; TLR-4; Der p 2; HDM; LPS; acute lung injury; allergic lung inflammation; innate immunity

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Introduction

Lipopolysaccharide (LPS), an essential component of the outer membrane of Gram-negative bacteria, induces a powerful inflammatory response that can lead to septic shock and death (1). LPS signals through toll-like receptor 4 (TLR4) by binding to its co-receptor, myeloid differentiation protein-2 (MD-2, LY96), and other accessory molecules, including LPS-binding protein (LBP) and CD14. Among these molecules, LBP and CD14 are important for enhancing LPS binding to MD-2 but are not essential for triggering TLR4 signaling (2, 3). MD-2 is an essential component of the signaling receptor complex that recognizes and initiates the innate immune response to bacterial LPS (4). MD-2 is a secreted glycoprotein that binds both LPS and the ectodomain of TLR4, forming the TLR4/MD-2/LPS complex (5, 6). Loss-of-function approaches (knockdown and mutation) demonstrated that MD-2 is indispensable for LPS-induced immune cell activation (7–9) in both mouse and human models (10, 11). Upon LPS binding, a receptor multimer composed of two copies of the TLR4/MD-2/LPS complex is formed (12, 13), which triggers a downstream signaling cascade, culminating in the activation of proinflammatory transcription factors such as NF-κB and the interferon regulatory factors (IRFs). We have shown that MD-2 interacts with Lyn kinase and is tyrosine phosphorylated following LPS-induced activation of the TLR4 signaling pathway. We have demonstrated that this posttranslational modification is required for TLR4/MD-2/LPS signaling (14).

Activation of the innate immune response is a critical step in the response to infection. LPS plays a major role in sepsis and septic shock pathogenesis (15, 16) with 57.2% of patients having either intermediate or high endotoxin levels on the first day of admission (17). As an acutely overactive innate immune responses can contribute to the pathogenesis of many inflammatory diseases (18), it is critical that innate immunity be tightly controlled, activated when necessary and kept inactive when not. In addition to a complex regulatory mechanism that controls innate immune activation, there are also several negative regulatory pathways of the innate immune system that limit the initial response and the potential damage due to uncontrolled overactive chronic inflammation (19–24). Several negative regulatory pathways have been reported, including proteins that bind an inactivate TLR signaling (19–24); microRNAs that regulate expression of TLR signaling genes (25); and of innate immune genes such as IRAK2, TLR4 and MyD88 that inhibit TLR signaling Another method of down regulating TLR4 signaling is to produce an inhibitory isoform by alternatively splicing specific genes encoding essential signaling components such as IRAK2, TLR3, MD2, and MyD88 (26–30). For instance, an alternatively spliced short form of MyD88 acts as a negative regulator of IL-1R/TLR/MyD88-triggered signals, leading to transcriptionally controlled negative regulation of innate immune responses (31, 32).

We recently identified a novel alternatively spliced isoform of human MD-2 - MD-2s - that lacks the region encoded by exon 2 of the *MD2* (*LY96)* gene (REF) gene. This human isoform differs from one found in mice (26, 27). We determined that MD-2s is upregulated by IFN-γ, IL-6, and TLR4 signaling, is a negative regulator of LPS-mediated TLR4 signaling, and competitively inhibits binding of full length MD-2 to TLR4 (33). We hypothesized that MD-2s would have beneficial effects *in vivo* in mitigating TLR4-mediated lung injury and inflammation models.

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To investigate the *in vivo* effects of MD-2s on TLR4-mediated lung inflammation, we used two mouse models dependent on MD2/TLR4 signaling: LPS-induced acute lung injury (ALI) and house dust mite (HDM)-induced allergic airway inflammation. In both models, we found that over-expression of MD-2s led to marked reduction in markers of tissue injury and inflammation. These data suggest that MD-2s may serve as an effective inhibitor and a potential therapeutic candidate to treat human diseases induced or exacerbated by TLR4 signaling.

Methods

Animals

C57BL/6 mice were used for all the experiments in this study. For all ALI experiments, C57BL/6 mice were bred in house (originally from Jackson Labs,Bar Harbor, ME). For all HDM studies, C57BL/6 mice were purchased from Jackson Labs. All animal experiments were performed according to the guidelines and approved protocols of the Institutional Animal Care and Use Committee, Cedars-Sinai Medical Center (CSMC). Laboratory animals are maintained in accordance with the applicable portions of the Animal Welfare Act and the guidelines prescribed in the DHHS publication, Guide for the Care and Use of Laboratory Animals. CSMC is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International) and abides by all applicable laws governing the use of laboratory animals.

Cell culture

The murine macrophage/monocyte cell line RAW264.7 (BH-AC71; ATCC, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% FBS (Hyclone, USA), 100 U/mL penicillin, and 100 U/mL streptomycin. Primary SAEC (normal human small airway epithelial cells) as well as all the basal media and growth supplements were obtained from Lonza (Walkersville, MD). Cells were cultivated according to the instructions of the manufacturer on plastic dishes or flasks (BD Bioscience, Heidelberg, Germany). Passage number was kept to less than four passages from original stocks. SAEC cells were maintained in small airway epithelial cell basal medium (SAGM) supplemented with 52 μg/ml bovine pituitary extract, 0.5 ng/ml human recombinant EGF, 0.5 μg/ml hydrocortisone, 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid (RA), 6.5 ng/ml triiodothyronine, 50 μg/ml Gentamicin/Amphotericin-B (GA-1000) and 50 μg/ml fatty acid-free bovine serum albumin (BSA).

