Metal allergens nickel and cobalt facilitate TLR4 homodimerization independently of MD2

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Development of contact allergy requires cooperation of adaptive and innate immunity. Ni²⁺ stimulates innate immunity via TLR4/MD2, the bacterial LPS receptor. This likely involves receptor dimerization, but direct proof is pending and it is unclear if related haptens share this mechanism. We reveal Co²⁺ as second metal stimulating TLR4 and confirm necessity of H456/H458 therein. Experiments with a new TLR4 dimerization mutant established dimerization as a mechanism of metal- and LPS-induced TLR4 activation. Yet, in interaction studies only LPS- but not metal-induced dimerization required MD2. Consistently, soluble TLR4 expressed without MD2 inhibited metal- but not LPS-induced responses, opening new therapeutic perspectives.

Keywords: contact allergy; dermatitis; inflammation; innate immunity; Toll-like receptor

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INTRODUCTION

Allergic contact dermatitis (ACD), defined as a T-cell-mediated delayed-type skin hypersensitivity (type IV) response to low molecular weight substances, represents a considerable environmental and occupational health hazard in developed countries [1]. Relevant agents causing occupation-induced ACD comprise the ubiquitous transition metals cobalt (Co²⁺) and nickel (Ni²⁺) [2].

Co²⁺ is broadly used in metal and electronic industries, magnetic alloys production and the construction sector. Sources further include ceramics, paints, dental prostheses, jewellery, hair dyes and fertilizers [3]. Co²⁺ induces ACD by a T-cell-mediated reaction associated with production and release of various cytokines [4]. However, similar to Ni²⁺, Co²⁺ also has irritant capacity and triggers a direct innate immune signal resulting in activation of proinflammatory cascades such as the IKK2/NF- κ B pathway in primary human endothelial cells (ECs) [5]. Enhanced

NF- κ B activity in turn drives expression of endothelial proinflammatory adhesion molecules, such as VCAM-1 and E-selectin, required for leucocyte recruitment to the site of exposure [6]. Early observations established that co-stimulation with irritants or unrelated haptens during the sensitization or elicitation phase allows induction of contact hypersensitivity to spurious amounts of contact allergens normally insufficient to trigger contact hypersensitivity in mice [7]. Accordingly, the intensity of the induced innate immune signal critically determines the responsiveness to a given contact allergen [8]. For instance, innate immune stimulation during the sensitization phase is essential for activation and mobilization of dendritic cells (DCs), which mediate clonal expansion of hapten-specific T-effector cells in the dermal lymph node [9]. Inappropriate DC activation leads to a failure to produce hapten-specific T cells triggering tolerance [9]. Thus, understanding the mechanism by which haptens activate the innate immune system holds great therapeutic potential.

Recently, we demonstrated that human Toll-like receptor 4 (TLR4), an innate immune receptor detecting bacterial lipopolysaccharide (LPS), has a crucial role in Ni²⁺-mediated inflammatory reactions [10]. On the basis of structural modelling and mutagenesis data we proposed a model, in which two Ni²⁺ ions bind a histidine triplet (H431, H456 and H458) provided by two opposing TLR4 molecules. Similar to LPS, which initiates TLR4 activation via a dimerization-dependent mechanism requiring its co-receptor MD2 [11], Ni²⁺ thus might induce proinflammatory signalling by crosslinking two TLR4 molecules. Yet, this concept remains unproven and the role of MD2 in Ni²⁺-induced TLR4 signalling is obscure. Moreover, it is now unclear if the mechanism of innate immune activation by Ni²⁺ is unique or shared by related metal allergens.

Here, we present Co²⁺ as second metal hapten triggering species-dependent TLR4/MD2 activation. We reveal that metal allergen-induced TLR4 activation occurs via MD2-independent TLR4 dimerization requiring its non-conserved histidines H456 and H458.

