

Methods to Investigate the Role of Toll-Like Receptors in Allergic Contact Dermatitis

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Abstract

Allergic contact disease is a common inflammatory skin disease resulting from hyperresponsiveness to harmless nonprotein substances such as metals, fragrances, or rubber. Recent research has highlighted a prominent role of Toll-like receptors, particularly TLR4 in contact allergen-induced innate immune activation that crucially contributes to the pathogenesis of this disease.

Here we describe several methods to investigate the role of Toll-like receptors in contact allergen-induced pro-inflammatory responses. These include expansion of disease-relevant human primary cells including endothelial cells and keratinocytes and their manipulation of TLR signaling by transfection, retroviral infection and RNA interference, basic methods to induce contact hypersensitivity in mice, and protocols for adoptive transfer of hapten-stimulated dendritic cells and T cells from TLR-deficient mice to wild-type mice and vice versa wild-type mice to TLR-deficient mice in order to explore cell-specific roles of TLRs in contact hypersensitivity responses.

Key words Allergic contact dermatitis, Contact hypersensitivity, TLR4, Hapten, Metal allergen

1 Introduction

Allergic contact dermatitis (ACD) is one of the most prevalent skin disorders. It is triggered by epicutaneous contact with low molecular weight (<500 Da) chemicals (haptens) that act as allergens [1, 2]. More than 4000 defined substances are known that are capable of inducing ACD [3]. Prominent examples are nickel (a frequent component of fashion jewelry and many consumer products) or fragrances. For studying the mechanisms of contact allergy, animal models have been extremely useful, especially investigating contact hypersensitivity (CHS), the mouse equivalent of ACD. Development of ACD (and CHS) requires a sensitization phase during which haptens are processed and presented by antigen-presenting dendritic cells (DCs) to prime for an adaptive T lymphocyte-mediated immune response; this phase is clinically inapparent. Upon reexposure to the same hapten, the so-called elicitation phase is initiated,

in which hapten-specific T lymphocytes locally proliferate in the skin [4] and orchestrate a clinically visible inflammation in dermis and epidermis. Belonging to the delayed-type (type IV) hypersensitivity diseases, the clinical signs of hypersensitivity typically emerge late after exposure, i.e., ~72 h after hapten contact in sensitized humans or after ~24–48 h in sensitized mice, respectively. They manifest as characteristic erythematous, scaling and itchy skin lesions, clinically known as eczema (dermatitis). Pathophysiologically, these result from cytotoxicity and inflammation triggered by a complex interplay of locally activated skin-resident cells including keratinocytes, endothelial cells (EC) and DCs as well as locally proliferating and infiltrating leukocytes [5, 2]. The latter most importantly comprise cells of the adaptive immune system such as hapten-specific cytotoxic T (Tc) cells and different T helper (Th) populations but also include innate immune cells such as neutrophils and macrophages that aggravate the inflammatory response.

While ACD and CHS have long been considered to be entirely dependent on the adaptive immune system it has recently become clear that generation of adequate innate immune signals is a prerequisite for initiation of both the sensitization and elicitation phase [1]. These can either be delivered by direct stimulation of innate immune receptors by the hapten itself, or result from indirect activation of membrane-bound or cytosolic pattern recognition receptors (PRR) by damage-associated molecular patterns (DAMPs) that are released in response to metabolism or hapten reactivity against endogenous cell components, respectively [1, 6]. An important consequence of hapten-induced innate immune activation is the stimulation and mobilization of hapten-loaded DCs to the skin-draining lymph node where they encounter and educate naïve T cells to respond to a given hapten [5]. Additionally, hapten-induced innate immune activation results in release of pro-inflammatory cytokines such as IL-1 by keratinocytes and licences local ECs to express various cell surface receptors and chemokines that support recruitment of leukocytes from the blood into the skin [1]. Thus, innate immune activation of skin resident cell populations is crucial for development of ACD.

Recently, evidence has accumulated that Toll-like receptors (TLR) play a key role in hapten-dependent innate immune activation and CHS induction. The first hint came from the observation that TLR2/4 double-deficient mice were protected from CHS induction by the model hapten 2,4,6-trinitro-1-chlorobenzene (TNCB), suggesting a critical role of those two PRRs in TNCB-induced CHS [7]. The indispensable requirement of TLR2 and TLR4 likely relies on TNCB-induced oxidation and enzymatic degradation of the extracellular matrix component hyaluronic acid triggering release of low-molecular hyaluronan [7, 8], which acts as DAMP for TLR2 and TLR4 [9–11] and induces the necessary innate immune activation for efficient T-cell priming.

In contrast to indirect innate immune activation via DAMPs, the metal allergens nickel, cobalt, and recently also palladium were shown to trigger innate immune activation via direct stimulation of TLR4 signaling [12–14]. In case of nickel and cobalt this requires the presence of two non-conserved histidine residues, H456 and H458, at the dimerization interface of human TLR4 [12, 13]. Metal binding to those residues supports cross-linking of two TLR4 receptor dimers as demonstrated by co-immunoprecipitation experiments with differently tagged TLR4 receptors exogenously expressed in TLR4-deficient HEK293 cells [13]. Notably, mouse TLR4 lacks equivalents of the above-mentioned metal-responsive histidines H456 and H458. As a result, wild-type mice are insensitive to nickel-induced TLR4 activation and fail to induce a significant innate immune signal and CHS upon nickel treatment [12]. However, the missing innate immune signal in mice can be restored by transgenic expression of human TLR4 [12], co-treatment with the natural TLR4 agonist lipopolysaccharide (LPS) [15], or co-incubation with other adjuvants [16] allowing CHS induction by nickel. This provides evidence that (a) innate immune activation is crucial for nickel-induced CHS but can be replaced by alternative TLR4/PRR activation and (b) that reconstitution with human TLR4 is sufficient to restore responsiveness to nickel in mice [17].

TLR-deficient cells sustaining intact TLR-dependent intracellular signaling circuits provide valuable tools to analyze direct stimulatory effects of haptens on TLR-mediated pro-inflammatory gene expression, as they are amenable for reconstitution experiments. A well-established human cell line suitable for reconstitution experiments with TLRs and other innate immune receptors is the human embryonic kidney cell line HEK293. This cell line has the advantage that it is deficient for most TLRs including TLR2 and TLR4 [18], is easy to cultivate and manipulate, and expresses enormous amounts of exogenously expressed proteins, which facilitates, e.g., co-immunoprecipitation experiments. Another extremely useful primary epidermal cell for contact allergy research are normal human foreskin epithelial keratinocytes (NHEK), which express a variety of endogenous TLR receptors as well as the TLR4 co-receptor MD2 but are deficient for TLR4 under undifferentiated, differentiated, and inflammatory conditions [13], respectively. Albeit more challenging with respect to culture conditions and manipulation, these cells can be transiently transfected to rates up to 50 % with appropriate reagents and are thus likewise well suited for reconstitution experiments with TLR4 [13]. Moreover, manipulated NHEKs can be used for in vitro 3D skin reconstitution models, which can be transplanted onto mice for further analysis.