Preparation of adenoviral vector expressing MD-2s-Myc

The human MD-2s gene was cloned into the pEntry CMV-IRES-mCherry shuttle vector using AgeI-NotI sites. After sequence verification, the pEntry-CMV-MD-2s-IRES-mCherry plasmid was recombined with pDest-Ad plasmid (Life Technologies). The recombinant pAd-MD-2s-mCherry plasmid was selected in DH10B *E. coli* using Ampicillin. For generating recombinant Ad-MD-2s-mCherry virus, the pAd-MD-2s plasmid DNA was transfected into HEK-293 cells. At 10–14 days post-transfection viral plaques appeared and the infected cell lysates were harvested. The Ad-MD-2s-mCherry virus underwent two additional rounds of amplification. The amplified virus was subjected to two rounds of CsCl

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gradient ultra centrifugation and dialysis. The viral vector titer was measured in HEK-293 cells by serial dilution. A control Ad-mCherry virus was generated as described above.

Mouse Model of LPS induced ALI

Male, 6 to 8 weeks old (C57BL/6J mice (n=8 in each group) received a first-generation replication-deficient adenovirus serotype 5 containing human short MD-2s cDNA (Ad-MD-2s) (1×10^9 viral particles in 50 µl PBS) or an empty vector lacking a transgene (Ad-EV) intratracheally (i.t.) 2 days before i.t. instillation of LPS (*Escherichia coli* serotype O:111; Alpha Chemical and Plastics Co., Hollister, MO) at 0.25 mg/kg. The mice were sacrificed 6 h later to evaluate lung injury. For BALF transfer experiments, 12 mice in each group treated with 1×10^9 pfu/mice of Ad-EV or Ad-MD-2s for 48 h and BALF collected, pooled and concentrated by using centrifugal filter devices (Amicon Ultra-15, Merck Millipore, Ireland) in sterile condition. A total of 100 µl of BALF was administrated i.t. into six naïve mice in each group and 1 h later mice were treated with 0.25 mg/kg of LPS i.t. and sacrificed 6 h later.

Mouse model of HDM-induced allergic airway inflammation

Female, 6 to 8 weeks old C57BL/6J mice (n = 10 in each group) received Ad-MD-2s (5×10^8 viral particles in 50 µl PBS), (Ad-EV) or PBS. In 48 h later, each of them treated with 50 µg of HDM extract of *Dermatophagoides pteronyssinus.* (GREER Laboratories, Lenoir, NC) once by i.t. for sensitization. Then mice were challenged with 50 µg/mice of HDM by i.t. route on day 15 and 18 and sacrificed on day 20.

Bronchoalveolar lavage fluid (BALF) and lung homogenate

BALF was obtained by cannulating the trachea with a needle and by infusing the lungs three times with 0.3_mL of PBS. After centrifugation at 2,000 ×*g* for 10 min, cell-free BALF was collected and kept -80 °C. Total protein quantification in cell-free BALF was accomplished on aliquots of supernatants from all samples with the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). The cell pellet was resuspended in cold PBS and the total cell counts were determined using a hemacytometer. The number of eosinophils determined by FACS using by PE-conjugated anti-Siglec F antibody. Lung tissues were homogenized in PBS, centrifuged and supernatant of each sample were kept −80 °C.

Inflammatory mediators and total protein measurement

Inflammatory mediators IL-6, TNF-α, KC, MIP-2, IL-5, and IL-8 were measured in BALF, supernatants of lung homogenate and culture medium by using Enzyme linked Immunosorbent Assay (ELISA) kit (BD Biosciences, San Jose, CA and R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Myeloperoxidase (MPO) Assay

MPO activity was measured in the supernatants of lung tissue homogenate. Briefly, the lung tissues were homogenized in PBS and centrifuged. The MPO activity was assayed by measuring the absorbance spectrophotometrically at 450_nm, using 0.167_mg/mL O-

dianisidine hydrochloride and 0.0005% hydrogen peroxide. Data were expressed as fold increase comparing to PBS group.

Immunohistochemistry

For paraffin sections, tissues were fixed in 10% neutral-buffered formalin and embedded using standard techniques. Sections (5 μm) were cut, deparaffinized, and were stained with standard H&E methods to evaluate the tissue histological alterations. For our allergic airway inflammation model, lung sections were also stained with Alcian blue reagent (American MasterTech, Lodi, CA) for detecting airway mucus production. Representative images were obtained using BZ-9000 microscope (Keyence, Itasca, IL). For immunofluorescence, the tissues were fixed in 2.5% PFA following dehydration with 30% sucrose overnight at 4°C. Transverse lung sections (30μm) were obtained with a cryostat and processed for immunofluorescence staining. The tissues were then permeabilized and blocked with protein block (Dako, City, DK). The sections were stained with combinations of antibodies against E-cadherin (BD Biosciences, San Jose, CA) and anti-Myc conjugated with Alexa Fluor 488 in blocking solution overnight at 4°C. We used the secondary Alexa Fluor 589-conjugated polyclonal donkey anti-rabbit antibody (1:2500; Jackson ImmunoResearch Laboratories, City, ST) for 1 h incubation at 37°C.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted by RNeasy extraction kit (Qiagen, City, CA) and reverse transcribed iScript™ cDNA Synthesis Kit (Biorad, Hercules, CA). Real-time polymerase chain reaction (qPCR) was performed using SYBR Green Master Mix (Takara Bio, City, JP). GAPDH served as a loading control. PCR primers for hIL8 : Fw 5’-tctggcaaccctagtctgct-3’, Rv : 5’-gcttccacatgtcctcacaa-3’ and hCCL2: Fw 5’-atccagctccttccaggatt-3’, Rv: 5’ – acacacccaccctctcttg -3’

