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Heat Shock Protein (HSP) 90's Mechanistic Role in Contact Hypersensitivity

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Abstract

Despite the known dangers of contact allergens and their long-lasting use as models in immunology, their molecular mode of action largely remains unknown. In this study, we report that a contact allergen, 1-chloro-2,4-dinitrobenzene (DNCB), elicits contact hypersensitivity through binding the protein we identify. Starting from an unbiased sampling of proteomics, we found nine candidate proteins with unique DNCB-modified peptide fragments. More than half of these fragments belonged to HSP90, a common stress-response protein and a damage-associated molecular pattern (DAMP), and showed the highest probability of incidence. Inhibition and shRNA knockdown of HSP90 in human monocyte cell line THP-1 suppressed DNCB's potency by more than 80 %. Next, we successfully reduced DNCB-induced contact hypersensitivity in HSP90-knockout mice, which confirmed our findings. Finally, we hypothesized that DNCB-modified HSP90 activates the immune cells through HSP90's receptor, CD91. Pre-treatment of CD91 in THP-1s and BALB/c mice attenuated DNCB's potency, consistent with the result of HSP90-knockout mice. Altogether, our data show that DNCB-HSP90 binding plays a role in mediating DNCB-induced contact hypersensitivity, and the activation of CD91 by DNCB-modified HSP90s could mediate this process.

Introduction

Unlike many types of common allergies that are IgE-mediated hypersensitivity responses, allergic contact dermatitis is a result of a complex immune mechanism involving allergen-specific T cells that develop through repeated exposure to the same allergen (1, 2). Allergic contact dermatitis, or contact hypersensitivity, is usually characterized by two distinct phases: the sensitization phase and the elicitation phase. During the sensitization phase, activated Langerhans cells and dermal dendritic cells migrate to the nearest lymph node, where they present allergens to naïve T cells. Upon re-exposures to the same allergen and latent period after the first exposure, the elicitation phase causes clinically apparent

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Declaration of Competing Interest

No competing financial interests have been declared.

Supplementary Information

Supplementary Information on this manuscript can be found as a separate attachment.

symptoms (3-6). In this pathology of contact hypersensitivity, we are still limited by the unknown mechanisms by which allergens activate Langerhans cells.

One trigger of the sensitization phase, and the most common model, is a group of highly reactive compounds often referred to as contact allergens. Contact allergens, commonly found in nature or as ingredients of manufactured goods, are usually small molecular weight compounds that are hypothesized to bind self-protein/s to become immunogenic (7-11). In fact, many studies have shown contact allergen's ability to modify peptides and proteins(12-16). Recently, our lab reported a non-linear correlation of the degree of sensitization and the chemical reactivity of contact allergens (17), leading to the hypothesis that contact hypersensitivity occurs via the activation of a group of proteins that act as receptors of contact allergens.

Clinically, contact dermatitis is commonly suspected based on the patterning of skin lesions, suggesting sites of contact with the allergen. It is commonly diagnosed by patch testing, in which potential allergens in a standardized dose and form are applied to the skin and the allergic reactions are noted through serial assessments during the week after application. While subsequent avoidance of that allergen tends to result in improvements, it is not an option for patients of environmental or occupational allergens, such as poison ivy or nickel. Though the use of topical steroids and corticosteroids can improve the clinical symptoms of contact dermatitis, the ultimate treatment or cure for contact dermatitis is still unavailable because the molecular mechanism of contact allergens still remains unclear.

In this work, we sought to elucidate the mechanism of the sensitization phase using a well-established model contact allergen, 1-chloro-2,4-dinitrobenzene (DNCB). To identify DNCB's protein targets in an unbiased manner, we characterized DNCB-modified proteins via immunoprecipitation, western blotting, and mass spectrometry. We identified DNCB-modified heat shock protein (HSP) 90 peptide fragments via mass spectrometry. To confirm its functional relevance in skin sensitization, we knocked down gene expression of HSP90 and other identified proteins by stable transduction with lentivirus-packaged shRNA and assessed the DNCB-induced IL-8 response. Through this series of experiments, we report that HSP90 binds to DNCB and is associated with DNCB-induced contact hypersensitivity. We further confirmed attenuation of DNCB-induced contact hypersensitivity when HSP90 expression is depleted or inhibited. Through this work, we provide the first direct evidence of DNCB-protein modification in living systems, contributing to the understanding of the molecular pathology of allergic contact dermatitis in greater detail and offering insights into new potential therapeutic targets for individuals suffering from allergic contact dermatitis.

Material and Methods

Cell Culture

Human monocyte cell line THP-1 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Carlsbad, CA) supplemented 10 % with fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA) and 1 % antibiotic-antimycotic (Thermo

Fisher Scie3ntific) at 37 °C in 5 % CO_2 incubator. Cells were passed every 3-4 days and plated at a density of 5 x 10^5 cells/mL.

Cell Lysate Preparation

DNCB, ≥ 99 % was purchased from Millipore Sigma and was dissolved in DMSO (Molecular Biology Grade, Millipore Sigma) to prepare a 1 M DNCB stock solution. The stock was sterile filtered and stored in a −80 °C freezer.

To prepare cell lysate, THP-1 cells were incubated with 50 μM DNCB which was diluted in cell culture medium from 1 M DNCB stock solution. About 18 x 10^6 THP-1 cells were resuspended in 18 mL medium with either buffer or 50 μM DNCB and seeded in a 6-well plate (3 mL per well). After incubation, the cells were harvested, washed with ice-cold 1X PBS (300 g, 5 min, 2x), lysed in 1X cell lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 % Triton X-100) containing 1 tablet of protease inhibitor (cOmplete™ ULTRA Tablets, Mini, EASYpack Protease Inhibitor, Millipore Sigma) for 30 min on ice, sonicated (15 s, 3x), and centrifuged at 16,000 g for 20 min to collect protein in supernatant. Protein samples were stored in the −80 °C freezer if not used immediately.

Bicinchoninic acid (BCA) assay was performed to quantify protein samples. We used Pierce™ BCA Protein Assay Kit (Invitrogen) and the assay was performed following the manufacturer's protocol with few changes. Protein samples were diluted 1:9 in 1X PBS and bovine serum albumin (BSA) was run along with the protein sample to generate a standard curve.

Protein Separation by Gel Electrophoresis and Western Blotting

Protein samples were analyzed by gel electrophoresis and western blotting. To recite briefly, we used 4-15 % Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, Hercules, CA) by running at 100 V for 60 min. Separated proteins were transferred to Immuno-Blot® PVDF Membrane (Bio-Rad) in the tank-blotting system for 16 h at 30 V. The membrane was blocked in blocking buffer (5 % w/v nonfat milk (Cell Signaling Technology, Danvers, MA) in 1X Tris Buffered Saline (TBS, Bio-Rad) with 0.1 % Tween-20 (Fisher Scientific)) for 1 h rt, rinsed in 1X TBS with Tween-20 (5 min, rt, shaking, 3x), stained with primary antibody overnight at 4 °C with shaking, rinsed in 1X TBS with Tween-20 (5 min, rt shaking, 3x), stained with a secondary antibody for 1 h rt with shaking in dark, rinsed in 1X TBS with Tween-20 (5 min, rt shaking, 3x), and dried on a clean paper towel in dark. The primary and secondary antibodies were diluted in the blocking buffer. Detailed information on the antibodies and their dilution factors are listed in the next section. Dried membranes were imaged using Azure Biosystems c600 (Thermo Fisher).

