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# Mechanisms of chemical-induced innate immunity in allergic contact dermatitis

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## Abstract

Allergic contact dermatitis (ACD) is one of the most prevalent occupational skin diseases and causes severe and long-lasting health problems in the case of chronification. It is initiated by an innate inflammatory immune response to skin contact with low molecular weight chemicals that results in the priming of chemical-specific, skin-homing $CD8^+$ Tc1/Tc17 and $CD4^+$ Th1/Th17 cells. Following this sensitization step, T lymphocytes infiltrate the inflamed skin upon challenge with the same chemical. The T cells then exert cytotoxic function and secrete inflammatory mediators to produce an eczematous skin reaction. The recent characterization of the mechanisms underlying the innate inflammatory response has revealed that contact allergens activate innate effector mechanisms and signalling pathways that are also involved in anti-infectious immunity. This emerging analogy implies infection as a potential trigger or amplifier of the sensitization to contact allergens. Moreover, new mechanistic insights into the induction of ACD identify potential targets for preventive and therapeutic intervention. We summarize here the latest findings in this area of research.

## Allergic contact dermatitis – hypersensitivity induced by harmless chemicals

In our daily life, we are confronted with a plethora of natural and synthetic chemicals. Many of them are foreign to our organism, so-called xenobiotics. The inhalation, ingestion or skin contact with such xenobiotics often occurs without any obvious consequences. However, some low molecular weight (MW) chemicals interact with host cells and molecules and influence biologic processes such as signal transduction. In some cases, xenobiotics are chemically reactive, and in other cases, they are metabolized to yield reactive compounds. They occasionally exhibit toxic, mutagenic or carcinogenic effects, or they modulate immune responses. This may lead to pathologies such as allergic contact dermatitis (ACD), respiratory diseases or autoimmunity. The outcome depends on the chemical properties, i.e. the mechanism of action, the target tissues and the molecular target structures and pathways.

One group of low MW (< 500 Dalton) xenobiotics are contact allergens. Examples are metal ions and salts such as nickel, cobalt and chromate, fragrances, dyes and preservatives. Contact allergens cause ACD, a common inflammatory skin disease characterized by pruritic eczematous lesions. In

**Abbreviations**
ACD, allergic contact dermatitis; AMP, antimicrobial peptide; APC, antigen-presenting cell; BMDC, bone marrow–derived dendritic cell; CHS, contact hypersensitivity; DAMPs, damage-associated molecular patterns; DC, dendritic cell; DNFB, 2,4-dinitrofluorobenzene; ECM, extracellular matrix; HA, hyaluronic acid; IL-1R, interleukin-1 receptor; $Ni^{2+}$, nickel; NLR, NOD-like receptor; PAMPs, pathogen-associated molecular patterns; PRR, pattern recognition receptor; TCR, T-cell receptor; TLR, Toll-like receptor; TNCB, 2,4,6-trinitrochlorobenzene; Treg, regulatory T cell.

contrast to irritant contact dermatitis (ICD), which evolves as a consequence of direct toxic effects of physical or chemical agents resulting in keratinocyte damage and local inflammation, ACD critically depends on adaptive immunity. In the clinically inapparent *sensitization phase* of ACD, epidermal Langerhans cells or dermal dendritic cells (DCs) migrate to skin-draining lymph nodes and present contact allergens to naïve T lymphocytes. This leads to the generation of skin-homing $CD8^+$ Tc1/Tc17 and $CD4^+$ Th1/Th17 effector T cells that enter the blood circulation (1, 2). Upon re-exposure of the skin, the contact allergen triggers a cascade of events resulting in the infiltration of neutrophils, monocytes and effector T cells as well as to epidermal vesiculation (spongiosis). During this *elicitation phase*, clinically apparent skin lesions develop. Depending on the degree of sensitization and the strength of the contact allergen, acute ACD develops within hours to a few days after exposure. Clinically, ACD is characterized by infiltrated erythematous pruritic patches and papules with or without vesicular reactions. These lesions develop primarily in the area of direct contact but can, in contrast to ICD, extend beyond this area. Chronic lesions are typically characterized by pruritus, lichenification, erythema, scaling, fissures and excoriations.

More than 3000 contact allergens are known (3). The risk of developing ACD largely depends on exposure patterns and habits. Occupational exposure to contact allergens frequently results in ACD and is a major cause of occupational disability. Among the most common inducers of occupational ACD are vulcanization accelerators such as thiuram, nickel ($Ni^{2+}$), epoxy and other resins, aromatic amines and chromate (4). On the other hand, personal habits influence the risk of developing ACD. For example, women show higher sensitization rates to $Ni^{2+}$ than men (5). This has primarily been attributed to the habit of wearing $Ni^{2+}$-containing jewellery rather than to genetic factors. Other important factors affecting allergen exposure refer to geographic or legislative aspects. In the United States, ACD to urushiol-containing plants of the genus *toxicodendron* is frequent while it is negligible in Europe. Notably, national and European Union (EU) legislative interventions that regulate the contents and release of $Ni^{2+}$ by-products coming into contact with skin such as the EU Nickel Directive issued in 1994 resulted in a decrease in sensitization rates to $Ni^{2+}$ (6). Surprisingly enough, however, Euro coins contain significant amounts of $Ni^{2+}$ (7).