Western Blot Assay

Protein concentration was determined by a BCA Protein Assay Kit (Pierce, Rockford, IL) with bovine serum albumin used as a standard. Equal amounts of protein were mixed with sample buffer and were separated by 10% SDS-PAGE and transferred onto to PVDF membrane. Membranes were then incubated in blocking buffer [5% skim milk in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20 (TBST)] for 1 h. Primary antibodies were applied in blocking buffer overnight at 4°C. Membranes were washed 3 times, for 5 min each with TBST, and incubated with a secondary antibody conjugated with horseradish peroxidase (GE Healthcare) in blocking buffer for 1 h at room temperature. After washing the membranes with TBST 4 times (5 min each), signal was detected by chemiluminescence using ECL Plus Western Blotting Detection kit (GE Healthcare Life Sciences, Piscataway, NJ) and a BioSpectrum UVP Imaging system (Bio-Rad, City, CA).

Flow cytometry

To check the overexpression of MD-2s *in vitro*, the cells were treated with 50:1 MOI of Ad-EV or Ad-MD-2s for 1h, changed culture medium and continuously incubated for 48h. The cells were dislodged by scraping and washed with PBS. After fixation and permeabilization

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with Cytofix/Cytoperm kit (BD Biosciences), the cells were stained with anti-Myc-Alexa Fluor 488 (Santa Cruz Biotechnologies, Santa Cruz, CA) on ice for 30 min. Cells were washed twice with PBS before fluorescence measurement. Eosinophil cell numbers were determined by staining with PE-conjugated anti-Siglec F. Fluorescence was assessed with a CyAnTM flow cytometer (Beckman Coulter) and data was analyzed using Summit (Dako, Carpinteria, CA, USA) software.

Statistical analysis

Results are expressed as mean ± SD. Data were analyzed using Student's *t* test. Significant differences was set at $p < 0.05$ for all studies. For multiple comparisons, statistical significance was evaluated by one-way ANOVA with Tukey's post-hoc test.

Results

Ad-MD-2s mediated MD-2s expression and inhibition of LPS/TLR4 signaling in mouse macrophages

We previously reported that MD-2s impedes LPS signaling *in vitro* by competitively inhibiting MD-2 binding to TLR4 (18). To investigate the potential therapeutic effects of MD-2s *in vivo*, we generated a Myc-tagged MD-2s adenoviral overexpression vector, Ad-MD-2s. Expression was confirmed by flow cytometry using an anti-Myc antibody compared to cells infected with an empty vector control (Ad-EV) (Supplemental Fig. 1A). We next infected RAW 264.7 cells to assess if Ad-MD-2s would inhibit LPS-induced signaling and inflammatory cytokine production. In response to 6-h LPS stimulation, the levels TNF-α and IL-6 were significantly reduced in Ad-MD-2s infected cells compared to Ad-EV infected cells (Supplemental Fig. 1B and C). These data demonstrate that over-expression of MD-2s blunts LPS-mediated proinflammatory responses in cells.

Expression of MD-2s protein after Ad-MD-2s administration *in vivo*

To elucidate the immunomodulatory effect of MD-2s in LPS-induced lung damage, we examined if i.t. administration of Ad-MD-2s could elevate MD-2s levels in mouse lung. We administered C57BL/6 mice with 1×10^9 pfu Ad-EV or Ad-MD-2s and 48 h later probed for the Myc tag in BALF and lung homogenates. Myc-tagged MD-2s was detected in both the BALF and lung homogenate as two major bands (33, 34) (Fig. 1A), indicating that MD-2s was differentially glycosylated as expected (18). We used immunofluorescence to determine which cells expressed MD-2s in the lungs. Frozen sections were stained with antibodies specific for Myc conjugated with Alexa Fluor-488 (green) and the epithelial cell marker E-cadherin, followed by secondary antibody conjugated with Alexa Fluor-569 (red). MD-2s was seen in the bronchial and alveolar epithelial cells of the lung. There was no detectable Myc signal in Ad-EV infected lungs (Fig. 1B).

Overexpression of MD-2s protein attenuates LPS-induced inflammatory lung injury

To assess the therapeutic effects of MD-2s *in vivo*, we infected mice with Ad-EV or AD-MD-2s, then 48 h later challenged them with LPS and assessed pulmonary inflammation 6 h later. In Ad-EV group, LPS treatment resulted, as expected, in thickened alveolar walls, alveolar congestion, and massive cell infiltration. These pathological changes were clearly

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attenuated in lungs of MD-2s overexpressing mice (Fig. 2A). Mice that received Ad-MD-2s had significantly reduced levels of protein and cells in the BALF compared to Ad-EV controls (Fig. 2B and C). As a proxy for neutrophil infiltration into the lungs, we measured MPO levels in the lung homogenates and found a significant reduction in enzyme levels in mice that received Ad-MD-2s (Fig. 2D). We next measured the proinflammatory cytokine IL-6, as well as the neutrophil chemokines MIP-2 and KC, and found that all three were significantly reduced in both BALF and lung homogenates in mice that received Ad-MD-2s compared to mice that received the empty vector (Fig. 2E-G). Importantly, adenoviral vector alone did not alter the effects of LPS induced ALI as measured by cellular recruitment and cytokine production (Supplemental Fig. 2). These observations indicate that Ad-MD-2s treatment attenuated lung injury in response to LPS by reducing vascular leak and decreasing neutrophil influx and production of proinflammatory cytokines and chemokines.