RESULTS AND DISCUSSION

Distinct skin cell types show divergent responses to Co²⁺

Generation of hapten-specific T cells critically requires innate immune activation of resident skin cells [9]. We thus compared

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Fig 1 | Keratinocytes fail to induce proinflammatory responses to Co²⁺. (A) Enzyme-linked immunosorbent assay-based quantification of IL-8 in the supernatants of normal human epidermal keratinocytes, human primary umbilical vein endothelial cells (HUVEC) (B) or human iDCs (C). Cells were stimulated for 8 h with Co²⁺ (1.5 mM), Ni²⁺ (1.5 mM) or medium (Ctrl). Data represent mean values of three independent experiments $\pm$ s.d. In (C), averages are derived from three analysed donors. Statistical differences are indicated by solid lines (** $P < 0.01$, *** $P < 0.001$, t -test). HUVEC, human primary umbilical vein endothelial cells; iDCs, immature dendritic cells; IL, interleukin.

different primary human skin cell types for responsiveness to Co²⁺. Besides DCs, the cell type directly involved in the generation of hapten-specific T cells, we analysed keratinocytes, the predominant epidermal cell type and ECs, which are among the first cells to respond to metal allergens *in vivo* [8] and have a pivotal role for leucocyte recruitment at later stages of ACD [7]. Unexpectedly, neither Co²⁺ nor Ni²⁺ was able to induce interleukin (IL)-8 production in normal human epidermal keratinocytes (NHEKs) (Fig 1A). By contrast, Co²⁺ induced considerable IL-8 protein release from ECs and human immature DCs (iDCs) (Fig 1B,C). This suggests a specific defect of keratinocytes, which is not shared by other skin cells.

NHEKs lack TLR4 and fail to respond to Ni²⁺/Co²⁺

We suspected the lack of functional TLR4/MD2 expression to account for the insensitivity of NHEK to Co²⁺ and Ni²⁺. While several reports support this view [12–14], the prevailing opinion is that keratinocytes contain endogenous TLR4 [9]. However, even groups claiming that keratinocytes express TLR4 failed to induce proinflammatory activation by LPS unless unusually high concentrations of $>100 \mu\text{g ml}^{-1}$ were employed [15], raising the possibility that activation resulted from contamination, for

example, with TLR2 agonists found in some LPS preparations [12]. We thus assessed the responsiveness of NHEKs to TLR4 stimulation. Consistent with an incapability to initiate TLR4 activation, NHEK failed to enhance IL-8 production when exposed to moderate LPS doses of $1 \mu\text{g ml}^{-1}$ (Fig 2A), a concentration inducing prominent IL-8 responses in TLR4/MD2-positive cells [10] (Fig 2D–F). This was not owing to a defect in the IKK2/NF κ B module or inability of NHEK to release IL-8 as IL-1 β or tumour-necrosis factor (TNF) readily triggered IL-8 production (Fig 2A). Quantitative real-time PCR (qRT–PCR) revealed a lack of detectable TLR4 but low MD2 messenger RNA expression in keratinocytes (Fig 2B). Neither induction of differentiation by addition of 1.0 mM CaCl₂ (supplementary Fig S1A online, top) [16] as verified by increased expression of the differentiation marker loricrin (supplementary Fig S1A online, bottom) nor stimulation with different proinflammatory stimuli was able to raise TLR4 mRNA expression above detection levels (supplementary Fig S1B online), suggesting that lack of TLR4 is not an exclusive feature of undifferentiated or non-activated keratinocytes. Consistent with the view that low/lacking TLR4 expression confined the responsiveness of keratinocytes, introduction of TLR4 alone conferred sensitivity to metal allergens and LPS (Fig 2C).

Co²⁺ induces several proinflammatory genes in ECs

To learn more about the proinflammatory signature induced by Co²⁺, we used a qRT–PCR-based array system to study the expression of 84 human cytokine genes and receptors in primary human ECs. To avoid secondary effects, we restricted Co²⁺ treatment to 5 h. Four individual experiments were analysed independently to ensure high statistical significance. Table 1 lists the transcripts significantly regulated by Co²⁺. These included strictly NF κ B-dependent genes such as *CXCL3* (*Gro- γ*) and *IL-8* [17], as well as *CXCL10* (*IP-10*), which rather indicates a type I interferon response [18]. A comparison of the Co²⁺-regulated inflammatory genes with those identified in a previous gene-chip microarray of Ni²⁺-regulated transcripts [17] revealed a striking overlap, indicating a similar mode of action.

Co²⁺-induced TLR4 activation requires MD2

To clearly demonstrate that TLR4/MD2 expression was responsible for the observed innate immune activation by Co²⁺, we employed small interfering RNA (siRNA). Depletion of either TLR4 or MD2 significantly decreased both LPS- and Co²⁺-induced IL-8 production in ECs (Fig 2D). Supplementation experiments in human HEK293 cells, which lack endogenous TLR4 and MD2 expression [19], confirmed a requirement of both receptor components for Co²⁺-induced NF κ B activation and proinflammatory gene expression (Fig 2E; supplementary Fig S2A,B online). Experiments with HEK293 stably expressing TLR4/MD2 (HEK293 TLR4/MD2) revealed a dose dependency of Co²⁺-induced IL-8 production (supplementary Fig S2C online) and excluded LPS contamination as source as treatment with the LPS-inactivating drug polymyxin B sulphate failed to block the response to Co²⁺ (supplementary Fig S2D online).

