UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

PhD Thesis

Jonas Damgård Schmidt

Investigations of Local Immunological Mechanisms in Contact Allergy

Principle supervisor: Associate Professor Charlotte Menné Bonefeld

Co-supervisor: Professor Jeanne Duus Johansen

This thesis has been submitted to the Graduate School of the Faculty of Health and Medical Sciences, University of Copenhagen, 30/06/2016

1 Preface

This PhD thesis aims to investigate tissue resident memory T cells in the context of contact hypersensitivity. The studies were mainly conducted *in vivo* using a murine contact hypersensitivity model, but also translational experiments were performed in patients with contact allergy. Moreover, the induction of hair dye allergy and the development of tolerance, were examined *in vivo*.

The experimental work presented here was carried out in the T cell biology group at the Department of Immunology and Microbiology, University of Copenhagen and the Department of Dermato-Allergology, National Allergy Research Centre, Gentofte University Hospital.

This thesis was financially supported by a full PhD grant from the Faculty of Health and Medical Sciences.

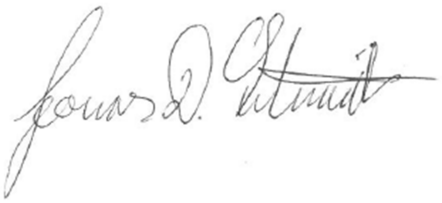
This work could not have been possible without the help of others. Foremost I thank my principle supervisor Associate Professor Charlotte Menné Bonefeld for competent guidance in the scientific disciplines of contact allergy and T cell biology and for an enthusiastic and inspiring attitude. Also I thank my co-supervisor Professor Jeanne Duus Johansen for her helpful spirit and for introducing me to the international network of the CARGO meetings. Also, I thank the members of the T cell biology group and Professor Carsten Geisler for providing a strong scientific, pleasant and encouraging atmosphere in the everyday work. I also wish to credit Trine Hilkjær Petersen and Terkild Brink Buus for much valued critical proofreading of this thesis.

Also I thank my parents for their great support and cheering.

Finally, I thank my dearests, Amelia, Isalina and Kiri for their love and support throughout my studies and especially in the last dreary period of concluding this thesis.

Copenhagen, 2016-06-30

Jonas Damgård Schmidt



2 Table of Contents

3 Abstract

The skin is exposed to a wide array of environmental substances during everyday life. Some of these can cause an inflammatory skin condition known as allergic contact dermatitis (ACD) and these substances are thus called contact allergens. ACD is a common disease in the developed world and is mainly considered as a T cell driven delayed type hypersensitivity reaction.

This PhD thesis is based on three studies.

Study I: Tissue resident memory T (T_{RM}) cells have in the recent years been found to provide local skin memory against virus infections. This study focuses on the involvement of T_{RM} cells in local skin memory in contact allergy. Here we describe a mouse model for investigating the development of local skin memory to an experimental allergen. Following 3 weeks of skin sensitization, we identify strong clinical symptoms to allergen challenge in the local sensitized skin. Furthermore, we detect two phenotypes of epidermal $CD8^+$ T_{RM} cells capable of fast production of IL-17 and IFNγ in the sensitized skin, which induce significant expression of IL-1β upon allergen exposure. Furthermore, we show that the local skin memory translates to ACD patients with nickel allergy.

Study II: Oxidative hair dyes can contain strongly sensitizing chemicals. This study investigates two commercial available hair dyes containing toluene-2,5-diamine (PTD) in a mouse model for sensitization. We measure the PTD content of the two hair dyes and demonstrate that they induce strong proliferation of T and B cells compared to corresponding PTD concentrations alone. When applied in a consumer like pattern with bi-weekly exposures, only the hair dye with high PTD content induces a significant inflammatory response. Following four exposures, the response reaches a plateau. Finally, we demonstrate that regulatory $CD4^+CD25^+FoxP3^+$ T cells are generated in the draining lymph nodes following sensitization and follow the general proliferation pattern of T and B cells when mice are exposed to the hair dye having a high content of PTD.

Study III: Occupational exposure to sensitizing hair dye ingredients entails a repetitive exposure to chemicals like *p*-phenylenediamine (PPD). From study II, we find that a prolonged exposure regimen may induce tolerance. In this study, the long-term exposure to increasing sub-sensitizing concentrations of PPD is examined. We find that the local exposed skin become unresponsive to PPD exposure following four weeks of topical treatment. Following a resting period, challenge responses to high doses of PPD, showed that the immune suppression was intact in the skin, whereas lymphocytes proliferated significantly in the draining lymph nodes.

4 Dansk resume

Huden udsættes for en bred vifte af miljømæssige stoffer i hverdagen. Nogle af disse kan forårsage en inflammatorisk hudtilstand kendt som allergisk kontakteksem (ACD), og disse stoffer derfor kaldes kontaktallergener. ACD er en almindelig sygdom i den vestlige verden og er primært betragtet som en T-celledrevet, forsinket hypersensitivitetsreaktion.

Denne ph.d.-afhandling er baseret på tre studier.

Studie I: Hud-residente hukommelses T (T_{RM})-celler har i de senere år vist sig at danne lokal hudhukommelse imod virusinfektioner. Dette stidue fokuserer på T_{RM} celler i dannelsen af lokal hudhukommelse i kontaktallergi. Her beskriver vi en musemodel og undersøger udviklingen af lokal hukommelse i huden overfor et eksperimentelt allergen. Efter hudsensibilisering, måler/finder vi stærk hudinflammation ved allergenprovokation i den lokal-sensibiliserede hud. Endvidere identificerer vi to fænotyper af epidermale $CD8^+$ T_{RM} celler, og viser at disse kan respondere med hurtig produktion af IL-17 og IFNγ, der inducerer signifikant ekspression af IL-1β i keratinocytter. Slutteligt viser vi, at den denne mekanisme for lokal hudhukommelse kan translateres til ACD hos patienter med nikkelallergi.

Studie II: Oxidative hårfarver kan indeholde mange forskellige stærkt allergifremkaldende kemikalier, men der fastsættes kun grænseværdier i produkter baseret på de enkelte kemikaliers sensibiliseringsegenskaber. I dette studie undersøges to kommercielt tilgængelige hårfarver indeholdende toluen-2,5-diamin (PTD) i en musemodel for sensibilisering. Vi måler PTD indholdet af de to hårfarver og viser, at de inducerer stærk proliferation af T- og B-celler sammenlignet med tilsvarende PTD-koncentrationer alene. Når hårfarverne påsmøres i et forbrugermønster med ugentlige eksponeringer, er det kun hårfarven med højt PTD indhold, der inducerer en signifikant inflammatorisk reaktion. Efter fire eksponeringer, når responserne et plateau. Slutteligt viser vi, at regulatoriske $CD4^+CD25^+FoxP3^+$ T-celler rekrutteres til de drænerende lymfeknuder og følger proliferation- og infiltrationsmønsteret for T- og B-celler efter sensibilisering ved eksponering for hårfarven med højt PTD indhold.

Studie III: Erhvervsmæssig eksponering over for allergifremkaldende hårfarvekemikalier, som p-phenylendiamin (PPD) indebærer gentagne eksponeringer. Fra studie II finder vi, at et langvarigt udsættelsesregime kan fremkalde tolerance. I dette studie undersøger vi langtidseksponering for PPD i stigende koncentrationer. Vi finder, at den lokalt eksponerede hud udvikler tolerance efter fire uger, og at mekanismen er opretholdt efter en hvileperiode.

5 List of abbreviations

Ab	Antibody
ACD	Allergic contact dermatitis
APC	Antigen presenting cell
BMDC	Bone marrow derived dendritic cells
CA	Contact allergy
CD	Contact dermatitis
DETC	Dendritic epidermal T cell
dLN	Draining lymph nodes
DNBS	2,4-dinitrobenzenesulphonic acid
DNCB	2,4-dinitrochlorobenzene
DNFB	2,4-dinitro-1-fluorobenzene
FITC	Fluorescein isothiocyanate
HICC	Hydroxylisohexyl-3-cyclohexene carboxaldehyde
i.v.	Intravenous
LLNA	Local lymph node assay
LPS	Lipopolysaccharide
ME-PPD	2-Methoxy-methyl-p-phenylene- diamine
MHC	Major histocompatibility complex
OXA	Oxazolone
PAMP	Pathogen associated molecular patterns
PNG	Peptidoglycan
PPD	p-phenylenediamine
PPRs	Pattern-recognition receptors
PTD / TDA	Toluene-2,5-diamine
ROS	Reactive oxygen species
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzenesulphonic acid
T_{RM}	Tissue resident memory T cell

6 Introduction

6.1 The anatomy of the skin

The skin is a complex organ that provides a physical barrier, which protects the body from harmful environmental substances and pathogens, thus the skin is a highly immunological organ. The overall architecture of the skin is divided into two layers called the epidermis and the dermis. The epidermis is the outermost layer and consists mainly of keratinocytes (KCs), which continuously grow and terminally differentiate from the stratum basale, which is attached to the basal membrane. The newly generated KCs form the stratum spinosum and further develop into the stratum granulosum. The latter, form a dense protein network of keratin cross-bound by filaggrin[1–3], and in addition tight junction proteins enhance cell-to-cell contact. Ultimately, the stratum corneum is the terminal layer consisting of enucleated KCs[4]. Although the epidermis mainly consists of KCs, it also contains melanocytes and immunological cells such as the dendritic Langerhans cells (LCs) and $CD8^+$ T cells under steady-state conditions[5,6]. Hair follicles consist of complex cell structures, which reach to the lower dermal layers and thus provide a point of entry for pathogens and environmental substances[4]. The dermis is less dense in cells and subdivided into two compartments: the upper and lower stratum papillae. They are both constituted of extracellular matrix, collagen and fibrin molecules acting as a framework for blood vessels and lymphatic vessels that provide access of nutrition and immune surveillance from circulating lymphocytes[4]. Mice are frequently used for studying skin diseases due to similar immune systems to humans, however there are anatomical differences; mice have faster cell turnover in the epidermis, which is signified by having faster wound healing[4]. In addition, human epidermis is about four times thicker compared to mice[6].

6.2 Allergic Contact Dermatitis

Allergic contact dermatitis (ACD) is a common inflammatory skin condition that affects 20-27% of the general European population[7–9]. From a socio-economical perspective, the consequences of ACD range from loss of quality of life to leave of absence from work, and the Danish Health Authorities have estimated the direct annual costs of ACD in the range of 800 million DKK including healthcare and absence from work[10]. ACD is characterized as a delayed type IV hypersensitivity reaction following skin contact to specific molecules, called

contact allergens or haptens. The disease development is divided into two phases: (I) The sensitization and (II) the elicitation phase (Figure 1). The sensitization phase is induced when a hapten penetrates the skin barrier and reacts with skin proteins to form an immunogenic antigen. Cutaneous dendritic cells take up the hapten and following activation, these cells migrate to the draining lymph nodes and present the hapten-peptide-major histocompatibility complex (MHC) to hapten-specific T cells that become activated and generate T cell memory[11]. Once sensitization has been induced, the affected individual may react with dermatitis to minute amounts of allergen on re-exposure[12,13]. The resulting inflammatory skin reaction is caused by distinct effector T cell subsets that release inflammatory cytokines and cytotoxic granules, which attract non-specific monocytes and ultimately result in clinical symptoms such as erythema, edema and vesicle formation (Figure 1)[14–16].

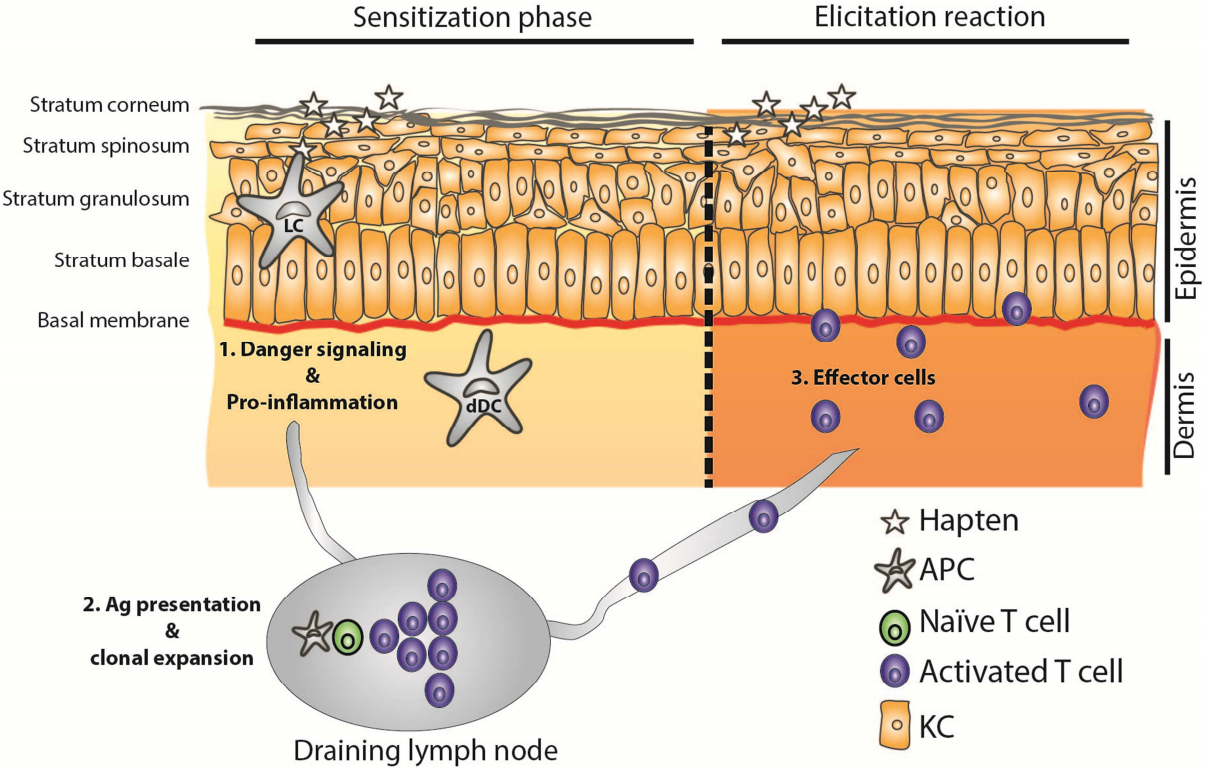


Figure 1: Schematic model for immunological mechanism in Allergic Contact Dermatitis
ACD is initiated in sensitization phase where haptens penetrate the skin barrier and bind to self-proteins. In this process, **(1)** danger signaling and pro-inflammation is induced and skin-resident APCs are stimulated to take up haptens and migrate to the dLNs. **(2)** Here, naïve specific T cells are activated by APCs clonal expansion generate **(3)** effector T cells that react promptly with skin inflammation upon hapten re-exposure in the challenge phase.

6.3 Mechanisms of sensitization

6.3.1 Properties of contact allergens

Contact allergens constitute a large heterogeneous group of low-weight molecules below 500 Dalton that are able to pass through the outer stratum corneum[17]. Thus contact allergens range over electrophilic organic molecules and inorganic metal ions, and in the context of ACD these are called haptens[11]. Given the small size, haptens are in themselves too small to provide an antigen but rather they bind skin proteins. Landsteiner and Jacobs originally proposed the idea that contact allergens need a carrier in order to provide an antigen[18]. They showed that the contact allergen, 2,4-dinitrocholorobene (DNCB), covalently bound to skin proteins to form what is generally referred to as a hapten-peptide complex[18]. The capacity of a hapten to form an immunological epitope depends on its physical-chemical properties such as redox potential and hydrophobicity that can allow a contact allergen to bind intracellular proteins. Some molecules need to be activated prior to skin penetration e.g. by sunlight or atmospheric oxygen and these are called pre-haptens, such as linalool and geraniol[19]. Pro-haptens e.g. cinnamyl alcohol and urushiol are activated by metabolic processing in Langerhans cells and keratinocytes[20,21]. Contact allergens can bind proteins by non-covalent interactions e.g. metal ions, such as cobalt and nickel, create hapten-peptide complexes by chelation of amino acid residues[22–24].

6.3.2 Irritation and inflammation in sensitization and elicitation responses

In order for sensitization to be induced, an inflammatory threshold must be reached, similar to the adjuvant effect in vaccinations, whereby the innate immune system is activated, and the resulting inflammatory reaction has been shown critical to skin sensitization. Thus, Kligman *et al* demonstrated that the effective concentration for sensitization to nickel, *p*-phenylenediamine (PPD) and Neomycin in human subjects was lowered when the skin sites were pretreated with sodium lauryl sulphate (SLS)[25]. In accordance with these findings, irritation has been shown to boost the elicitation response as demonstrated by Agner *et al*[26]. Here, ACD patients sensitive to nickel, responded with synergism to nickel challenge when SLS was co-administered[26]. In another study, ACD patients with contact allergy to two fragrances reacted synergistically following patch testing with both haptens in combination compared to patch testing with the fragrances separately applied[27]. Murine studies have demonstrated that mice sensitized with oxazolone (OXA), respond to sub-irritant levels of OXA in the elicitation phase when co-administrated with 2,4,6-trinitrochlorobenzene

(TNCB) used in concentrations above irritant levels[12]. Furthermore, it was shown that TNCB in combination with croton oil, a known irritant[28], enhances the elicitation response[12]. In accordance, a murine study showed that the inflammatory level in the sensitization response correlated with the strength of the elicitation response[29].

The importance of irritant effects is further highlighted by the different responses seen toward DNFB and DNTB, which generate identical haptens but have highly different irritant effects. Mice sensitized with DNTB do not respond to challenge with DNFB unless the irritant sodium dodecyl sulphate (SDS) is co-administered in the sensitization. In the reversed setup, mice sensitized with DNFB respond significantly to challenge with DNTB[30].