To determine if MD-2s functioned specifically by inhibiting TLR4 signaling and not other TLR pathways, we evaluated the effect of MD-2s on the inflammatory cell accumulation and cytokine production in the lungs following an i.t. challenge with Pam3CSK4, a potent activator of TLR2/TLR1 pathway. Pam3CSK4 induced marked infiltration of inflammatory cells into the alveolar space and alveolar collapse (Fig. 3A and B) and increased levels of MPO, IL-6, MIP-2, and KC (Fig. 3C–F). However, none of these endpoints differed between Ad-EV or Ad-MD-2s infected mice. These data demonstrate that overexpression of MD-2s did not inhibit TLR2-mediated inflammation and support our conclusion that MD-2s acts specifically to blunt TLR4 signaling.

Secreted MD-2s in the BAL inhibits LPS-induced lung injury

A soluble form of MD-2 (sMD-2) is markedly elevated in plasma from patients with severe infections and in other fluids from inflamed tissues (35, 36). The sMD-2-LPS complex plays a crucial role in the LPS response by activating epithelial and other TLR4 positive MD-2 negative cells in the inflammatory microenvironment. We reported that MD-2s exists also as a secreted form that retains its TLR4-inhibitory activity in cell models (33). To assess if soluble MD-2s produced *in vivo* from Ad-MD-2s could block TLR4 signaling, we transferred BALF from Ad-MD-2s or Ad-EV infected mice into naïve mice then challenged with LPS (Fig. 4A). Soluble MD-2s in BALF was detected by immunoblotting using anti-Myc antibody (Fig. 4B). Concentrated BALF was administrated i.t. 1 h before instillation of 0.25 mg/kg LPS and the mice were sacrificed 6 h later. In the group that received Ad-EV BALF, LPS challenge led to increased total cell number and levels of MPO, IL-6, and KC (Fig. 4C–F). The pretreatment of BALF prepared from Ad-MD-2s treated mice significantly blunted these proinflammatory responses (Fig. 4C–F). The pretreatment of BALF obtained from naïve animals had no effect on LPS induced inflammation (Supplemental Fig. 3A–D). While it is possible that an unknown factor in the BALF is contributing the inhibition of LPS signaling, the data indicate that it is likely to be the secreted form of MD-2s.

Ad-MD-2s inhibits HDM-induced lung allergic inflammation in mice

Recent publications have described the MD-2 related lipid recognition family (ML) of proteins of which MD-2 is the prototypical member (37, 38). More recently, the house dust mite major antigen Der p 2 was found to have many common structural and functional

characteristics with MD-2 (12). In mouse models of allergic asthma, the effects of Der p 2 are markedly reduced in *Tlr4* knockout mice and can be prevented in wild-type mice by administration of a TLR4 antagonist (39–41). The bronchiolar-alveolar epithelium (BAE) is a primary target site for inhaled agents that cause lung injury and releases a broad range of mediators that influence other cell populations. New findings showed epithelial cells are in a pivotal position in the development of allergic inflammation though the activation of the TLR4 signaling pathway (39, 40, 42–44). However, in addition to its proinflammatory effects, due to its structural similarities to MD-2, Der p 2 can functionally replace MD-2 and facilitate TLR4 signaling *in vitro* (11). To determine if MD-2s inhibits HDM induced activation of epithelial cells, we measured mRNA expression of CCL2 and IL-8, and the release of IL-8 protein from human primary small airway epithelial cells (SAEC). The cells were transduced with Ad-EV or Ad-MD-2s at 50:1 MOI for 1 h, washed and incubated for 48 h. The cells were then stimulated with HDM or PBS. As expected, HDM stimulated the expression of CCL-2 and IL-8 mRNA as well as IL-8 secretion. However, transduction with Ad-MD-2s significantly inhibited the induction of these cytokines (Fig. 5). We also performed similar experiments using the human bronchial epithelial cell line (BEAS-2b) and obtained the same results (data not shown). These data demonstrate that MD-2s inhibits the expression of inflammatory mediators, CCL-2 and IL-8, by airway epithelial cells exposed to HDM.

To explore further if MD-2s has a protective effect on allergic airway inflammation *in vivo*, we utilized the HDM induced allergic inflammation model in mice. This model exhibits a number of features of human allergic airway inflammation including, cellular infiltration into the lungs, increase in eosinophil numbers, and elevated levels of inflammatory cytokines in BAL fluid. As shown in Fig.6A, mice were treated with Ad-EV or Ad-MD-2s (n=10 for each group) at 5×10^8 pfu/mice for 48 h, followed by sensitization with 50 μg HDM given by i.t. route. Mice were then challenged with 50 μg HDM on days 15 and 18 post adenoviral administration and sacrificed on day 20 (Fig. 6A). Sections of the lungs stained with H&E showed marked histological alterations such as peribronchial and perivascular cell infiltration in the airway after HDM challenge in the PBS or Ad-EV pretreated groups. However, these changes were reduced in the mice that were treated with AD-MD-2s (Fig. 6B). MD-2s overexpression during sensitization also significantly reduced the influx of eosinophils into the airways, as well as the level of the Th2 associated cytokine, IL-5 ($p< 0.01$) (Fig.6 C and D). Finally, MD-2s overexpression significantly reduced goblet cell hyperplasia and airway mucus production in murine lung when compared to mice treated with PBS or Ad-EV prior to HDM sensitization (Fig 6E and F). It should be noted while there were no differences found between PBS groups and empty vector, there was a trend for reduced eosinopils with the vector control. It is possible that adenoviral transduction slightly inhibited Th2 responses by the induction of type I IFN. However, it is clear that MD-2s significantly reduced the overall allergic airway inflammation compared with both controls. These results suggest that in addition to inhibiting LPS induced ALI, MD-2s can also inhibit HDM induced allergic lung inflammation in mice.