Co²⁺ activates human TLR4 but not mouse Tlr4

In case of Ni²⁺, TLR4 activation is species-dependent and requires the presence of two potential metal-binding histidines

Fig 2 | Co²⁺ mediates its proinflammatory effects via TLR4. (A–C) Keratinocytes lack functional TLR4 expression. (A) Enzyme-linked immunosorbent assay showing IL-8 production in NHEK exposed for 8 h to LPS (*S. minnesota* R595; 1 μg ml⁻¹), IL-1β (100 U ml⁻¹), TNF (10 ng ml⁻¹) or medium (Ctrl). (B) Quantitative real-time PCR analysis of human MD2 (black bars) and TLR4 (grey bars) expression in unstimulated NHEK as compared with TLR4/MD2-negative HEK293 cells or TLR4/MD2-positive HUVEC. Data are presented as average-fold mRNA expression ± s.d. related to the HUVEC-positive control (set to 100%). (C) Enzyme-linked immunosorbent assay, showing IL-8 protein release from NHEK transfected with human MD2, TLR4 or both in combination. Cells were stimulated for 8 h with 1.5 mM Co²⁺, 1.5 mM Ni²⁺, 1 μg ml⁻¹ LPS or medium (Ctrl). (D–F) Co²⁺ requires TLR4/MD2 for proinflammatory signalling. Cells were stimulated as in (C). (D) IL-8 production by HUVEC transfected with the indicated small interfering RNAs for 48 h before stimulation. (E) Co²⁺-induced IL-8 protein expression in the indicated cell lines as determined by western blot analysis of total lysates. (F) Co²⁺-induced activity of a transfected 6 × κB-luciferase (luc) reporter on transfection of HEK293 MD2 cells with TLR4, TLR4 H456A/H458A, Tlr4 or a ‘humanized’ Tlr4 mutant with histidine substitutions at the corresponding positions of the mouse sequence (Tlr4 Y454H/N456H). Bar diagrams represent averages (A–D) or mean fold values (F) of three to four independent experiments ± s.d. The immunoblot in (E) is representative of three independent experiments. *P* > 0.05 not significant (NS), **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *t*-test. HUVEC, human primary umbilical vein endothelial cells; IL, interleukin; LPS, lipopolysaccharide; NHEKs, normal human epidermal keratinocytes; NS, not significant; TLR4, Toll-like receptor 4; TNF, tumour-necrosis factor.

in TLR4 that are conserved in primates but not other species [8]. Similarly, Co²⁺ upregulated IL-8 in human THP-1 monocytes (supplementary Fig S3A online) but failed to induce the NFκB target gene TNF in murine Raw 264.7 macrophages (supplementary Fig S3B online). The inability of mouse cells to respond to Co²⁺ was neither owing to the absence of mouse Tlr4 (Tlr4) or Md2 as LPS stimulation readily triggered TNF induction in Raw 264.7 (supplementary Fig S3B online) nor did it result from species-dependent sequence variations in MD2 as HEK293 TLR4 cells expressing human or mouse MD2 were equally responsive to Co²⁺ (supplementary Fig S3C,D online).

We next evaluated if alanine mutation of the non-conserved histidines H456 and H458 affected Co²⁺-induced proinflammatory gene expression. Double mutation of H456 and H458 significantly reduced Co²⁺-mediated proinflammatory activation in various readouts (Fig 2F; supplementary Fig S4A–C online). Conversely, expression of a ‘humanized’ Tlr4 (Y454H-N456H) in HEK293 MD2 cells restored Co²⁺ sensitivity (Fig 2F; supplementary Fig S4A online). Notably, all mutants were still able to confer LPS responsiveness (Fig 2F; supplementary Fig 4A–C online), indicating that LPS and Co²⁺ have different sequence requirements for activation.