Despite similar exposure, only a minority of exposed individuals develop ACD suggesting an influence of genetic predisposition (8, 9). While genome-wide association studies are still not available, polymorphisms and mutations of various candidate genes have been associated with ACD (10). These include factors essential for the skin barrier function, such as filaggrin (11), or factors influencing inflammatory and immunological processes, such as cytokines. However, published data are in part inconclusive or have not yet been reproduced in independent cohorts.

Prevention or treatment of contact eczema primarily relies on the identification of the hazardous chemicals and the causative contact allergen(s) as well as the close avoidance of exposure (12). Personal protective equipment and the use of emollients may help reduce irritancy and stabilize the barrier function of the skin avoiding the penetration of contact allergens. Interestingly, a recent study reported that topical application of $Ni^{2+}$-chelating nanoparticles consisting of $CaCO_3$ or $CaPO_4$ in a glycerine emollient may prevent $Ni^{2+}$ penetration into the skin (13).

Contact eczema is mostly treated with topical glucocorticosteroids. The potency of the steroids and the vehicle to be used depend on the state of eczema, its anatomical localization and the identity of the causative contact allergen(s). Systemic corticosteroid administration is only rarely necessary. Novel approaches for the treatment of hand eczema refractory to topical glucocorticoids include the use of alitretinoin, an agonist of all six known retinoid receptors. Topical calcineurin antagonists such as tacrolimus and pimecrolimus are occasionally used off-label. However, agents that specifically interfere with key steps of ACD pathogenesis have not been developed and introduced yet.

## Characteristics of chemical contact allergens

Most organic or inorganic chemicals that cause ACD are small compounds with a MW of $< 500$ Dalton. As this is too small to be immunogenic, contact allergens must bind to carrier proteins to serve as antigens for the adaptive immune system (hapten–carrier concept). Therefore, protein reactivity is mandatory for contact allergens, however, not only for the formation of T-cell epitopes (14) but, importantly, also for the activation of the innate immune system (1). Some non-reactive chemicals, so-called prohaptens, enter the xenobiotic metabolism pathway in skin cells. There, they are converted to reactive contact allergens by enzymes of the cytochrome P450 (CYP) family in skin cells such as keratinocytes and DCs and can then be exported out of the cell via multidrug-resistance-related family proteins (MRPs) (15–17). Another mechanism for the generation of reactive contact allergens is the (auto-) oxidation of prehaptens (18).

A particular feature of contact allergens is their irritancy or adjuvanticity, i.e. their ability to activate innate immune and stress responses, which in combination cause skin inflammation (1, 19) (Figs 1 and 2). This clearly distinguishes them from conventional protein antigens, which require additional innate signals or addition of exogenous adjuvants to induce immune responses. In general, inflammation induced by xenobiotic chemicals can be termed *xenoinflammation* as recently proposed (20). Xenoinflammation is required in the sensitization phase of ACD for the activation of contact allergen–armed skin DCs that migrate to the local lymph nodes where they prime contact allergen–specific T cells and imprint a skin-specific homing receptor profile (21–23). In the elicitation phase, the inflammatory response triggers the recruitment of effector T cells and other leukocytes to the skin. Importantly, the innate inflammatory immune response is a prerequisite for the activation and shaping of the adaptive immune response (24).

Recent results from basic research employing human cells or the mouse model for ACD, the contact hypersensitivity (CHS) model, have elucidated some of the molecular mecha-

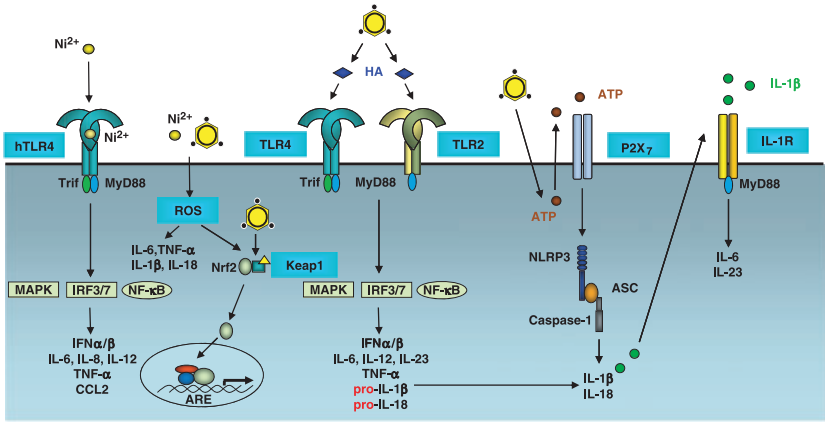


**Figure 1** The innate immune response to contact allergens. $Ni^{2+}$ interacts with human TLR4 to induce proinflammatory signalling, while 2,4,6-trinitrochlorobenzene (TNCB) and other organic contact allergens indirectly activate TLR2 and TLR4 via degradation of hyaluronic acid. Both types of contact allergens induce reactive oxygen species (ROS), which contribute to skin inflammation. Contact allergens may activate Nrf2 in the antioxidant phase 2 response either via ROS-mediated oxidation of or by direct chemical modification of critical cysteines in Keap1. 2,4,6-trinitrochlorobenzene and other organic contact allergens cause the release of ATP from skin cells. By triggering the purinergic receptor $P2X_7$, ATP mediates contact allergen–dependent activation of the NLRP3 inflammasome and caspase-1-dependent processing of pro-IL-1β and pro-IL-18.