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Discussion

In this study, we demonstrated that the alternatively spliced form of human MD-2 has clear beneficial effects on TLR4 mediated lung injury using the murine models of LPS induced ALI and HDM induced allergic airway inflammation. MD-2s markedly reduced the numbers of inflammatory cells, total proteins, MPO activity and IL-6, KC and MIP-2 levels in BALF and lung homogenate after LPS exposure to the lungs. Additionally, the results of the BALF transfer experiment showed that the soluble form of MD-2s also ameliorated LPS induced ALI in mice.

TLR4 alone is not sufficient for conferring LPS responsiveness. MD-2 is absolutely required for TLR4 signaling and is considered the coreceptor for triggering of TLR4 signaling (45, 46). Indeed, addition of exogenous MD-2 protein could reverse LPS hyporesponsiveness in some experiments (34, 47). In addition, MD-2 knockout mice do not respond to LPS and survive against endotoxic shock (48). Hence MD-2 is logical target for pharmacological intervention against triggering of TLR4 signaling (49, 50). As LPS recognition is the first step of the signaling pathway, inhibiting this process could be an efficient way to suppress inflammatory responses before the signal has been transmitted into the downstream pathways. Some effective approaches targeting this step has already been described, but most of them are mainly focused on TLR4 and LPS, while few antagonists directed towards MD-2 has been reported (51–53). Extensive work has suggested that a number of endogenous molecules such as nuclear protein HMGB1 or oxLDL may be potent activators of TLR4 and capable of inducing proinflammatory cytokine production by the monocyte-macrophage system and the activation and maturation of dendritic cells (54, 55). Thus it is possible that MD-2s might also be able to downregulate this endogenous signaling. Further investigations into the scope of MD-2s inhibition is required.

As we described in our previous publication (33), MD-2s is generated by alternatively splicing out exon 2 of the MD-2 gene, which leads to an in-frame deletion of 30 amino acids spanning at positions 39–69. Based on the earlier studies (56–58) describing the essential residues in LPS or TLR-4 binding regions of the MD-2 protein, which are intact in our MD-2s. However, recent publications reported the importance of several amino acids in LPS induced TLR-4/MD-2 mediated cell activation, which are missing in MD-2s (9, 59, 60). Kawasaki et al. reported that the mouse MD-2 mutant G59A can form the cell surface TLR4/MD-2 complex, but it's ability to confer LPS responsiveness was reduced (61). Expression of the polymorphic variant G56R MD-2 reduces transfer of endotoxin from CD14 to MD-2, thereby reducing TLR4-dependent cell activation (62). These data suggest that amino acids encoded in exon 2 of MD-2 gene play key roles in the triggering and activating LPS signaling.

Particular innocuous environmental proteins act as allergens in susceptible hosts, but clear mechanistic explanations of allergenicity are still lacking. Concentrated in HDM fecal pellets, Der p2 has the highest rates of skin test positivity compared to other mite allergens in HDM allergic patients (63). Der p 2 belongs to the MD-2-related lipid-recognition domain family of proteins, binds LPS, and also interacts directly with the TLR4 complex, facilitating LPS signaling (11). Der p 2 drives Th2 inflammation in the airway in a TLR4-

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dependent fashion by mimicking MD-2 in the absence of MD-2. Thus, Der p 2 has auto-adjuvant activity (38, 64). This is of special importance because airway epithelial cells express TLR4 but little or no MD-2 (65). Several studies have demonstrated a role for TLR4 signaling in type-2 mediated inflammation. In response to the major allergen HDM, specifically Der p2, the mucosal epithelium induces pro inflammatory mediators such as IL-8, TSLP and IL-6 that causes the selective recruitment, retention, and accumulation of cells in the lung. Here, we showed *in vitro* that MD-2s clearly suppresses HDM induced IL-8 release in human primary SMALL airway epithelial cells and also human epithelial cell line. In our allergic lung inflammation model, MD-2s effectively inhibited the increase of T helper 2 cytokine, IL-5 and eosinophil count in BALF and histological studies found that MD-2s substantially inhibited HDM induced goblet cell hyperplasia in the airway. These results suggest that MD-2s may be useful for the treatment of HDM related allergic lung inflammation via its inhibition of TLR4 signaling.

MD-2 also plays an important role in certain viral infections. During influenza infection, ROS production leads to oxidized lipids that can potently signal though TLR4, requiring MD-2 in the process (66). Additionally, during RSV infection, the F protein also signals through MD-2/TLR4 (67). In both of these cases, excess inflammation can lead to damaging pneumonia, which, when inhibited by TLR4 agonists, may help resolve the inflammation (66, 67). Finally, the HIV Tat protein has also been shown to bind the TLR4-MD-2 complex, leading to its activation and dysregulation (68). Thus these viral infections may also present attractive targets for MD-2s based immune regulation and future experimental studies investigating the potential beneficial effects of MD2s in these viral infections are warranted.The complexities involved in averting a prolonged and dysregulated immune response to LPS still need to be investigated further. Sepsis remains a leading cause of death worldwide. Despite years of extensive research, effective drugs that inhibit the pro-inflammatory effects of LPS and improve outcome when added to conventional sepsis treatments are lacking (69, 70). A naturally occurring alternatively spliced isoform, such as MD-2s, may behave like a decoy coreceptor to form a nonfunctional complex that negatively regulates downstream signaling (33). In this study, we showed that MD-2s attenuates TLR4 mediated inflammatory responses *in vivo* suggesting that MD-2s may be useful in the treatment of inflammation associated with TLR4 and LPS. Thus further investigations into the potential therapeutic aspects of MD-2d will be needed as well as a comprehensive examination of the regulation of the native MD-2s. In the meantime, our data suggest that in addition to several other alternatively spliced inhibitory check points, an MD2s-mediated negative feedback loop also ensures that innate immunity is self limiting; this could be relevant to sepsis, asthma, cancer and several other diseases with an inflammatory component.