Antibodies Used

For primary antibodies, we used mouse anti-DNP antibody clone 9H8.1 (Millipore Sigma, 1:10,000 dilution), HSP90 alpha monoclonal antibody clone 5G5 (Invitrogen, 1:1,000 dilution), purified anti-LRP1 (CD91) antibody (Biolegend, 1:500 dilution), and phosphotyrosine monoclonal (pY20) antibody (Invitrogen, 1:500 dilution). For secondary antibodies, we used goat anti-mouse IgG (H+L) Secondary Antibody, DyLight 650

(Invitrogen, 1:10,000 dilution). For CD91 blocking in vivo experiments, we used InVivoMAb anti-mouse/human/rat LRP (CD91) antibody and InVivoMAb IgG1 Isotype Control (Bio X Cell).

Immunoprecipitation

For immunoprecipitation, we purchased μMACS™ Separator from Miltenyi Biotec (Germany) and performed following the manufacturer's protocol with few changes. About 1 mg of protein was mixed with 1 μg monoclonal antibody and 50 μl μMACS Protein G MicroBeads (Miltenyi Biotec). After vigorous vortexing, the mixture was placed in ice for 30 min. To purify antibody-bound proteins, the mixture was applied to μ Columns (Miltenyi Biotec). The columns were primed with 200 μl 1X cell lysis buffer before applying the mixtures. Then, the column was washed with 200 μl high salt wash buffer (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 % NP-40 alternative, 4x) and 100 μl low salt wash buffer (20 mM Tris-HCl (pH 7.5)). For elution, we used 2X Laemmli Buffer (Bio-Rad) heated to 95 °C. The eluent was directly loaded on 4-15 % MiniPROTEAN® TGX™ Precast Protein Gels for separation and western blotting analysis.

Sample Preparation for Mass Spectrometry

About 3 mg of protein samples were shipped to Kendrick Labs, Inc. (Madison, WI, US) for 2D gel electrophoresis and western blotting. Briefly summarizing, proteins were first separated according to the carrier ampholyte method of isoelectric focusing in a glass tube using 2.0 % pH 3-10 Isodalt Servalytes.(18, 19) Then, each tube gel was sealed to the top of a stacking gel that overlaid 10 % acrylamide slab gel (1.0 mm thick) for the secondary separation by molecular weight. The gel was transblotted on a PVDF membrane, blocked in a blocking buffer, stained with anti-DNP antibody, and then stained with anti-mouse IgG-HRP for two hours. After treating with ECL, the membrane was exposed to x-ray film. Proteins separated from the gel were excised and sent to Northwestern Proteomics for mass spectrometry.

Generation of Knockdown Cell Lines with shRNA

To generate knockout cells, we purchased MISSION® shRNA Custom Lentiviral Particles from Millipore Sigma, three shRNA template oligonucleotides targeting each of mRNA sequences. The shRNA templates were designed with the puromycin selection marker for positively selecting for cells with shRNA. Briefly summarizing transduction procedure, 2×10^4 THP-1 cells were seeded in a 96 well plate with 105 μl cell culture medium containing 8 μg/ml hexadimethrine bromide and 15 μl lentiviral particles. The supernatant was replaced with the fresh cell culture medium on the next day. Successfully transduced cells were selected with 5 μg/ml puromycin beginning day 5 post-transduction. Empty vectors, MISSION® PLKO.1-puro Empty Vector Control Transduction Particles were used as a control for the transduction procedure.

Real-Time PCR (qPCR)

Stably transduced cells were seeded in a 6 well plate (3×10^6 cells per well, 10^6 cells per ml) and incubated in 37 °C incubator under two conditions: 1) cell culture medium

only and 2) cell culture medium containing 50 μM DNCB. Then, total RNA was isolated using Direct-zol RNA Miniprep Kit with TRI Reagent Treatment (Zymo Research, Irvine, CA) following the manufacturer's protocol with one change: RNA was eluted with 10 μl of DNase/RNase-Free Water included in the kit. Elution was repeated once to produce 20 μl of total RNA eluent.

Recovered RNA samples were reverse-transcribed using SuperScript™ IV First-Strand Synthesis System (Invitrogen). To produce an unbiased cDNA template, we used Oligo(dT)$_{20}$ primer provided with the First-Strand Kit. Synthesized cDNA templates were quantified with Qubit 4 Fluorometer (Invitrogen) using 1X dsDNA HS Assay Kit (Invitrogen) and stored in −20 °C freezer if not used immediately.

For qPCR, we used RT^2 SYBR Green ROX qPCR Mastermix (Qiagen, Hilden, Germany) following the manufacturer's protocol with several changes. Per run, approximately 50 ng of cDNA was mixed with 200 nM primers and qPCR Mastermix. Primers were purchased from Integrative DNA Technologies (Coralville, IA). Beta-actin (*ACTB*) was used as endogenous control and sequences for IL-8 and beta-actin were selected based on previous publications (20-22). A complete list of used primers can be found in Supplementary Information (Table S1). qPCR was repeated 3x and statistical significance was calculated based on ddCt values.

Contact Allergen Treatment on THP-1 Cells

To screen additional contact allergens in HSP90-knockdown THP-1 cells, we purchased citral (95 %), cinnamaldehyde (natural, ≥ 95 %), eugenol ≥ 98 %, and 2-methyl-4-isothiazolin-3-one (MI) from Sigma Aldrich, and 1-fluoro-2,4-dinitrobenzene (DNFB) from Acros Organics. Before contact allergens were added to cells, they were dissolved in DMSO to make 1 M solutions, and further diluted in the cell culture medium. Cells were placed in the incubator for 3 h before we isolated RNAs for qPCR as described above. We used 50 μM DNFB to match the concentration of DNCB. We used 100 μM citral, cinnamaldehyde (CA) and eugenol, and 75 μM MI because it was the optimal concentration to induce IL-8 response (data not shown).

Co-Dosing of Chemical Inhibitors and Contact Allergens

Geldanamycin (GA) ≥ 98 % purity was purchased from Stemcell Technologies (Vancouver, Canada). It was dissolved in 180 μl DMSO to make 10 mM stock solution, aliquoted, and stored in a −20 °C freezer. Before usage, GA was further diluted in DMSO to reach 1,000X more concentrated than the desired final concentration. For example, GA stock solution was diluted to 1 mM in DMSO and 1 μl of this was added to the cell culture to reach 1 μM GA treatment. DNCB was diluted to 500 μM in the cell culture medium. For the assay, 10^6 THP-1 cells were seeded in a 24 well plate with 900 μl of cell culture medium and 1 μl of 1,000X GA solution. After 5 min in the incubator, 100 μl of 500 μM DNCB was added to make the final concentration of 50 μM. After incubating for 16-20 h, the release of IL-8 chemokine in the supernatant was measured using the human IL-8 ELISA (BioLegend, San Diego, CA).

CD91 Blocking Assay

For the wells with anti-CD91, THP-1 cells were pre-incubated with 1 μg of anti-CD91 antibody for 1 h in an ice-bath. The cells were washed in 300 μl 1X PBS (3x), resuspended in the cell culture medium containing 50 μM DNCB, and incubated for 16-20 h. The release of IL-8 was measured using human IL-8 ELISA kit (BioLegends).

Contact Hypersensitivity Animal Model

This experiment was performed following previously published protocols with several minor changes (23, 24). All the animal studies and mice maintenance were approved by the Institute of Animal Care and Use (IACUC #72517). Generally, the experimental procedure goes as follows. The abdomen of the mice was shaved with a razor. On Day 0 and 1, the mice were sensitized with either 25 μl of solvent (1:4 v/v olive oil/acetone) or 25 μl of 0.5 % w/v compounds. After 3 days of resting period, the mice were challenged for 5 consecutive days with 10 μl of either solvent or solutions. The experiment was terminated 24 h after the final day of the challenge.