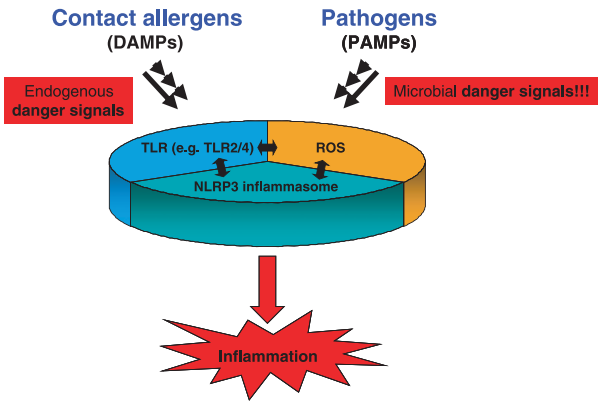


**Figure 2** Analogies of innate immune responses induced by contact allergens and pathogens. Contact allergens directly or indirectly trigger innate immune receptor signalling and inflammation via TLR2, TLR4, $P2X_7$ and the NLRP3 inflammasome. While $Ni^{2+}$ interacts with human TLR4, 2,4,6-trinitrochlorobenzene (TNCB) induces TLR2/4 triggering via hyaluronic acid breakdown. They also induce ROS production. 2,4,6-trinitrochlorobenzene induces ATP release and $P2X_7$-dependent NLRP3 inflammasome activation. IL-1β is released and triggers IL-1R signalling. Microbial pathogens also activate these pathways. TLR, the NLRP3 inflammasome and ROS are nonredundant functionally coupled pathways that act in concert to induce inflammation in response to contact allergens or microbial pathogens.

nisms underlying contact allergen–induced xenoinflammation. A striking finding was the analogy between innate anti-infectious immune responses and the innate immune response to contact allergens (1).

Here, we summarize the recent advances in the mechanistic understanding of chemical-induced innate immunity and discuss their implications for the mechanisms of induction and for potential new causative treatments of ACD.

## Translating chemical reactivity into innate immune and stress responses

One of the most burning tasks is to elucidate the mechanisms underlying the activation of innate immune and stress responses by contact allergen–dependent chemical modification of proteins and, presumably, other macromolecules. It is conceivable that some of the protein modifications introduced by contact allergens have effects similar to conventional post-translational modifications (PTM) such as phosphorylation and glycosylation or they may interfere with such PTM. As a consequence, the function and localization of target proteins may be altered to activate or block signalling pathways and transcriptional regulation (1, 25). Future research aims to identify such functionally relevant target proteins and their relevant target sites for chemical modification by contact allergens. Up to now, it has been shown that the modification of surface thiols by contact allergens can trigger DC maturation (26–28), but the target proteins are so far unknown. In contrast, one good example for functional contact allergen targets is the cytosolic protein kelch-like ECH-associated protein 1 (Keap1) (29–37). In response to oxidative or electrophilic stress, cells upregulate the cytoprotective antioxidative phase II response. In unstimulated cells, Keap1 is associated with the transcription factor nuclear factor–erythroid 2-related factor 2 (Nrf2) and thereby promotes its ubiquitin conjugation and degradation by the proteasome. Upon oxidation of critical cysteine residues in Keap1, Nrf2 is released and translocates into the nucleus. There, it associates with small Maf proteins and other cofactors to activate the transcription of genes containing antioxidant response

elements (ARE) (31, 32). Electrophilic chemicals, among them drugs and contact allergens, can modify selective cysteines in Keap1, and this correlates with the elicitation of the antioxidant response (29, 30) (Fig. 1). As a consequence, a variety of proteins including glutathione synthetase and glutathione reductase, thioredoxin synthetase and thioredoxin reductase, heme oxygenase 1 (HMOX) and NADPH quinone oxidoreductase 1 (NQO1) are upregulated (30–32). Because irritants do not activate the Keap1/Nrf2 response, this system has been proposed as an *in vitro* assay to identify cysteine-reactive contact allergens (33–37).

Another interesting example is the p65 subunit of the transcription factor NF-κB. Sesquiterpene lactones from Arnica plants are weak contact allergens. They have potent anti-inflammatory effects that are able to suppress CHS to 2,4,6-trinitrochlorobenzene (TNCB) (38, 39). Moreover, they fail to induce CHS in mice unless immunosuppressive $CD4^+$ cells are depleted (38). Interestingly, these characteristics correlate with their ability to inhibit NF-κB activation owing to covalent modification of a cysteine residue in position 38 of the p65 subunit of NF-κB (40). These data suggest that the irritant effect of some weak contact allergens may be reduced by negative regulation of proinflammatory signalling owing to covalent modification of signal transduction molecules.

Proteomic analyses have begun to address the question of target proteins in a more systematic approach. Target proteins from human B cells interacting with $Ni^{2+}$ (41), from the human monocytic leukaemia cell line THP-1 modified with 2,4-dinitrofluorobenzene (DNFB) and analogues (42), from the murine macrophage cell line RAW264.7 modified with DNFB (43) as well as proteins with altered thiol content from THP-1 cells treated with diphenylcyclopropenone have been identified (44). The functional relevance of these modified proteins in contact dermatitis remains to be shown.

## Toll-like receptor signalling and oxidative stress responses in contact dermatitis

The so-called irritant effect of contact allergens describes their action as adjuvants, i.e. their ability to activate the innate immune system. In the murine CHS model, it was demonstrated that the strength of the irritant effect of a given contact allergen determines the strength of the CHS response and decides whether immunity or tolerance occurs (39, 45–48).