Ni²⁺/Co²⁺ induce TLR4 dimers independently of MD2

It is well documented that some TLRs function as homodimer and others as heterodimers [20]. Homodimerization of TLR4 is an initial step for receptor activation [21]. To explore if metal

allergens, similar to LPS, induce TLR4 dimerization, we performed co-immunoprecipitation (co-IP) experiments in HEK293 wild-type or HEK293 MD2 cells that were transfected with differently tagged TLR4 constructs (haemagglutinin (HA)-TLR4, FLAG-TLR4 or both in combination) and subsequently stimulated with Ni²⁺, Co²⁺ or LPS as control. Figure 3A shows the appearance of a slight basal interaction complex of HA-TLR4 and FLAG-TLR4 in α-FLAG IPs of HEK293 MD2 cells co-transfected with HA-TLR4/FLAG-TLR4 but not in IPs of cells transfected with the respective constructs alone. Yet, complex formation was strongly enhanced on stimulation of Ni²⁺, Co²⁺ or LPS. Unlike LPS, however, which did not raise dimerization above background, both metal allergens also facilitated complex formation in the absence of MD2 (Fig 3B).

The recently published crystal structure of TLR4 has shed light on its dimerization interface [22]. To confirm that dimerization is crucial for receptor activation, we generated a single alanine mutation of asparagine N433, which represents one of several residues in the TLR4 dimerization interface implicated to form direct contacts between opposing TLR4 molecules on dimerization [22]. Mutation of single or both N433 residues gradually decreased dimerization capacity of TLR4 as evident from co-IP experiments with HEK293 MD2 cells transfected with different combinations of HA- and FLAG-tagged TLR4 and TLR4 N433A constructs (Fig 3C). In fact, no basal or stimulus-dependent TLR4 complex was detectable when both N433 residues were disrupted, indicating that complete loss of N433 is incompatible with dimerization. Consistently, HEK293 MD2 cells transfected

Table 1 Cytokines and cytokine receptors regulated by Co^{2+} in primary human ECs

Gene symbol	Description	Fold up by Co^{2+}	P-value	Regulation by Ni^{2+}
<i>Upregulated genes</i>				
CXCL3 (Gro- γ)	Chemokine (C-X-C motif) ligand 3	36.28	0.042799	On
CCL20 (MIP3- α)	Chemokine (C-C motif) ligand 20	23.32	0.011699	On
CXCL2 (Gro- β)	Chemokine (C-X-C motif) ligand 2	14.84	0.048677	35.97
CXCL6 (GCP2)	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	14.28	0.000209	21.68
CCL5 (RANTES)	Chemokine (C-C motif) ligand 5	13.40	0.033357	No
IL8	Interleukin 8	11.72	4.47E-08	32.95
CXCL1 (Gro- α)	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	8.01	0.002375	23.70
CXCL5 (ENA 78)	Chemokine (C-X-C motif) ligand 5	7.36	0.020081	12.75
LTB (TNFC)	Lymphotoxin beta (TNF superfamily, member 3)	6.46	1.06E-07	On
IL1A	Interleukin 1, alpha	5.72	0.006050	On
TNF (TNF- α)	Tumour necrosis factor (TNF superfamily, member 2)	5.47	0.010384	No
CEBPB (NF-IL6)	CCAAT/enhancer-binding protein (C/EBP), beta	5.18	0.010484	4.27
LTA (TNFB)	Lymphotoxin alpha (TNF superfamily, member 1)	3.63	0.000125	On
CXCL10 (IP-10)	Chemokine (C-X-C motif) ligand 10	3.40	0.009645	On
<i>Downregulated genes</i>				
CXCL12 (SDF1)	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	- 17.71	0.005603	No
AIMP1 (SCYE1)	Aminoacyl tRNA synthetase complex-interacting multifunctional protein 1/small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)	- 3.49	0.001465	- 2.85
IL10RB	Interleukin 10 receptor, beta	- 2.67	0.000044	No

Abbreviations: ECs, endothelial cells; IL, interleukin; HUVEC, human primary umbilical vein endothelial cells; qRT-PCR, quantitative real-time PCR; tRNA, transfer RNA. HUVEC were stimulated with 1.5 mM Co^{2+} or medium as control for 5 h. RNA was isolated and the expression of 84 genes encoding cytokines and cytokine receptors was assessed using a commercially available qRT-PCR SYBR green array as described in the Supplementary Materials and Methods online. Listed genes represent transcripts showing an at least 2.5-fold mean up- or downregulation by Co^{2+} in relation to the unstimulated controls. Only genes with single amplification products as determined by melting curve analysis and statistically significant P-values of regulation (<0.05) in four independent experiments were considered. Coregulation by Ni^{2+} was analysed by comparison with a list of previously published Ni^{2+} -response genes identified in HUVEC [17].