Sensitization to mixtures of chemicals is highly relevant in the perspective of consumer-exposure to e.g. perfumes, as these are known to contain up to 100 fragrances. Whereas some are classified as weak sensitizers, indeed also moderate and strong sensitizers have been identified in perfume products[31]. The toxicological assessments of complex allergen mixtures are *per se* difficult to interpret statistically, as the available test methods only predict safe concentration for individual allergens[32]. Recently, data from our group showed that mice sensitized with a mix containing cinnamal, iso-eugenol and hydroxylisohexyl 3-cyclohexene carboxaldehyde (HICC) reacted with stronger elicitation response to challenge with cinnamal when compared to mice sensitized with cinnamal alone[33]. The mechanisms for the cocktail effect may involve the combined irritant effect of the hapten mixture in combination with enhanced T cell priming during sensitization, as demonstrated in a recent study from our group[34].

6.3.1 Toll-like receptors mediate innate responses in the sensitization phase

Besides from providing a physical barrier, the skin is indeed an immunological active barrier that can respond to invading pathogens and environmental substances. The skin cells express innate pattern recognition receptors (PRRs), e.g. Toll-like receptors (TLRs) and nod-like receptors (NLR) that can sense pathogen/danger associated molecular patterns (PAMPs/DAMPs) of exogenous and endogenous molecular motifs[6,35].

In mammals, 11 members of the TLR family have been identified with TLR1-9 being conserved between man and mouse[36]. The cytoplasmic parts of TLR contain a Toll/IL-1 receptor (TIR) domain, which resemble the IL-1 receptor domain, but the domains differ in their extracellular part with TLRs having a conserved leucine rich repeat motif that form a horseshoe ligand binding site[36]. TLRs are expressed by many cells and have distinct ligand affinity. Some are expressed on the cell surface (TLR1-2 and TLR4-5) whereas others are found in intracellular sub compartments (TLR3, TLR7-9)[37]. Upon ligand binding, the TLRs dimerize and recruit a number of adaptor proteins which initiates phosphorylation signals to promote cytokine production. MyD88 is a key adaptor protein that selectively binds the TIR domain of all TLRs except TLR3[37]. Thus, dimer formation of TLR1/6:TLR2 or TLR4:TLR4 mediates further signal transduction through IRAK1:TRAF6 and subsequently activates the nuclear factor kappa B (NF-κB), which promote IL-1β, IL-6 and TNF-α transcription[37]. Signaling through TLR3-ligand interaction occurs independently of MyD88, but through TRIF and activates the transcription factor interferon regulatory factor3 (IRF3) that promotes transcription of antiviral IFN-β. Interestingly, under specific conditions, TLR4 has been shown to induce Type I IFNs independently of MyD88[38].

In context of ACD, TLR2 and TLR4 have been shown to play a pivotal role in initiating the sensitization. In a series of studies from the lab of S. F. Martin, it was shown that mice genetically deficient for TLR2 and TLR4 were unresponsive to sensitization with TNCB, oxazolone and fluorescein isothiocyanate (FITC)[39]. Conversely, wild-type mice housed under germ-free conditions responded to sensitization with TNCB, indicating that the response was not dependent on microbes, and thus considered as a sterile inflammation. Constituents of the extracellular matrix can act as endogenous TLR ligands or DAMPs. For example, heparan sulphate biglycan and low molecular-weight breakdown products of hyaluronic acid are both ligands of TLR2 and TLR4[40,41]. Thus, it was suggested that the protein reactive property of TNCB could generate breakdown products of low molecular-weight hyaluronic acid (HA) stimulating TLR2/4 signaling[40,42]. This was supported by the finding that anti-HA administration to mice, held in germ-free conditions, impaired the CHS response to TNCB[39].

Although nickel does not bind skin proteins by covalent interactions, nickel has strong affinity for histidine amino acid residues; a property widely used in protein purification. Interestingly, nickel has been shown to dimerize human TLR4 by chelation of extracellular histidine residues. However, the murine TLR4 lacks histidine residues in its extracellular part

and render mice non-responsive to topical nickel sensitization, but conversely, intra-dermal injection with nickel co-administered with LPS (TLR4 ligand), induce sensitization in mice[23]. In line with this, it was shown that transgenic mice bearing the human TLR4 did indeed respond to percutaneous nickel sensitization[23]. However, recent data from our group have demonstrated that mice are indeed susceptible to nickel sensitization by epicutaneous nickel exposure[43]. This response was found to be dependent on MyD88 but independent on TLR4[43]. Although, the basis for this discrepancy remains unsettled, the studies used different CHS models. The latter study employed a re-exposure regimen on the ears for sensitization and challenge, which may promote a local CHS mechanism for sensitization over abdominal sensitization and ear challenge[23,43]. In support of TLR4-independent sensitization, in vitro experiments using primary human KCs were did indeed respond to nickel with significant IL-8 production[44]. However, these KCs only reacted to TLR3 ligands, whereas addition of know TLR4/5/7/8/9 ligands failed to induce IL-8 responses[44].

Although TLR2 and TLR4 seem to play pivotal roles in CHS, it is noticeable that a role for TLR3 involvement has been elucidated in a recent study[45]. Here, TLR3-deficient mice displayed reduced CHS responses both to TNCB and croton oil, whereas transgenic mice overexpressing TLR3 showed enhanced CHS as measured by ear thickness[45]. Furthermore, TLR3-KO mice responded with lower IL-1β and CXCL10 levels compared to the wild-type control group[45].

6.3.2 Activation of the inflammasome in CHS

One of the cytokines induced following skin exposure to haptens is interleukin-1β (IL-1β). Early studies by Katz and Enk have shown that IL-1β is induced 15 min after skin-exposure[46]. Moreover, intra-dermal injections of recombinant IL-1β, have been found to enhance LC activation as increased numbers of LCs migrate to the LNs upon allergen application[47]. Transgenic mice, deficient for the IL-1 receptor (IL-1R), show suppressed responsiveness in the CHS model[48], and administration of the IL-1R antagonist anakinra impairs the CHS response[49]. This underlines the pivotal role of signaling through IL-1R in CHS. Indeed IL-18 has also been shown as an important cytokine in the initiation of CHS, given that IL-18$^{-/-}$ mice respond with impaired CHS to DNFB and OXA, but could be restored by intra dermal injection of IL-18, IL-1β and TNF-α prior to sensitization, indicating that IL-18 is upstream of IL-1β and TNF-α[50]. Interestingly, induction of IL-1β is up-

regulated in KC cultures treated with SDS or DNFB but remains un-responsive to DNTB treatment[30]. This may explain why DNFB acts as a strong sensitizer, whereas DNTB is known to induce tolerance[51]. Mice sensitized with DNTB that received IL-1β intravenously *(*i.v.*)* reacts to DNFB challenge, underscoring the importance pro-inflammatory signal derived by IL-1β[30].

The production and secretion of IL-18 and IL-1β is a two-step process that involves priming signals that occurs by TLR signaling through a MyD88 pathway ultimately activating nuclear factor kappa B (NF-κB) that induce expression of the inactive proforms, which needs to undergo enzymatic activation by the NOD-like receptor 3 (NLRP3) inflammasome[52]. The NLRP3 inflammasome is a multi-protein complex situated in the cytoplasm, and it consists of a sensory protein, the NOD-like receptor (NLR), the adaptor protein, ASC, and the protease Caspase-1. The concomitant events of potassium efflux and generation of intracellular reactive oxygen species (ROS) tightly control activation of the NLRP3[52].

Extracellular ATP induced from haptens can activate $P2X_7$ receptors and induce potassium depolarization and accumulation of intracellular ROS and thereby trigger NLRP3 activation[30]. $P2X_7$ receptors has been shown important in CHS, as $P2X_7^{-/-}$ mice are unresponsive to OXA and TNCB[53]. Interestingly, BMDCs from $P2X_7^{-/-}$ mice are un-able to sensitize when transferred to wild-type mice, suggesting that ATP-$P2X_7$ interaction is an integral part of DC activation[53].

6.3.1 Dendritic cells of the skin

The cutaneous DCs are crucial to sensitization, and their activation leads to dLN homing where specific T cells in turn are activated by antigen-presentation. At least three cutaneous DC subsets exist; the Langerin$^+$CD103$^+$ Langerhans cells residing in the epidermis and the two dermis residing DC subsets: CD103$^+$ dermal DCs (dDCs) and CD103$^-$ dDC[54]. Their specific roles in CHS have been debated extensively in the scientific literature and an aggregate of the main findings is provided in this paragraph[46,54–56].

Langerhans cells (LCs) are the APCs of the epidermis and form a network of DCs that constitutes 2-5% of the epidermal cell population[57]. LCs have characteristic cytoplasmic organelles called Birbeck granules, which contain CD207/Langerin[58–60]. Functionally, the Birbeck granules are important in exogenous antigen uptake and presentation by MHC class

II and it has been suggested that LCs may prime CD8 T cells, which involves a mechanism that engage MHC class I presentation through an alternative pathway that depends on Birbeck granules[61]. Functional cross presentation for both human and murine LCs has been demonstrated *in vitro*[62]. Hence, LCs isolated from murine skin exposed to the protein antigen ovalbumin, induced activation of specific $CD4^+$ and $CD8^+$ T cell *in vitro*[63]. In addition human, LCs have been found to efficiently prime naïve $CD8^+$ T cells when incubated in the presence of virus antigen[64].

Moreover, Langerin has been shown to bind mannose and thus provide an uptake mechanism by endocytosis[58]. LCs have long been considered as important APCs in the sensitization phase: Given their proximal localization in the epidermis LCs are the first responders upon hapten exposure and in line with this, LCs have been found to induce IL-1β as early as 15 min after hapten application[46,47].

Migration of DCs to the draining lymph nodes involves a series of events that cause DCs to disentangle from the surrounding stromal cells. Here, autocrine IL-1β signaling induce IL-1β, IL-1α and TNF-α that co-operate in loosening skin integrity by down-regulating surface attachment molecules e.g. E cadherin[65]. DCs express the chemokine receptor CCR7 that engage them to migrate toward CCL19 and CCL21 chemokine gradient deriving from the draining lymph nodes[66,67]. During migration, DCs gradually mature and attain a dendritic morphology and stabilize MHC-II expression[68].

In recent years the involvement of LCs in CHS has been questioned, as different approaches for depleting LCs was undertaken. In one study, mice were treated with topical administration of glucocorticoid, which results in down-regulation of LCs in the epidermis. In the absence of LCs, an enhanced CHS response was observed, indicating that LCs may down-modulate the elicitation response or that their absence results in enhanced sensitization[69]. However, this treatment was not specific for LCs, as dermal DC subtypes are affected by glucocorticoid treatment[70].

However, with genetic engineering becoming available, new approaches allow conditional ablation of specific cell subsets. Thus, recombination of simian diphtheria toxin receptor (DTR) into the promotor region of the Langerin gene provides a technique for depletion of Langerin expressing cells. Following treatment with diphtheria toxin, dDCs $Langerin^+$ cells re-populate the skin after 7-14 days whereas LCs remain absent within this timeframe and the

resulting CHS responses is augmented, suggesting that LCs are immunosuppressive[71]. In contrast, Wang *et al* found that the absence of LCs reduce the CHS response[72]. The discrepancy may in part be influenced by differences in hapten (DNFB) concentration, 1% and 0.15% respectively. In addition, mice deficient for Baft3, are devoid of Langerin$^+$ dDCs and show unaffected CHS reponse[73].

The biology of the cutaneous DCs is seemingly complex. From *in vivo* studies it has been suggested that LCs induce tolerance, whereas the Langerin$^+$ dDC counterparts seem to promote Th1 CHS responses[54,70,74]. LCs are situated in the distal skin, and are the first to encounter environmental substances, therefore they may provide means for a tolerogenic function to prevent strong immune responses to common antigens and commensal bacteria. In contrast, LCs have been shown important for the development of Th17 CD4$^+$ T cells in a mouse model for *Candida albicans (C. albicans)* infection[75]. In wild-type mice, Th1 and Th17 immune responses develop in response to *C. albicans* infection. Transgenic mice, which constitutively express the diphtheria A subunit (DTA) in the Langerin$^+$ promotor lacking LCs, fail to produce Th17 cells following *C. albicans* infection however, CD8$^+$ cells are unaffected. Conversely, mice having only the LCs respond with Th17 reponse to *C. albicans* and *staphylococcus aureus* infection whereas Th1 cell are absent[75].

6.3.2 Dendritic epidermal T cells recognize keratinocyte stress signals

In addition to LCs, murine dendritic epidermal T cells (DETCs) also lodge in the epidermis and mediate the early events of the CHS response. DETCs have the γδ T cell receptor and respond with IL-17A production through IL-1β stimulation[49]. Nielsen *et al* have shown that KCs up-regulate the three NKG2D ligands (NKG2DL); mouse UL16-binding protein-like transcript 1 (Mult-1), histocompatibility 60 (H60) and retinoic acid early inducible-1 (Rae-1) following allergen-induced stress[76]. Blocking of NKG2D suppressed DETC activation and subsequent CHS induction[76]. In the same study, the human homologues of NKG2DL, MICA and MICB, were shown to be up-regulated in cultivated human KC following nickel stimulation, indicating that the mechanism translate from mice to humans.

6.3.3 T cell priming and differentiation

As naïve $CD4^+$ and $CD8^+$ T cells enter the dLNs through high-endothelial venules they encounter APCs in the paracortical area of the dLN and scan the peptide-MHC molecules on professional APCs through transient interactions. The activation and differentiation of T cells require three signals: (I) Specific interaction between the T cell receptor (TCR) and the MHC-peptide complex must overcome a threshold for intracellular signaling (II) co-stimulatory signaling and (III) cytokine signaling.

(I) The physical chemical properties of a given contact allergen determine the route of MHC presentation; thus extracellular haptens are presented by MHC-II. Haptens that bind to extracellular proteins may be internalized and degraded in proteolytic lysosomes and presented by MHC-II. Lipophilic haptens may pass the cell membrane by passive diffusion and thus bind intracellular protein, which are continuously processed and displayed by MHC-I[77]. Finally, it has been shown that nickel can act as a superantigen, by cross-binding the TCRβV17 chain to MHC and thus by-passing antigen presentation by DCs[78].

(II) Co-stimulatory molecules on matured DCs like B7-1 and B7-2 pair with CD28 on T cells and are important for T cell activation, as signified by the observation that CD28-deficient mice show impaired T cell responses in CHS[79–81]. Formation of the CD40-CD40L pair is known to lead to expression of OX40L on DCs and ultimately up–regulation of B7 molecules by a positive feedback mechanism[82]. The importance of OX40 ligation has been signified in CHS experiments, in which OX40L-deficient mice have shown poor T cell responses to oxazolone, FITC and DNFB[83]. Interestingly, a study showed that formation of CD40-CD40L is required for activation of $CD8^+$ T cells in the sensitization to DNFB[82]. Hence, mice treated with anti-CD40L prior to sensitization inhibited $CD8^+$ T cells but left $CD4^+$ T cells unaffected. Moreover, anti-CD40L treatment in CD4 knockout mice did not affect $CD8^+$ activation in the CHS response, suggesting that $CD4^+$ T cells support priming of CD8 T cells during sensitization via CD40-CD40L interaction that possibly increase IL-2 levels in the dLNs[82]. Following activation through co-stimulatory receptor ligand interactions, the T cell needs to down-modulate signaling to avoid overstimulation, this can occur through CTLA-4 on T cells by ligation of B7-1 and B7-2 on DCs[84,85]. In addition, inhibitory co-stimulatory molecules such as PD-L1 and PD-L2 on DCs bind PD-1 on T cells. Blockade of PD-L1, but not PD-L2, have been shown to exacerbate CHS responses with excessive T cell infiltration in the dLNs, indicating its immune dampening function in immune activation[86]. In addition, new members of the B7 family have been discovered; an interaction partner for B7-H3 has

yet to be identified[87]. Anti-B7-H4 treatment in mice has shown to impair T cell proliferation[88]. Interestingly, B7-H4 is found to be widely expressed in the periphery of mice on the transcriptional level and only on hematopoietic cells[88].

(III) The naïve $CD4^+$ T cell can differentiate and develop into distinct helper subsets depending on the cytokine environment and can roughly be divided into Th1, Th2, Th17, and regulatory T (Treg) cells (Treg cells are described in depth in a later paragraph). Thus, development of Th1 is favored in the presence of IL-12 and IFN-γ; Th2 cells are induced by IL-4, and Th17 cells by IL-1β, IL-6, IL-23 and TGF-β. These subsets are characterized by the capacity to produce unique sets of cytokines. Th1 cells produce IFNγ, IL-2 and TNFα and support macrophage functionality in response to intracellular bacterial infections and are controlled by the T-bet transcription factor[89]. The Th17 cell subset is important in the immune response to fungal infections and extracellular bacteria. The Th17 cells are under transcriptional regulation by the RORγt transcription factor that in turn produce IL-17A and IL-17F that induce CXCL8 chemokine production in stromal cells and thereby attract neutrophils through the CXCR2[90,91]. The Th2 subset support humoral immunity and is important for clearance of parasite infections. Classically, Th2 cells are described to support antibody production by direct interaction with B cells, and is characterized by the production of IL-4, IL5 and its signature transcription factor GATA-3[92,93]. Similar to the development $CD4^+$ T cell subsets, $CD8^+$ T cells can develop into Tc1, Tc2 and Tc17 that convey the identical cytokine expression profiles as their cognate $CD4^+$ T cell subsets[94].