Supplementary Material

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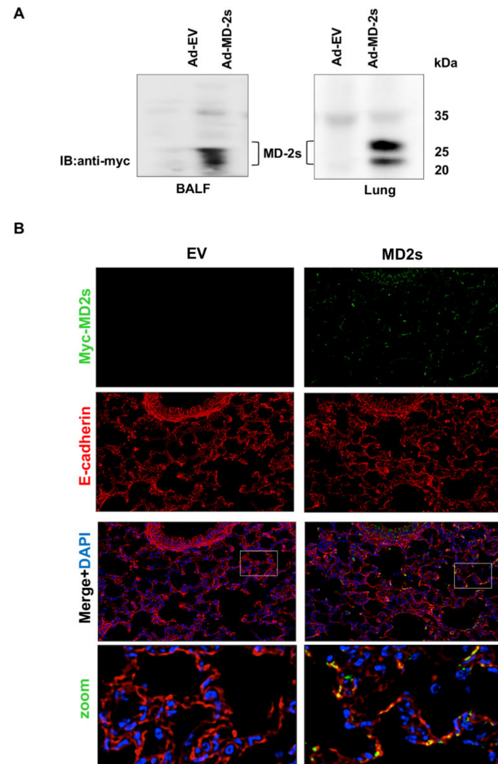

Figure 1. Expression of recombinant adenovirus-mediated MD-2s protein*in vivo*

Ad-EV or Ad-MD-2s-Myc was administered intratracheally (i.t.) to C57BL/6 mice at a dose of 1×10^9 plaque-forming units (pfu)/mice. **(A)** 48h later, BALF was pooled in each group and concentrated. Lung homogenate was prepared. Samples were subsequently analyzed by SDS-PAGE and immunoblotted with an anti-Myc antibody. **(B)** Immunofluorescent staining in lung sections. Green: Myc tag of MD-2s protein, red: epithelial cell marker, E-cadherin and blue: nuclei. Photographs are representative examples from each group. Images were taken at 40×.

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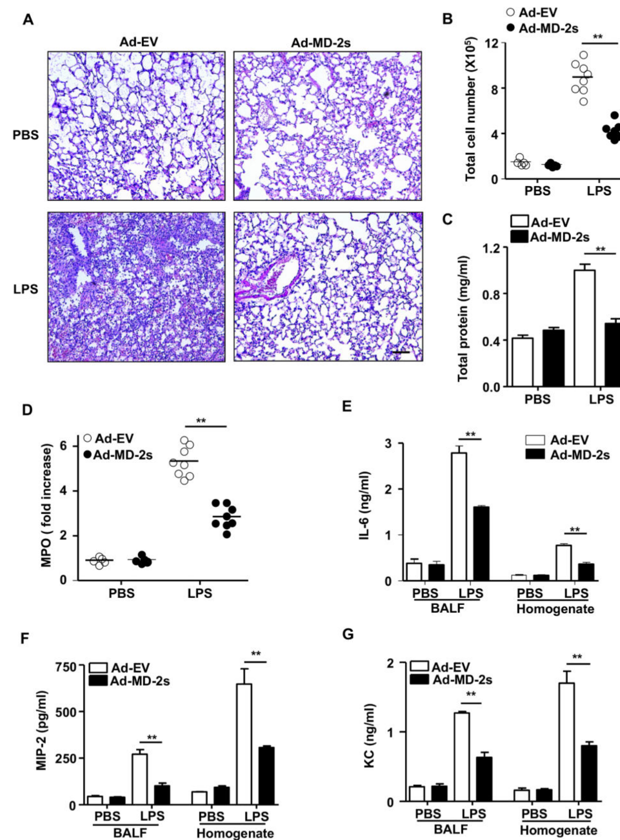

Figure 2. MD-2s over expression attenuates lung tissue damage in mice challenged with LPS
Mice (n=8 for each group) received i.t instillation of Ad-EV or Ad-MD2s at dose of 1×10^9 pfu/mice and 48h later, LPS (0.25 mg/kg) was administrated i.t. and sacrificed 6h later. **(A)** Lungs were collected and stained with H&E for histology (×400). **(B)** Total cell number and **(C)** Protein level in BALF were determined. **(D)** MPO content was assessed in lung tissue homogenates, as described in Materials and Methods section**. (E)** IL-6 and **(F)** MIP-2 and **(G)** KC concentrations were determined by ELISA. Data are presented as the mean ± standard error of the mean. **P < 0.05