For the GA inhibition study, BALB/c mice aged 6-8 weeks were obtained from Jackson Laboratory. The solution of 0.5 mM GA was prepared in 10 % DMSO in solvent. It was applied on the same spots of sensitization and challenge 30 min before they were treated with 0.5 % w/v DNCB. As a negative control, a group of mice was sensitized and challenged with 1:4 olive oil and acetone solution only.

For the HSP90 knockout experiment, mice deficient in HSP90-alpha proteins (colony strain C57BL/6N-*Hsp90aa1tm1(KOMP)Wtsi/Mmucd*) were obtained from Mutant Mouse Resource and Research Center (MMRRC) at University of California, Davis, and bred on site. For the wild-type (WT) group, we used C57BL/6 mice.

For the CD91 blocking *in vivo* experiment, C57BL/6 mice aged 6-8 weeks were obtained from Jackson Laboratory. During the sensitization phase, the mice received the injection (100 μg, 0.1 ml, i.p) 4 h before DNCB or solvent treatment. The rest of the procedures remained the same.

Cytokine Assessment from Mouse Ear Tissue

To measure cytokine levels, the excised ears were mechanically homogenized on ice in 1 ml of RPMI 1640 medium. The protein was retrieved by centrifuging at 20,000 g at 4 °C for 20 min and collecting the supernatant. Cutaneous cytokine level was measured using CBA Mouse Inflammation Kit, Mouse Th1/Th2/Th17 Kit (BD Biosciences) and NovoCyte Flow Cytometer (ACEA Biosciences, San Diego, CA).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism. Comparisons between two or more groups were performed using two-way ANOVA. Graphs report the mean with standard deviation unless noted otherwise in the caption. A p-value < 0.05 was considered statistically significant.

Results

DNCB modifies cytosolic proteins in 30 minutes

As mentioned above, contact hypersensitivity is hypothesized to begin when contact allergens covalently bind self-proteins, the event that activates Langerhans cells. Our model contact allergen, DNCB, undergoes nucleophilic aromatic substitution (S_NAr) reaction, which involves the addition of a nucleophilic functional group to an aromatic ring (25). In proteins, cysteine and lysine side chains serve as a nucleophilic functional group, producing DNCB-modified proteins that can be detected in mass spectrometry by shifts of 166 Da in a mass spectrum (Figure 1). In initial experiments, we sought to examine a general population of cellular proteins that reacted with DNCB. Human monocyte cell line THP-1 – a well-established screening platform and has a similar phenotype to dendritic cells (26, 27) – was treated with 50 μM DNCB for 30 min – the optimal condition to activate the innate immune response and yield the most modification (Figure S1). The cellular lysate was separated via gel electrophoresis and immunoblotted with anti-dinitrophenol (DNP) antibody that binds DNP residues of DNCB-modified proteins. From the western blot, we identified more than 30 distinct protein bands, indicating that DNCB reacts with many different proteins (Figure 2A). This observation aligns with what others have previously reported (28). However, we were surprised to observe this many protein modifications just from only 30 min of incubation because previous experiments were at least 2 h. This result suggests that protein modification by DNCB occurs in a rather short timeframe, and in proteins with free nucleophilic functional groups.

To narrow our search to protein with high DNP modification, we used immunoprecipitated the whole lysates using affinity-based purification with anti-DNP antibody. Anti-DNP antibody was first mixed with cellular lysates to select proteins with the most DNCB-modification. Then, it was added with magnetic beads to form a protein-antibody-bead complex, which was separated from the rest of the sample with a magnetic column. Although the number of protein bands reduced to five (Figure 2B), the recovered DNCB-modified proteins were insufficient for mass spectrometry analysis (data not shown). To address this issue, cellular lysates were separated by 2-dimensional gel electrophoresis and immunoblotted with anti-DNP antibody (Figure 2C), leading to recovery of sufficient DNCB-modified proteins for mass spectrometric analysis. To increase our likelihood of identifying DNCB-modified proteins, we excised spots that: (i) were clearly distinct from neighboring protein spots (no smearing), (ii) developed darker over time the membrane was exposed to HRP-substrates (Figure S2), (iii) corresponded to the molecular weight observed from the immunoprecipitation (Figure 2C).

Excised protein samples were trypsin-digested, and resulting peptides were analyzed using a nano-LC ESI MS/MS. Mass spectrometric data containing peptide masses and peptide fragment sequences were searched against Mascot database search software. Figure 3 shows a sample of our crude data. To summarize, we identified 270 potential protein-matched peptides from 6 spots (Table S1). Only 9 of these proteins revealed peptide fragments corresponding to the peaks indicating DNCB-modification (Table 1). All the proteins identified as putative DNCB-modified proteins were cytosolic proteins, suggesting that

DNCB not only modifies proteins quickly but is also internalized within 30 min. Each of our putative DNCB-modified proteins showed at least one unique DNCB-modification on a peptide fragment. These unique peptide fragments appeared at least twice in the mass spectrum. Among the identified DNCB-modified proteins, peptide fragments from HSP90 showed a high Mascot ion score (Table 1), and thus a higher probability of DNCB-modification. Furthermore, 13 of 21 DNCB-modified peptides belonged to proteins in the HSP90 family, with each variant of HSP90 having multiple sites of potential modification. To determine whether each of these proteins has the potential to contribute to the sensitization response in the skin, we examined the relative abundance of identified proteins in human monocytes and human skin tissue (29). All identified DNCB-modified proteins were in the top 25 % of protein expression in the skin. Our next question whether the self-proteins that elicit contact hypersensitivity were indeed common skin proteins that can elicit a general pro-inflammatory response, separately from an antigen-specific response or a limited subset of proteins.

HSP90 could be associated with contact hypersensitivity

The identified proteins, owing to their abundant presence on human skin, are also commonly identified contaminants of mass spectrometry (30). Though we revealed direct evidence of DNCB-modified proteins, the relationship to their functional relevance to contact dermatitis is yet to be elucidated. To validate mass spectrometry data and to understand the roles that candidate proteins play in skin sensitization, we generated knockdown THP-1 cell lines by stably transducing cells with the lentivirus-packaged shRNA for six of the nine candidate proteins: *DNAJB11*, *HSP90AA1*, *HSP90AB1*, *PDIA3*, *RNH1*, and *SPTBN1* (Figure S3). *RPL13* and *TUBA1C* were excluded from this study because they are ribosomal and cytoskeletal proteins that are essential for housekeeping functions. Endoplasmin (*HSP90B1*), despite its promising mass spectrometry result, was also excluded because previous studies reported that it is necessary for a cell's survival (31). In addition to gene targets, we used an empty vector, which is the lentivirus-packaged shRNA without any known gene target, as a negative control for transduction.

After each knockdown cell was treated with 50 μM DNCB for 3 h, we evaluated the degree of sensitization by measuring the interleukin (IL)-8 transcript level with qPCR (Figure 4A-F). Release of IL-8 is a key event in the sensitization phase and a marker that indicates the induction of the inflammatory response elicited by DNCB and other contact allergens (32-36). After exposure to DNCB, THP-1 cells with knockdown showed a significant reduction in *IL-8* mRNA transcript levels compared to THP-1 cells transduced with an empty vector or without transduction. Most notably, DNCB-treated *DNAJB11* and *HSP90AA1*-knockdown cells showed IL-8 mRNA transcript level indistinguishable from resting cells, implying that DNCB failed to induce sensitization and positioning DNAJB11 and HSP90AA1 as critical proteins associated with DNCB-induced contact hypersensitivity. *IL-8* mRNA transcript level of DNCB-treated *RNH1*-knockdown cells showed no significant differences from those of non-transduced or empty vector controls. Because RNH1 interacts with intracellular and extracellular ribonucleases to regulate their activity (37), we assume that suppression of RNH1 could cause uncontrolled RNase activity within the cell, which explains high variability in the error in data. Even partial suppression in response from

knockdown suggests that each of the other five proteins contribute to IL-8 production and thus play a role in contact hypersensitivity.