with HA-TLR4 N433A failed to produce more than basal IL-8 on stimulation with Ni^{2+} , Co^{2+} or LPS, suggesting that TLR4 dimerization is crucial for receptor activation by both metal allergens and LPS (Fig 3D).

sTLR4 can inhibit $\text{Ni}^{2+}/\text{Co}^{2+}$ -induced NF κ B activation

The putative metal-binding histidines H456 and H458 are structurally adjacent to N433 in the TLR4 dimerization interface [22]. Despite the dispensability of both residues for LPS-induced TLR4 activation, therapeutic approaches aiming to block metal binding by peptides or small-molecule inhibitors thus entail the risk of affecting LPS-induced dimerization and hence bacterial responsiveness [8]. We thus explored the possibility to take advantage of their different MD2 requirement for TLR4 dimerization. We generated a soluble form of TLR4 (sTLR4) encompassing its ectodomain (amino acids 27–627) fused N-terminally to the signal sequence of the Ig κ -chain to direct it to the secretory pathway [23]. Transfection of this mutant into HEK293 cells results in MD2-independent release of sTLR4

into the culture supernatant (Fig 4A). Remarkably, addition of conditioned medium containing MD2-free sTLR4 significantly decreased Co^{2+} - or Ni^{2+} -induced IL-8 production when transferred to HEK293 TLR4/MD2 (Fig 4B) or HUVEC recipient cells (Fig 4C) whereas LPS-induced IL-8 expression was unaffected. The observed inhibition required both metal-binding and dimerization capacity as neither sTLR4 H456A/H458A nor sTLR4 N433A was capable to block metal-induced IL-8 production in parallel experiments (Fig 4B, C). Hence, inhibition might rely on chelation of free metal ions by sTLR4 homodimers that form in the absence of free MD2.

Overall, our data validate TLR4 crosslinking as common mechanism of Co^{2+} - and Ni^{2+} -induced innate immune activation. Remarkably, unlike LPS, metal-induced TLR4 dimerization did not require MD2. While this might open new therapeutic opportunities as inferred by our sTLR4 blocking experiments, it also raises questions about the role of MD2 in metal-induced TLR4 activation. As MD2 was clearly indispensable for metal-induced proinflammatory gene expression, MD2 might perhaps serve to

Fig 3 | Metal-induced proinflammatory gene expression involves TLR4 dimerization and is initiated independently of MD2. HEK293 MD2 cells (A and C) or HEK293 WT cells (B) were co-transfected with the indicated combinations of HA- or FLAG-tagged TLR4 or TLR4 N433A constructs and stimulated with Ni²⁺, Co²⁺ or LPS for 30 min. Cell lysates were immunoprecipitated with α-FLAG antibody and formed complexes visualized by immunoblot with an α-HA antibody. Membranes were re-probed with α-FLAG antibody to confirm equal immunoprecipitation. α-HA and α-FLAG western blots with total lysate are shown to illustrate similar transfection efficiencies. Immunoblots are representative of three to four independent experiments. (D) Analysis of metal- or LPS-induced IL-8 production in supernatants from HEK293 MD2 cells transfected with HA-TLR4 or HA-TLR4 N433A, respectively. Cells were stimulated with Ni²⁺, Co²⁺ or LPS for 8 h. Data represent means ± s.d. of three independent experiments. ****P* < 0.001, *t*-test. HA, haemagglutinin; IL, interleukin; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; WT, wild-type.

Fig 4 | Soluble TLR4 can block Ni²⁺/Co²⁺-induced IL-8 production. (A) Western blot analysis of the indicated HA-tagged TLR4 proteins in supernatants and total lysates from transfected HEK293 WT cells. Data demonstrate MD2-independent release of truncated sTLR4 into the culture supernatant. (B–C) IL-8 enzyme-linked immunosorbent assay of HEK293 TLR4/MD2 cells (B) or HUVEC (C) incubated with undiluted supernatants produced by HEK293 WT cells transfected with the indicated constructs. Supernatants were taken 48 h after transfection with the indicated TLR4 constructs and freshly supplemented with diluent (ctrl), Ni²⁺, Co²⁺ or LPS before addition to the respective recipient cells and incubated for 8 h. Data represent means ± s.d. of three independent experiments. ***P* < 0.01, *t*-test. HA, haemagglutinin; HUVEC, human primary umbilical vein endothelial cells; IL, interleukin; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; WT, wild-type.

further shape the metal-bound TLR4 receptor complex to allow effective signal initiation. Future structural studies should resolve this issue.