Other useful primary cells for contact allergy research include endothelial cells. Vessel endothelium is among the first to respond

to hapten challenge in vivo and is highly responsive to proinflammatory activation by various stimuli including LPS, TNF, and IL-1 β [12, 17]. Since microvascular dermal endothelial cells are difficult to isolate human umbilical vein endothelial cells (HUVEC) are frequently used as substitutes. HUVEC endogenously express TLR4 along with MD2 as well as intracellular TLR3, but show no functional expression of other surface TLRs [19] making them an ideal model to test hapten-induced TLR4 activation. Similarly to NHEKs they are commercially available from various suppliers but unlike those are relatively easy to cultivate and can well be manipulated via small-interfering RNA (RNAi) as well as by retroviral gene transduction in order to interfere with TLR4 signaling [12].

As mentioned above, wild-type mice and mouse cells are poor models to study metal-induced CHS and innate immune activation due to the failure of mouse TLR4 to respond to metal haptens [12, 13]. However, this deficit turned out to be extremely handy to dissect the exact molecular requirements for metal-induced TLR4 activation since ex vivo isolated primary cells from wild-type mice could be used as reconstitution system to test the responsiveness of human TLR4 mutants to nickel-induced proinflammatory activation [12]. Moreover, the availability of human TLR4-transgenic *Tlr4*^{-/-} mice opens the unique opportunity to analyze the relative contributions of distinct lymphocyte or DC populations to metal-induced CHS in adoptive transfer experiments in which purified cell populations from human TLR4-transgenic *Tlr4*^{-/-} donor mice are transferred to wild-type or TLR4-deficient receptor mice.

The methods described below are categorized into two parts addressing different aspects of hapten-induced TLR activation: The first one deals with methods to analyze direct TLR activation and dimerization by haptens in human primary cells and HEK293 cells. In the second part we elaborate on methods of CHS-induction by haptens and provide methods for the in vitro generation of DCs and isolation of T cells for adoptive transfer. The latter is particularly designed to test the role of distinct cell populations isolated from hapten-sensitized donor mice with genetic alteration in TLR signaling components for the induction of CHS responses in nonresponsive wild-type or TLR-deficient recipient mice.

2 Materials

2.1 Cell Culture

1. NHEK and HUVEC cells from single donors (Promocell) (*see Note 1*).
2. Wild-type HEK293 and HEK293 stably expressing human MD2 and CD14 (HEK293-MD2/CD14) (Invivogen).
3. ϕ NX amphi virus producer cells [20] (The National Gene Vector Biorepository).

4. FLYRD18 human fibrosarcoma virus producer cells [21] (Sigma).
5. Culture medium for HEK293 cells, ϕ NX amphi, and FLYRD18: Dulbecco's modified Eagle's Medium (DMEM) GlutaMAX I™ with high glucose (Life Technologies) supplemented with 10 % fetal calf serum (FCS; GE Healthcare) (*see Note 2*).
6. HUVEC culture medium: mix 1 part endothelial growth medium (EGM™; Lonza) as specified under (a) and 2 parts of medium 199 Earle's Salts GlutaMAX I™ (Life Technologies) as detailed under (b).
 - (a) EGM: Endothelial basal medium (500 ml) is supplemented with EGM SingleQuot Supplements and Growth Factors (Lonza) as follows: 10 ml FBS, 0.5 ml of human epidermal growth factor, 0.5 ml hydrocortisone, 0.5 ml gentamycin/amphotericin B, and 2 ml of bovine brain extract with heparin.
 - (b) M199 medium with Earle's Salts GlutaMAX™ supplemented with 10 % FCS, 30 μ g/ml gentamycin, 15 μ g/ml amphotericin B, and 0.8 IU/ml heparin.
7. NHEK culture medium: A 1:1 mix of serum-free keratinocyte growth medium (KSFM) containing 50 μ g/ml bovine pituitary extract (BPE) and 5 ng/ml hEGF (Life Technologies) and calcium-free Eagle's Minimum Essential Medium (EMEM; Lonza) without supplementation. The final calcium chloride concentration of the mixture medium is 0.05 mM, which impedes spontaneous differentiation and keeps cells in optimal proliferative condition [22].
8. Chelex 100 Resin (analytical grade, 100–200 mesh; Bio-Rad), for removal of calcium from FCS in order to avoid calcium-induced differentiation of NHEK keratinocytes upon subculture.
9. Phosphate buffered saline (PBS).
10. Trypsin/EDTA (0.05 %/0.02 % in sterile PBS).
11. 0.05 % EDTA pH 8.0 (sterile filtered).
12. DMSO.
13. FCS or Chelex-treated FCS (in case of NHEK) for passaging and freezing of cells.
14. Antibiotics for selection: hygromycin (50 μ g/ml) for re-selection of stable HEK293-MD2/CD14; hygromycin (300 μ g/ml) and diphtheria toxin (1 μ g/ml) for co-selection of retroviral *gag/pol* and *env* genes stably expressed by ϕ NX amphi cells; Blasticidin S (4 μ g/ml) and Zeozin (Phleomycin, 10 μ g/ml) for re-selection of viral packaging genes of FLYRD18; puromycin (2 μ g/ml) for selection of stable viral producers

containing retroviral expression constructs for dominant-negative signaling components or small hairpin RNA (shRNA).

15. Metal salt stock solutions for stimulation:
 - (a) 150 mM Nickel Chloride ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, ACS grade; Merck Millipore), in aqua ad injectabilia (pyrogen-free water), sterile filtered and used at a final concentration of 1.5 mM.
 - (b) 150 mM Cobalt chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, premium quality; Sigma), in aqua ad injectabilia (pyrogen-free water), sterile filtered and used at final concentration of 1.5 mM.
16. LPS as positive control for TLR4 stimulation:
 - (a) Smooth LPS *E. coli* Serotype O55-B5 (TLR_{grade}; Enzo), used at 1 $\mu\text{g}/\text{ml}$.
 - (b) Rough LPS: LPS *E. coli* Serotype R515 or LPS *Salmonella minnesota* R595 (TLR_{grade}; Enzo), used at 1 $\mu\text{g}/\text{ml}$.
17. Polymyxin B-sulfate (PMB; Sigma) used at 50 $\mu\text{g}/\text{ml}$ to control for endotoxin contamination of metal solutions.