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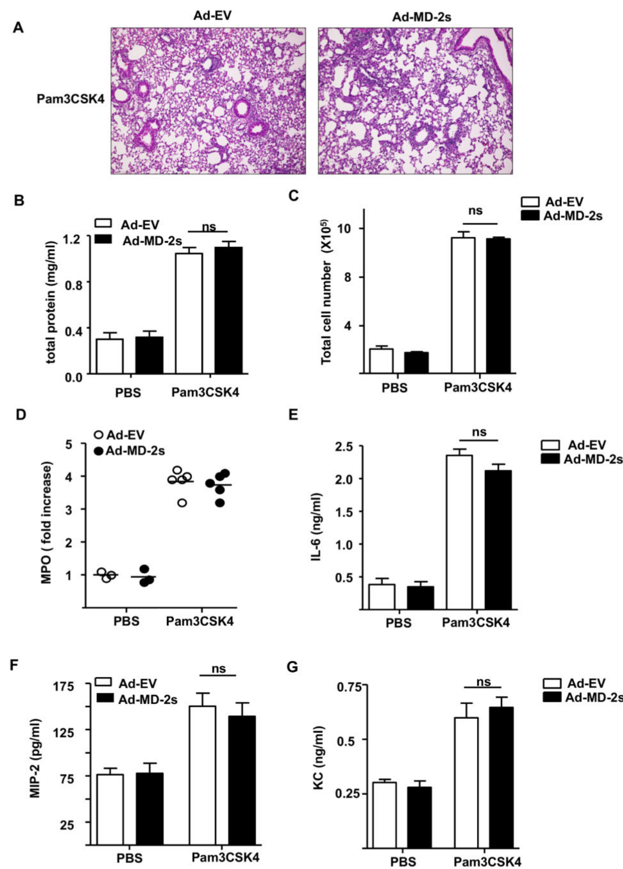

Figure 3. MD-2s does not prevent Pam3CSK4-induced pulmonary inflammation

Ad-EV or Ad-MD2s 1×10^9 pfu was delivered into lungs of mice by i.t. and 48h later Pam3CSK4 by 0.25 mg/kg or PBS instilled i.t. and sacrificed 6h later. **(A)** Lungs were collected and stained with H&E for histology. **(B)** Total protein **(C)** Total cell number in BALF, **(D)** MPO activity in lung homogenate was measured by MPO assay. **(E)** IL-6, **(F)** MIP-2 and **(G)** KC were determined in BALF by ELISA. Values are the mean ± SD; n=5 for each treatment group and vehicle. $^{**}P < 0.05$

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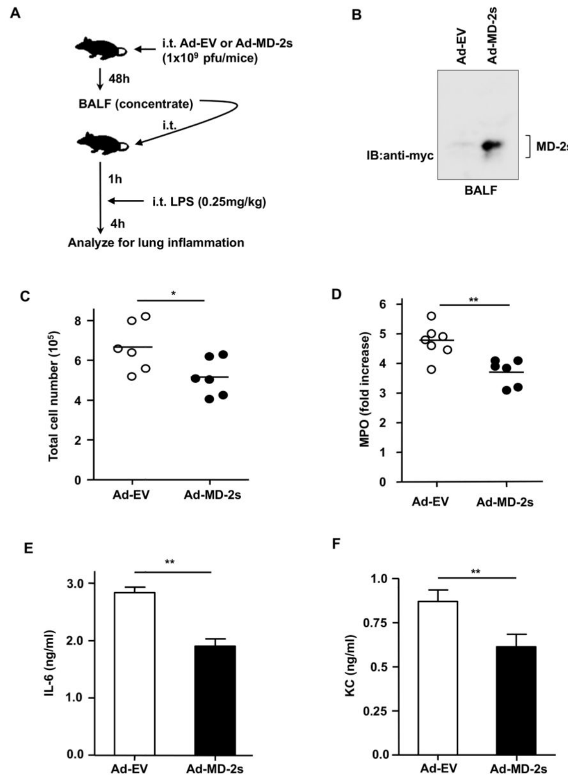

Figure 4. Soluble form of MD-2s protein inhibited LPS induced cytokines and chemokines

Ad-EV or Ad-MD-2s 1×10^9 pfu was delivered into lungs of mice (n = 12 for each group). 48h later mice were sacrificed and BALF was pooled in each group and concentrated. Mice were (n = 6 for each group) i.t. administrated with concentrated of BALF (100 µl) 1h before LPS (0.25 mg/kg, 50µl) i.t. instillation and sacrificed 6h later. **(A)** The schematic view of experiment. **(B)** The expression of Myc-tagged MD-2s protein in concentrated BALF. **(C)** Total cell number in BALF **(D)** MPO activity in lung homogenate was measured. **(E)** IL-6 and **(F)** KC concentrations were determined in BALF by ELISA. Values are the mean ± SD; *P < 0.05 ** P < 0.05