From the clinical perspective, our experiments not only promise new treatment opportunities using sTLR4 variants or sTLR4-derived peptides but also warrant caution about strategies directly targeting metal binding to inhibit metal-induced hypersensitivity

responses as such efforts are prone to affect LPS-induced TLR4 dimerization and bacterial responses as well.

METHODS

Cell culture. HUVEC and NHEK were obtained from Promocell and cultured as described [16,17]. HEK293 lines (InvivoGen) and murine RAW 264.7 macrophages were cultured in DMEM GlutaMAX (Gibco) supplemented with 10% FCS. Human THP-1 monocytes were grown in RPMI 1640 GlutaMAX (Gibco) containing 10% fetal bovine serum and 1 mM sodium pyruvate. Human iDCs were generated from CD14⁺ monocytes isolated from single donor blood as detailed in the Supplementary Information.

Reagents. CoCl₂·6H₂O (Sigma) and NiCl₂·6H₂O (Merck) were routinely used at 1.5 mM. Unless indicated, LPS R595 from *S. minnesota* (Alexis) was used and applied at 1 µg ml⁻¹.

Plasmids and transient gene transfer. Expression vectors for mouse and human MD2, HA-TLR4, HA-Tlr4, HA-TLR4 H456A/H458A and HA-Tlr4 Y454H/N456H have been described [10,24,25]. CMV1 FLAG-TLR4 was provided by Tularik (CA). FLAG-TLR4 N433A, HA-TLR4 N433A and the expression vectors for the different soluble variants of the TLR4 ectodomain (amino acids 27–627) (sTLR4, sTLR4 N433A and sTLR4 H456A/H458A) were generated from CMV1 FLAG-TLR4, pDisplay HA-TLR4 [25] or pDisplay HA-TLR4 H456A/H458A [10] by single or sequential site-directed mutagenesis (Quick-change II site-directed mutagenesis kit, Agilent) using codon-specific TLR4-complementary mismatch primers. Plasmids were transfected using calcium phosphate.

Promoter reporter gene studies. HEK293 cells were transfected with 6 × κB.luc and ubiquitin-dependent *Renilla* luciferase, and ratios of *Firefly* and *Renilla* luciferase activity were analysed using the DualGlo Luciferase Assay System (Promega) [17].

Detection of cytokines. Culture supernatants were assayed for human IL-8 and mouse TNF using enzyme-linked immunosorbent assay kits from BD according to the manufacturer's instructions.

Western blot. Western blots were performed as described [26] and proteins detected by ECL using adequate primary antibodies (α-IL-8 (G265, BD Biosciences); α-FLAG (600-401-383, Rockland); α-HA (3F10, Roche); α-tubulin (T5168, Sigma)) and horseradish peroxidase-coupled secondary antibodies.

Co-IPs. Transfected HEK293 and HEK293 MD2 cells expressing the different FLAG/HA-TLR4 proteins were lysed in E1A lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40 and 5 mM EDTA) freshly supplemented with 20 mM sodium glycerophosphate, 100 nM sodium orthovanadate, and complete protease inhibitor mixture (Roche) and 1.5 µg total lysate subjected to IP with mouse α-FLAG M2 (F3165, Sigma) antibody overnight at 4 °C. Immune complexes were precipitated with protein-G-agarose (Roche, 1 h, 4 °C), washed three times with high salt E1A lysis buffer containing 500 mM sodium chloride and visualized by western blot with α-HA or α-flag antibodies as described above.

Quantitative real-time PCR. qRT-PCR was performed as described [26] using commercially available Taq-Man probes from Invitrogen (TLR4: Hs00152939_m1; MD2: Hs00209770_m1; GAPDH: Hs99999905_m1; Loricrin: Hs01894962_s1). Test gene expression was normalized to the expression of the housekeeping gene *GAPDH* and related to an experimental control using the 2^{-ΔΔC(T)} method.

RNA interference. Scrambled siRNA (used as control) and validated siRNAs targeting TLR4 or MD2 (S1100151004, S103246446, Qiagen) were diluted to 200 nM final concentration and introduced into HUVEC using Oligofectamine (Invitrogen) as described [26].

Statistical analysis. At least three independent experiments were performed to calculate averages and s.d. Statistical significance was analysed by unpaired Student's *t*-test. *P*-values of <0.05 (*) were interpreted as significant and those >0.05 as nonsignificant (NS). Higher significances are designated accordingly (***P*<0.01, ****P*<0.001).

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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