2.2 Transfection

1. Oligofectamine™ and OptiMEM medium (Life Technologies) for transfer of small interfering RNA (siRNA) into HUVEC.
2. Scrambled siRNA and validated siRNA directed against TLR signaling compounds commercially available (Qiagen or Life Technologies).
3. Retroviral vectors pBABE puro [23] and pRetro Super (pRS) [24] with and without gene insert for generation of stable ϕNX amphi and FLYRD18 retroviral producer cell lines producing amphotropic retroviruses for expression of dominant-negative signaling components or shRNA directed against TLR signaling-relevant genes in primary HUVEC.
4. Polybrene (5 mg/ml in aqua destillata; Sigma).
5. 0.8 μm syringe filter (Sartorius) for filtration of virus supernatant.
6. Puromycin (10 mg/ml in aqua destillata; AppliChem). Use at 2 $\mu\text{g}/\text{ml}$ for selection of positive integration of retroviral constructs.
7. FuGENE HD® (Roche) and Lipofectamin® 2000 (Life Technologies) transfection reagents.

2.3 Co-immunoprecipitation

1. HA-tagged human TLR4 (pDisplay-HA-TLR4 [25]), FLAG-tagged TLR4 (pCMV-FLAG-TLR4) (Tularik Inc.), and mutagenized variants thereof [12, 13].
2. E1A Lysis Buffer (ELB): 150 mM Sodium chloride, 50 mM HEPES, pH 7.5, 5 mM EDTA, 0.1 % NP-40 in dd H_2O . Before use, freshly add 20 mM β -Glycerophosphate (as protein threonine phosphatase inhibitor), 0.5 mM sodium-ortho-vanadate

- (as protein tyrosine phosphatase inhibitor) [26], and 1× Complete® protease inhibitor (Roche) to obtain ELB⁺⁺⁺.
3. Mouse α-FLAG® (M2; Sigma) and Mouse α-HA (12CA5; Abgent) antibodies for immunoprecipitation used at 0.5 or 1 µg/ml, respectively.
 4. Protein G Agarose (Roche).
 5. 2× SDS-sample buffer: 125 mM Tris-HCl, pH 6.8, 4 % Sodium dodecyl sulfate (SDS), 20 % Glycerol, 5 % β-Mercaptoethanol, 0.005 % Bromophenol blue in dd H₂O.
 6. Rat α-HA (high affinity) (3F10; Roche) and Rabbit α-FLAG antibody (Rockland) used for Western blotting at 50 ng/ml or 0.5 µg/ml, respectively.
 7. Secondary horseradish peroxidase- (POD-)coupled antibodies: α-rat-POD (1:2000; Santa Cruz), α-rabbit-POD, and α-mouse POD (1:2000; GE Healthcare).
 8. Enhanced chemiluminescence (ECL) detection buffer. Freshly prepared 1:1 mixture of:
 - (a) Solution A: 0.1 M Tris-HCl, pH 8.5, freshly supplemented with 2.5 mM Luminol and 0.4 mM p-coumaric acid.
 - (b) Solution B: 0.1 M Tris-HCl, pH 8.5, freshly supplemented with 0.18 % H₂O₂.
 9. Antibodies for Western blots: mouse α-(human) IL-8 (G265-8) (1 µg/ml; BD Pharmingen), mouse α-alpha-Tubulin used as loading control (B-5-1-2) (1:10,000; Sigma).
 10. ELISAs: BD OptEIA human IL-8 ELISA Set and OptEIA human MCP1 (CCL2) ELISA Set (BD Pharmingen).
 11. FACS antibodies: mouse α-(human) IL-8 (G265-8) (1:100; BD Pharmingen), Mouse α-(human) ICAM1 (84H10) (1:100; Beckman-Coulter), mouse anti-human E-selectin (1.2B6) (1:100; Merck Millipore), and mouse α-(human) VCAM1 (1.G11B1) (1:100; AbD Serotec).

2.4 CHS Responses

1. Inbred mice: wild-type mice (C57BL/6, C57BL/10, or other), mutant mice (e.g., TLR-deficient) on the same genetic background, 6–8 weeks of age from an SPF facility.
2. Electric shaver to remove hair from abdominal skin.
3. Contact allergens: TNCB (Sigma), dissolve in acetone prior to use; 2,4,6-trinitrobenzene sulfonic acid (TNBS; Sigma), prepare 3 mM stock in PBS and adjust pH to 7.4, store aliquots at –20 °C; NiCl₂ (NiCl₂·6H₂O; ACS grade; Merck Millipore) prepare 1, 5, and 10 mM stock solutions in 0.9 % NaCl, store at 4 °C.

4. Irritant: croton oil (Sigma).
5. LPS *E. coli* Serotype O55-B5 (TLR_{grade}; Enzo), prepare 1 µg/ml stock solution.
6. 20, 200, and 1000 µl pipettes and pipette tips.
7. Thickness gauge, e.g., from Käfer Messuhrenfabrik GmbH, Villingen-Schwenningen, Germany.
8. 1 ml syringes with 0.3 mm × 12 mm needles for intracutaneous dendritic cell injections or 0.45 × 12 mm for i.v. injections.
9. RPMI medium: RPMI-1640 supplemented with 10 % heat-inactivated fetal calf serum (FCS), 2 mM l-glutamine, 25 mM HEPES buffer, 50 µg/ml penicillin-streptomycin (all from Life Technologies), and 10 µM 2-mercaptoethanol (2-ME) (Sigma).
10. PBS.
11. Acetone.

3 Methods

3.1 Cell Culture

All cell culture works are carried out under sterile conditions (under laminar air flow, using sterile solutions and media, sterile consumables). All primary cells and cell lines are grown at 37 °C, 5 % CO₂ and high humidity and should regularly be tested for negativity of mycoplasma infection.

Primary human NHEK and HUVEC cells from single donors can either be isolated freshly from donor material or purchased from a commercial supplier (e.g., Promocell) as frozen cell aliquots (passage 1 or 2 for HUVEC or NHEK, respectively) for expansion (*see Note 1*).

Below we provide a protocol for expansion of primary human NHEK keratinocytes or endothelial cells.