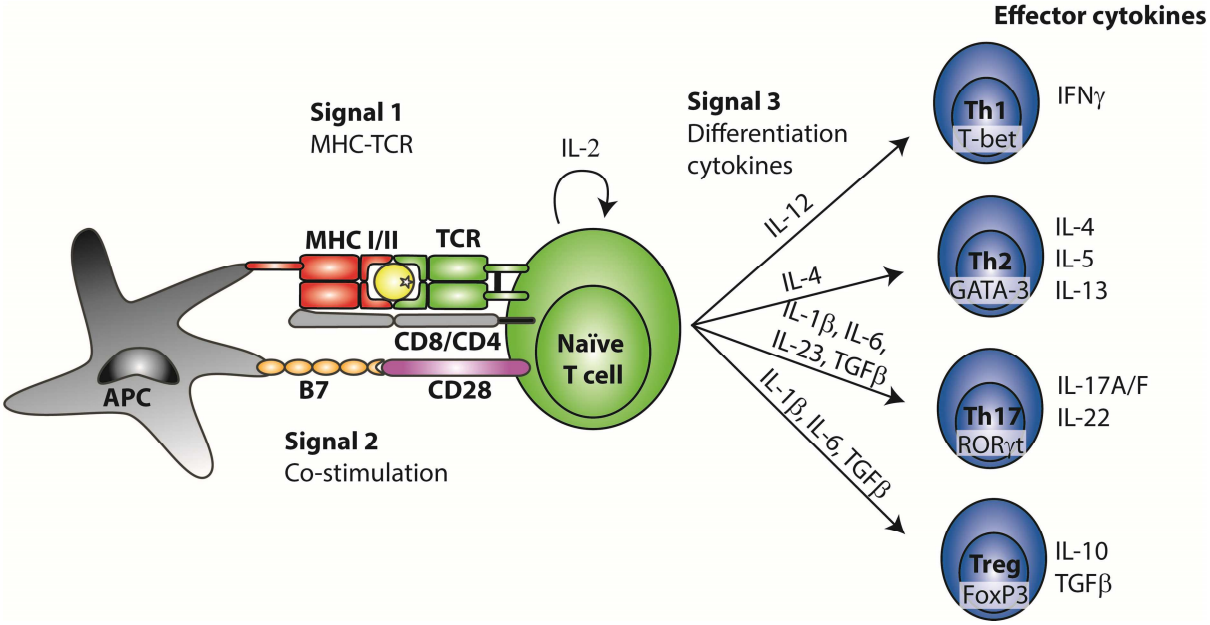


Figure 2: Schematic drawing of essential event in T cell activation and differentiation. Activation and differentiation of T cells critically depends on three consecutive events. **Signal 1.** Interaction and match between MHC-peptide and TCR and CD4/8, engage formation of the immunological synapse. **Signal 2.** Ligation between co-stimulatory molecules interact on APC and TCR form and induce intracellular signaling that initiates IL-2 secretion that engage a positive feedback loop by autocrine signaling and expression of CD25 that enhance IL-2 receptor affinity. **Signal 3.** The differentiation of CD4+ T cells into effector subsets depend on cytokines in the environment. These can induce stabile expression of key transcription factors that manifests into distinct T helper subsets, characterized by having unique cytokine expression. Th1 cells are induced by IL-12 derived from APC are characterized by the T-bet and IFN-γ, which are important against intracellular bacterial and viral infections. Th2 cells are promoted by IL-4 and recognized by GATA-3 and can produce IL-4, IL-5 ad IL-10 are important in humoral immunity and helminth infestions. Th17 can be generated by IL-1β IL-6, IL-23 and TGF-β in response to fungal and extracellular bacterial infections. Th17 have the RORγt transcription factor and can produce IL-17A, IL-17A and IL-22. Treg cells are immune regulators and can be induced by IL-1β, IL-6 and TGF-β. Treg cells are characterized by the FoxP3 transcription factor and the cytokines IL-10 and TGF-β.

6.4 Elicitation and effector T cells in CHS

Once sensitized, an individual may react with dermatitis upon challenge exposure to low amount of a given allergen in the elicitation phase. From CHS studies, it is clear that the elicitation phase is initiated by hapten-unspecific secretion of cytokines and chemokines that occurs within the first two hours of the reaction. Ultimately the activation and attraction of $CD4^+$ (Th1, Th2 and Th17) and $CD8^+$ (Tc1, Tc2 and Tc17) T cells, which mediate the hapten-specific responses involving attraction of neutrophils and macrophages to the site of inflammation, peak 24-48 hours post challenge[95–97].

KCs and LCs are the first to respond with secretion of IL-1β and TNFα through inflammasome activation and TLR2/4 signaling[30,48], much like the sensitization phase. In response to these cytokines, the endothelial cells enhance ICAM-1 and E- and P-selectin expression allowing leukocytes to enter the tissue by adhesion molecules[98,99]. Neutrophil attraction to the hapten exposed skin is important for both the sensitization and elicitation response, where they provide pro-inflammatory mediators such as ROS, and their ablation or depletion impair CHS responses[100–102]. In the elicitation phase, neutrophils are recruited by KC derived CXCL1 and CXCL2 (both are homologous to human CXCL8), which are strongly up-regulated as early as three hours following challenge[103] and Ab-mediated depletion of both chemokines reduce the elicitation response[100,104]. Studies have shown that neutrophils and T cells co-operate in infiltrating the skin[103]. Thus, Kish *et al* showed that IL-17 and IFNγ producing $CD8^+$ T cells induce CXCL1 and CXCL2[103]. Whereas IL-17 is known to indirectly attract neutrophils through CXCL8[105], it was found that Ab-mediated depletion of IFNγ significantly reduced CXCL1 and CXCL2 levels within the hapten challenged skin[103]. IFNγ induce CXCL9, CXCL10 and CXCL11 production and these are known ligands for the CXCR3 found on effector T cells, which ultimately establish a positive feedback loop to attract more effector T cells[106].

The cellular contributors in the elicitation is complex and involves many cell types, however the reaction is mainly considered as a T cell driven hypersensitivity reaction as $RAG^{-/-}$ mice, devoid of clonal repertoire, show greatly diminished CHS reponses[107]. In line with this, antibody-mediated depletion of both $CD4^+$ and $CD8^+$ T cells results in impaired CHS responses[108]. However, the specific involvement of $CD4^+$ and $CD8^+$ T cells has been debated. As $CD8^+$ T cells are classically characterized by cell-to-cell mediated cytolysis by releasing Perforin and Granzyme B, these were considered to convey the classical lesions of the CHS response[109–111]. In support of this hypothesis, MHC-II-deficient mice that fail to develop $CD4^+$ T cells, show enhanced CHS responses to DNFB, and thus an immune dampening role of $CD4^+$ T cells have been proposed[112,113].

6.5 Local skin memory

Inflammatory flare-up reactions in the skin are known to occur in previously affected skin areas and a known phenomenon in inflammatory skin diseases in e.g. psoriasis, atopic dermatitis and ACD. In humans, flare-up reactions are known to occur at the primary site of

sensitization, when effector T cells develop and enter circulation, where they encounter residual hapten at the site of sensitization[114]. Patients with ACD, can experience allergen-specific flare-ups in skin areas previously affected following allergen challenge on an anatomical distant site[115] or by oral administration[116]. The phenomenon of spontaneous flare–up reactions is indicative of a local skin memory and investigated in an early study by Grolnick *et al* (1941). Here it was demonstrated that spontaneous flare-up reactions occurred at previous patch test areas following re-patch testing with weekly intervals at other areas, which indicates a local immunological skin memory[115].

6.5.1 T cells in local skin memory

The generation of local memory and the involvement of T cells have been assessed by Scheper *et al*, who showed that locally sensitized skin reacted with faster kinetics in sensitized animals when measured by erythema scoring [117]. In accordance with these findings, ACD patients have been shown to retain $CD4^+$ T cells in the dermis up to 21 days following skin sensitization[118]. A possible mechanism behind $CD4^+$ T cell retention is through persistent chemokine attraction of CCL27 derived from the epidermis that signal to CCR10 expressed on the retained $CD4^+$ T cells[118]. Although, the $CD4^+CCR10^+$ T cells were indeed found in the upper dermis, this subset was mainly found in perivascular clusters in the lower dermis[118]. In this context, it has been shown that perivascular clusters of DCs and $CD8^+$ T cells form following challenge with DNFB in the murine CHS response[119]. Within these perivascular clusters, antigen presentation is evident as blockade of the immunological synapse with anti-LFA-1 lowered the $CD8^+$ T cell content within the perivascular cluster and furthermore reduced the CHS response. Furthermore, macrophages are required for assembly of the DC-T cell cluster, and their depletion result in lack of T cell derived IFN-γ expression[119].

6.5.2 Tissue resident memory T cells

T cell memory provide immune protection toward invading pathogens, and after clearance a fraction of T cells persist and circulate between blood and lymph systems where they can facilitate fast recall responses to antigen recognition. It has been conceptualized that T cell memory is comprised of two T cell memory subsets[120]. Thus central memory T cells (T_{CM}) re-circulate between blood and the T cell zones of the secondary lymphoid organs, whereas

effector memory T cells (T_{EM}) re-circulate between secondary lymphoid organs, blood and non-lymphatic tissues e.g. the skin[121]. T_{EM} and T_{CM} cells are distinguished by expression of the lymph node homing markers, T_{EM}: $CCR7^{-}CD62L^{-}$ and T_{CM}: $CCR7^{+}CD62L^{+}$[120].

In recent years, a special subset of memory T cells named tissue resident memory T cells (T_{RM}) has emerged. T_{RM} cells can populate non-lymphoid tissues and have been found to populate the skin following infections to some virus where they exert specific long-term immune protection to virus re-infection[122–125]. Two T_{RM} cell phenotypes have been described in murine models for virus infection, with $CD8^{+}$ being predominantly in focus, the $CD4^{+}$ T_{RM} cells have been found important in HSV-2 infections[126], whereas $CD8^{+}$ T_{RM} cells have been characterized in HSV-1 and *vacciania* infection models[125,127]. The development of T_{RM} cells has been shown to occur from a $KLRG1^{-}$ T cell precursor subset, that upon epidermis entry respond to KC-derived TGF-β, by sequentially expressing the two membrane proteins CD69 and CD103[125,126,128]. These two membrane proteins serve to retain T_{RM} cells in the epidermis, the integrin CD103 interacts with E-cadherin that is expressed by KCs[128], and CD69 impairs expression of the Sphingosine-1-phosphate receptor 1 (S1PR1), that normally promote tissue egress toward the blood S1P gradient[129]. While situated in the tightly packed epidermis, T_{RM} cells are dendritic in morphology and are indeed able to migrate in a lateral motion[130]. Here they interact with LCs, but constitutive signaling of TGF-β, IL-7 and IL-15 have been shown to be essential for T_{RM} cell survival[130,131].

Functionally, T_{RM} cells have been thoroughly investigated in virus murine infection models. Thus, $CD8^{+}$ T_{RM} cells have mainly been found to be up-regulated in the skin following infection with herpes simplex virus (HSV)[122]. Skin from immunized mice retained viral protection when skin grafts were transplanted to naïve mice[122]. In another study, mice infected with *vaccinia* virus up-regulated circulating $CD8^{+}$ T_{CM} and skin resident $CD8^{+}$ T_{RM} cells. Following pathogen clearance, mice were subjected to parabiosis with naïve mice by surgically joining peripheral the blood vessels, which allow equilibration of circulating T cells[125]. After separation, the immunized mice were immune protected against virus re-infection, whereas the naïve recipient mice had poor protection[125]. Moreover, treatment with the LN egress blocking pharmaceutical agent FTY720 did not affect the response in immunized animals, indicating that circulating T cells were dispensable[125].

In a recent paper by Gaide *et al*, it was shown in a mouse model that sensitization with DNFB induce development of T_{RM} cells, which shared clonally origin to circulating resident T_{CM}

cells when comparing the TCR sequence[132]. In accordance with virus studies[126,128,133], T_{RM} populated skin sites showed fast kinetics to DNFB challenge whereas skin sites non-sensitized skin showed delayed inflammatory kinetics, which were ascribed to the T_{CM} cells being recruited from the LNs[132]. Importantly, these findings translated to human experiments by showing that T_{RM} phenotypic cells formed in the skin following sensitization with diphenylcyclopropenone (DPCP) and were retained as long as 4 months after DPCP application[132].

Whereas conventional effector $CD8^+$ T cells exert cytotoxic interactions by cell-to-cell contact with cells bearing cognate MHC-peptide complexes, $CD8^+$ T_{RM} cells are in scarce within the epidermal cell population, and thus primarily function by releasing the cytokines TNFα, IFNγ and IL-2 by direct Ag-TCR interaction[134–136]. In terms of contact allergy, IL-17A producing T cells are known as key players of pathogenesis, therefore it is intriguing that both epidermal $IFN\gamma^+$ and $IL\text{-}17A^+$ T_{RM} cells have recently been identified in psoriatic plaques[137]. Following remission of the skin plaques, T_{RM} cells were still detected but molecular signatures cleared following treatment with UV radiation.

Taken altogether, T_{RM} cells are important in protection against virus re-infections, as they act as antigen specific sentinels that can react promptly by releasing pro-inflammatory cytokines that precedes inflammation.

6.6 Immunological tolerance in contact allergy

An important aspect of the immune system is self-regulation, which is required for resolution following inflammation. In addition, tolerance to self-antigens is required to maintain homeostasis. In terms of contact allergy, the allergen exposure regimen, such as tissue exposure, allergen concentration and number of exposures are determining factors each affecting the balance of tolerance *versus* allergy.

6.6.1 Regulatory T cells

Over the recent years it has become clear that regulatory T (Treg) cells, signified by $CD4^+CD25^+Foxp3^+$, play an important role in modulating immune responses. Natural occurring Treg (nTreg) cells are formed in the thymus during T cell development these form a

central tolerance toward auto antigens, inducible Treg (iTreg) cells are formed in the periphery from naïve T cells under specific conditions[138].

Treg cells can exert their immune regulating functions by cytokine signaling mainly through IL-10, and under some conditions TGF-β mediates immune suppression as well[139]. In addition, Treg cells convey immune suppression by cell-to-cell contact by expression of CTLA-4 that down-modulate DCs and also glucocorticoid-induced TNF receptor-related gene (GITR) can interact with the GITR ligand on DCs[140–142]. Treg cells can, also down modulate immune responses via surface expression of two ectoenzymes, CD39 and CD73 that convert extracellular ATP to AMP and AMP to adenosine, respectively. Adenosine is anti-inflammatory and inhibit effector T cells via production of TGFβ derived from Treg cells[143]. Adenosine has been shown to impair the challenge response in CHS experiments, to TNCB when administered i.v. prior to sensitization[144]. Furthermore, the transfer of $CD39^{+}$ Treg cells were required for tolerance development in adoptive transfers, whereas Treg cells from $CD39^{-/-}$ mice failed to tolerize[144]. The authors proposed a mechanism for adenosine mediated suppression, which involve a down-regulation of E- and P-selectins in the stromal cells that ultimately decrease infiltration of effector T cells[144].

6.6.2 Oral tolerance

Whereas allergens can induce, strong sensitization to skin contact, oral ingestion of contact allergens prior to sensitization has been shown to induce suppression to specific allergens. Thus, early epidemiological studies have shown a significant association between ear piercings and nickel allergy, whereas having braces in early life protects against nickel sensitivity[145,146]. In accordance, experimentation in guinea pigs has shown that oral administration of nickel prior to sensitization suppressed the elicitation responses[147].

In a study by Artik *et al*, desensitization to nickel was induced in mice by oral administration of nickel[148]. In this study, T cells from dLNs of desensitized mice did not respond to *in vitro* re-stimulation with Ni^{2+}. The authors showed that adoptive transfer of as few as 100 T cells to sensitized animals induced tolerance, but both $CD4^{+}$ and $CD8^{+}$ T cells were required[148]. Although, only a low number of T cells were needed to transfer nickel tolerance, the induction of $CD25^{+}$ T cells was required a high dose as shown in a follow-up study[149].

From recent studies, it has been demonstrated that oral tolerance induction to DNFB in mice involved generation of plasmacytoid DCs in the gut-associated lymphoid tissues[150]. In another study, plasmacytoid DCs where shown to induce killing of hapten specific $CD8^+$ T cells in the liver and in secondary lymphoid organs[150]. In addition, the authors found that $CD4^+$ Treg cells suppressed residual $CD8^+$ T cells, which rendered the animals unresponsive. Based on these studies, it seems likely that crosstalk DCs cells may populate or circulate between gut-associated lymphoid tissues and skin LNs due to expression of LN homing molecule, e.g. CCR7.

6.6.3 Low zone tolerance

Low zone tolerance (LZT) has been described as the repetitive exposure to low concentration of haptens, which cause specific tolerance. It has become clear that LZT involves induction of dLN-resident $CD8^+$ T cells, as transfer of these cells adopts hapten specific tolerance to naïve animals[151]. The development of a $CD8^+$ T cell Tc2 phenotype produced IL-4 to *in vitro* re-stimulation and depends on IL-10 derived from $CD4^+$ T cells in the dLN. This was supported by the finding that transfer of $CD8^+$ T cells from IL-$10^{-/-}$ did not develop LZT[152]. In line with these findings, it has been shown that the presence of $CD4^+CD25^+Foxp3^+$ Treg cells are required for induction of tolerogenic DCs, that yet in turn generate LZT $CD8^+$ T cells in the dLN[153].

In addition, another study demonstrated that TNFα is essential in the induction of LZT. They found $CD8^+CD11c^+$ tolerogenic DCs induced TNFα specific killing of $CD8^+$ suppressor T cells in the dLN through signaling of the TNF receptor subunit p75[154]. Although the details in this interplay between Treg cells, LZT $CD8^+$ T cells and DCs are not fully elucidated, it seems that they cooperate and are important in LZT development.

In summary, Treg cells convey important functions in preventing immune responses to innocuous environmental substances that are exposed to the skin or the oral mucosa.