To directly confirm that DNCB modifies HSP90, cell lysates samples were immunoprecipitated with the anti-DNP antibody followed by western blotting with the anti-DNP antibody. Western blotting revealed higher band intensity from the DNCB-treated sample, supporting our mass spectrometry result (Figure 4G). Next, we wondered whether HSP90 was a universal target that mediates contact hypersensitivity of all contact allergens. We screened five more contact allergens of a diverse reaction mechanism on the *HSP90*-knockdown cells (Figure 4H). One of these, 1-fluoro-2,4-dinitrobenzene (DNFB), is an analog of DNCB and binds proteins via S_NAr reaction that ultimately results in the DNP-modification. In line with the DNCB result, HSP90-knockdown reduced the inflammatory response to DNFB. Other contact allergens (citral, cinnamaldehyde (CA), methylisothiazolinone (MI), and eugenol) do not form DNP-modification when they bind proteins. HSP90-knockdown did not reduce the inflammatory response to these allergens. These results suggest that HSP90 is a target specific to DNCB and its analogs, but it is not a universal receptor of contact allergens.

Inhibition of HSP90 attenuates contact hypersensitivity

Next, we sought to validate the knockdown results. Although RNAi is a powerful technique, off-target effects may lead to false discovery (38), necessitating further validation of these results via a secondary confirmation. To confirm the knockdown results, we sought to explore attenuation of DNCB-induced contact hypersensitivity using HSP90's chemical inhibitors, such as geldanamycin (GA). HSP90 requires energy from ATP for function and GA selectively binds the ATP-binding site of HSP90, blocking its overall activation (39, 40). We hypothesized that DNCB-HSP90 modification would be hindered by GA and the subsequent reduction in IL-8 secretion. THP-1 cells were treated with 0.1 μM GA for 30 min to allow initial binding of GA to HSP90 and then stimulated with 50 μM DNCB. As determined by ELISA, IL-8 release decreased by 76 % compared to those incubated without GA (Figure 5A). While we could not confirm that GA hindered DNCB-HSP90 interaction, this experiment suggested that inhibiting HSP90 could attenuate DNCB-induced contact hypersensitivity.

Following this clear trend, we then tested whether HSP90 might play a role in contact hypersensitivity in vivo (41) (Figure 5B). For this experiment, DNCB was dissolved in 1:4 olive oil and acetone (solvent) at a concentration of 5 mg/ml (24, 41, 42). GA solution (0.5 mM) was prepared in the same solvent, but containing 10 % DMSO to increase the solubility of GA. The control group received After challenge with DNCB, the average change in the ear thickness of the DNCB-treated group was 0.372 mm, 9-fold higher than the control group where mice were sensitized and challenged with the solvent. When mice received 0.5 mM GA along with DNCB (DNCB+GA/DNCB+GA), the average change in the ear thickness reduced to 0.26 mm, showing a 30.1 % decrease in thickness relative to the DNCB group (Figure 5C). To account for pro-inflammatory response due to DNCB that are solely separate from that of contact hypersensitivity, we included a group where the mice received 0.5 mM GA during the sensitization phase and then were challenged with

0.5 % DNCB (GA/DNCB). The average ear thickness of this group was within the range of error of the DNCB+GA/DNCB+GA group, implying that GA reduced DNCB-induced contact hypersensitivity. These results altogether provide evidence that disrupting interaction between DNCB and HSP90 from DNCB activation reduces contact hypersensitivity. While it does not appear that HSP90 accounts for all the activity of DNCB, these data provide evidence for the first direct protein target in the sensitization phase. The limited changes in inflammatory responses may be due to non-specific protein modification in the elicitation phase.

These results provided further evidence that contact hypersensitivity has a molecular dependence on HSP90. To further confirm this dependence, we evaluated it in HSP90 knockout mice that express a complete deletion of the hsp90aa1 gene. We hypothesized that mice without HSP90 would experience a reduction in sensitization and possibly a reduction in elicitation. C57BL/6 (wild-type) and HSP90-knockout mice (hsp90aa1$^{-/-}$) were each sensitized with 0.5 % DNCB for 2 days and challenged in the ear with the same compound for 4 consecutive days. To provide further controls, we also included animals that were heterozygous for hsp90aa1. On average, the HSP90-knockout mice showed a reduction of 0.099 mm in change of ear thickness, a 31 % reduction compared to the wild-type mice (Figure 5D). HSP90-knockout mice also expressed less cutaneous TNF-α and IL-6 levels compared to the wild-type mice (Figure 5E). The heterozygotes also showed modest reductions, but with less consistency in cytokines, it indicates the importance of a complete removal of HSP90 for elimination of the elicitation activity. These results, altogether, indicate that HSP90 is a protein associated with contact hypersensitivity.

DNCB-modified HSP90 proteins activate CD91 pathway

Next, we sought to elucidate the mechanism by which DNCB-modified HSP90 activates the immune system. HSP90 has many functions (31, 43, 44), including facilitating antigen presentation during an immune response. The most common pathway for HSP90 to elicit an inflammatory response is via a cell surface protein CD91, which is known to activate HSP90, gp96, and HSP70 (45-47). We hypothesized that modification of HSP90 by DNCB might alter either the availability or binding affinity of HSP90 to CD91.

To test if DNCB-modified HSP90 undergoes the CD91-mediated signaling pathway, we conducted a series of CD91 blocking assays. In the first experiment, we directly blocked CD91 interaction with an anti-CD91 antibody and then administered DNCB. THP-1 cells were pre-incubated with 60 ng/ml of anti-human CD91 antibody for 1 h at 4 °C, washed with PBS to remove any excess antibody, resuspended, and incubated with 50 μM DNCB for 20 h (Figure 6A). Measuring secreted IL-8 in the supernatant with IL-8 ELISA, we observed an approximately 80 % reduction in DNCB-induced IL-8 when pre-incubated with CD91 antibody. Cells incubated with lipopolysaccharide (LPS), an innate immune activator that does not undergo the CD91 pathway, showed a negligible change in the IL-8 response, strengthening support for the hypothesis that CD91 activation was critical in the elicitation of contact hypersensitivity. To test whether the interaction between DNCB-modified HSP90 and CD91 activated signaling, THP-1 cells were incubated with 50 μM DNCB for 2 h, lysed to collect cellular proteins, and the lysate was subjected to immunoprecipitation with the

anti-CD91 antibody. Western blotting of the eluents showed phosphorylation of the CD91 β-chain of the DNCB-treated, but not the untreated sample (Figure 6B).