1. Thaw frozen aliquots of NHEK or HUVEC (containing $\sim 5 \times 10^5$) cells as quickly as possible at 37 °C in a water bath (requires about 90 s) and add about 1 ml of the appropriate pre-warmed medium (*see Subheading 2.1*) to gently resuspend cells.
2. Transfer immediately to one T175 flask (NHEK) or three T75 flasks (HUVEC) containing at least the tenfold volume of pre-warmed medium (*see Note 3*).
3. At 70–80 % density (this takes about 5 days) wash cells twice with pre-warmed PBS.
4. Pre-incubate for 12 min in 0.05 % sterile EDTA, pH 8.0, followed by a short trypsin/EDTA (0.05 %/0.02 % in sterile PBS) treatment (NHEK) or trypsinize directly (HUVEC) to detach cells from the dish (*see step 5*).

5. Add trypsin/EDTA carefully to the culture vessel and swirl gently to cover the cells.
6. Remove Trypsin/EDTA solution immediately and incubate cells briefly at 37 °C (for no longer than 3 min!).
7. Neutralize trypsin by addition of 5 ml of 10% FCS (HUVEC) or 10 % Chelex-treated FCS (NHEK) (*see Note 4*).
8. Rinse cells gently off the culture vessel, and transfer to 15 ml tubes for centrifugation at $250\times g$ (~1100 rpm in a common table centrifuge) to obtain cell pellets.
9. After resuspension in fresh medium seed cells onto 18 T175 culture flasks (HUVEC) or count and seed at a density of ~3000 cells/cm² onto new culture vessels (NHEK).
10. Culture cells with media replacement every second day until they reach 70–80 % confluency (after about 4–6 days).
11. Detach cells as described above, pool and count cells prior to distribution into 50 ml tubes and centrifugation at $250\times g$.
12. Gently resuspend in an appropriate amount of ice-cold freezing medium containing 10 % FCS (for NHEK: 10 % Chelex-treated FCS), 10 % DMSO, and 80 % of the respective growth medium and distribute cells to precooled cryowells at portions of 5×10^5 , 1×10^6 , 2×10^6 , and 4×10^6 cells.
13. Put cryowells into styroracks and place at –80 °C for freezing.
14. After 2–3 days transfer the frozen cell aliquots to liquid nitrogen for long-term storage (*see Note 5*). For experiments or manipulation an appropriate amount of cells (*see Subheading 3.2* below) is thawed and either used directly (HUVEC) or passaged once prior to use (NHEK).

3.2 Transfection

Below we describe three transfection methods to manipulate human primary cells for analysis of TLR-dependent signaling: (a) knockdown of essential signal transducers of TLR signaling by siRNA transfection (*see Subheading 3.2.1*), (b) retroviral expression of dominant-negative signaling mediators or shRNA constructs (*see Subheading 3.2.2*), or (c) TLR4 reconstitution experiments by transient transfection (*see Subheading 3.2.3*).

3.2.1 siRNA Transfection

1. Seed expanded HUVEC (passage 3) onto 6-well dishes at a density of 1.2×10^5 cells/well.
2. On day 2, check cell morphology and density by phase contrast microscopy. If cells have reached the desired cell density of ~70–80 % proceed with **step 3**, otherwise grow cells for one more day. Discard altogether if cultures show increased cell death or do not reach the above specified density by Day 3.

3. Dilute 10 μ l of a 20 μ M siRNA oligo solution (*see Note 6*) directed against a TLR4-relevant signaling component such as MD2 and in parallel a scrambled siRNA (as negative control) with each 90 μ l prewarmed OptiMEM (without supplements and FCS) and for each siRNA 10 μ l Oligofectamine™ with 90 μ l OptiMEM (*see Note 7*) in separate polypropylene tubes.
4. Incubate the solutions for 10 min at room temperature.
5. Add the RNAi-containing solutions to the respective tubes containing the Oligofectamine™ dilution and mix gently by tapping.
6. Incubate another 30 min and then add 800 μ l OptiMEM (without supplements) to obtain 1 ml solution for each transfection.
7. Wash cells twice with prewarmed OptiMEM medium and then add the Oligofectamine™/RNAi mixture directly to the cells (final concentration of the RNAi oligo is 200 pmol/ml).
8. Incubate for 4 h at 37 °C, then replace by fresh culture medium.
9. After about 2–3 days (*see Note 8*) stimulate cells with a hapten such as 1.5 mM NiCl₂ or CoCl₂ for 5 h to test the direct effect of a hapten on proinflammatory gene expression at RNA level by quantitative real-time PCR or for 8–16 h if protein-based readouts such as ELISA, Western blot, or FACS are used [12, 13]. Typical readout genes expressed by HUVEC are the chemokines CXCL8 (IL-8) and CCL2 (MCP-1) and the surface receptors E-selectin, ICAM or VCAM-1 as indicators of IKK2/NF κ B-dependent gene expression [27] and CCL5 (RANTES) and CXCL10 (IP-10) as genes regulated by TLR4 via the TRAM/TRIF pathway [28, 29].

3.2.2 Retroviral Infection

HUVEC are rather difficult to transfect by conventional methods but can well be manipulated by retroviral gene expression, which usually yields transfection rates of >90 % (*see Note 9*). In order to produce the appropriate retrovirus required, we employ a stable amphotropic ϕ NX cell line (for overexpression of dominant-negative signaling components) or stable high-titre producing FLYRD18 cell lines (for shRNA constructs) (*see Note 10*) established by standard methods as described elsewhere [30]. The following protocol describes the procedure using an established ϕ NX producer cell line for dominant-negative IRAK1 (IRAK1 dn) or an FLYRD18 cell line for shRNA-expressing IRAK1.

1. On day 1, seed amphotropic ϕ NX producer cells with stable integration of a retroviral expression vector (e.g., pBABE puro [23]) for a gene of interest (e.g., IRAK1 dn) or FLYRD18 producer cells stably expressing a specific shRNA construct (e.g., pRS-IRAK1) along with matched producer lines for the

respective empty retroviruses at densities of $\sim 3.5 \times 10^4$ cells/cm² (ϕ NX amphi) or 1×10^4 /cm² (FLYRD18) into DMEM containing 10 % FCS and a selection antibiotic for the integrated retroviral backbone (e.g., puromycin at a concentration of 2 μ g/ml). Choose appropriate culture vessel sizes corresponding to the amount of HUVEC cells to be infected (e.g., for infection of each 10 cm dish of HUVEC seed one 10 cm dish containing 2×10^6 ϕ NX amphi or 6×10^5 FLYRD18 cells).