6.7 Risk assessments of contact allergens

Although ACD symptoms can be treated with corticosteroids, no cure is currently available which leaves allergen prevention and limitation as best options. For many years *in vivo*

models have been validated for screening of hazardous compounds, like guinea pig maximization test, which classifies allergens into weak, mild, moderate, strong or extreme based on the proportion of positive reactions observed in a number of test animals[155]. Another approach that was been widely used is the murine local lymph node assay (LLNA). The LLNA has been widely used to estimate safe levels of allergens and has been part of the organization of economic cooperation and development (OECD) guideline in hazard evaluation of chemicals introduced to consumers[32]. As specific T cell responses are a key marker of skin sensitization, the LLNA measures a proliferative response in the dLNs following exposure of a test substance, in dilution series, applied topically on the ear dorsum for three consecutive days followed by analysis of the dLNs on day 5. Incorporation of tritiated thymidine is measured as an indication of proliferation of lymphocytes in single cell suspensions prepared from dLNs[156]. Extensive inter-laboratory studies have been conducted to validate the LLNA[157]. Together, they show that a proliferative response above 3-fold (SI=3) compared to the empty vehicle (solvent), classifies the tested substance as a human skin sensitizer. By extrapolation of the dose–response linearity the effective concentration (EC) of a given substance can be calculated to predict the sensitizing concentration that results in SI=3. Although the LLNA provides a strong model for screening sensitizers, false positive results to the known, irritant sodium lauryl sulphate (SLS), has been described[158]. Of notice, Gerberick *et al* developed a model to discriminate irritants from sensitizers. They found that an up-regulation of $B220^+$ cell percentage in draining lymph nodes by a 1.25 factor compared to the vehicle control validated the tested substance as a contact sensitizer, indicating that B cells, which express B220, are highly involved in sensitization[159,160].

Recently, animal testing of cosmetic products have been banned within the European union. In response, various *in vitro* models have been developed and validated for screening for sensitizing chemicals. These models include, human skin explants[161], peptide reactivity assays[162] but also analysis of the quantitative structure-activity relationship models (QSAR) compares chemical similarities and predicts sensitization potency based on LLNA data from similar motifs[163].

6.8 Hair dyes and hair dye allergy

Contact allergy to oxidative hair dyes is well-known and can involve severe contact dermatitis with facial edema and even hospitalization in some cases[164]. The main principle of oxidative hair dying occurs by mixing a dye component, containing both dye molecule and

chemical couplers, with an oxidization component, which often contain hydrogen peroxide. The oxidation induces dye molecules to polymerize, which subsequently binds irreversibly to hair straws. The chemistry of hair dying is complex given the fact that over 200 ingredients can be found in commercial available hair dye products and about 100 potential contact sensitizers are allowed in hair dye products, e.g. dyes, couplers and fragrances[165–167]. The two dye precursor molecules, PPD and PTD are alike in their chemical structure and known as strong contact sensitizers, and are frequently found in black shade hair dye products on the Spanish and Swedish marked, respectively[166,168]. The major concern regarding hair dye allergy, is due to the severity of elicitation responses that the affected patient can experience, e.g. strong facial edema and rashes in the scalp area[164].

PPD has been used as permanent hair dye ingredient for more than 100 years, and its properties are ideal for hair dyeing since low-molecular weight and protein reactivity allows passage into the hair structure where PPD can polymerize via chemical couplers and oxidizers, and the resulting products bind irreversibly to hair proteins[169]. Given these properties, PPD fulfills the profile of a contact allergen[170]. PPD is itself a poor hapten, but can upon oxidation e.g. by atmospheric oxygen or reactive amino acid residues bind irreversibly to proteins[171]. Recently, PPD has been shown to induce IL-18 in reconstituted human epidermal cell cultures[172]. The IL-18 production was found to be with ROS dependent as antioxidants in the culture reduced IL-18 in response to PPD[172]. From *in vitro* studies, it has been shown that the PPD derivative, Bandrowski's base (BB), is generated following oxidation[173]. BB is a strong contact sensitizer in mice and can elicit strong reactions in PPD sensitive ACD patients[173,174]. Of notice, a recent advance in the development of hair dye molecules have introduced the methoxymethylside chain to PPD (ME-PPD) that display significantly less activation of innate immune responses when assessed by peptide reactivity and B7.2 expression in DC cultures[175]. In addition, EC3 value of ME-PPD obtained from LLNA, was found to be 4.3% in contrast to PPD and PTD EC3 values of 0.1% and 0.17%[175]. Moreover, patch testing with ME-PPD in PTD and PPD allergic patients showed tolerization in 29 of 43 the included hair dye allergic patients[176].

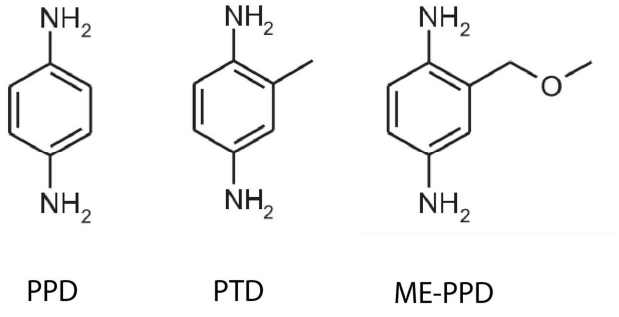


Figure 3: Chemical structures of PPD, PTD and ME-PPD

6.8.1 Development of tolerance to hair dyes

It seems counter-intuitive that hair dyes contain mixtures of strong allergens, but yet the clinical frequency of hair dye allergy is about 1% of the general population[165,177]. In line with this, questionnaire based studies have revealed that about 50% of the general European and Danish population have used hair dye at least one time in their lifetime, with women more frequent users (75%) than men (18%), and the overall median age of first-time hair dyeing was reported to be 16 years[177,178].

It has been proposed excessive couplers in the hair dye mixture neutralize the sensitization propensity of hair dye molecules[179]. PPD alone has been shown to sensitize 100% of an experimental group following repetitive exposure[180]. In accordance, LLNA assays ranks PPD and PTD as extreme sensitizers[167].

Recent data from our group demonstrated that a PPD containing commercial available hair dye, resulted in strong inflammatory responses in a mouse model in response to sensitization, measured by ear thickness and T and B cell recruitment and proliferation in the draining lymph nodes[114]. Noteworthy, the non-oxidized PPD-containing color component, did itself induce immune responses comparable to the hair dyes[181]. In another study from our group it was demonstrated that repetitive hair dye exposures induce regulatory T cells in the dLNs[183]. The aforementioned hair dyes were applied every two weeks for a total of 10 weeks. Following four exposures the inflammatory responses reached a plateau when measuring T cell proliferation. Interestingly, this plateau correlated with up-regulation of $CD4^+CD25^+FoxP3^+$ Treg cells in the draining lymph nodes and ear IL-10[183].

7 Thesis objectives

This thesis aims to elucidate two areas of interest in contact allergy: First, we wished to gain insight into the development of local memory and the involvement of T cells governing specific skin memory. To his end, we wanted to establish a murine model to examine the local effects of sensitization and analyze T cells in the affected skin following sensitization and challenge. Based on the murine CHS model we wished to investigate the similar setup in ACD patients in order to translate the mechanism.

Secondly, we sought to shed a critical light upon regulations that dictate the use of potent contact sensitizers in consumer available hair dye products containing PTD. The limitations of chemicals in cosmetic products are based on quantitative risk assessments for individual substances. As oxidative hair dyes are known to contain several strong contact allergens, we wanted to investigate the immune responses commercial available hair dyes to different exposure regimens in murine CHS responses. Furthermore, we wanted to elaborate previous findings that repetitive hair dye exposures possibly induce a tolerance mechanism.

8 Included studies

The PhD thesis is based on three studies, listed below:

I. Jonas D. Schmidt, Malin G. Ahlström, Jeanne D. Johansen, Beatrice Dyring-Andersen, Christina Agerbeck, Morten M. Nielsen, Steen S. Poulsen, Anders Woetmann, Niels Ødum, Allan R. Thomsen, Carsten Geisler & Charlotte M. Bonefeld. **Skin-resident memory CD8$^+$ T cells induce IL-1β production following allergen-exposure.** Manuscript submitted to Contact Dermatitis, 29 June, 2016.

II. J.D. Schmidt, J.D. Johansen, M.M. Nielsen, E. Zimersson, C. Svedman, M. Bruze, K. Engkilde, S.S. Poulsen, C. Geisler and C.M. Bonefeld. **Immune responses to hair dyes containing toluene-2,5-diamine.** *Br J Dermatol* 2014; **170**:352–9.
    a. *Corrigendum.* DOI: 10.1111/bjd.12676

Study III, is based on preliminary results and is not intended for publication in its present form. However, it extents to the findings of study II, that repetitive hair dye exposure may induce tolerance.

III. J.D. Schmidt, J.D. Johansen, E. Nielsen, C. Geisler & C.M. Bonefeld. **Repeated exposures to low concentration of p-phenylenediamine induce local tolerance.** Manuscript in preparation.

CONTACT DERMATITIS
ENVIRONMENTAL AND OCCUPATIONAL DERMATITIS

Rapid allergen-induced IL-17 and IFN-γ secretion by skin-resident memory CD8+ T cells

Journal:	*Contact Dermatitis*
Manuscript ID	Draft
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	n/a
Complete List of Authors:	Schmidt, Jonas; Kobenhavns Universitet Sundhedsvidenskabelige Fakultet, Dept of Immunology and Microbiology Ahlström, Malin; Copenhagen University Hospital Gentofte, National Allergy Research Centre, Department of Dermato-Allergology Johansen, Jeanne Duus; National Allergy Research Centre, Department of Dermato-allergology Dyring-Andersen, Beatrice; Gentofte Hospital, Department of Dermato-Allergology Agerbeck, Christina; Kobenhavns Universitet Sundhedsvidenskabelige Fakultet, Dept of Immunology and Microbiology Nielsen, Morten; Universitiy of Copenhagen, ISIM Poulsen, Steen; Universitiy of Copenhagen, ISIM Woetmann, Anders; Universitiy of Copenhagen, ISIM Ødum, Niels; Universitiy of Copenhagen, ISIM Thomsen, Allan; Universitiy of Copenhagen, ISIM Geisler, Carsten; Kobenhavns Universitet Sundhedsvidenskabelige Fakultet, Immunology and Microbiology Menne Bonefeld, Charlotte; Universitiy of Copenhagen, ISIM;
Keywords:	allergic contact dermatitis, Tissue-resident T cells, IL-1β, IFN-γ, IL-17A, Local memory

SCHOLARONE™
Manuscripts

Rapid allergen-induced IL-17 and IFN-γ secretion by skin-resident memory CD8$^+$ T cells

Jonas D. Schmidt[1,2]; Malin G. Ahlström[2]; Jeanne D. Johansen[2]; Beatrice Dyring-Andersen[1,2]; Christina Agerbeck[1]; Morten M. Nielsen[1]; Steen S. Poulsen[3]; Anders Woetmann[1]; Niels Ødum[1]; Allan R. Thomsen[1]; Carsten Geisler[1] & Charlotte M. Bonefeld[1,*]

[1]Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark. [2]National Allergy Research Centre, Department of Dermato-Allergology, Copenhagen University Hospital Gentofte, Denmark. [3]Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark.

[*]Corresponding author: Charlotte Menné Bonefeld, Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Blegdamsvej 3, DK-2200, Copenhagen, Denmark. Phone: + 45 3532 7866, e-mail: cmenne@sund.ku.dk

Funding sources: LEO Pharma Research Foundation, Kgl. Hofbuntmager Aage Bangs Foundation, Danish Medical Research Council, Novo Nordisk Foundation and Lundbeck Foundation

Conflicts of interest: None.

Contact Dermatitis - Manuscript Copy

Summary

Background Skin-resident memory T (T_{RM}) cells are associated with immunological memory in the skin. Whether immunological memory to allergens in the skin is solely localized to previously allergen-exposed sites or is found globally in the skin is not clear. Furthermore, the mechanisms whereby T_{RM} cells induce rapid recall responses needs further investigations.

Objectives To study if contact allergens induce local and/or global memory and to determine mechanisms involved in memory responses in the skin.

Methods To address these questions, we analyzed responses to contact allergens in mice and humans sensitized to 2,4-dinitrofluorobenzene and nickel, respectively.

Results Challenge responses in both mice and humans were dramatically increased at sites previously exposed to allergens compared to previously unexposed sites. Importantly, the magnitude of the challenge response correlated with the epidermal accumulation of IL-17A- and IFN-γ-producing T_{RM} cells. Moreover, IL-17A and IFN-γ enhanced allergen-induced IL-1β production in keratinocytes.

Conclusions We show that sensitization with contact allergens induces a strong, long-lasting local and a weaker, temporary global immunological memory to the allergen that is mediated by IL-17A and IFN-γ-producing $CD8^+$ T_{RM} cells.

(178 words, max 200 words)

Contact Dermatitis - Manuscript Copy

Introduction

Skin-resident memory T (T_{RM}) cells play an important role during recall responses in the skin, but whether the generation of memory T cells results in a localized or a wide-spread global immunological memory in the skin is still unclear [1-9]. Following skin infection with *vaccinia* virus, specific T_{RM} cells were detected both locally at the area of infection and at distant sites [6]. Likewise, it has been described that T_{RM} cells are recruited both to allergen-exposed and -unexposed skin [2]. In contrast, it was shown that *herpes simplex* virus infections only lead to generation of local memory at the site of infection [4]. The existence of isolated, site-specific memory to antigens is supported by the local flare-up reactions that can occur at previously affected skin areas in patients with allergic contact dermatitis (ACD) following systemic re-exposure to the allergen [10;11]. ACD is a T cell mediated skin disease. It is induced following exposure of the skin to contact allergens, and it is a common disease affecting 27% of the European population [12]. More than 30 years ago, Scheper *et al.* showed that local memory to specific allergens could develop in a guinea pig model for ACD and that this memory most likely was mediated by T cells [13]. However, whether the local memory was due to resident or circulating memory T cells was not determined.

IL-1β is a central cytokine in ACD [14-16]. Exposure of the skin to contact allergens leads to a rapid induction of *IL1β* expression [17]. IL-1β is produced by various cells in the skin including keratinocytes, Langerhans cells, dendritic cells and mast cells [18]. Contact allergens can trigger Toll-like receptors (TLR) leading to IL-1β production and inflammation [19;20]. Interestingly, T cell subsets capable of producing IL-17 and IFN-γ have been associated with ACD [21-25]. The importance of these cytokines in ACD is underscored by an impaired response to allergens in IL-17 and IFN-γ knockout mice [22].

Contact Dermatitis - Manuscript Copy

In the present study, we investigated the role of T_{RM} cells in the response to contact allergens. By use of a mouse model for ACD, we found a much stronger memory response in skin areas previously exposed to the allergen compared to skin areas that had not previously been exposed to the allergen. The memory responses most probably were mediated by $CD8^+$ T_{RM} cells via their rapid production of IL-17A and IFN-γ as both cytokines enhanced allergen-induced IL-1β production in keratinocytes. We show that similar mechanisms are involved in memory responses to nickel in humans. Thus, in patients with allergy to nickel, we found a rapid response to nickel after re-exposure to nickel only at the exact same areas of the skin that previously had been exposed to nickel. Furthermore, we found that the magnitude of the challenge responses to nickel in humans correlated with local recruitment of epidermal $CD8^+$ T cells and production of IL-17A and IL-1β.

Contact Dermatitis - Manuscript Copy

Material and Methods

Study subjects and skin samples

Ten individuals with previous positive patch test to nickel were enrolled after obtaining informed, written consent in accordance with the Declaration of Helsinki principles for research involving human subjects. The study was approved by the ethics committee of Copenhagen (H-15004317) and the Danish Data Protection Agency. The individuals were patch tested twice using 8 mm Finn chambers® on Scanpor® tape with petrolatum or nickel sulfate 5% in petrolatum (20 mg, TRO-LAB® Almirall Hermal, Reinbek, Germany) as indicated in figure 5a. Twenty-four hours after the second patch test, the reactions were scored following the guideline of The European Society of Contact Dermatitis [26]. Skin biopsies (4 mm) were subsequently taken from all 4 skin sites.

Mice

C57BL/6 and B6(Cg)Rag2tm1.1Cgn (RAG2-KO) mice were purchased from Taconic (Ry, Denmark) and Jackson (San Diego, California), respectively. Mice were housed in specific pathogen-free facilities in accordance with national animal protection guidelines (license number: 2012-15-2934-00663). Four mice were used in each experimental group in at least two independent experiments.

Contact sensitization and challenge to allergens

Mice were sensitized for three consecutive days by topical administration on the dorsum of the ears using 25 µl of 0.15% DNFB(Sigma, Brøndby, Denmark) diluted in vehicle (olive oil:acetone mixed 1:4). For sensitization on the abdominal skin, mice were shaved 48 hours prior to topical application of 50 µl or 100 µl 0.15% DNFB for three consecutive days. In some experiments, mice were chal-

Contact Dermatitis - Manuscript Copy

lenge by topical application of 25 µL 0.15% DNFB and the mice were euthanized by cervical dislocation.

Immunohistochemistry

Paraffin embedded ears were cut in slide sections of 5 µm and stained with rabbit-anti-mouse IL-1β antibodies (H-153, Santa Cruz Biotechnology, TX, USA). Followed by secondary staining with biotinylated goat anti-rabbit Ig antibodies (Vector Laboratories, CA, USA) and counterstained with Mayers Hemalun. Sections of 3 µm from human skin embedded in paraffin were incubated with mouse anti-CD8 (Clone: C8/144B, Nordic Biosite, Denmark), anti-CD3 Optibody™ (Clone: BS103, 1:200, Nordic Biosite, Denmark) or rabbit anti-IL1β (Polyclonal, Proteintech, IL, USA).

For visualization of DNFB-generated DNP-hapten, sections were treated with microwaves for 15 min in a 10 mmol citric-buffer at pH 9 followed by a pre-incubation in 2% BSA for 10 min and an overnight incubation at 4° C with rabbit anti-DNP antibodies (D9656, Sigma-Aldrich, Denmark). The samples were washed and incubated with goat anti-rabbit antibodies conjugated to Hilyte flour 488 (Novus Biologicals, CO, USA).