Intrigued by the potential of CD91 and its role in DNCB-mediated contact hypersensitivity, we explored how CD91 blockade would translate *in vivo*. To accomplish this, we established an *in vivo* inhibition model. From the day before the start of the experiment to the end of the sensitization phase, each mouse was injected intraperitoneally with one of the following: PBS, 0.5 mg/ml of purified mouse anti-CD91 antibody, or 0.5 mg/ml of purified mouse isotype antibody (Figure 6C). At the end of the challenge phase, the anti-CD91 group treated with DNCB showed an average ear thickness of 0.794 mm, which was significantly lower (approximately 50 %) than the average change of the DNCB-treated group with PBS injection (Figure 6D). Similarly, we observed a slight decrease in cutaneous cytokine levels between the groups with PBS and anti-CD91 antibody injection (Figure 6E). While we saw a statistically significant reduction in the ear thickness, we could not confirm a difference in the cutaneous cytokine level in between the PBS and anti-CD91 antibody injection groups, suggesting that the CD91 pathway activation is only part of the mechanisms by which cytokine expression is increased despite the reduction in swelling related to the hypersensitivity. Regardless, the cellular and animal model data strongly suggest that CD91 is important for the immune signaling cascade of contact hypersensitivity, but that CD91 may not be the only receptor or may play a role in the sensitization, but not the elicitation phase. Further experiment will be required to elucidate these distinctions, but our results imply that discovery of more molecular targets could lead to new therapeutic options for contact hypersensitivity in those in whom allergen elimination is not possible.

Discussion

In this study, we report that DNCB binds HSP90, which, in turn, acts as a target and mediator of the contact hypersensitivity response. Characterizing DNCB-modified proteins with proteomics, we discovered that many DNCB-modified peptides of the highest Mascot scores belonged to the HSP90 family, suggesting the high probability of DNCB-HSP90 binding. The knockdown and small-molecule inhibition of HSP90 almost completely suppressed DNCB's potency in human cells. In line with this result, *HSP90*-knockout mice and mice co-treated with the HSP90 inhibitor showed reduced ear thickness and cytokine release to DNCB treatment. Though we could not confirm that the HSP90 inhibitor hindered the DNCB-HSP90 binding, our results confirmed the functional relevance of HSP90 in contact hypersensitivity.

HSP90, a stress-responsive and molecular chaperone protein that plays a role in maintaining homeostasis, recently gained attention for its involvement in the innate immune response and inherent adjuvanticity.(48-51) HSP90s participate in the antigen-presentation pathway involving major histocompatibility complex (MHC) class I molecules. Though not consistent, several studies reported that injection of antigenic peptides with HSP90s modulated both cellular and humoral responses against pathogens that carry those antigens. (52) HSP90 also serves as a danger signal. During a stress event such as pathogen invasion, cells increase the synthesis of HSP90s, which are released to the extracellular components and activate antigen-presenting cells (APCs) via the CD91 signaling cascade.

Based on this fact and our evidence of HSP90's association with hypersensitivity, we tested if DNCB treatment activated the CD91 signaling cascade. The immunoprecipitation and western blot experiment showed that DNCB treatment phosphorylated CD91, confirming the activation of the CD91 signaling in cells. Taking this further, we tested whether inhibition of CD91 could attenuate DNCB-induced contact hypersensitivity. THP-1 cells that were pre-incubated with anti-CD91 antibody secreted approximately 80 % less IL-8 to DNCB stimulation. In the in vivo model, administration of anti-CD91 antibody before DNCB sensitization resulted in a statistically significant reduction in the ear thickness. Our results, altogether, indicate that DNCB-HSP90 binding initiates contact hypersensitivity via activating the CD91 signaling cascade.

CD91 is a scavenger receptor class-A (SR-A) expressed on APCs and a known receptor for immunogenic heat shock proteins like HSP70 and HSP90. In our experiments, though the blockade of CD91 with its antibody decreased the IL-8 response in human cells and ear thickness in vivo, the cutaneous cytokines associated with contact hypersensitivity, such as IL-6 and TNF-α, did not change significantly between PBS and CD91 antibody injected groups. We think it could be due to the availability of other intake mechanisms that compensate for the inaccessibility of CD91. In support of this hypothesis, one report showed that deletion of SR-A in bone marrow-derived dendritic cells (BMDCs) impaired binding and internalization of exogenous HSP90s and formation of MHC class I by 50 %. However, the effector function of CD8+ cells did not alter between the control and SR-A knockout groups, suggesting that there are compensatory mechanisms that overcome the lack of SR-A.(53) DNCB is immunogenic, and CD91 signaling could be one of many ways that DNCB and contact allergens activate the immune response. In considering how DNCB might induce HSP90-CD91 binding, we predict the possibility that DNCB could alter the availability of HSP90s extracellularly, which could influence the affinity of HSP90s to CD91. In future work, we plan to explore the potential of contact allergens and chemical sensitizers to alter the composition of HSP90 and other DAMP-associated proteins and their binding affinities to receptors.

Identification of protein targets of contact allergens has been a long-term question, (54-56) and we hope that our work provided a mechanism by which they elicit contact hypersensitivity. One limitation of this work, however, is the lack of generalizability. In addition to DNCB, we screened several other contact allergens in *HSP90*-knockdown THP-1 cells. Contact allergens that were chemically and structurally different from DNCB showed unchanging IL-8 potency in the knockdown cell. It suggests that there may be more than one immunological pathway that contact allergens mediate hypersensitivity, and more work can be done to identify proteins of other contact allergens. Nevertheless, these seminal results pave a way for studying protein modification of allergens, potentially allowing sub-classification of contact allergens based on the mechanism of contact hypersensitivity. Ultimately, a better understanding of the molecular mechanism of hypersensitivity promises to improve the diagnostic test, phenotyping of the disorder, and individualized treatment beyond allergen avoidance for patients suffering from allergic contact dermatitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article:

2D	2-dimensional
Ab	antibody
APC	antigen-presenting cell
BMDC	bone marrow-derived dendritic cells
CA	cinnamaldehyde
CBA	cytometric bead array
DAMP	damage-associated molecular pattern
DMSO	dimethyl sulfoxide
DNAJB11	DnaJ heat shock protein family (Hsp40) member B11
DNCB	1-chloro-2,4-dinitrobenzene or dinitrochlorobenzene
DNFB	1-fluoro-2,4-dinitrobenezene or dinitrofluorobenzene
DNP	dinitrophenol
ECL	enhanced chemiluminescent
ELISA	enzyme-linked immunosorbent assay
ESI	electronspray ionization
GA	geldanamycin
HSP90	heat shock protein 90
HSP90AA1	heat shock protein 90 alpha family class A member 1
Hsp90aa1$^{-/-}$	HSP90-knockout mice homozygote

HSP90AB1	heat shock protein 90 alpha family class B member 1
HSP90B1	endoplasmin
IgE	immunoglobulin E
IL-6	interleukin-6
IL-8	interleukin-8
ip	intraperitoneally
IP	immunoprecipitation
kDa	kilodalton
LC	liquid chromatography
LRP	low density lipoprotein receptor-related protein 1 or CD91
MI	methylisothiazolinone
MS	mass spectrometry
PDIA3	protein disulfide isomerase family A member 3
pTyr	phosphotyrosine
RNAi	RNA interference
RNH1	ribonuclease inhibitor 1
RPL13	ribosomal protein L13
shRNA	short-hairpin ribonucleic acid
S_NAr	nucleophilic aromatic substitution
SPTBN1	spectrin beta 1
SR-A	scavenger receptor class-A
TNF-α	tumor necrosis factor alpha
THP-1	human monocyte cell line
TUBA1C	tubulin alpha 1c
WB	western blot
WT	wild-type

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Key Points

- A contact allergen, DNCB, binds proteins in a short timeframe.
- DNCB directly binds HSP90, which appears to contribute to contact hypersensitivity.
- Inhibition of HSP90 can attenuate DNCB-induced contact hypersensitivity.