The molecular mechanisms of innate immune activation by contact allergens were long unresolved. We hypothesized that contact allergens may trigger innate immune activation by employing the same pathways as microbial pathogens. Microbes activate the innate immune system by providing danger signals in the form of so-called pathogen- or microbe-associated molecular patterns (PAMPs) (49–51). These can be bacterial or fungal cell wall components, microbial nucleic acids or proteins and sugars. Both hematopoietic and nonhematopoietic cells express pattern recognition receptors (PRR) such as the membrane-associated Toll-like receptors (TLR) and the cytotosolic NOD-like receptors (NLR) which detect such danger signals and activate inflammatory signalling pathways (52–54). These include the activation of NF-κB, mitogen-activated protein kinases (MAPK) and IRF3/7 leading to the production of proinflammatory cytokines, chemokines and antimicrobial peptides (AMPs). Furthermore, phagocytosis of pathogens results in a respiratory burst by the production of reactive oxygen species (ROS) that serve to kill pathogens. In addition, ROS act as signalling molecules that are now recognized as important inducers of proinflammatory mediators (55–57). Importantly, PRR can also be triggered by nonmicrobial endogenous danger signals, so-called damage-associated molecular patterns (DAMPs) (54, 58, 59). These include extracellular matrix (ECM) components such as hyaluronic acid (HA) and biglycan, heat shock proteins and uric acid crystals.

We have focussed on the putative role of PRR such as the TLR and NLR and on the role of ROS in xenoinflammation. Our studies on the role of both TLRs in the CHS model revealed a crucial role for TLR2 and TLR4 (60) (Fig. 1). The combined absence of these TLRs in mice was sufficient to prevent sensitization to the contact allergens TNCB, oxazolone and fluorescein isothiocyanate (FITC). Resistance to CHS was also observed in mice lacking functional IL-12Rβ2 and TLR2 or IL-12Rβ2 and TLR4 (60). Importantly, contact allergen–modified bone marrow–derived DCs (BMDCs) from these CHS-resistant mouse strains failed to induce sensitization in wild-type mice, while wild-type BMDCs were able to sensitize resistant animals for CHS (60). Moreover, our study uncovered two possible mechanisms of sensitization to contact allergens: an IL-12-dependent one in which a high degree of redundancy between TLR2 and TLR4 exists and a more efficient, IL-12-independent mechanism that requires both functional TLR2 and TLR4. These data also reveal a crucial role of IL-12 in CHS, most likely as an autocrine factor for DCs (61) and as a third signal for T-cell priming and polarization (62, 63).

The importance of TLR signalling in CHS was further demonstrated in mice lacking the TLR/IL-1 receptor (IL-1R) associating adaptor molecule MyD88. MyD88-deficient mice, in contrast to mice lacking the adaptor protein Trif that is also involved in signalling via TLR4, failed to mount CHS responses to DNFB (64) and TNCB (S.F. Martin and F.C. Weber, unpublished data).

The obvious question then was whether microbial ligands for TLR2 and TLR4, e.g. bacterial lipopeptides and lipopolysaccharide (LPS) from skin bacteria, could play a role in CHS induction. However, experiments in germ-free mice showed that CHS to TNCB can develop normally in the absence of bacterial flora. This result strongly suggests that in the case of contact allergens such as TNCB, the generation of endogenous TLR2 and TLR4 ligands in the skin is crucial for the innate immune response that precedes the T-cell activation in ACD.

Presentation of contact allergens to naive T cells by activated DCs is a prerequisite for successful sensitization (14, 65). Notably, we demonstrated that contact allergen treatment *in vitro* only partially activated mouse and human DCs (60) (S.F. Martin and P.R. Esser, unpublished data). Mouse

DCs from both wild-type and CHS-resistant mice upregulated the costimulatory molecules CD40, CD86 and the chemokine receptor CCR7 but failed to produce cytokines such as IL-6, IL-12p70, IL-23 and IFN-α/β. This suggested that full activation of DCs requires a contact allergen–dependent endogenous danger signal that is generated in the skin and delivered via TLR2 and TLR4 (60). In this context, it is important to note that contact allergens are able to induce ROS production in monocytes and DCs *in vitro* (26, 66, 67) (Figs 1 and 2). Reactive oxygen species induce ECM degradation and are capable to trigger proinflammatory cytokine responses (57, 68, 69). Therefore, it is tempting to speculate that contact allergens induce ROS and the respective consequences in the skin. The derivatives of degraded ECM might then provide endogenous PRR ligands driving full activation of DCs under sterile inflammatory conditions (70). A possible candidate is biglycan, an ubiquitous leucine-rich repeat proteoglycan of the ECM. Biglycan has been recently described as a TLR2/4 ligand and activator of the NLRP3 inflammasome (71, 72). Moreover, the oxidative degradation of high MW HA might provide further endogenous activators of the innate immune system (68, 69, 73). While high MW HA is known to exert anti-inflammatory function (74) and to play a role in the maintenance of immune tolerance (75), low MW HA acts as a proinflammatory TLR2/4 ligand as shown in human DCs *in vitro* and in the models of chemical-induced lung inflammation (74, 76–78). Interestingly, we could show that blocking HA function in germ-free mice significantly reduced sensitization for CHS (60). This identifies a role for degraded HA as endogenous activator of TLR2/4 signalling in CHS (Fig. 1).