Detection of DNP-hapten by Western blot analysis

The ears were recovered from the animals, snap-frozen in liquid nitrogen and homogenized in lysis buffer (5 mM Tris-base, 250 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, pH 7.4) with protease complete inhibitor cocktail (Roche) using the Precellys® tissue homogenizer system at 4°C. Protein concentrations of the homogenates were determined using the Bradford assay. Samples were adjusted to 50 µg pr well and subjected to 10% acrylamide gel SDS-PAGE (Biorad, Copenhagen, Denmark) under reducing conditions and blotted onto a polyvinylidene fluoride membrane (Biorad, Copenhagen, Denmark) by wet-electrotransfer. Subsequent antigen probing was done using rabbit anti-DNP-BSA antibodies

Contact Dermatitis - Manuscript Copy

(Sigma-Aldrich, Brøndby, Denmark) followed by incubation with secondary HRP-conjugated swine anti-rabbit Ig antibodies (Dako, Glostrup, Denmark) and development with Western ECL substrate (GE healthcare, Uppsala, Sweden).

Stimulation of PAM2.12 keratinocytes

The murine keratinocyte cell line PAM2.12 was maintained as previously described [14]. 1.5 x 10^5 cells were seeded in each well of a 6-well plate in 2 ml medium. After resting for 24 hours 10 ng/µl IFNγ (Peprotech), 10 ng/µl IL-17A (R&D systems, Minneapolis, USA) and 0.25 µg/ml 2,4-dinitrobenzenesulfonic acid hydrate (DNBS) (Sigma-Brøndby, Denmark) were added as indicated to the cells. After incubation for 24 hours the cells were harvested and analyzed.

IL-1β measurements

Ears homogenates were prepared as previously described [14] and analyzed for IL-1β by ELISA (eBioscience, San diego, USA).

Preparation of single cell suspensions from the epidermis and FACS analysis

Single cell suspensions were prepared from epidermis as previously described [14]. The cells were stained with CD69 (clone H1.2F3), CD8a (clone 53-6.7), TCRβ-BV421 (clone H57), TCRγδ-BV711 (clone GL3), IFN-γ (clone XMG1.2), IL-17A (clone TC11-18H10) and fixable viability dye efluor 780 (all from BD Pharmingen, Albertslund, Denmark). For intracellular cytokine staining, cells were stimulated with PMA (1.25 µg/ml; Sigma, Brøndby, Denmark), ionomycin (625 ng/ml; Sigma, Brøndby, Denmark) and monensin (2.08 µg/ml; Sigma, Brøndby, Denmark) in complete RPMI 1640 for 4 h at 37°C.

Contact Dermatitis - Manuscript Copy

Real-time polymerase chain reaction (qPCR)

mRNA was purified as previously described [27]. qPCR was done with 1 µg RNA using the Revert Aid First strand cDNA synthesis kit (Thermo Scientific, Copenhagen, Denmark). For qPCR the TaqMan® Universal Master Mix II with UNG (Applied Biosystems, Nærum, Denmark) was used according to the suppliers instructions with the GAPDH (mouse: Mm03302249_g1 or human: Hs04420697_g1) Interleukin-1β (mouse: Mm00434228_m1 or human: Hs01555410_m1) Interferon-γ (Mm01168134_m1), Interleukin-17A (mouse: Mm00439618_m1 or human: Hs00174383_m1)and CD8 (Mm01182107_g1) Taqman probes (Life Technologies).

Contact Dermatitis - Manuscript Copy

Results

Local and global immunological memory to DNFB following exposure to DNFB

To investigate whether exposure to contact allergens leads to development of local or global immunological memory in the skin, we compared the magnitude of the challenge responses in ears from mice sensitized on either the ears or the abdomen (figure 1a). Compared to control mice treated with olive oil:acetone (OOA), we found a significant increase in ear thickness after challenge with 2,4-dinitrofluorobenzene (DNFB) both in mice sensitized on the ears and in mice sensitized on the abdomen (figure 1b). However, the increase in ear thickness was 3-fold higher in mice sensitized on the ears compared to mice sensitized on the abdomen. The local memory lasted for at least 6 weeks after sensitization, whereas global memory could not be detected at this point (figure 1c). Furthermore, the local memory seems to be dominantly hapten specific as only a minor ear swelling was seen after challenge to DNFB in mice sensitized to oxazolone (Oxa) compared to mice sensitized to DNFB (figure 1d). We could not detect DNFB in the skin 21 days after sensitization and could thereby exclude the possibility that local retention of the allergen could explain our results (figure 2).

Next, we assessed the IL-1β levels induced in the skin by exposure to DNFB. We found a massive induction of IL-1β following DNFB challenge on the ears in mice previously sensitized to DNFB on the ears compared to control mice (figure 1e). Only a minor increase in IL-1β was found in the ears of mice following a single exposure of the ears to DNFB (figure 1e). To determine if memory to DNFB was dependent on the adaptive immune system, we determined IL-1β production in the ears following exposure and re-exposure to DNFB in RAG2KO mice. In contrast to wild-type C57BL/6 mice, RAG2KO mice mounted only a minute IL-1β production following DNFB challenge, indicating that the adaptive immune system is required for the strong memory response to DNFB in wild-type mice (figure 1e).

Contact Dermatitis - Manuscript Copy

To determine whether the site of sensitization influenced the local production of IL-1β, we sensitized mice with DNFB on either the ears or the abdomen and challenged them on the ears (figure 1a). Correlating with the ear thickness, we found a significantly higher production of IL-1β in the ears of mice that had been sensitized on the ears than in the ears of mice that had been sensitized on the abdomen (figure 1 f). We found that the majority of IL-1β was located in the epidermis independently of whether mice were sensitized on ears or abdomen (figure 1g and h).

Increased numbers of CD8$^+$ T_{RM} cells in the epidermis following DNFB sensitization

Next, we wanted to investigate whether the site-specific challenge response could be ascribed to allergen-induced accumulation of CD8$^+$ T_{RM} cells. To do this, we exposed mice to OOA or DNFB for three consecutive days on the ears, and 21 days after we purified the cells from the epidermis and determined the distribution of TCRβ$^+$ T cells. We found a significant increase of TCRβ$^+$ T cells in the epidermis of mice sensitized to DNFB compared to control mice (figure 3a). In addition, we determined the expression level of *CD8* in the epidermis by qPCR. We found a significant increase in the *CD8* expression level in the epidermis from mice sensitized to DNFB (figure 3b). To characterize the CD8$^+$ T cells found in the epidermis, we co-stained αβTCR$^+$CD8$^+$ T cells for CD69 and CD103 known to be expressed by CD8$^+$ T_{RM} cells [4;28]. We found that the majority of the αβTCR$^+$CD8$^+$ T cells in epidermis exposed to DNFB expressed both CD69 and CD103, indicating that these cells were CD8$^+$ T_{RM} cells (figure 3c).

DNFB-induced CD8$^+$ T_{RM} cells produce IL-17A and IFN-γ

It is known that IL-17A and IFN-γ augment allergen-induced production of IL-1β [22;23]. Consequently, we wanted to determine if DNFB-induced CD8$^+$ T_{RM} cells produce these cytokines and thereby could be responsible for the strong memory response. Because relatively few cells can be purified

Contact Dermatitis - Manuscript Copy

from the epidermis of the ears, we sensitized mice on the abdomen for 3 consecutive days with DNFB or with OOA as control. After 21 days, we purified cells from the epidermis and analyzed them. As observed in the ears, the fraction of $TCR\beta^+$ cells was markedly increased in the epidermis from the abdomen after DNFB sensitization (figure 4a). Likewise, $CD8^+$ T cells represented the vast majority of the allergen-induced accumulation of $TCR\beta^+$ cells in the epidermis (figure 4a and b). Next, we determined the capacity of the epidermal T cells to produce IL-17A and IFN-γ. A 2-fold increase in the percentage of $CD8^+$IL-$17A^+$ T cells was found in epidermis exposed to DNFB compared to epidermis exposed to OOA (figure 4c and d). In parallel, we found a 4-fold increase in the frequency of IFN-γ-producing $CD8^+$ T cells in epidermis exposed to DNFB compared to epidermis exposed to OOA (figure 4e and f). Interestingly, DNFB treatment did not change the distribution of either $CD4^+$IL-$17A^+$ T cells or $CD4^+$IFN-γ^+ T cells found in the epidermis (figure 4c-f).

IL-17A and IFN-γ levels increase rapidly during memory responses and augment allergen-induced IL-1β production in keratinocytes

To further investigate the interplay between IL-1β, IFN-γ and IL-17A during the local memory response to contact allergens, we monitored the time-course of *IL1β, IFN-γ* and *IL17A* expression in the epidermis following exposure to DNFB in mice that were or were not sensitized to DNFB. Both *IL1β* and *IFN-γ* expression were detectable in the epidermis of non-sensitized and sensitized skin prior to the challenge with DNFB (0 h) with the highest expression levels found in sensitized skin. In contrast, *IL17A* expression was not detectable at any time-points in non-sensitized mice (figure 5a-c). An increase in *IL1β* expression was found 4 hours after challenge in both non-sensitized and sensitized skin (figure 5a). In sensitized skin, *IL1β* expression further increased after 24 hours, whereas a plateau was reached after 4 hours in non-sensitized epidermis (figure 5a). Interestingly, whereas the expression of *IFN-γ* did not change in non-sensitized epidermis upon DNFB challenge,

Contact Dermatitis - Manuscript Copy

a 50-fold increase in *IFN-γ* expression was found 4 hours after DNFB challenge in sensitized epidermis (figure 5b). Furthermore, whereas *IL17A* expression could not be detected in non-sensitized epidermis upon DNFB exposure, *IL17A* expression could be detected in sensitized epidermis 4 hours after DNFB exposure, and this level was stable at least 24 hours after the challenge (figure 5c).

Finally, we determined if IFN-γ and/or IL-17A had an effect on dinitrobenzene sulfonic acid (DNBS)-induced IL-1β expression in keratinocytes. We stimulated cultures of PAM2.12 cells with IFN-γ, IL-17A, DNBS or combinations of these for 24 hours and then determined *IL1β* expression. Whereas IFN-γ treatment did not induce *IL1β* expression (figure 5d), IL-17A induced a 2-fold increased *IL1β* expression (figure 5d). As expected from our previous results, DNBS induced an increased expression of *IL1β* (figure 5d) [14]. Addition of IFN-γ, IL-17A or especially the combination of the two during DNBS stimulation increased the *IL1β* expression compared to *IL1β* expression induced by DNBS stimulation alone (figure 5d).

Patients with nickel allergy have distinctly localized memory to nickel at previously exposed sites

Next we wanted to determine whether the local memory response to DNFB in mice translated to similar conditions in humans. To investigate this, we recruited 10 patients with allergy to nickel and patch tested them with either nickel or petrolatum (Veh) as control at areas previously unexposed to nickel as shown in figure 6a. After 21 days, the patients were challenged at exactly the same skin areas with a second patch test as indicated in figure 5a, and after 24 hours the reactions were scored. Visible reactions were only found at skin areas that had been exposed to nickel both in the first and second patch test (figure 6b and table 1). Next, we examined the expression level of *IL1β* in the different test areas. Interestingly, we found increased *IL1β* in all areas that had been exposed to nickel either during the first, the second or both tests (figure 6c). However, the expression level of

Contact Dermatitis - Manuscript Copy

IL1β was highest in skin that had been exposed to nickel both during the first and second patch test (figure 6c). As in the ACD mice model, we found that IL-1β in human ACD primarily located to the epidermis (figure 6d). Interestingly, *IL17A* was found in 4 out of 7 skin areas that had been exposed to nickel both during the first and second patch test but only in 1 out of 14 skin areas that had been exposed to nickel only once (table 1 and figure 6c). The presence of *IL17A* correlated with the clinical score and the levels of *IL1β*. Finally, we found that $CD8^+$ T cells were recruited to the interphase between the epidermis and the dermis after exposure to nickel during the first patch test (figure 6d).

Contact Dermatitis - Manuscript Copy

Discussion

In this study, we show that exposure to contact allergens leads to a strong, site-specific and a weaker, global immunological memory to the allergen in the skin. Thus, we found increased ear-swelling and levels of IL-1β in the ears of mice that were both sensitized and challenged on the ears compared to mice that were sensitized on the abdomen and challenged on the ears. We demonstrate that allergen-induced local memory is associated with the accumulation in the epidermis of $CD8^+$ T_{RM} cells able to produce IL-17A and IFN-γ. Furthermore, we found that IFN-γ and especially IL-17A enhanced allergen-induced IL-1β production in keratinocytes. Finally, we demonstrate the existence of distinct local memory to nickel in the skin of patients with allergy to nickel. The inflammatory memory response in the patients correlated with accumulation of $CD8^+$ T cells in the interphase between the dermis and epidermis and with the presence of increased levels of *IL1β* and *IL17A* in the affected skin.

In accordance with a recent study by Gaide et al., we found that T_{RM} cells accumulate in the skin following DNFB sensitization [2]. Studies using DNFB, virus, or *L. major* infections have shown that T_{RM} cells not only are recruited locally but also become globally seeded in the skin [2;6;29;30]. However, even though T_{RM} cells were seeded globally in the skin, the challenge responses were more pronounced in skin areas previously exposed to the specific antigen [2;4;6]. In contrast, another study found that following infections with herpes simplex virus only a localized seeding of T_{RM} cells in the infected skin took place [4]. We found that sensitization to DNFB induced a strong and long-lasting local memory and a weaker, temporary global memory to DNFB in the skin. In contrast to us, Gaide et al. found that the global skin memory to DNFB was long-lasting [2]. Several conditions may explain these differences. First of all Gaide et al. determined the response by looking at the presence of various T cell clones, whereas we determined the inflammatory response [2]. Further-

Contact Dermatitis - Manuscript Copy

more, Gaide et al. used a higher dose of DNFB and a smaller area of exposure than we did, and these parameters are known to be critical for sensitization [2;31;32]. In addition, Gaide et al. boosted the response, which most likely affected the generation of long lasting global memory [2]. Our results from the patients with allergy to nickel strongly supported the existence of very distinct local memory to allergens. Although these patients had been exposed to nickel many times at other sites of the skin, they did not show a memory response at previously unexposed skin areas, but developed a strong memory response exactly at the site that had been exposed for nickel previously.

$CD8^+$ T_{RM} cells are known to produce IFN-γ rapidly in response to antigen challenge, thereby setting the local tissue in an alerted state via induction of pro-inflammatory cytokines and chemokines [1;33]. We found that although the expression of *IL1β* increased following exposure to DNFB in both non-sensitized and sensitized skin, the increase was 10-fold higher in sensitized skin than in non-sensitized skin. This is in good agreement with the ability of contact allergens to induce *IL1β* expression via two pathways, namely by an innate pathway involving TLRs and by a pathway dependent on effector cytokines produced by T cells [22;34]. In contrast to *IL1β* expression, an increased expression of *IFN-γ* and induction of *IL17* after DNFB exposure was only found in sensitized skin, indicating that these cytokines are produced by memory cells recruited to the skin as a consequence of the sensitization. As we find an increased percentage of $CD8^+$ T_{RM} cells producing IL-17A and IFN-γ in the epidermis 21 days after sensitization, we find it likely that these cells are the cells mediating the increased expression level of *IL17A* and *IFN-γ* found in sensitized skin following challenge with DNFB. Although, only a minority of T_{RM} cells produce IL-17A compared to IFN-γ, these might still play an important role in enhancing the allergen-induced IL-1β production as IL-17A seems to be a potent enhancer of DNBS-induced IL-1β production. This is also supported by the observation that blocking IL-17A signaling has a stronger effect on allergen-induced IL-1β produc-

Contact Dermatitis - Manuscript Copy

tion than blocking IFN-γ signaling [22;23]. The correlation we found between the expression levels of *IL1β* and *IL17* in the skin of the patients with allergy to nickel also support the importance of IL-17A-producing T_{RM} cells in memory responses to allergens.

In their response to skin infections, T_{RM} cells most probably play an important and beneficial role [1;4;6]. It has been shown that virus-specific T_{RM} cells in the skin can trigger a state of pathogen alert, increasing the response to unrelated pathogens following co-exposure to the specific and unrelated pathogen [1]. In contrast to the beneficial role during infections, one might expect that the same mechanism increases the risk of developing ACD to contact allergens when simultaneously exposed to a mixture of contact allergens. E.g. fine fragrances often consist of 10-100 or even more potential contact allergens [35] and perfume allergy could thus be an example of "ACD-spreading". In accordance, it is surprising that as many as 4.1% of the population have contact allergy against one or more fragrance allergens as these are generally classified as weak to moderate contact allergens [36]. We suggest that once allergen-specific T_{RM} cells are present in the skin, they will increase the risk of developing additional allergies as they readily induce a strong inflammatory response when new allergens are presented together with the allergen that originally induced the ACD.