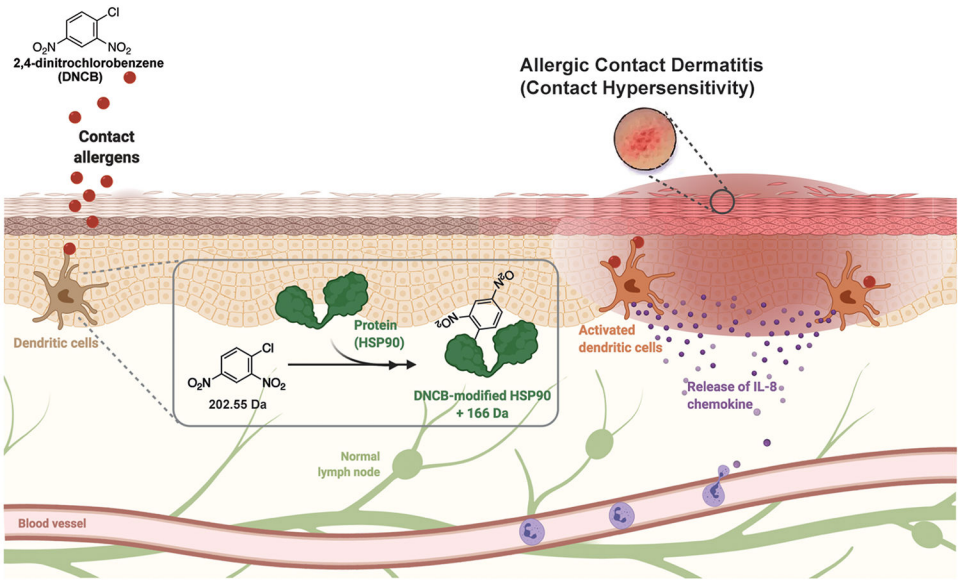

Figure 1.

Overview of allergic contact dermatitis (contact hypersensitivity) in the skin. DNCB modifies lysine and cysteine residues of the protein to give a shift of 166 Da, detectable by mass spectrometry. Modification of proteins activates the skin dendritic cells.

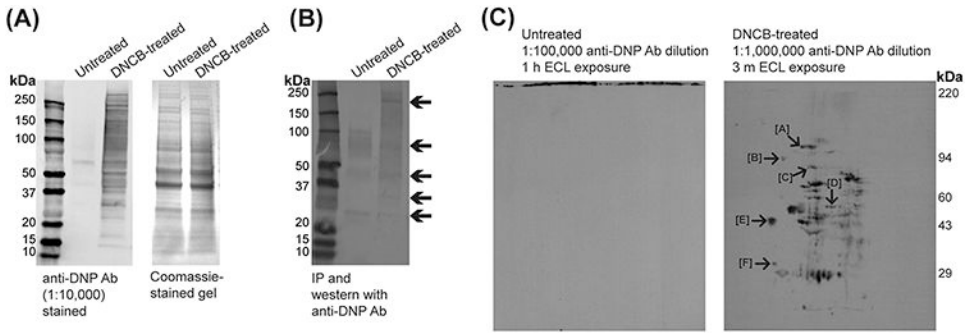

Figure 2.

DNCB modifies many proteins in an unbiased manner. **(A)** Untreated and DNCB-treated samples were western blotted with the anti-DNP antibody (1:10,000 dilution). On the right is Coomassie-stained gel that reveals whole protein lysates. **(B)** The samples were immunoprecipitated with the anti-DNP antibody (1 μg), followed by western blot with the same antibody (1:10,000 dilution). The arrows indicate the position of protein bands. **(C)** The samples were separated in the 2D gel electrophoresis, followed by western blot with the anti-DNP antibody (1:100,000 dilution for untreated and 1:1,000,000 for DNCB-treated). Spots labeled [A] to [F] were excised and digested for proteomics.

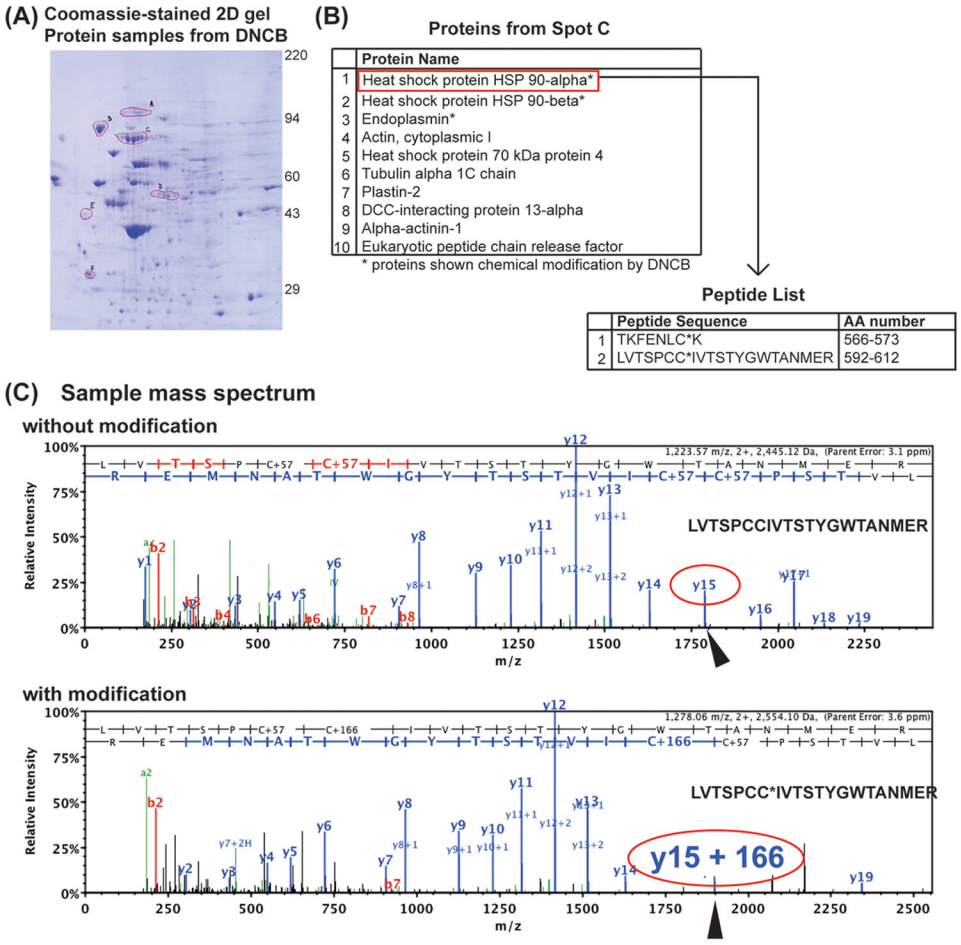

Figure 3.

Representative mass spectrometry data. **(A)** The Coomassie-stained 2D gel of the DNCB-treated protein sample. Spots [A] to [F], circled in red, were excised and digested for proteomics. **(B)** Proteomics revealed ten proteins from Spot C, 3 of the ten proteins were DNCB-modified (marked with asterisks on the list). As an example, we show a list of peptides recovered from HSP90-alpha (Peptide List). The amino acid with DNCB-modification is marked with asterisks. **(C)** For this peptide fragment LVTSPCCIVTSTYGWTANMER, the y15 ion has a mass of 1,788 Da in the unmodified condition. The same ion of the DNCB-treated protein sample is 1,897 Da (black arrows). This is an example of DNCB-modification and the mass shift.