Recently, ROS have been shown to enhance hyaluronidase-2-mediated HA degradation in a p38 MAPK-dependent manner (79). The breakdown of ECM components is even enhanced by the HA-induced transcription of matrix metalloproteinase (MMP) genes (80). It also results in the generation of AMPs, which are necessary to protect the skin from infections. Such AMPs can also interfere with innate immune responses. One example is the enhanced production of the AMP β-cathelicidin in response to low MW HA-triggered TLR2/4 activation (81). Upregulated cathelicidin expression serves as a counter-regulatory signal for inflammatory processes as mice lacking cathelicidin expression show increased CHS responses to repeated DNFB application (82). Interestingly, cathelicidin prevents HA-mediated cytokine and chemokine release (83).

Taken together, the picture emerges that initial contact allergen–induced ROS production plays an essential role in the generation of endogenous TLR ligands by oxidative HA breakdown (68, 69, 73) and subsequent upregulation of hyaluronidase activity resulting in further enzymatic HA degradation (79). The breakdown of high MW HA to proinflammatory low MW HA fragments may further enhance ECM degradation by an increased transcription of MMP genes that may result in the release of free biglycan. Biglycan release also triggers TLR2/4 as well as NLRP3 inflammasome activation contributing to the proinflammatory innate immune response (72) and may therefore play a role in CHS. In contrast, the low MW HA-mediated release of AMPs like β-cathelicidin prevents overwhelming bacterial infections and provides a counter-regulatory mechanism by limiting inflammation at sites where barrier defects occur owing to ECM degradation.

## The case of nickel as the first inorganic TLR4 ligand

Among the more than 3000 known contact allergens, $Ni^{2+}$ by far is the most relevant one in terms of reported incidences of contact eczema and sensitization rates: alone in Europe, an estimated 65 million individuals suffer from $Ni^{2+}$ allergies (9, 84). In addition, $Ni^{2+}$ allergies have a considerable socioeconomic impact because they can result in occupational disability.

$Ni^{2+}$ is considered the prime example for a contact allergen as it can efficiently activate both innate immune responses and adaptive T-cell responses. The first evidence for a direct proinflammatory action of this metal hapten dates back to the early 1990s when it was shown that treatment of primary human endothelial cells with $Ni^{2+}$ results in a rapid induction of the surface adhesion molecules ICAM1, VCAM1 and E-selectin, which all contribute to leukocyte adhesion (85). Intriguingly, endothelial expression of proinflammatory cytokines such as the monocyte-attracting chemokine CCL2/MCP1 was observed as early as 12–24 h after elicitation of ACD in $Ni^{2+}$-sensitized individuals, a time point well before $Ni^{2+}$-reactive T cells are detectable in the skin (86). This suggested the existence of a clinically inapparent proinflammatory signal induced by $Ni^{2+}$ that precedes the arrival of hapten-specific T cells. Further experiments revealed that $Ni^{2+}$ directly induces the activation of a conserved proinflammatory cascade known as the IKK2/NF-κB pathway in endothelial cells (87) and elicits global gene expression patterns overlapping with those triggered by the proinflammatory cytokine TNF-α (88). Besides regulating NF-κB, $Ni^{2+}$ furthermore stimulates activity of the p38 MAPK, an important coregulator of various NF-κB target genes such as CCL2/MCP1 in endothelial cells (89). In addition, it induces NF-κB-independent stabilization of the proangiogenic transcription factor hypoxia-inducible factor-1 alpha (HIF1-α) (88).

While these early reports demonstrated that $Ni^{2+}$ can directly activate proinflammatory cascades such as the IKK2/NF-κB- and p38 MAPK pathways to a degree comparable to proinflammatory cytokines in human cells (87, 89), its exact mechanism of action remained enigmatic until very recently human TLR4 (hTLR4) was shown to be the crucial receptor for $Ni^{2+}$-induced proinflammatory gene expression (90) (Fig. 1). Intriguingly, the development of $Ni^{2+}$ allergies in humans appears to be a caprice of nature. Binding of $Ni^{2+}$, but not of the natural ligand LPS, to hTLR4 critically requires the presence of two nonconserved histidines (H) in hTLR4, H456 and H458, which are missing in the mouse TLR4 (90). This species difference in the sequence of TLR4 accounts for the long-known natural resistance of mice to $Ni^{2+}$-induced CHS. Transgenic expression of hTLR4 in TLR4-deficient mice can restore $Ni^{2+}$-induced direct proinflammatory activation and confers sensitivity of mice to $Ni^{2+}$-induced CHS, underscoring that species-dependent differences in TLR4 signalling are responsible for the strikingly different susceptibilities of both species to $Ni^{2+}$ (90). It

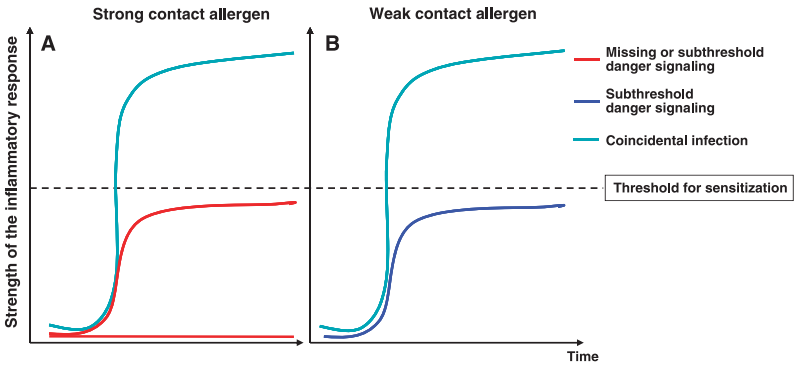


**Figure 3** Hypothetical role of infections as cofactor for sensitization to contact allergens. (A) Mutations or polymorphisms in innate signalling components prevent the activation of the innate immune response by contact allergens. Coincidental infection delivers the missing or amplifies the subthreshold innate stimulus via alternative pathways. (B) Weak contact allergens trigger a subthreshold inflammatory response. Coincidental infection amplifies this response sufficiently to promote sensitization to contact allergens.