Contact Dermatitis - Manuscript Copy

Figure legends

Figure 1. Local immunological memory to DNFB develops following exposure to DNFB

C57BL/6 mice were sensitized and challenged as shown in a. b. Increase in ear thickness 3 weeks and 6 weeks (c) after sensitization. d. Increase in ear thickness in mice sensitized with either DNFB or oxazolone (OXA) following DNFB challenge. e. and f. Mice were sensitized with DNFB as described above and challenged on the ears after 21 days. IL-1β levels were determined in ear homogenates as determined by ELISA is shown. g. The level of IL-1β in epidermis and dermis of challenged ears h. The distribution of IL-1β determined by IHC staining of ear sections, scale bars indicate 100 µm. The experiments were performed using 4 animals per group in two independent experiments. Error bars signify standard error of the mean. Asterisks indicate the level of statistical significance calculated by unpaired two-way student's t-test: ****: P<0.0001; ***: P<0.0005; **: P<0.001; *: P<0.05

Figure 2. The local memory response is not mediated by retention of DNFB in skin

C57BL/6 mice were sensitized and challenged 21 days after with either DNFB or vehicle. (A) Quantified presentation of the amounts of DNFB in ear homogenates determined by Western blot analyses using anti-DNP antibodies to detect DNFB-modified proteins. (B) IHC staining with anti-DNP antibodies of cross-sectioned ears. Scale-bars indicate 200 µm. The data shown are based on two independent experiments, each with 4 animals per group. Error bars signify standard error of the mean. Asterisks indicate the level of statistical significance calculated by an unpaired two-way student's t-test *: P<0.05

Figure 3. Increased numbers of $CD8^+$ T_{RM} cells in the epidermis following DNFB sensitization

Contact Dermatitis - Manuscript Copy

C57BL/6 mice were sensitized on the ears with DNFB or OOA as control. After 21 days, single cell suspensions from the epidermis were analyzed. a. Cells were purified from the epidermis and the frequency of $TCR\beta^+$ cell was determined by flow cytometry. b. CD8 mRNA expression relative to GAPDH expression as determined by qPCR. c. Representative FACS plot of CD8 and TCRβ expression on epidermal single cell suspensions and histograms of CD69 and CD103 expression on $TCR\beta^+$ cells. Red histograms represent $CD8^+TCR\beta^+$ cells and blue histograms represent $CD4^+TCR\beta^+$ cells. Error bars signify standard error of the mean. Asterisks indicate the level of statistical significance calculated by an unpaired two-way student's t-test: **: P<0.001; *: P<0.05

Figure 4. DNFB-induced $CD8^+$ T_{RM} cells produce IL-17A and IFN-γ

C57BL/6 mice were sensitized on the abdomen for three consecutive days. After 21 days, the cells were purified from the epidermis and $TCR\beta^+$ cells were analyzed further by flow cytometry. a. The frequency of $TCR\beta^+$ cells. b. The distribution of $CD4^+$ and $CD8^+$ T cells (gate as $CD8^-TCR\beta^+$ and $CD8^+TCR\beta^+$ cells, respectively) in the $TCR\beta^+$ cell population. c. The frequency of IL-17A-producing $CD4^+$ and $CD8^+$ $TCR\beta^+$ cells. d. Representative dot plots of $TCR\beta^+$ cells stained for CD8 and IL-17A. e. The frequency of IFN-γ-producing $CD4^+$ and $CD8^+$ $TCR\beta^+$ cells. f. Representative dot plots of $TCR\beta^+$ cells stained for CD8 and IFN-γ. Error bars signify standard error of the mean. Asterisks indicate the level of statistical significance calculated by an unpaired two-way student's t-test: ****: P<0.0001; ***: P<0.0005; **: P<0.001

Figure 5. IL-17A and IFN-γ levels increase rapidly during memory responses

Mice were either left untreated or sensitized with DNFB for three consecutive days on the ears. After 21 days, the epidermis from groups of non-sensitized (gray bars) or sensitized (black bars) mice were analyzed before challenge (0h) or after 4 or 24 h after challenge with DNFB for expression of

Contact Dermatitis - Manuscript Copy

a. *IL1β*, b. *IFN-γ* and c. *IL17A* by qPCR. d. PAM2.12 cells were treated with 0.25 µg/ml DNBS, 10 ng/ml IFN-γ or 10 ng/ml IL-17A either alone or in the indicated combinations. After 24 h, the induction of *IL1β* expression was assessed by qPCR. Error bars signify standard error of the mean. Asterisks indicate the level of statistical significance calculated by an unpaired two-way student's t-test: ***: P<0.0005; **: P<0.001; *: P<0.05

Figure 6. Patients with nickel allergy have distinctly localized memory to nickel at previously exposed sites

Ten individuals with allergy to nickel were patch tested twice with either 5% $NiSO_4$ in petrolatum (Ni) or petrolatum (Veh) at four precisely defined skin areas with a 21 days interval. 24 hours after application of the second patch test the local immune responses were analyzed. a. Schematic model for the experimental setup. b. Photo of the test area from two representative individuals 24 hours after application of patch test II. c. Expression levels of *IL1β* and *IL-17A* in the punch biopsies from seven individuals. d. Histological staining for IL-1β, CD8 and CD3 were made on biopsies from three individuals. Arrows indicate cells positive for CD8 and CD3 staining. Representative histological stains are shown. Asterisks indicate the level of statistical significance calculated by an unpaired two-way student's t-test: *: P<0.05.

Contact Dermatitis - Manuscript Copy

References

1. Ariotti S, Hogenbirk MA, Dijkgraaf FE *et al*. T cell memory. Skin-resident memory CD8(+) T cells trigger a state of tissue-wide pathogen alert. *Science* 2014; **346**: 101-5.

2. Gaide O, Emerson RO, Jiang X *et al*. Common clonal origin of central and resident memory T cells following skin immunization. *Nat Med* 2015; **21**: 647-53.

3. Cheuk S, Wiken M, Blomqvist L *et al*. Epidermal Th22 and Tc17 cells form a localized disease memory in clinically healed psoriasis. *J Immunol* 2014; **192**: 3111-20.

4. Gebhardt T, Wakim LM, Eidsmo L *et al*. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol* 2009; **10**: 524-30.

5. Heath WR, Carbone FR. The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells. *Nat Immunol* 2013; **14**: 978-85.

6. Jiang X, Clark RA, Liu L *et al*. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature* 2012; **483**: 227-31.

7. Mackay LK, Stock AT, Ma JZ *et al*. Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. *Proc Natl Acad Sci U S A* 2012; **109**: 7037-42.

8. Mackay LK, Rahimpour A, Ma JZ *et al*. The developmental pathway for CD103(+)CD8+ tissue-resident memory T cells of skin. *Nat Immunol* 2013; **14**: 1294-301.

9. Zhu J, Peng T, Johnston C *et al*. Immune surveillance by CD8alphaalpha+ skin-resident T cells in human herpes virus infection. *Nature* 2013; **497**: 494-7.

10. Grolnick M. The Spontaneous Flare-up of Negative Test-Sites in Experimental Sensitization in Man. *J Immunol* 1941; **41**: 127-42.

11. Jacob SE, Barland C, ElSaie ML. Patch-test-induced "flare-up" reactions to neomycin at prior biopsy sites. *Dermatitis* 2008; **19**: E46-E48.

12. Diepgen TL, Ofenloch RF, Bruze M *et al*. Prevalence of contact allergy in the general population in different European regions. *Br J Dermatol* 2016; **174**: 319-29.

13. Scheper RJ, von BM, Boerrigter GH *et al*. Induction of immunological memory in the skin. Role of local T cell retention. *Clin Exp Immunol* 1983; **51**: 141-8.

14. Nielsen MM, Lovato P, MacLeod AS *et al*. IL-1beta-dependent activation of dendritic epidermal T cells in contact hypersensitivity. *J Immunol* 2014; **192**: 2975-83.

15. Vennegaard MT, Dyring-Andersen B, Skov L *et al*. Epicutaneous exposure to nickel induces nickel allergy in mice via a MyD88-dependent and interleukin-1-dependent pathway. *Contact Dermatitis* 2014; **71**: 224-32.

Contact Dermatitis - Manuscript Copy

16. Watanabe H, Gaide O, Petrilli V *et al*. Activation of the IL-1beta-processing inflammasome is involved in contact hypersensitivity. *J Invest Dermatol* 2007; **127**: 1956-63.

17. Enk AH, Katz SI. Early molecular events in the induction phase of contact sensitivity. *Proc Natl Acad Sci U S A* 1992; **89**: 1398-402.

18. Honda T, Egawa G, Grabbe S *et al*. Update of immune events in the murine contact hypersensitivity model: toward the understanding of allergic contact dermatitis. *J Invest Dermatol* 2013; **133**: 303-15.

19. Martin SF, Dudda JC, Bachtanian E *et al*. Toll-like receptor and IL-12 signaling control susceptibility to contact hypersensitivity. *J Exp Med* 2008; **205**: 2151-62.

20. Schmidt M, Raghavan B, Muller V *et al*. Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nat Immunol* 2010; **11**: 814-9.

21. Dyring-Andersen B, Skov L, Lovendorf MB *et al*. CD4(+) T cells producing interleukin (IL)-17, IL-22 and interferon-gamma are major effector T cells in nickel allergy. *Contact Dermatitis* 2013; **68**: 339-47.

22. He D, Wu L, Kim HK *et al*. CD8+ IL-17-producing T cells are important in effector functions for the elicitation of contact hypersensitivity responses. *J Immunol* 2006; **177**: 6852-8.

23. He D, Wu L, Kim HK *et al*. IL-17 and IFN-gamma mediate the elicitation of contact hypersensitivity responses by different mechanisms and both are required for optimal responses. *J Immunol* 2009; **183**: 1463-70.

24. Nakae S, Komiyama Y, Nambu A *et al*. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* 2002; **17**: 375-87.

25. Kish DD, Volokh N, Baldwin WM, III *et al*. Hapten application to the skin induces an inflammatory program directing hapten-primed effector CD8 T cell interaction with hapten-presenting endothelial cells. *J Immunol* 2011; **186**: 2117-26.

26. Johansen JD, Aalto-Korte K, Agner T *et al*. European Society of Contact Dermatitis guideline for diagnostic patch testing - recommendations on best practice. *Contact Dermatitis* 2015; **73**: 195-221.

27. Bonefeld CM, Nielsen MM, Gimenez-Arnau E *et al*. An immune response study of oakmoss absolute and its constituents atranol and chloroatranol. *Contact Dermatitis* 2014; **70**: 282-90.

28. Masopust D, Vezys V, Wherry EJ *et al*. Cutting edge: gut microenvironment promotes differentiation of a unique memory CD8 T cell population. *J Immunol* 2006; **176**: 2079-83.

29. Glennie ND, Yeramilli VA, Beiting DP *et al*. Skin-resident memory CD4+ T cells enhance protection against Leishmania major infection. *J Exp Med* 2015; **212**: 1405-14.

30. Liu L, Fuhlbrigge RC, Karibian K *et al*. Dynamic programming of CD8+ T cell trafficking after live viral immunization. *Immunity* 2006; **25**: 511-20.

Contact Dermatitis - Manuscript Copy

31. Friedmann PS. Contact sensitisation and allergic contact dermatitis: immunobiological mechanisms. *Toxicol Lett* 2006; **162**: 49-54.

32. Friedmann PS. The relationships between exposure dose and response in induction and elicitation of contact hypersensitivity in humans. *Br J Dermatol* 2007; **157**: 1093-102.

33. Schenkel JM, Fraser KA, Vezys V *et al*. Sensing and alarm function of resident memory CD8(+) T cells. *Nat Immunol* 2013; **14**: 509-13.

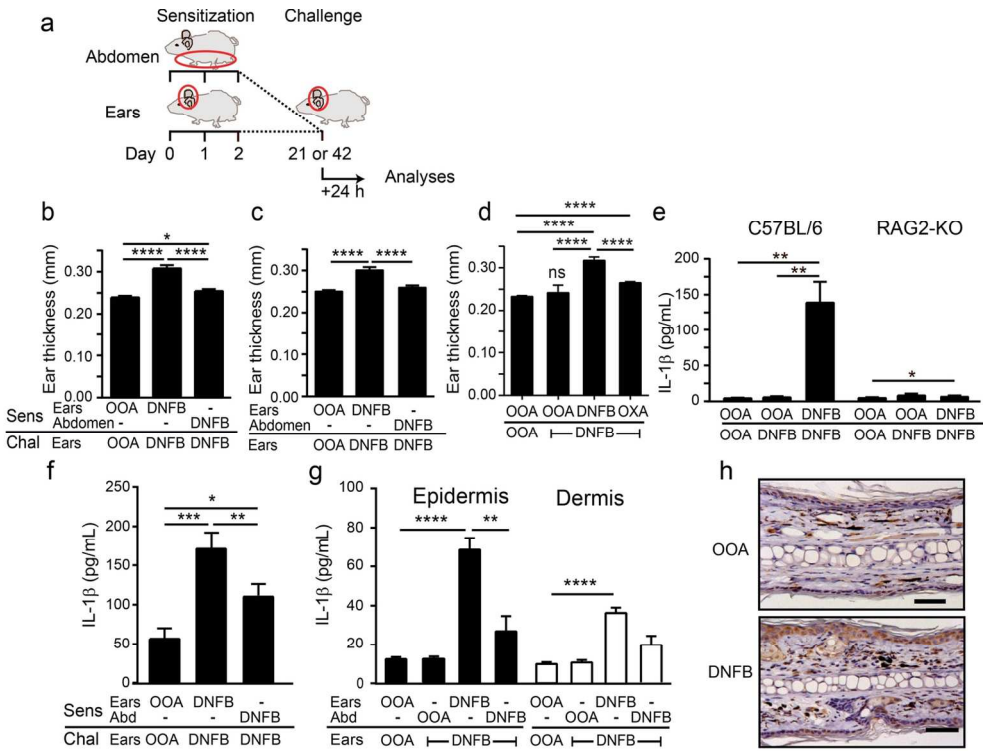
34. Martin SF, Esser PR, Weber FC *et al*. Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. *Allergy* 2011; **66**: 1152-63.

35. Johansen JD, Lepoittenvin J-P. Fragrances. In: *Contact Dermatitis*, Fifth edition edn. Springer, 2011: 607-28.

36. Johansen JD. Fragrance contact allergy: a clinical review. *Am J Clin Dermatol* 2003; **4**: 789-98.

Contact Dermatitis - Manuscript Copy

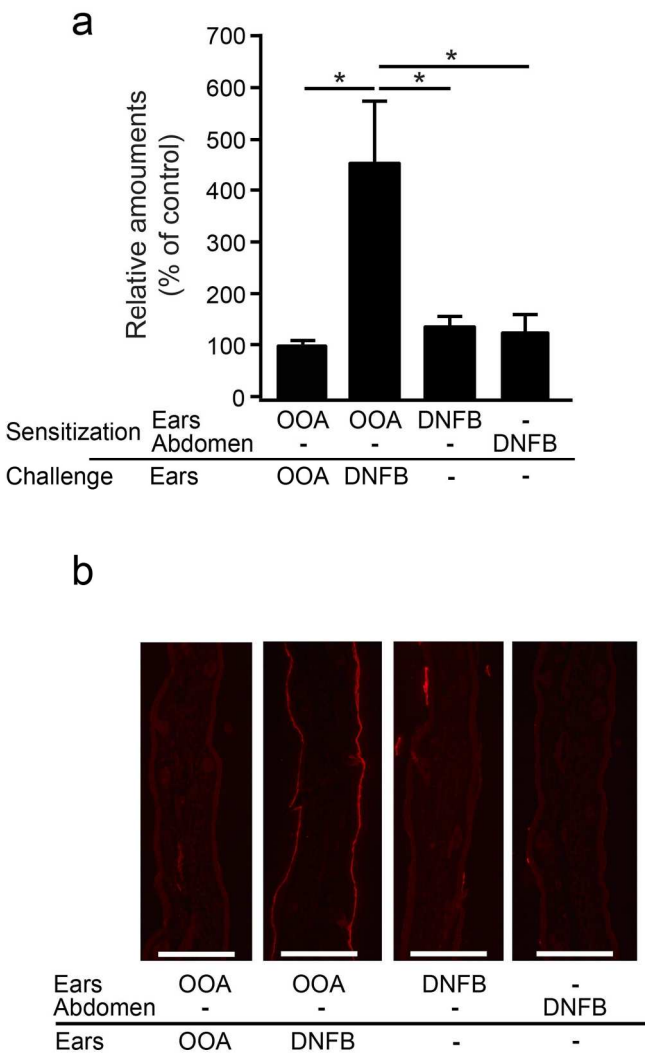
Figure 1



137x116mm (300 x 300 DPI)

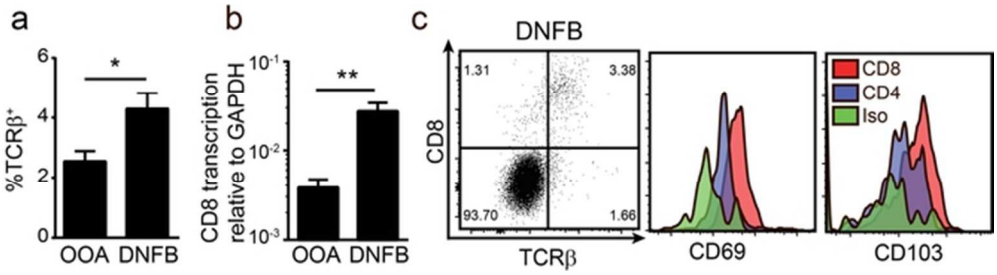
Manuscript Copy

Figure 2



123x213mm (300 x 300 DPI)

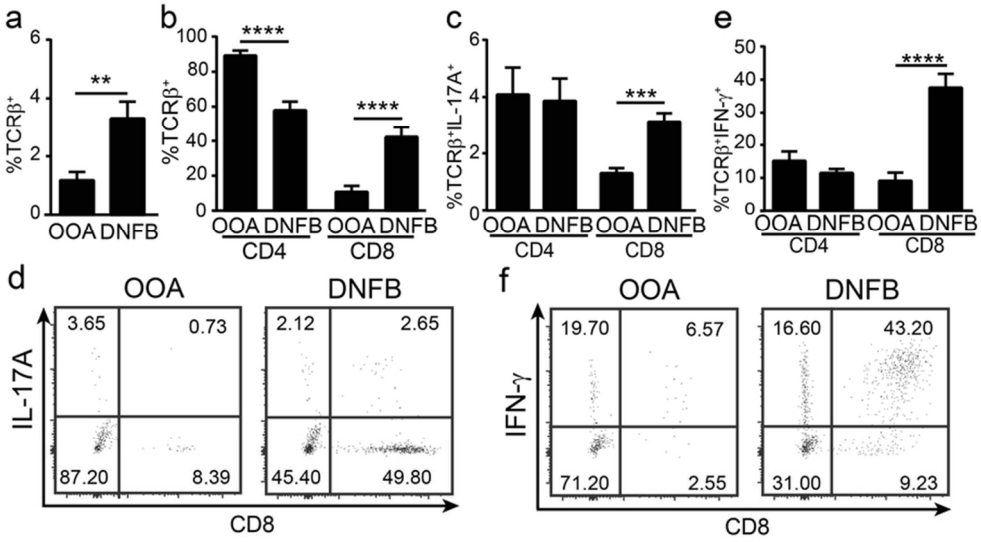
Figure 3



56x25mm (300 x 300 DPI)