2. On day 2, replace medium of producer cultures by fresh medium lacking antibiotics (*see* **Note 11**).
3. Thaw appropriate amounts of HUVEC from a frozen aliquot (*see* **Note 3**) and seed them at densities of 4300–5200 cells/cm² into HUVEC-Mix (for an experiment with three stimulation conditions as shown in Fig. 1 typically seed one 10 cm dish with $2.5\text{--}3 \times 10^5$ cells per infection). Seed one additional dish to control for efficiency of the employed selection antibiotic used at later steps for enrichment of infected HUVEC (optional).
4. On day 3, replace medium of producer cell lines by an appropriate volume of HUVEC-Mix (e.g., add 6 ml to a 10 cm dish) for the first virus production. Change medium from HUVEC cultures to remove traces of DMSO and sustain HUVEC under optimal growth conditions.
5. In the morning of day 4, pipet virus supernatants from the different producer cell lines into separate 50 ml tubes (for infection of multiple dishes pool supernatants containing identical viruses to ensure equal infection efficiency) and immediately add fresh HUVEC-Mix to the producer cells for production of the second virus harvest (*see* below).
6. Filtrate the virus supernatant through a sterile 0.8 μ m syringe filter (*see* **Note 12**) into a new 50 ml tube and add Polybrene at a final concentration of 5 μ g/ml to facilitate infection.
7. Invert several times to mix, aspirate medium from the endothelial cells and immediately add the virus solution.
8. Incubate at 37 °C (in a CO₂ incubator) for 6 h and repeat **steps 5–7** for the second infection in the evening. Keep second virus supernatant on the cells overnight and add HUVEC-Mix to the producer cells for a third harvest (*see* **Note 13**).
9. On day 5, perform a third infection as described in **step 8**. Discard producer cells and incubate HUVEC at 37 °C for 6 h before replacing the virus supernatant with fresh HUVEC-Mix (8 ml per 10 cm dish).
10. On day 7–8, start with the selection procedure for positive infection using an appropriate antibiotic (e.g., add 2 μ g/ml puromycin to the cells for 16–18 h) (*see* **Note 14**).

11. On day 8–9, reseed the infected HUVEC cells at a density of 6000 to maximally 15,500 cells/cm² according to the requirements of your experiment. For instance, in order to test the impact of shRNA expression on hapten-induced expression of pro-inflammatory genes on RNA level seed approximately 1 × 10⁵ cells/well on 6-well dishes and stimulate cells the following day for 5 h with a hapten (e.g., 1.5 mM NiCl₂) along with a positive control for TLR activation (e.g., 1 µg/ml LPS from *Salmonella minnesota* for TLR4 activation in HUVEC) and a diluent control [13] (see **Note 15**). For protein-based readouts such as Western blot or FACS for IL-8 [12] seed cells into 10 cm dishes (e.g., 5 × 10⁵) and stimulate for 8–16 h the following day. An exemplary knockdown experiment in HUVEC using retrovirally expressed shRNA for IRAK1 and nickel-induced IL-8 expression as readout is shown in Fig. 1.

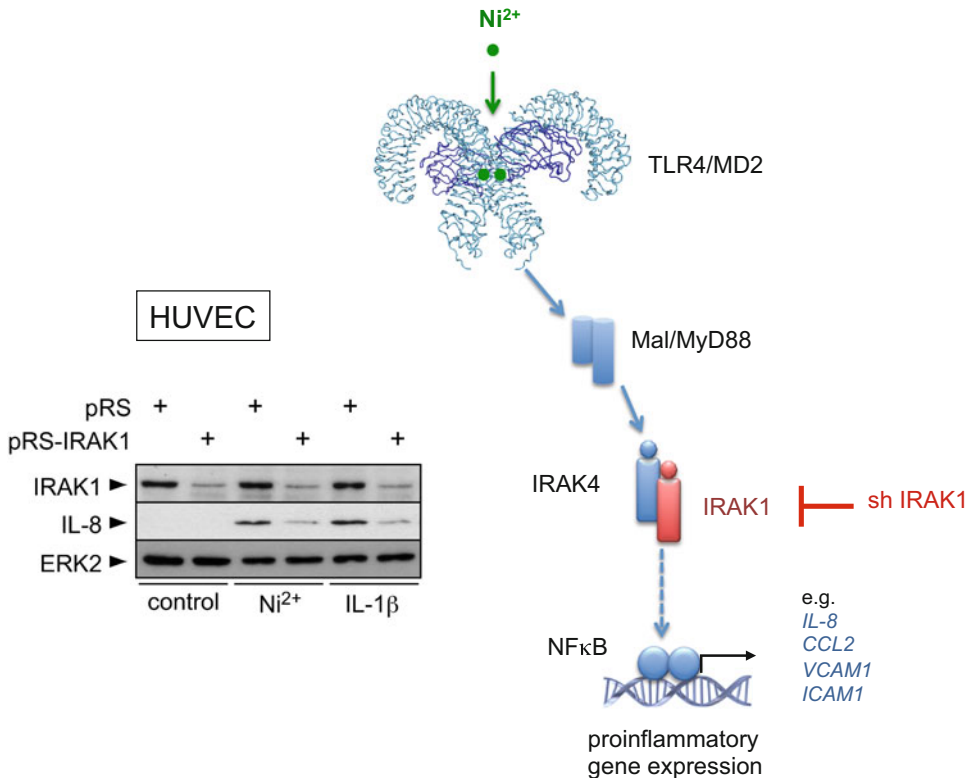


Fig. 1 Retrovirus-mediated knockdown of IRAK1 demonstrates decreased nickel responsiveness in HUVEC cells. HUVEC were retrovirally infected with either an empty retrovirus (pRS) or an shRNA construct for IRAK1 (pRS-IRAK1) and 96 h post infection stimulated for 8 h with the TLR4-activating metal allergen nickel (Ni²⁺) or IL-1 (as positive control), respectively. Western blot analysis demonstrates a reduction of nickel-induced IL-8 production in IRAK1-depleted cells. The schematic shows the central position of IRAK1 downstream of TLR4 signaling and illustrates the proposed mechanism of nickel-induced TLR4 activation by triggering TLR4/MD2 heterodimers [12, 13]

3.2.3 TLR4 Reconstitution

We provide a procedure to test hapten responsiveness upon reconstitution of TLR4 or a point-mutated TLR4 variant into NHEK cells, which lack detectable TLR4 expression but express endogenous MD2 [13].