remains to be determined whether other strong contact allergens may likewise directly generate the necessary innate immune signals required for sensitization to contact allergens. Interestingly, earlier findings suggest that coadministration of either $H_2O_2$, IL-12, SDS, PMA or the bacterial adjuvants LPS or complete Freund's adjuvant (CFA) allows for the induction of CHS by $Ni^{2+}$ also in wild-type mice (91, 92). This implies that alternative TLR4 activation, for example, via endogenous TLR4 ligands as in the case of the contact allergen TNCB (60) or by microbial TLR4 ligands may substitute for a missing or subthreshold contact allergen–mediated TLR4 activation. This also implies that concomitant infections may serve as triggers or amplifiers of the innate inflammatory immune response to contact allergens (Figs 2 and 3).

The generation of $Ni^{2+}$-reactive T cells may not necessarily require $Ni^{2+}$-induced TLR4 activation. $Ni^{2+}$ is capable of direct interaction with amino acids within the TCR of $Ni^{2+}$-responsive T cells and of the restricting human histocompatibility leukocyte antigen (HLA) DR molecules on antigen-presenting cells (APCs) such as DCs (65, 93, 94). Thus, TLR4 activation during the sensitization phase may primarily serve to activate and mobilize skin-relevant APCs that present $Ni^{2+}$ on HLA molecules to T cells in the skin-draining lymph nodes. The role of TLR4 in the elicitation phase is currently unclear. In case of ACD to $Ni^{2+}$, TLR4 is most likely required to trigger the activation of the endothelium (85–88), which is a prerequisite for the entry of blood leukocytes to sites of allergen challenge.

Importantly, the striking species dependency of $Ni^{2+}$-induced ACD warrants the careful interpretation of animal-derived data with human contact allergens and advises the analysis of contact allergens in alternative test systems such as organ cultures of human cells (12, 95).

## Inflammasome activation in CHS

A crucial role of the cytokines IL-1β and IL-18 in CHS has been demonstrated (96–98). In line with these findings, the NLRP3 inflammasome and IL-1R signalling are required for CHS. The NLRP3 inflammasome activates caspase-1. This protease cleaves the biologically inactive proforms of IL-1β and IL-18, which are produced, for example, upon TLR2/4 signalling, to their biologically active and secreted forms. Mice lacking either NLRP3 or the adaptor protein ASC showed impaired CHS responses to TNCB and DNFB (48, 99, 100). Similarly, caspase-1 or IL-1R deficiency or treatment of mice with the IL-1R antagonist anakinra prevented CHS (48, 97, 100, 101).

The important role of inflammasome activation in CHS was further underlined by using 2,4-dinitrothiocyanobenzene (DNTB). 2,4-dinitrothiocyanobenzene is a weak contact allergen or a tolerogen. This property correlates with its inability to efficiently activate the inflammasome (36, 48). However, upon coapplication of the irritant SDS or of IL-1β DNTB was able to sensitize mice for CHS elicited with the strong contact allergen DNFB. Preliminary evidence suggests that the failure of DNTB to elicit sufficient irritancy results in the presentation of haptenated peptides on tolerogenic DCs, and this induces tolerance via induction of $CD4^+CD25^+$ regulatory T cells (Tregs) (48).

The mechanism of NLRP3 inflammasome activation by contact allergens was unclear until recently. Besides HA fragments triggering TLR2 and TLR4 (Fig. 1), extracellular nucleotides are potent endogenous danger signals. ATP is the main energy carrier within cells. Yet under physiological conditions, the concentration of extracellular ATP is as low as 10 nM, while the intracellular concentration ranges between 3 and 10 mM. Stressed, damaged or dying cells release ATP, which interacts with purinergic receptors on different immune and structural cells. These receptors bind extracellular nucleotides and their degradation products ADP, AMP and adenosine. The family of purinergic receptors is divided into two subgroups: the P2Y receptors that are G-protein-coupled 7-transmembrane receptors and the P2X receptors that are ligand-gated ion channels (102, 103). Because extracellular nucleotides are potent inducers of inflammation, they are tightly regulated by ectonucleotidases such as CD39 and CD73 and also by active uptake into cells. CD39 and CD73 are highly expressed on Tregs and contribute to nonspecific immune regulation by the removal of proinflammatory ATP

and by the production of anti-inflammatory adenosine (104, 105). CD39-deficient mice displayed stronger T-cell-independent irritant CHS responses to irritants such as croton oil or benzalkonium chloride but, surprisingly, showed attenuated T-cell-dependent allergic CHS to the contact allergen oxazolone. Contact hypersensitivity was also reduced upon sensitization of wild-type mice with CD39-deficient BMDCs modified with 2,4,6-trinitrobenzene sulphonic acid (TNBS) as compared to similarly treated wild-type BMDCs (106). A recent study demonstrated that CD39-dependent adenosine produced by Tregs prevented the adhesion of effector T cells to the endothelium of blood vessels by downregulation of the adhesion molecules E- and P-selectin and thereby suppressed CHS to TNCB (107).