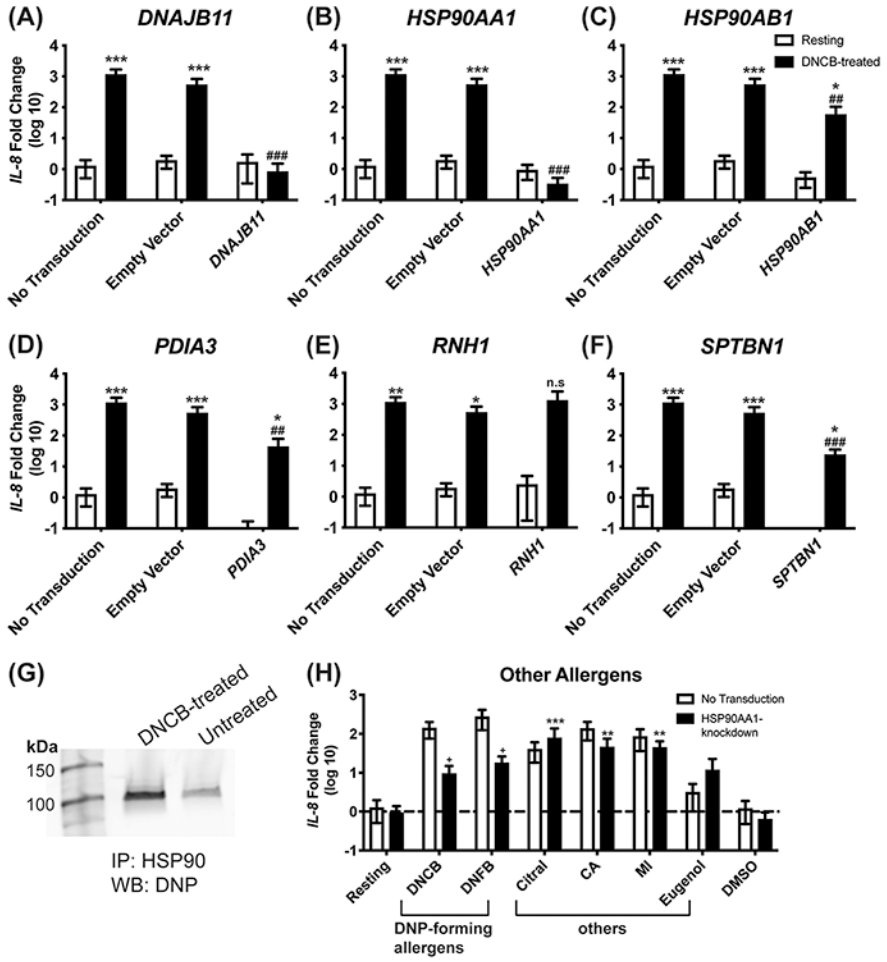

Figure 4.

(A-F) Protein knockdown with shRNA shows that *DNAJB11* and *HSP90AA1* can be associated with DNCB-induced hypersensitivity. For all graphs, white bars indicate the IL-8 level of untreated or resting samples and the black bars indicate the IL-8 level after 3 h incubation of 50 μM DNCB. Fold change was calculated by normalizing to the resting of No Transduction (first white bar of each graph). All experiments were repeated 3 times. The graph shows median + SEM. Statistical significance was calculated based on ddCt value. Gene abbreviations: *DNAJB11*, DnaJ heat shock protein family (Hsp40) member B11; *HSP90AA1*, heat shock protein 90 alpha family class A member 1; *HSP90AB1*, heat shock protein 90 alpha family class B member 1; *PDIA3*, protein disulfide isomerase family A member 3; *RNH1*, ribonuclease inhibitor 1; *SPTBN1*, spectrin beta 1 **(G)** The DNCB-treated cell lysate was immunoprecipitated with the anti-HSP90 antibody and western blotted with the anti-DNP antibody. **(H)** To test the specificity of HSP90 in DNCB-induced contact hypersensitivity, additional contact allergens were screened in HSP90AA1-knockdown THP-1 cells. The IL-8 response was measured with qPCR were cultured with contact allergens. DNFB: dinitrofluorobenzene, CA: cinnamaldehyde, MI: methylisothiazolinone, DMSO: dimethyl sulfoxide. * indicates statistical significance to the resting of No Transduction. *$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$. # indicates statistical

significance to the DNCB-treated of No Transduction. #p < 0.01, ##p < 0.001, ###p < 0.0001. + indicates statistical significance to the IL-8 response of its No Transduction +p < 0.05.

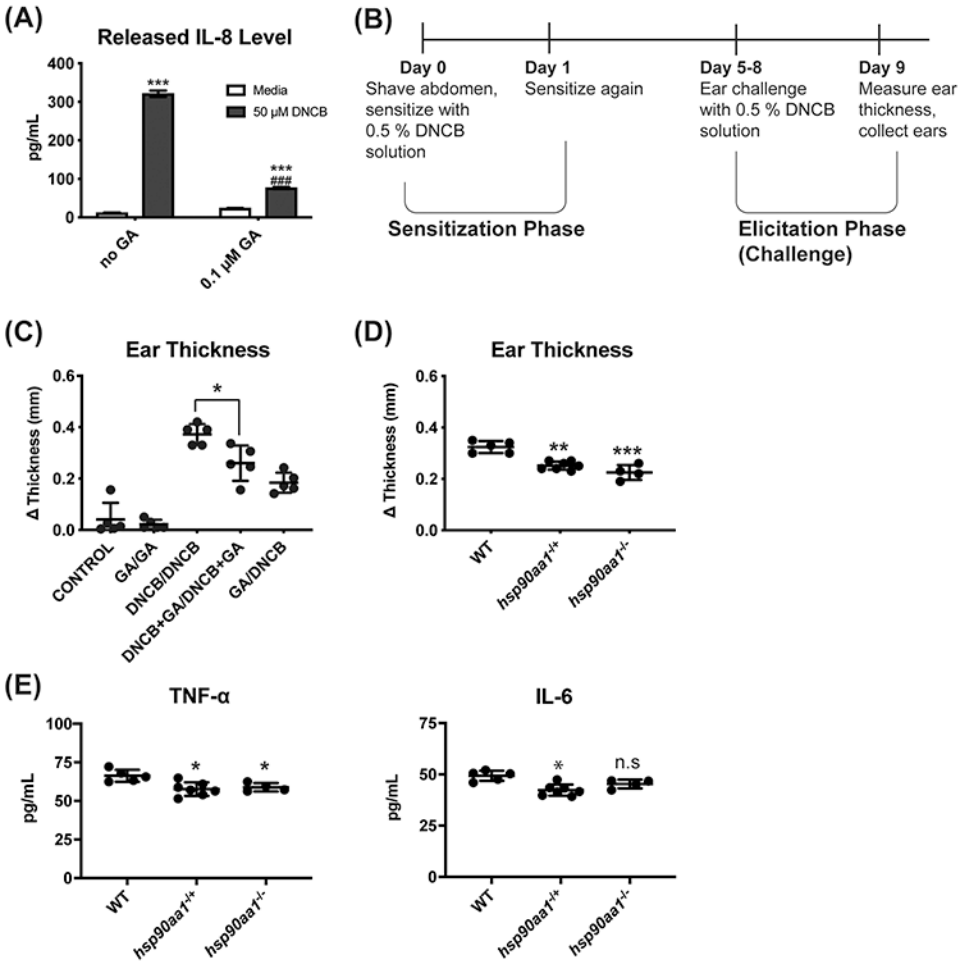

Figure 5.