1. Recover NHEK cells from frozen stocks (*see Note 3*) and plate at a density of $\sim 1.7 \times 10^4/\text{cm}^2$ (e.g., 1×10^6 cells onto a 10 cm dish).
2. Passage cells once prior to seeding at a density of 1×10^5 cells per well into 6-well culture dishes for transfection.
3. After 1–2 days (cells should be 70–80 % dense) transiently transfect cells with each 2 μg total DNA and 12 μl of FuGENE[®] HD reagent (Roche) as follows:
4. Replace medium by fresh growth medium lacking antibiotics.
5. Prepare two polypropylene tubes per transfection and stimulation condition (for instance to test the responsiveness of a TLR4 point mutant in comparison to wild-type TLR4 and empty vector, prepare six tubes per stimulation condition).
6. To the first tube add 2 μg of the DNA solution to be transfected (*see Note 16*); to the second one add 12 μl FuGENE[®] HD to 88 μl calcium-free EMEM (without supplements) (*see Note 7*).
7. Incubate for 5 min and then add the FuGENE[®] HD solution dropwise to the DNA under gentle shaking.
8. Incubate for another 15 min at room temperature.
9. Add transfection mixture dropwise to the cells and swirl to mix.
10. Change medium 6 h post-transfection and grow in culture medium for at least 40 h prior to stimulation with hapten and appropriate controls such as LPS for 5 or 8–16 h in order to analyze TLR-dependent gene expression (e.g., of IL-8 as readout for TLR4-dependent NF κ B activation) at RNA or protein level, respectively (*see Note 17*).

3.3 Co-immunoprecipitation

Hapten-induced dimerization of TLRs can be investigated by transfection of differently tagged TLR constructs into HEK293 cells and subsequent analysis of basal or hapten-induced interaction of the two co-expressed TLR molecules by co-immunoprecipitation (Fig. 2). The following protocol describes how to analyze TLR4 dimerization in response to nickel or cobalt stimulation in the presence or absence of its co-receptor MD2 using HEK293 cells or HEK 293-MD2/CD14 cells, respectively [13] but can easily adapted to address different questions.

1. Seed HEK293 and HEK293 hMD2 cells into antibiotic-free culture medium at a density of 5×10^6 cells per 10 cm culture dish, 1 day prior to transfection.
2. Transfect at least one dish each with 24 μg empty vector, a combination of empty vector and each one of the tagged TLR4 constructs (12 μg each, 24 μg DNA in total), or a 1:1 mixture of FLAG-tagged TLR4 and HA-tagged TLR4 and for each combination 60 μl of Lipofectamine® 2000 (Life Technologies) according to manufacturer's suggestions (*see Note 18*).
3. The following day split transfected cells 1:3 for stimulation with a hapten such as nickel, LPS, or diluent control.
4. After 30 min of stimulation, harvest cells by washing twice with ice-cold PBS and lyse directly on the dish by adding 500 μl supplemented ELB (*see Note 19*).
5. Calculate protein concentration and per sample distribute a volume equivalent to 1.5 mg total protein into separate 1.5 ml microcentrifuge tubes for immunoprecipitation.
6. To each tube add 500 μl of supplemented ELB containing 1 μg of mouse α -FLAG antibody (if necessary adjust total volumes with supplemented ELB).
7. Tumble over night at 4 °C using an end-over-end rotary shaker.
8. Add 25 μl of protein G agarose beads and precipitate immune-complexes by centrifugation at 14,000 rpm using a bench top microcentrifuge.
9. Wash three times at 4 °C by addition of 1 ml high salt ELB buffer containing 500 mM sodium chloride and subsequent centrifugation at 14,000 rpm.
10. Resuspend beads by the addition of 25 μl 2 $\times$ SDS buffer.
11. Boil at 95 °C for 5 min and centrifuge briefly to pellet beads.
12. Subject 10 μl of the supernatant to SDS-PAGE.
13. Perform Western blotting using a rat- α -HA high affinity primary antibody (50 ng/ml) and a POD-coupled α -rat secondary antibody to detect co-precipitated HA-TLR4 (indicating TLR4 dimerization) by ECL.
14. Control for equal precipitation of FLAG-tagged TLR4 by reprobing membranes with 0.5 $\mu\text{g}/\text{ml}$ rabbit α -flag antibody.

3.4 CHS Responses

Here, we describe the measurement of ear swelling as a readout for the CHS model. It is, however, necessary to analyze immune responses to contact allergens or irritants in detail with respect to TLR-dependent differences. This is done by using skin, lymph nodes, spleen, blood, etc. to study innate and adaptive cellular

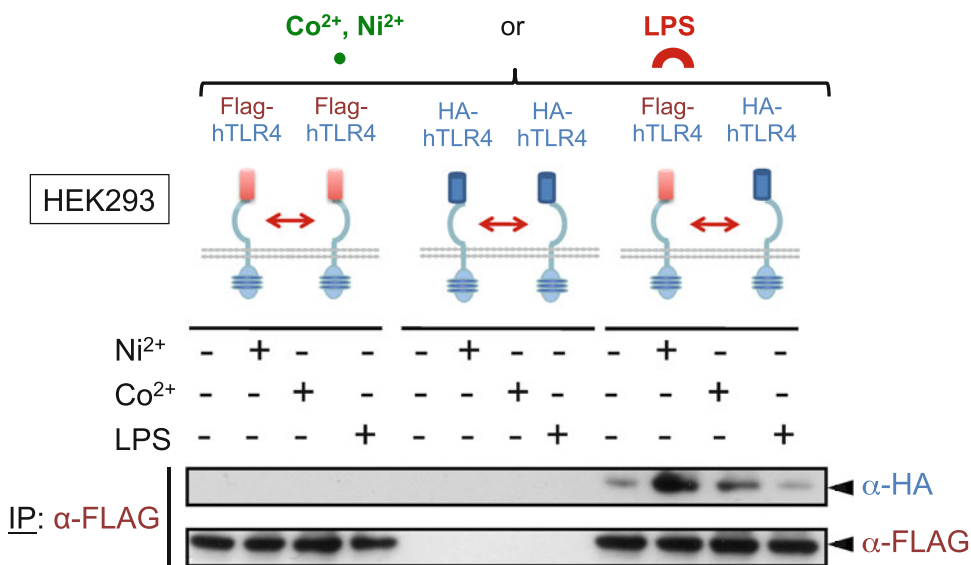


Fig. 2 Co-immunoprecipitation demonstrates hapten-induced TLR4 dimerization. HEK293 cells were transiently transfected with empty vector (not shown), a combination of empty vector plus FLAG-tagged human TLR4 (*left*), empty vector plus HA-tagged human TLR4 (*middle*), or a combination of both (*right*), respectively. After 48 h cells were stimulated for 30 min with nickel (Ni²⁺), cobalt (Co²⁺), LPS or diluent and subjected to immunoprecipitation with a FLAG-antibody. Co-immunoprecipitated HA-TLR4/FLAG-TLR4 dimers were then detected by immunoblot using an HA-specific antibody. An immunoblot for FLAG-tagged TLR4 using a FLAG-tag-specific antiserum is shown as control for equal precipitation of FLAG-TLR4. Equal transfection of the tagged TLR construct was confirmed by Western blotting of total lysate with Tag-specific antibodies (not shown). Data demonstrate that, unlike LPS, which requires MD2 to stimulate TLR4 dimerization above basal levels [13], the metal allergens nickel and cobalt are capable of inducing TLR4 dimerization independently of MD2. Modified from [13]