Triggering of the $P2X_7$ receptor by ATP results in a pannexin-1-dependent $K^+$ efflux from the cell, which is a prerequisite for the activation of the NLRP3 inflammasome (54). A previous study showed that intradermal injection of non-hydrolysable ATP-γS at the time point of sensitization enhanced CHS to DNFB (108). We could now demonstrate that contact allergens such as TNCB and oxazolone induce the release of ATP from skin cells *in vivo*. This results in potent activation of the NLRP3 inflammasome via $P2X_7$ as demonstrated by the absence of CHS in $P2X_7$-deficient mice (101) (Fig. 1). Moreover, contact allergen–modified $P2X_7$-deficient BMDCs lacked the ability to sensitize both wild-type and $P2X_7$-deficient recipients, while similarly modified wild-type BMDCs could sensitize both wild-type and $P2X_7$-deficient recipients. $P2X_7$-deficient BMDCs failed to release mature IL-1β upon a combined LPS and ATP stimulation. This defect could be restored by replacing ATP with the $P2X_7$-independent inflammasome activator alum. In addition, the CHS-resistant phenotype of $P2X_7$-deficient mice was rescued by sensitization with contact allergen–modified alum prestimulated $P2X_7$-deficient BMDCs or by intracutaneous administration of recombinant IL-1β. Experiments using ASC- and NLRP3-deficient BMDCs demonstrated that the alum- and ATP-mediated effects are dependent on the NLRP3 inflammasome. BMDCs from these mouse strains also failed to induce the sensitization of wild-type mice (101). Again, as shown for TLR2, TLR4 and IL-12Rβ2 (49), $P2X_7$, ASC and NLRP3 are crucial for sensitization to contact allergens and have to be functional in DCs.

These findings underscore the importance of $P2X_7$- and inflammasome-dependent IL-1R signalling in CHS (Fig. 1). IL-1β appears to be the main caspase-1-dependent inducer of inflammation in CHS as IL-1R-deficient mice are resistant to CHS, while IL-18-deficient mice still develop CHS. In line with this observation, CHS can be prevented by administration of the IL-1R antagonist anakinra before sensitization (48, 101). Administration of the $P2X_7$ antagonist KN-62 also led to abrogated sensitization (101) (Fig. 4).

Besides the NLRP3 inflammasome that regulates inflammatory cytokine release, NLRP12 plays an important role in CHS (109). This NLR is highly expressed in DCs and neutrophils. NLRP12-deficient mice showed severely attenuated CHS responses to oxazolone and FITC and reduced neutrophil infiltration as well as impaired lymph node homing of DCs. These data demonstrate a role of NLRP12 in the regulation of chemotaxis in neutrophils and DCs in CHS (109). Although previous studies showed a role of NLRP12 in IL-1β production upon overexpression (110) and demonstrated a negative regulation of the noncanonical NF-κB pathway downstream of the TNF receptor (111), in the CHS model, no change in the production of IL-1β and TNF-α was found in the ear tissue of NLRP12-deficient mice (109).

## Implications for *in vitro* assays to identify contact allergens

The understanding of the innate immune mechanisms in ACD allows their implementation in the development of

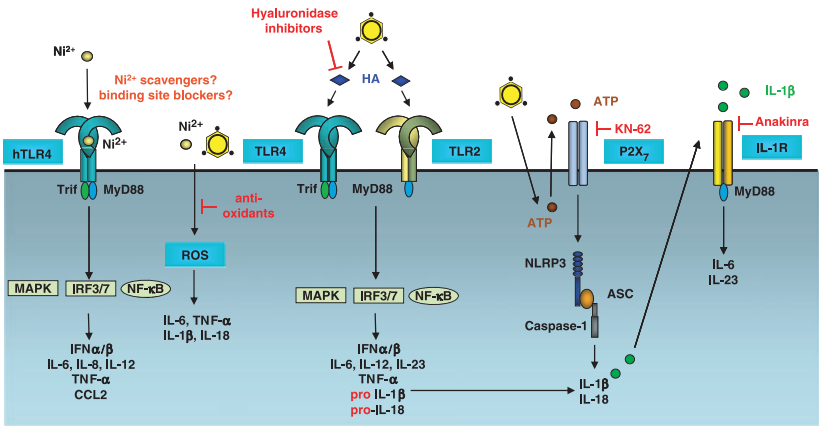


**Figure 4** Possible therapeutic approaches of anti-inflammatory intervention to prevent or treat allergic contact dermatitis. Nickel contact dermatitis may be prevented by the development of $Ni^{2+}$ scavengers or small molecules that block the $Ni^{2+}$ binding sites in human TLR4. In mice, contact hypersensitivity (CHS) is prevented by blocking the breakdown of hyaluronic acid or by inhibiting the formation or action of ROS with antioxidants. Contact hypersensitivity is also blocked by inhibiting NLRP3 inflammasome activation using antagonists for $P2X_7$ (KN-62) or IL-1R (Anakinra). Because of the functional coupling, the blockade of a single pathway is sufficient to prevent sensitization for CHS in mice.