Blocking HSP90's activity reduces skin sensitization potential. **(A)** Co-treating THP-1 cells with 0.1 μM GA and 50 μM DNCB reduces secretion of IL-8 chemokine. Cells were cultured for 20 h. n = 3. The released IL-8 was reduced by 76 %. * indicates statistical significance to the "no GA" media group. # indicates statistical significance to the "no GA" 50 μM DNCB group. ***p < 0.001, ###p < 0.0001. **(B)** Schematics of the in vivo experiment. Mice were sensitized on the shaven abdomen on Day 0 and 1. After 3 days of resting, mice were challenged on the ear for 4 consecutive days. Approximately 24 h after the last challenge, we measured the ear thickness and harvested ear tissue. **(C)** GA's efficacy in vivo. The reported graph shows the change in ear thickness before and after the challenge (n = 5). Control: treatment with 1:4 olive oil and acetone (solvent) only. GA/GA: sensitize and challenge with 0.5 mM GA. DNCB/DNCB: sensitize and challenge with 0.5 % w/v DNCB. DNCB+GA/DNCB+GA: sensitize and challenge with both GA and DNCB. GA/DNCB: apply GA during the sensitization phase and challenge with 0.5 % DNCB. **(D)** Contact hypersensitivity on hsp90aa1-knockout mice. The graph shows the difference in ear thickness before and after the challenge. **(E)** Cutaneous TNF-α and IL-6 levels in the ear tissue of the knockout mice collected on Day 9. * statistical significance to WT. *p < 0.05, **p < 0.005, ***p < 0.0005.

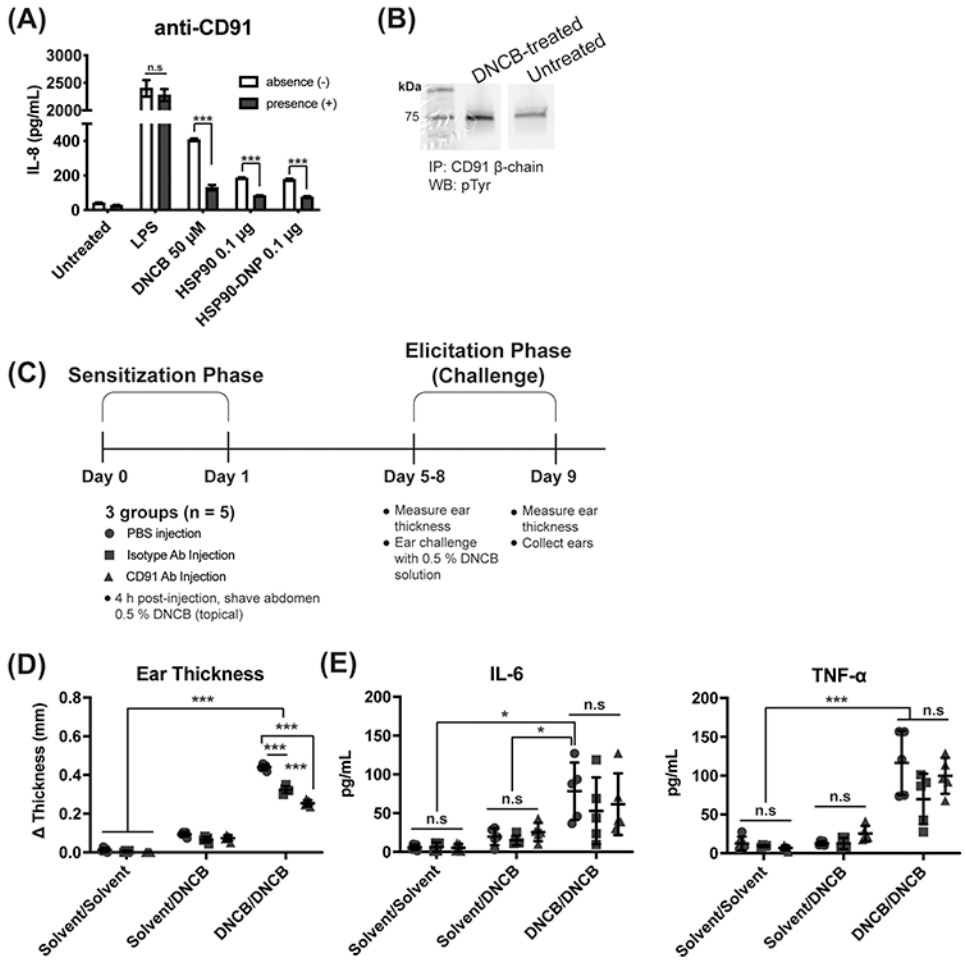

Figure 6.

Blocking CD91 attenuates DNCB-induced contact hypersensitivity. **(A)** THP-1 cells were incubated with 60 ng/ml anti-human CD91 antibody at 4 °C for 1 h, washed in PBS (3X), replenished with the cell culture medium containing 50 μM DNCB. After 20 h incubation, the supernatant was collected for human IL-8 ELISA. The above graph shows the comparison of IL-8 response between the group incubated without (white bar) and with (dark gray bar) the CD91 antibody. CD91 blockade decreased the IL-8 response to DNCB. **(B)** Phosphorylation of intracellular β-chain of CD91 protein. Cell lysate samples were immunoprecipitated with LRP antibodies that bind the β-chain of CD91. The resulting protein samples were stained with pTyr antibodies. DNCB treatment phosphorylated CD91. **(C)** A scheme of in vivo CD91 blocking experiment. During the sensitization phase, mice received injections (100 μg, ip) 4 h before sensitization. The rest of the experiment was the same as described previously. **(D)** The difference in ear thickness before and after the challenge. The anti-CD91 Ab injection significantly decreased the ear thickness. **(E)** Cutaneous cytokines in the ear were quantified using a cytometric bead array (CBA) kit. Within DNCB/DNCB groups, anti-CD91 Ab injections did not decrease cutaneous cytokine levels, suggesting that some inflammation still occurred.

Table 1

List of DNP-modified Proteins

	Name of protein	Gene ID	Location of protein	Relative abundance (ppm)[27]		Peptide sequence found	Mascot score	MW (kDa)
				Monocyte	Skin			
1	60 S ribosomal protein L13	RPL13	Cytosol	232	42.2	RNKSTESLQANVQRLK*	19.2	24
2	DnaJ heat shock protein family (HSP40) member B11	DNAJB11	Cytosol, ER	318	194	QLLK*QGSVQK	29.7	41
3	Endoplasmin	HSP90B1	Cytosol, ER	766	1399	ELISNASDALK*IR	100.5	92
						NLGTIAK*SGTSEFLNK	77.1	
						EFEPLLNWMK*DK	33.0	
						LTESPC*ALVASQYGWSGNMER	98.6	
						IMK*AQAYQTGK	40.5	
4	Heat shock protein HSP90-alpha	HSP90AA1	Cytosol, ER	663	864	TKFENLC*K	15.2	85
						LVTSPCC*IVTSTYGWTANMER	61.0	
5	Heat shock protein HSP90-beta	HSP90AB1	Cytosol, ER	1057	832	ADLINNLGTIAK*SGTK	75.8	83
						VILHLK*EDQTEYLEER	25.9	
						VFIMDSC*DELIPEYLNFIR	83.0	
						LVSSPC*CIVTSTYGWTANMER	98.6	
						QK*AEADKNDK	35.3	
6	Protein disulfide-isomerase A3	PDIA3	ER	1025	1174	VDC*TANTNTCNK	30.2	57
						FIQENIFGIC*PHMTEDNK	45.4	
7	Ribonuclease inhibitor	RNH1	Cytosol	2332	264	DSPC*QLEALK	42.2	50
						LGDVGMAELC*PGLLHPSSR	33.5	
8	Spectrin beta chain, non-erythrocytic 1	SPTBN1	Cytosol	200	236	K*QQMLENQMEVR	15.6	275
9	Tubulin alpha-1C	TUBA1C	Nucleus, cytoskeleton	1042	401	TIQFVDWC*PTGFK	28.8	50