immune responses and the production of mediators such as cytokines and chemokines using a great variety of common techniques such as ELISA, flow cytometry, or qRT-PCR. These protocols apply to general research in immunology and the field of ACD/CHS, and their description is beyond the scope of this chapter.

3.4.1 Conventional CHS

The mouse CHS model is suitable to study the general role of TLRs for the disease. According to standard protocols [31], different contact allergens can be used for sensitization and challenge of wild-type mice or mice lacking single or multiple TLRs or components of the TLR signaling pathways (e.g., the adaptor proteins MyD88 or TRIF). In addition, irritants such as croton oil can be used to study the role of TLRs in irritant contact dermatitis, which is T cell-independent. The so-called mouse ear swelling test (MEST) represents the most common readout for studying CHS. Below we provide a standard protocol that can easily be modified for different haptens. Ideally, experimental groups of five mice should be used (*see Note 20*).

1. Sensitize mice by “painting” (slow pipetting and distribution with the pipet tip) 100 μ l of a hapten solution (e.g., 3 % TNCB in acetone) on the shaved abdominal skin with a pipette.
2. On Day 5 measure basal ear thickness and elicitate the CHS response by painting 20 μ l of a lower concentration of the same hapten (e.g., TNCB 1 % in acetone) on the dorsal side of both ears.
3. Determine the increase in ear thickness over time by measuring with an engineer’s micrometer. Measurements are typically performed at 24 and 48 h. In addition, the increase in ear thickness can be measured 6 h after sensitization. The early ear swelling response is dependent on histamine released by mast cells [32].

In the case of metal ions such as nickel and cobalt, solutions of metal salts (10 mM NiCl_2 or CoCl_2 in 0.9 % NaCl solution) are injected into the abdominal skin for sensitization ($2 \times 50 \mu\text{l}$ as described in Subheading 3.4.2 for DCs) and into the ear skin for elicitation (20 μ l per ear) as described for human TLR4 transgenic mice [12]. LPS must be added for the sensitization of wild-type mice due to the lack of binding sites for the metal ions in the murine TLR4. Suitable concentrations of nickel and LPS must be determined experimentally and different combinations and protocols have been tested [15, 33]. As a standard protocol, mice are sensitized by intraperitoneal injection of 250 μ l of a 1:1 (v/v) mixture of 1 mM NiCl_2 and 1 $\mu\text{g}/\text{ml}$ LPS stock solutions. Elicitation of CHS is then done 10 days later by injection of 20 μ l of a 5 mM NiCl_2 solution into the ear pinnae. Ear swelling peaks at 48 h. The use of ultrapure metal salts is recommended to avoid immunogenic contaminants [33].

3.4.2 Sensitization by DC Injection

The advantage of DC injection for sensitization lies in the possibility to address the role of TLRs specifically in DCs. Bone marrow-derived DCs are generated *in vitro* according to standard protocols [31].

1. One half of the DCs harvested at the end of the culture is left unmodified, the other half is hapten-modified *in vitro* by resuspension of the pellet after centrifugation in 1 ml of 3 mM TNBS (the water-soluble form of TNCB) in PBS and incubation for 7 min in a water bath in the dark.
2. Fill up the tube with RPMI medium containing 10 % FCS to prevent further reaction and toxicity.
3. Wash the TNBS-modified DCs twice in medium to remove residual TNBS and then once in PBS.
4. Resuspend and adjust cell concentration to $3 \times 10^6/\text{ml}$ in PBS for injection.

5. On Day 0, inject 3×10^5 unmodified or TNBS-modified DCs from wild-type or TLR-deficient mice into the skin at two sites (100 μ l per mouse: inject 2×50 μ l of the respective cell suspensions) left and right of the median of the shaved abdomen for sensitization. Formation of a subcutaneous bubble indicates the correct injection.
6. Perform elicitation as usual on Day 5 with 1 % TNCB applied on the dorsal side of the ears (*see* **Notes 20–22**). Pretreatment of DCs with e.g., TLR ligands can be done prior to the injection. In our studies this allowed us to demonstrate that triggering of TLR9 with CpG-oligodeoxynucleotides (CpG-ODN) as synthetic TLR9 ligands in vitro restored the absent sensitizing potential of DCs lacking TLR4 and IL-12R β 2 [7].

3.4.3 Adoptive T-Cell Transfer

The transfer of lymph node cells or purified T cells can address the role of TLRs for the T-cell response both in the sensitization and the elicitation phase of CHS. The former requires the transfer of T cells from wild-type or TLR-deficient mice into wild-type recipients. This experiment provides information about the dependency of T-cell priming on the TLR of interest. The latter requires the transfer of T cells from wild-type mice into wild-type or TLR-deficient recipients. This experiment is designed to investigate the role of TLRs for the recruitment and activation of the transferred wild-type T cells. Challenge of the recipient mice is performed as usual following i.v. injection of the T cells.

1. Sensitize donor mice by painting of not only the shaved abdominal skin with 100 μ l 3 % TNCB but also the dorsum of both ears with 20 μ l 1 % TNCB to obtain enough cells from the local skin draining lymph nodes.
2. Sacrifice the mice 5 days later.
3. Dissect the superficial inguinal and auricular lymph nodes, pool and prepare a single-cell suspension.
4. Inject 2×10^7 lymph node cells in 200 μ l PBS i.v. into the tail vein of recipient mice.
5. Directly after the injection, challenge the recipient mice on the dorsum of both ears with 20 μ l 1 % TNCB.
6. Measure the increase in ear thickness as described above at 24 and 48 h. As controls, lymph node cells from solvent-treated or untreated donor mice can be used.