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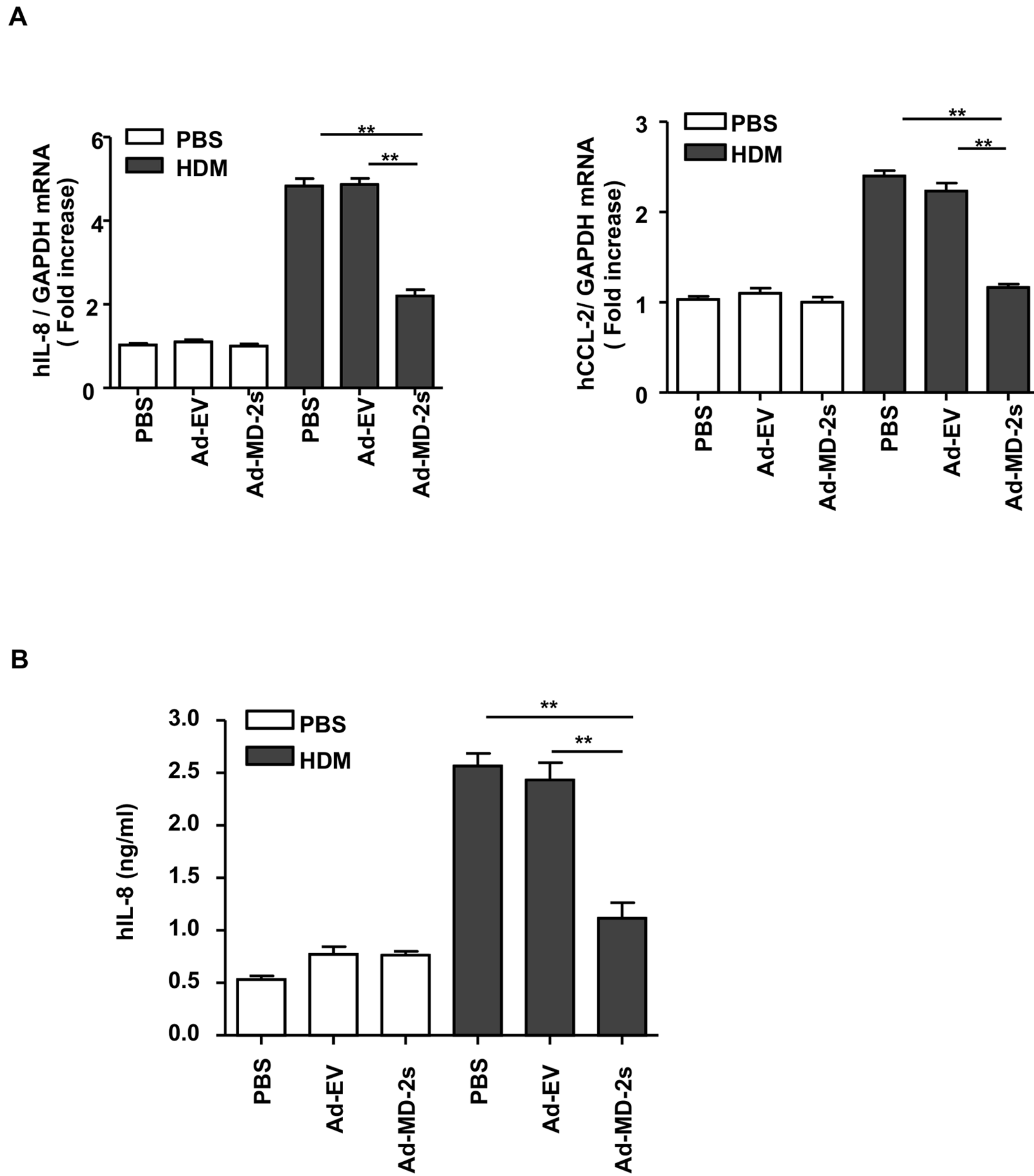

Figure 5. The effect of MD-2s on HDM induced human small airway epithelial cell (SAEC) activation

SAECs were transduced with Ad-EV or Ad-MD-2s-Myc of MOI 50:1 for 1h and after changing culture medium, incubated further for 48h. **(A)** The cells were stimulated with 100 μg/ml of HDM for 8h. mRNA for IL-8 and CCL-2 expression were examined by qPCR. GAPDH served as loading control. **(B)** After 24h treatment of HDM, culture medium were collected and IL-8 production was measured by ELISA. A representative result of three independent experiments. Value are the mean ± SD. **P<0.01.

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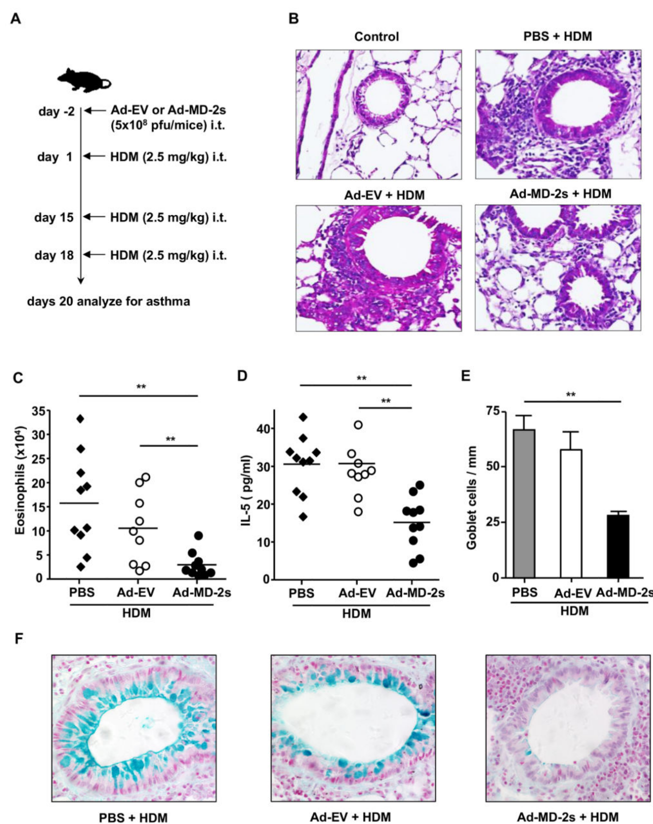

Figure 6. Alleviation of allergic lung inflammation in HDM challenged mice pretreated with Ad-MD2s

(A) Schematic protocol. The mice were treated with PBS, Ad-EV or Ad-MD2s (n=10 for each group) at 5×10^8 pfu/mice for 48h. Sensitization in day 1 and challenge in days 14 and 18 with HDM at a dose 50 μg/mice were performed by i.t. administration. Then at day 20, mice were sacrificed. **(B)** Lung sections were stained with H&E for measurement of cellular infiltration of the peri-airway region. Original magnification (×40×). (C) The number of eosinophils measured by FACS. **(D)** Level of IL-5 in the BALF by ELISA. **(E)** The number of goblet cells in airway. Data are the mean ± SEM and are representative of eight mice evaluated in each group (one-way ANOVA [Tukey post hoc]; $^{**}P < 0.001$. **(F)** A representative picture of each group.