Contact Dermatitis - Manuscript Copy

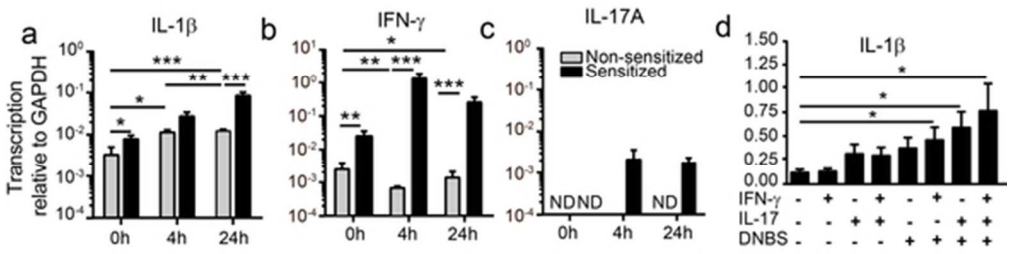
Figure 4



78x48mm (300 x 300 DPI)

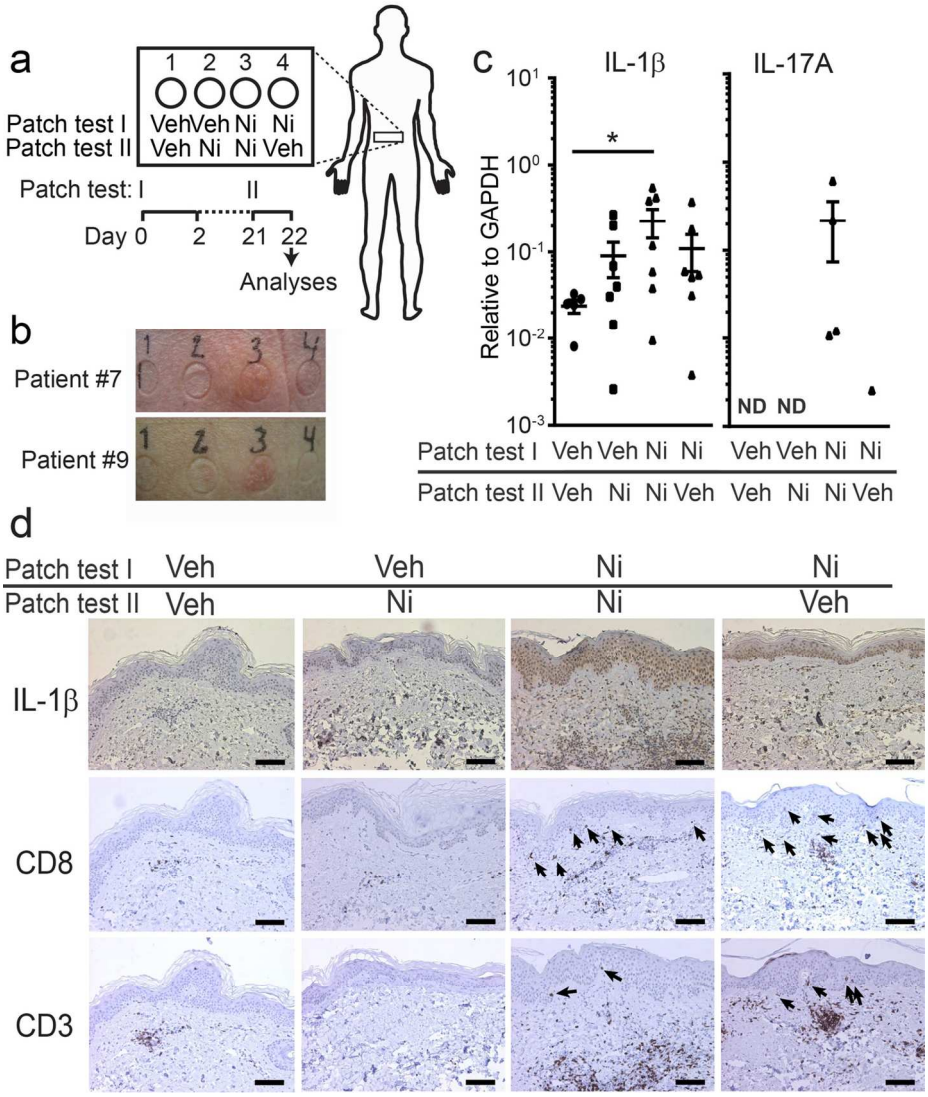
Contact Dermatitis - Manuscript Copy

Figure 5



49x17mm (300 x 300 DPI)

Figure 6



123x160mm (300 x 300 DPI)

Table 1. Correlation between clinical score, IL-17A and IL-1β

			Clinical score Patch test II				Ni/Ni Transcription rel. to GAPDH	
Patient #	Age	Gender	Veh/Veh	Veh/Ni	Ni/Ni	Ni/Veh	IL-17A	IL-1β
1	58	Female	-	-	-	-	0.669	0.392
2	69	Female	-	-	-	-	Not detectable	0.038
3	43	Female	-	-	++	-	0.235	0.551
4	45	Female	-	-	-	-	Not detectable	0.010
5	55	Female	-	-	++	-	0.011	0.119
6	67	Male	-	-	-	-	Not detectable	0.060
7	56	Female	-	-	+++	-	0.012	0.426
8	50	Female	-	-	+	-	Used for IHC	Used for IHC
9	75	Female	-	-	+	-	Used for IHC	Used for IHC
10	56	Female	-	-	-	-	Used for IHC	Used for IHC

155x180mm (300 x 300 DPI)

BJD
British Journal of Dermatology

Immune responses to hair dyes containing toluene-2,5-diamine*

J.D. Schmidt,[1,2] J.D. Johansen,[2] M.M. Nielsen,[1] E. Zimersson,[3] C. Svedman,[3] M. Bruze,[3] K. Engkilde,[2] S.S. Poulsen,[4] C. Geisler[1] and C.M. Bonefeld[1]

[1]Department of International Health, Immunology and Microbiology and [2]National Allergy Research Centre, Department of Dermato-Allergology, Copenhagen University Hospital, Gentofte, Denmark

[3]Department of Occupational and Environmental Dermatology, Malmo University Hospital, Malmo, Sweden

[4]Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

Correspondence
Charlotte Menné Bonefeld.
E-mail: cmenne@sund.ku.dk

Accepted for publication
5 October 2013

Funding sources
Danish Environmental Protection Agency, A.P. Møller Foundation for the Advancement of Medical Sciences, LEO Pharma Research Foundation, Danish Medical Research Council, Novo Nordisk Foundation, Lundbeck Foundation.

Conflicts of interest
None declared.

*Plain language summary available online

DOI 10.1111/bjd.12676

Summary

Background Toluene-2,5-diamine (PTD) is the most frequently used dye in oxidative hair dyes on the Scandinavian market. However, little is known about immune responses to PTD-containing oxidative hair dyes.

Objectives To study immune responses induced by PTD-containing hair dyes in mice.

Methods Immune responses against two different permanent hair dye products containing 1·60% (w/w) and 0·48% (w/w) PTD within the colour gel, and various concentrations of pure PTD were studied. The local inflammatory response was measured by ear swelling and cell infiltration, and T- and B-cell infiltration and proliferation was determined in the draining lymph nodes.

Results Concentration-dependent immune responses were seen to PTD both in the skin and draining lymph nodes. The hair dye containing 1·60% PTD induced strong local inflammation and caused T- and B-cell infiltration and proliferation as well as an increased number of regulatory T cells in the draining lymph nodes. In contrast, the hair dye containing 0·48% PTD induced skin inflammation but only minor responses in the draining lymph nodes.

Conclusions Consumer-available PTD-containing permanent hair dyes can be potent immune activators inducing both pro- and anti-inflammatory responses. The outcome of the response is dependent on allergen dose, amount of additional allergens and exposure regime.

What's already known about this topic?

- Use of permanent hair dyes can induce severe contact allergic reactions.
- Toluene-2,5-diamine (PTD) is more frequently used in hair dyes in the Scandinavian market than *p*-phenylenediamine (PPD).

What does this study add?

- PTD-containing consumer-available hair dyes can induce severe immunological reaction in mice.
- The immune response to PTD-containing hair dyes is dependent on PTD concentration and additional allergens within the hair dye mixture.

Permanent hair dyes are well-known skin sensitizers, and consumers may develop contact allergic reactions following exposure. The allergen of most concern in permanent hair dyes is *p*-phenylenediamine (PPD). However, several other hair dye substances have been shown to be potent skin sensitizers; among these is toluene-2,5-diamine (also known as *p*-toluenediamine, or TDA) (PTD).[1,2] A Danish case investigation showed that eight of nine hair dye products in the Danish market causing clinical dermatitis contained PTD, whereas only one contained PPD.[2] Recent analyses of oxidative hair

© 2013 British Association of Dermatologists

dye products on the Swedish and Spanish markets revealed that PPD was present in 16% and 50% and PTD was present in 80% and 49% of the products, respectively.[3,4] Taken together, PTD seems to be more frequently used in hair dye products in Scandinavia than PPD.

We have recently investigated immune responses to PPD-containing permanent hair dyes using mouse models.[5,6] We found that hair dye mixtures are potent inducers of primary immune responses. This is interesting, as a questionnaire study showed that only 5·3% of individuals reported allergic reaction following hair dye exposure.[7] It is therefore likely that hair dye use can lead to tolerance development. We analysed this by using a mouse model in which mice were exposed repetitively to PPD-containing hair dye. Repeated exposure to hair dye induced both pro- and anti-inflammatory responses.[6] However, it is unknown whether similar immune responses are induced by PTD-containing hair dyes.

The purpose of this study was to investigate immune responses against PTD-containing permanent hair dyes by employing two mouse models previously used to study immune responses to PPD-containing permanent hair dyes.[5,6]

Materials and methods

Mice

Age-matched female C57BL/6 mice were purchased from Taconic (Ejby, Denmark) and housed in the specific pathogen-free animal facility at the University of Copenhagen in accordance with national animal-protection guidelines (licence number 2007/501-1357). Four mice were used in each experimental group in two independent experiments.

Chemicals

The following chemicals were used:

1. Acetone (Sigma 534064-500 mL, CAS no.: 67-64-1),
2. Olive oil (Fluka 75343-1L, CAS no.: 8001-25-0),
3. p-phenylenediamine (PPD) (Fluka 100 g, CAS no.: 101-54-2, purity 98%),
4. PTD-base obtained from an acidified (HCl) PTD-sulfate (Acros Organics cat. no.: 167541000, CAS no.: 615-50-9) solution and extracted with ethyl acetate and subsequently dried by vacuum evaporation; PTD purity was confirmed by gas-chromatography mass spectrometry,
5. Hair dye 1 (L'Oréal Féria, 20·01 Black spirit; Batch: 10H700),
6. Hair dye 2 (Schwarzkopf Brillance, Black 890; Batch: 0205Z80184).

Contact sensitization

We used a modified version of the local lymph node assay (LLNA) for testing the sensitizing potential of the test substances as previously described.[8] The following substances were tested: 25 μL of 0·25% PTD (w/v), 1% PTD (w/v), 4% PTD (w/v) and 4% PPD (w/v) diluted in acetone : olive oil (3 : 1), 25 μL of hair dye mixture consisting of colour gel and oxidizer (1 : 1). Control mice were exposed to acetone : olive oil (3 : 1). The hair dye mixture was applied on the ears and after 30 min the hair dye mixture was washed off with cotton wool soaked in tempered water until no more colour could be removed from the ears. The commercial hair dye products tested were from two leading international brands, obtained in September 2012 from the retail market in Denmark. Both products were of a dark shade and contained PTD, according to the ingredients label. Hair dye 1 (HD1) contained PTD declared as free base, hair dye 2 (HD2) contained PTD as a sulfate salt. Mice were exposed to the allergens for three consecutive days on both ears (days 0–2). At day 3, mice were given 0·8 mg mL^{-1} 5-bromo-2-deoxyuridine (BrdU) (0·8 mg mL^{-1}) (Sigma Aldrich, Broendby, Denmark) in their drinking water. BrdU is a thymidine analogue, which is incorporated into the DNA of the proliferating cell, and thus a marker of proliferation. At day 5, mice were euthanized. Subsequently, skin inflammation was quantified by the increase in ear thickness as measured in a blinded manner using an engineer's micrometre (Mitutoyo, Tokyo, Japan). Ears were removed and prepared for histology as described below. The draining auricular lymph nodes were subsequently removed for further analyses.

Repeated hair dye exposure

For testing the effect of regular use of hair dyes on the immune response, 25 μL hair dye mixture was applied to the dorsum of the mice ears. The hair dye was mixed as described above. The mixture was washed away after 30 min with cotton wool soaked in lukewarm water until no more colour could be removed from the ears. Control mice were exposed to aqua : acetone : olive oil in the ratio 2 : 2 : 1 [this was done before we implemented acetone : olive oil (3 : 1) vehicle to solubilize PTD]. Mice were exposed to the hair dyes every second week for a maximum 10 weeks. Groups of mice were given 0·8 mg mL^{-1} BrdU in their drinking water 24 h before the second, fourth and sixth exposure. The mice in each group were euthanized 24 h after the second, the fourth and the sixth treatment, respectively. Subsequently, skin inflammation was quantified by the increase in the thickness of the ears as measured in a blinded manner using an engineer's micrometre (Mitutoyo). Ears were subsequently removed and prepared for histology. The draining lymph nodes were removed for further analyses with flow cytometry.

Histology

Mouse ears were fixed in 4% formalin, and embedded in paraffin. Five-micrometre sections were cut on a microtome and after deparaffination the sections were stained with haematoxylin and eosin. The tissue sections were analysed by means

© 2013 British Association of Dermatologists

of an Axioskop 2 Plus Zeiss microscope fitted with a Coolsnap Camera (Photometrics, Tucson, AZ, U.S.A).

Flow cytometry

Single cell suspensions were prepared from the draining lymph nodes. Cells were counted and adjusted to 1×10^7 cell mL^{-1} and plated in 96-well plates (100 μL per well). To determine the distribution and proliferation of T and B cells, cells were incubated with fluorochrome-conjugated anti-CD4 (clone RM4-5), anti-CD8a (clone 53–6.7), or anti-CD19 (clone ID3) monoclonal antibodies (mAb), all from BD Biosciences (BD, Albertslund, Denmark). Cells were incubated with the antibodies diluted (1 : 100) in ice-cold phosphate-buffered saline containing 2% fetal calf serum and 0·1% NaN_3 for 30 min on ice. The cells were stained for surface expression of CD4, CD8 and CD19 as described above. After washing, the cells were fixed, permeabilized and treated with 300 μg mL^{-1} DNase for 60 min at 37 °C. Finally, the cells were stained with anti-BrdU mAb (APC BrdU Flow Kit, BD Pharmingen™) and analysed by flow cytometry using a FACS Calibur (BD Biosciences) with FlowJo software. To determine the number of $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells, the cell suspensions were surface stained with fluorochrome-conjugated anti-CD4 and anti-CD25 (clone PC61) followed by intracellular staining with anti-Foxp3 mAb using the Foxp3 kit from eBioscience (San Diego, CA, U.S.A.) according to the manufacturer's instructions (cat. no. 88-8111-40).

Statistics

In the figures, asterisks signify the outcome of a two-tailed unpaired Student's *t*-test within 95% confidence interval, of groups treated compared with the coherent control: not significant (n.s.): $P > 0{\cdot}05$; levels of significance: $^*P \leq 0{\cdot}05$; $^{**}P \leq 0{\cdot}01$; $^{***}P \leq 0{\cdot}001$.

Results

To test the sensitization potential of PTD we sensitized mice with pure PTD and two different permanent hair dyes containing PTD and compared the responses to the response following exposure to 4% PPD. Surprisingly, treatment with 1% PTD caused the same degree of ear swelling (115 ± 2%) as treatment with 4% PPD, indicating that PTD is a strong activator of immune responses (Fig. 1a). HD1 induced a strong skin inflammation as seen in massive ear swelling and cellular infiltration (Fig. 1a,b). Even though sensitization with HD2 induced less skin inflammation than HD1, it was still stronger than seen after exposure to pure PTD or PPD (Fig. 1). Thus, PTD-containing hair dyes seem to be potent inducers of skin inflammation as we have previously shown for PPD-containing hair dyes.[5]

Accordingly, when studying the draining lymph nodes we found a concentration-dependent increase in the infiltration of $CD4^+$ and $CD8^+$ T cells and $CD19^+$ B cells following PTD exposure (Fig. 2a). Exposure to either HD1 or HD2 induced only 50–75% T-cell infiltration compared with 1% PTD (Fig. 2a). Interestingly, whereas HD1 induced B-cell infiltration equal to the B-cell infiltration seen following exposure to 4% PTD, HD2 induced only 50% of this B-cell infiltration (Fig. 2a).