urgently needed *in vitro* alternatives to animal-based tests for the identification of contact allergens (12). The regulatory basis for that is the 7th Amendment to the EU Cosmetics Directive that prohibits sensitization testing of cosmetic ingredients in animals since March 2009 and the EU regulation REACh requiring testing of at least 40 000 already marketed chemicals for their sensitizing potential. Ideally, testing should be carried out with human cells or organ cultures employing *in vitro* assays to replace the extensive use of animals for sensitization testing. However, up to now, there are no validated *in vitro* assays that immediately replace the gold standard, the mouse local lymph node assay (LLNA) (95, 112). This assay measures the capacity of chemicals applied three times to mouse ear skin to induce the proliferation of lymph node cells. Besides identification of contact allergens, the LLNA also allows potency assessment of contact allergens, an important issue when it comes to the use of potential contact allergens in consumer products in subsensitizing concentrations. The LLNA reflects the sensitization process from the skin contact of the chemical to the induction of a T-cell response in the lymph node. It is conceivable that no single *in vitro* assay may reliably warrant contact allergen identification given the complexity of the *in vivo* sensitization process and the necessarily highly reductionist nature of *in vitro* assays. Therefore, current strategies aim at the combination of assays that reflect key steps of the sensitization process in a tiered approach as proposed by Jowsey et al. (113). The current assays in development address several key steps of the sensitization process: the reactivity of the test substance with model peptides (114, 115), the activation of DCs by the test substance measuring upregulation of CD86 and CD54 and production of IL-8 (116, 117), the production of IL-18 in keratinocytes (118), the migration of DCs (119), the activation of the Keap1/Nrf2 antioxidant response in HaCaT keratinocyte reporter cells (36) and the priming of contact allergen–specific human T cells by DCs (120–122). However, the overall reliability, accuracy and further optimization of such assays depend on our ability to close the still significant gaps of knowledge (95, 123).

Genomic and proteomic approaches now identify common characteristic gene signatures and protein profiles for contact allergens based on the regulation of these parameters in monocytes, DCs or keratinocytes treated with the test substances. The power of these global approaches is the identification of pathways that are regulated by contact allergens. Interestingly, many of the current assays employ the activation of TLR/IL-1R-dependent pathways, inflammasome activation, oxidative stress and antioxidant responses. With our now much more detailed mechanistic understanding of the sensitization process, the existing assays can be improved by including new read-out parameters, and new assays can be developed. This will allow to integrate these *in vitro* alternatives to animal testing as useful tools for hazard identification and risk assessment in immunotoxicology. Furthermore, they may aid the diagnosis and prevention of ACD and of other chemical- and drug-related hypersensitivities (12, 95, 122).

## Infections and ACD – analogous mechanisms and implications

The realization that contact allergens activate similar innate immune and stress responses as infectious agents (Figs 1 and 2) has interesting implications regarding the interplay of infections and contact allergens. We have already demonstrated that the inability of TLR4- and IL-12Rβ2-deficient BMDCs to mediate sensitization can be restored by treatment with a synthetic bacterial DNA, CpG-oligodeoxynucleotides, via TLR9 (60). Moreover, alum pretreatment that bypasses $P2X_7$ and directly activates the NLRP3 inflammasome restores the sensitization capacity of $P2X_7$-deficient BMDCs (101). These findings as well as the observation that the sensitization of mice for CHS to $Ni^{2+}$ can be induced efficiently upon coadministration of LPS or CFA (91, 92) suggest that susceptibility to ACD can be acquired by coincidental infection. Thus, triggering of TLRs, the NLRP3 inflammasome and ROS formation by infection may provide missing danger signals or amplify insufficient inflammatory stimuli (Figs 2 and 3). Deduced from the findings in the CHS model, this could result from an impaired function of innate signalling receptors or other components of the signalling pathways owing to polymorphisms. Thus, a loss of function or impairment of TLR2 and TLR4 signalling may be compensated by triggering alternative TLR activation by infectious agents to feed the inflammatory cascade (Fig. 3A). Moreover, weak contact allergens may exert an inefficient adjuvant effect that leads to subthreshold inflammation. In that case, a coincidental infection may amplify the inflammatory response over the critical sensitization threshold via TLR signalling, inflammasome activation and ROS production (Fig. 3B).

## Potential relevance for the treatment of ACD

An important observation from the mechanistic studies of the sensitization process of CHS is the nonredundant collaborative action of the innate immune and stress pathways (Fig. 2) and their crucial importance for DCs (1). Innate immune signalling-incompetent DCs fail to induce sensitization to contact allergens. Notably, it does not matter which of the pathways is compromised as shown by us using BMDCs lacking either TLR2/IL-12Rβ2, TLR2/TLR4, TLR4/IL-12Rβ2, NLRP3, ASC or $P2X_7$, respectively (60, 101). As a consequence, it is sufficient to block one of these pathways or the degradation of HA or production or action of ROS to prevent sensitization as clearly shown in the CHS model (48, 60, 101) (Esser, P.R. et al., unpublished data) (Fig. 4). Moreover, these innate signalling pathways may also be relevant for the elicitation of CHS and most likely play a role in cell types other than DCs, for example endothelial (85–88) and epithelial cells (100, 124).

The findings made in the CHS model may help identify potential drug targets for the causative treatment of ACD and also for other inflammatory diseases (Fig. 4). However, anti-inflammatory strategies need to be carefully designed to prevent uncontrollable infections because the defence against

microbial pathogens might use the same components of innate immunity that are targeted by the respective drugs. In the case of $Ni^{2+}$, interaction with TLR4 does not interfere with the binding of LPS (90). Thus, an optimal drug should block the $Ni^{2+}$ binding site of hTLR4 while leaving the antimicrobial response untouched (Fig. 3). Altogether, the current exciting results from basic research should encourage the development of new and causative approaches for the prevention and treatment of ACD.

## Conflict of interest

The authors declare no conflict of interest.

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