4 Notes

1. Each batch should be individually tested for basal and induced pro-inflammatory activation by analysing expression of appropriate activation markers (e.g., FACS staining for the endothelial

surface receptors VCAM1, E-selectin and ICAM1 in case of HUVEC). For experiments we routinely use expanded frozen aliquots stored in liquid nitrogen from passage 3 or 4, respectively (*see* Subheading 3.1). Primary cells can also be freshly prepared from donors using appropriate protocols but this is a quite cumbersome and time-consuming procedure. In our experience, the expanded cultures of NHEK and HUVEC from commercial suppliers are comparable to those of home-made cells, but expansion of commercially produced cell preparations is more convenient and economic (particularly for small labs that do not routinely use those cells on a daily basis) and has the advantage that cells have routinely been tested for HBV, HCV, and HIV infection by the supplier. Moreover, suppliers usually keep greater cell batches with different growth characteristics (e.g., doubling time, seeding efficiency) in stock that can be tested for proliferation and pro-inflammatory responsiveness before larger amounts of cell aliquots are ordered.

2. Each batch of FCS should be tested for the absence of endotoxin contaminants. We routinely monitor this by confirming lack of pro-inflammatory VCAM1 and E-selectin expression 16 h after switching HUVEC cultures from medium containing a tested FCS batch to one supplemented with a new FCS batch.
3. Do not centrifuge prior to seeding as this reduces viability. For NHEK, medium should be removed as quickly as possible as they are sensitive to DMSO (e.g., thaw in the morning and replace by fresh medium upon adherence in the evening). HUVEC withstand DMSO quite well but it is recommended to replace medium the day after seeding in order to increase viability. Never warm up more medium than required and always keep supplemented media at 4 °C. Do not use any of the supplemented media for longer than 4 weeks.
4. As FCS contains a substantial amount of calcium, which triggers differentiation of keratinocytes at levels as low as 0.1 mM [22], for NHEK culture we treat all our FCS with Chelex resin to remove calcium. We use the “batch method” suggested by the supplier, filter sterilize after incubation, and store the Chelex-treated FCS aliquoted at –20 °C until use.
5. Do not keep primary cells at –80 °C for longer periods of time as this may result in enhanced cytotoxicity at re-thawing.
6. In our experience pools of up to three (functional) siRNAs against one target work significantly more efficient than single oligos. This approach also reduces the risk of off-target effects. In the case where multiple oligos are used, reduce the amount of the individual oligos accordingly to keep the total siRNA

amount constant (e.g., if a pool of two oligos is used, add 5 μ l of oligo 1 and 5 μ l of oligo 2).

7. Oligofectamine™/FuGENE® transfection reagents should not come into contact with plastic in order to retain optimal efficiency. Thus, directly pipet transfection reagents into the media (without touching the wall) and not vice versa. Mix gently by tapping against the tube (do not pipet up and down).
8. Required times for efficient knockdowns should be optimized for different gene products using appropriate readouts. Usually knockdown is best between 48 and 72 h after siRNA transfection, so the total time of the experiment should preferentially not exceed 72 h. If longer times are required use retroviral approaches to stably express small-hairpin RNA (shRNA) in the cells (*see* Subheading 3.2.2).
9. This procedure uses amphotropic retroviruses, which can infect human (dividing) cells. Make sure that adequate lab facilities and legal permissions are in place.
10. FLYRD18 cells produce extremely high virus titres and in our experience provide superior infections with pRetro Super (pRS) backbone-based shRNA vectors.
11. This step is necessary to remove traces of the selection antibiotic and dead cells from the culture as well as keeping producer cells in the log phase of proliferation. You may add 5 μ g/ml Polybrene at this stage to facilitate self-infection of the employed producer cell line to boost infection efficiency (optional).
12. This step is meant to remove potential contaminations of the supernatant with detached virus producer cells. Do not use pore sizes smaller than 0.45 μ m as this significantly reduces infection rates for unknown reasons.
13. This step is optional for infection with regular expression constructs but in our experience enhances knockdown levels when using shRNA-containing retroviruses.
14. Selection is not mandatory for ectopic expression of signaling components since infection rates are usually >90 % but still enhances expression levels and functionality when dominant-negative constructs or shRNAs are expressed. For infection with shRNA constructs, we recommend waiting one extra day before selection. Alternatively, select shRNA-infected HUVEC at 1 μ g/ml puromycin for 40 h prior to reseeding cells for experiments. Do not keep antibiotic on cells for longer than suggested as this might interfere with responsiveness to stimuli. If strong signs of cell death are observed you may give cells an extra day for recovery prior to reseeding.
15. To exclude contamination of hapten solutions with endotoxin it is mandatory for initial experiments to include additional

controls in which 50 µg/ml Polymyxin B-sulfate is added prior to hapten- and LPS-stimulation [12].

16. For co-transfections of two (e.g., TLR4 and MD2) or more plasmids reduce the amount of each construct (e.g., 1 µg each for two constructs) to maintain a total amount of 2 µg.
17. Depending on your requirements and readout you may consider positive selection of your transfected cells prior to stimulation. This can be done by co-transfection of limiting amounts of an additional plasmid containing a resistance marker for a fast working antibiotic such as puromycin that is suitable for overnight selection. For enrichment of selected cells we usually add 0.25–0.5 µg of pBABE puro vector, and select puromycin-resistant cells two days after transfection by 16 h treatment with 2 µg/ml puromycin. Make sure to reduce the amount of the construct of interest (e.g., TLR4) accordingly to maintain a total amount of 2 µg.
18. The classical calcium phosphate-based transfection is an option, but in our experience Lipofectamin-based reagents are superior for co-transfection experiments.
19. Adding β-glycerophosphate and sodium orthovanadate to the lysis buffer is essential when examining the phosphorylation status of intracellular signaling proteins. However, this may not be necessary for analysis of TLR dimerization.
20. In order to assess antigen specificity of the MEST, only one ear can be painted with the contact allergen used for sensitization. The second ear can be painted with solvent or with a different contact allergen. In that case the group sizes must be doubled.
21. Different kinetics may be observed if TNCB is used at different concentrations or in a different mouse strain or if other contact allergens are used.
22. The standard CHS protocol works with plateau concentrations of contact allergen. In order to avoid compensation of subtle effects on CHS due to mutations in TLR etc. it is recommended to titrate the sensitizing and/or elicitation dose of the contact allergen. Similarly, when DC injection is used for sensitization, titration of the DC number used for sensitization may be worthwhile.

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