We then analysed T- and B-cell proliferation in the draining lymph nodes following sensitization with PTD, PPD, HD1 or HD2 as determined by $BrdU^+$ cells. We found that $CD4^+$ T cells dominated the proliferative response, whereas less proliferation of $CD8^+$ T cells and B cells was induced and primarily following exposure to HD1 (Fig. 2b). Interestingly, exposure to HD2 induced only an approximately threefold increase in $CD4^+$ T-cell proliferation, whereas HD1 induced an approximately 10-fold increase $CD4^+$ T-cell proliferation compared with control mice (Fig. 2b). Taken together, HD1

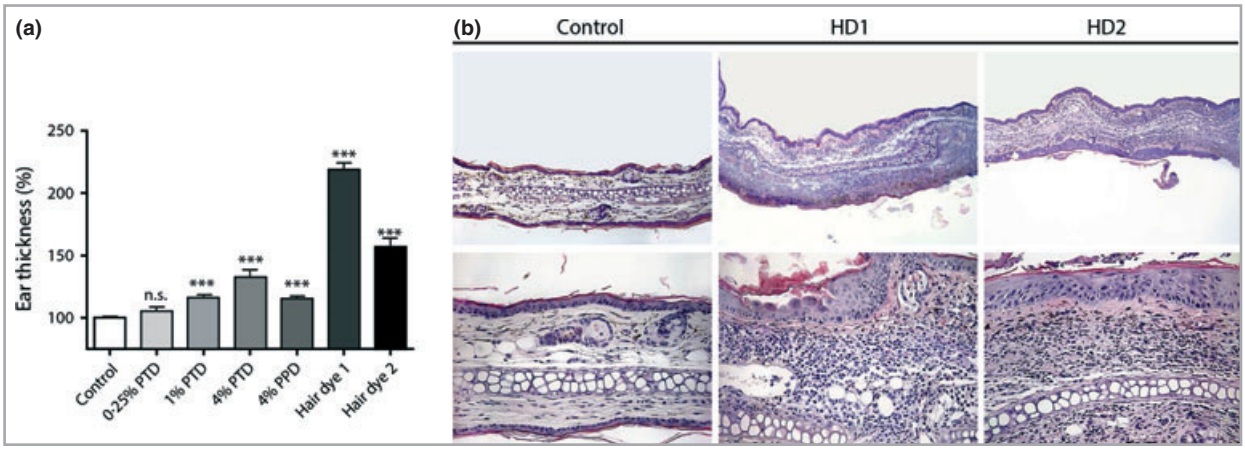


Fig 1. Toluene-2,5-diamine (PTD)-containing hair dyes induce a strong local inflammatory response. (a) The primary inflammatory response in mice treated with vehicle (control), 0·25% PTD, 1% PTD, 4% PTD, 4% PPD, hair dye 1 (HD1) or hair dye 2 (HD2) as measured by relative ear thickness normalized to ear thickness of mice treated with vehicle. Mean ± SD, n = 8 based on two independent experiments. (b) Representative haematoxylin and eosin staining of cross-sectioned ears from mice treated as indicated. n.s., not significant ($P > 0{\cdot}05$). $^{***}P \leq 0{\cdot}001$.

© 2013 British Association of Dermatologists

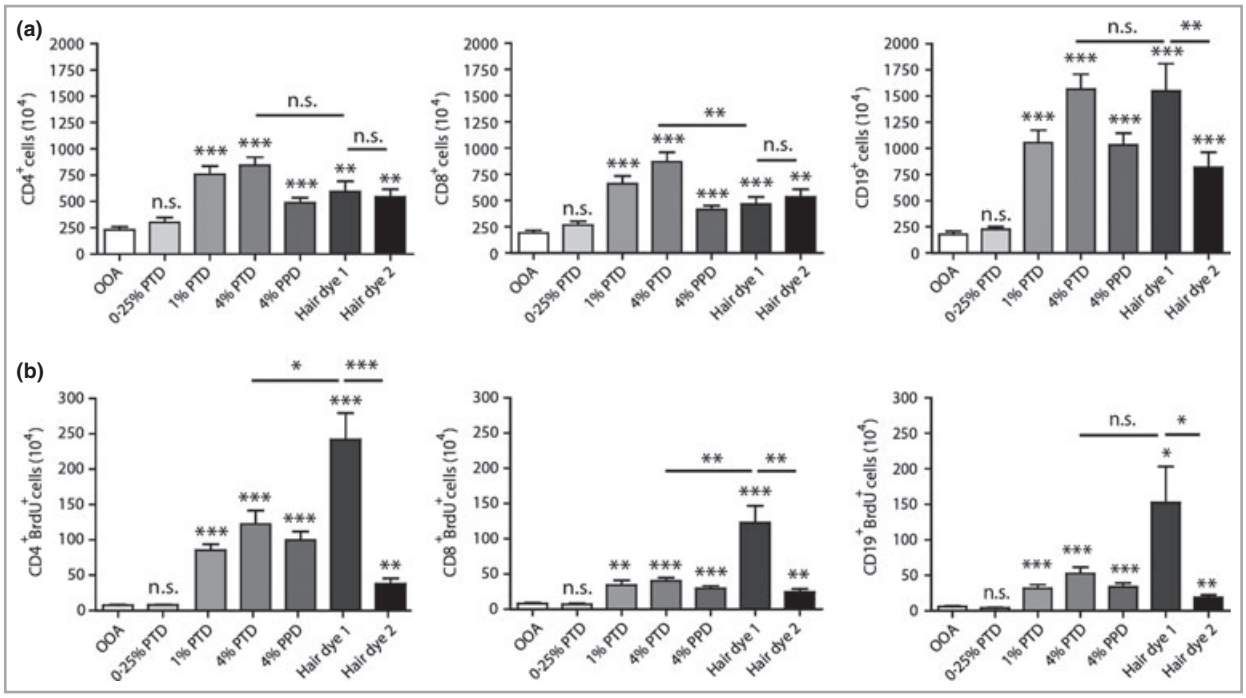


Fig 2. An adaptive immune response is induced by PTD- and PTD-containing hair dyes. The adaptive immune response to mice treated with vehicle (OOA, oliveoil : acetone (1 : 3)), 0·25% PTD, 1% PTD, 4% PTD, 4% PPD, hair dye 1 or hair dye 2. Total numbers of $CD4^+$, $CD8^+$ and $CD19^+$ cells (a), $CD4^+BrdU^+$, $CD8^+BrdU^+$ and $CD19^+BrdU^+$ cells (b) in the draining lymph nodes quantified from flow cytometry analysis. Mean ± SD, $n = 8$ based on two independent experiments. PTD, toluene-2,5-diamine; PPD, *p*-phenylenediamine. n.s., not significant ($P > 0{\cdot}05$). *$P \leq 0{\cdot}05$; **$P \leq 0{\cdot}01$; ***$P \leq 0{\cdot}001$.

seems to constitute a more potent immune activator than HD2.

To further investigate the immune response to PTD-containing hair dyes, three different groups of mice were exposed to HD1, HD2 or vehicle (control) once every second week. The immune responses were analysed 24 h following the second, fourth or sixth exposure. An increase in ear thickness was observed following treatment with HD1 (Fig. 3a) with a maximum after the fourth exposure (144 ± 3%) and a subsequent decrease to 137 ± 2% after the sixth exposure, when compared with the control group (Fig. 3a). Ear swelling as a result of cell infiltration was confirmed by histology (Fig. 3b). Interestingly, HD2 treatment induced only a minor increase in the ear thickness, which appeared to be exposure-independent indicating that this could represent an irritant response (Fig. 3). The immune responses in the draining lymph nodes reflected the local inflammatory response in the exposed ears. Exposure to HD1 induced an exposure-dependent infiltration and proliferation of both T and B cells, peaking after the fourth exposure (Fig. 4a,b). In contrast, neither infiltration nor proliferation was seen in mice exposed to HD2 at any time point (Fig. 4a,b).

We have previously shown that repetitive exposure to PPD-containing hair dyes leads to an increased number of Treg cells in the draining lymph nodes.[6] As repetitive exposure to HD1 induced a response similar to what we have seen for PPD-containing hair dyes, it is likely that exposure to HD1 also leads to an increased number of Treg cells within the draining lymph nodes. To investigate this, we used the experimental setup described in Figure 1 and examined the number of $CD4^+CD25^+FoxP3^+$ Treg cells in the draining lymph nodes. For PTD-treated mice we found a concentration-dependent increase in $CD4^+CD25^+FoxP3^+$ Treg cells (Fig. 5a). Exposure to HD1 induced the highest number of infiltrating $CD4^+CD25^+FoxP3^+$ Treg cells, which was approximately two-fold higher than seen after exposure to HD2 (Fig. 5a). To further investigate the induction of Treg cells following hair dye exposure, we quantified $CD4^+CD25^+FoxP3^+$ Treg cells in the draining lymph nodes of mice exposed repetitively to the hair dyes as described in Figure 3. We found an increase in the number of $CD4^+CD25^+FoxP3^+$ cells to the fourth exposure in mice treated with HD1, whereas no change in the number of $CD4^+CD25^+FoxP3^+$ cells was seen in HD2-treated mice (Fig. 5b). Thus, the number of $CD4^+CD25^+FoxP3^+$ Treg cells in the draining lymph nodes seems to follow the inflammatory response.

The differences in the immune responses seen after treatment with HD1 and HD2 could be mediated by differences in the PTD concentration and/or by the number of other allergens present in the two hair dye mixtures. We consequently determined the concentration of soluble PTD in the hair dyes. HD1 contained 1·60% PTD (w/w) and HD2 contained 0·48% PTD (w/w) within the colour gel (Table 1). In addition to PTD, HD1 and HD2 contained five and four colour substances, and three and two fragrance allergens, according to the manufacturing label, respectively (Table 1).

© 2013 British Association of Dermatologists

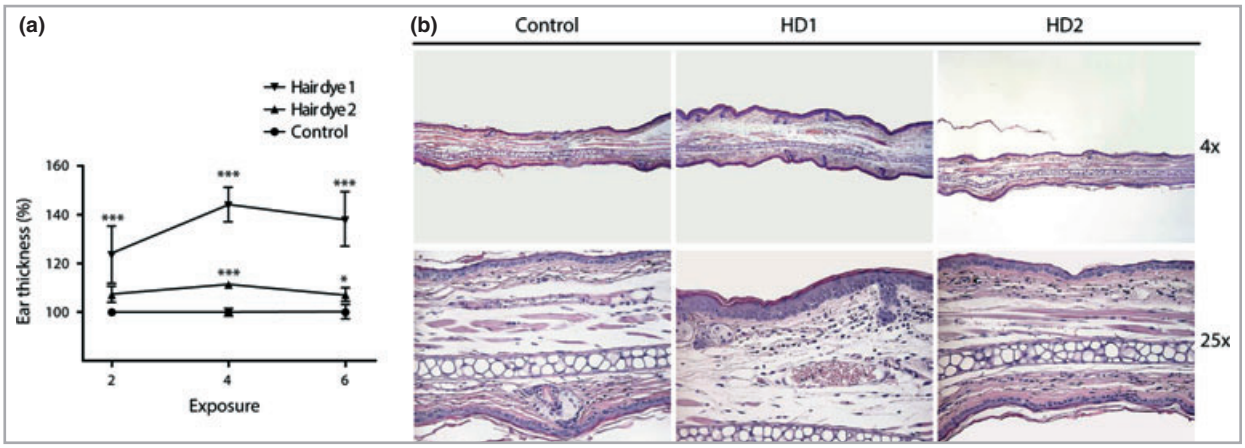


Fig 3. Repeated hair dye exposure induces local inflammation. (a) Local inflammation as measured by ear thickness following repetitive exposure to hair dye 1 (HD1, ▼) or hair dye 2 (HD2, ▲) normalized to control (vehicle, ●). The mice were exposed once every second week up to six times. The number of exposure is indicated at the abscissa. Mean ± SD, n = 8 based on two independent experiments. (b) Representative haematoxylin and eosin staining of cross-sectioned ears from mice treated as indicated. *P ≤ 0·05; ***P ≤ 0·001.

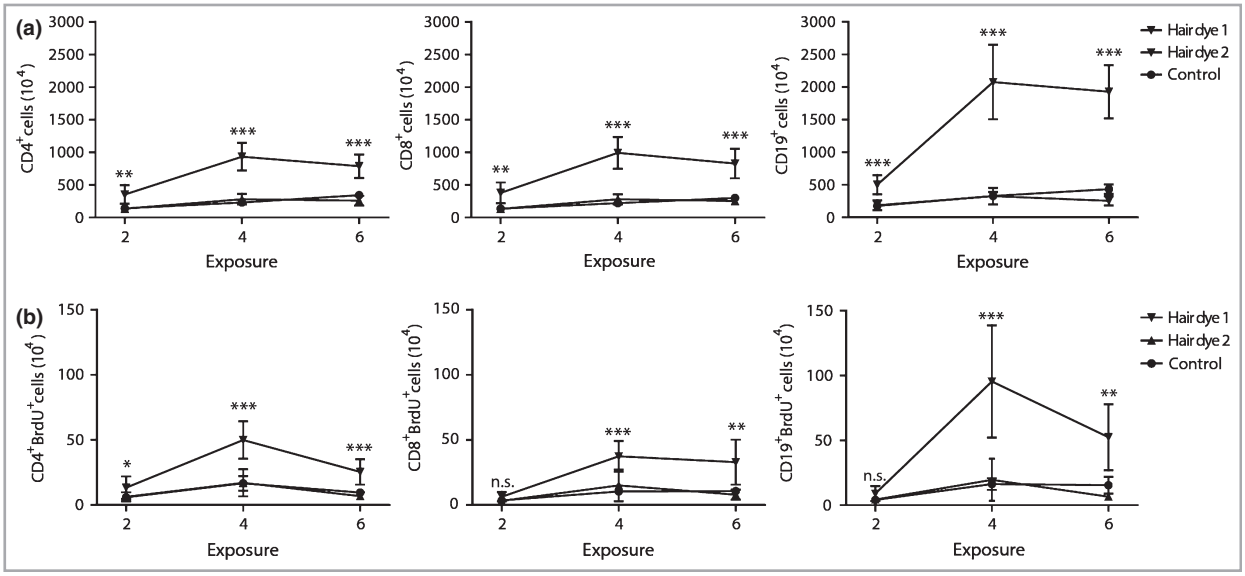


Fig 4. Repetitive toluene-2,5-diamine (PTD) exposure activates adaptive immune responses. Number of $CD4^+$, $CD8^+$ and $CD19^+$ cells (a), and $CD4^+BrdU^+$, $CD8^+BrdU^+$ and $CD19^+BrdU^+$ cells (b) in the draining lymph nodes following repetitive exposure to hair dye 1 (▼) or hair dye 2 (▲) or control (vehicle, ●). The mice were exposed once every second week up to six times. The number of sensitization is indicated at the abscissa. Mean ± SD, n = 8 based on two independent experiments. n.s., not significant (P > 0·05). *P ≤ 0·05; **P ≤ 0·01; ***P ≤ 0·001.

Discussion

In the present study, we investigated immune responses to two PTD-containing permanent hair dyes. By using a modified version of the LLNA, we found that both hair dyes (HD1 and HD2) induced a primary immune response as measured by local skin inflammation and T- and B-cell infiltration and proliferation within the draining lymph nodes. In contrast, we found that only HD1 induced an adaptive immune response when a repetitive model for allergen exposure was used, whereas HD2 induced only a minor skin inflammation. Furthermore, we found that the number of $CD4^+CD25^+FoxP3^+$ Treg cells within the draining lymph nodes correlated with the level of skin inflammation and the number of proliferating T and B cells in the lymph nodes. Finally, the concentration of PTD within the two hair dyes was analysed, showing that HD1 contained 1·60% (w/w) PTD and HD2 contained 0·48% (w/w) PTD within the colour gel.

These results support our previous data on PPD-containing hair dyes, which showed that hair dyes are potent immune activators inducing both pro- and anti-inflammatory responses depending on the exposure regime.[5,6] However, whereas HD1 induced a response very similar to the response we have

 © 2013 British Association of Dermatologists

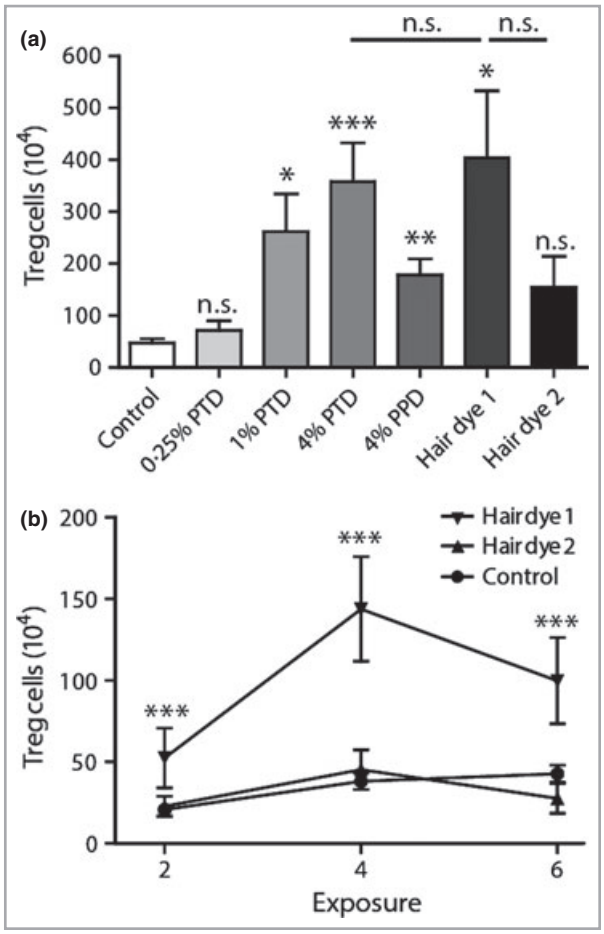


Fig 5. Induction of regulatory T (Treg) cells by PTD-containing hair dyes. Number of Treg cells ($CD4^+CD25^+FoxP3^+$ cells) in the draining lymph nodes of (a) mice treated with vehicle (control), 0·25% PTD, 1% PTD, 4% PTD, 4% PPD, hair dye 1 or hair dye 2 in the modified local lymph node assay setup or (b) mice exposed once every second week up to six times with hair dye 1 (▼), hair dye 2 (▲) or control (vehicle, ●). Mean ± SD, n = 8 based on two independent experiments. PTD, toluene-2,5-diamine; PPD, *p*-phenylenediamine. n.s., not significant ($P > 0·05$). *$P \leq 0·05$; **$P \leq 0·01$; ***$P \leq 0·001$.

reported for PPD-containing hair dyes, HD2 seemed less potent in inducing an immune response.[5,6] Exposure to HD2 induced skin inflammation in both models used in this study but to a lesser degree than HD1 (Figs 1 and 3). Furthermore, in contrast to HD1, repetitive exposure to HD2 did not lead to T- or B-cell proliferation. The different immune responses to HD1 and HD2 could be caused by the lower concentration of PTD in HD2 compared with HD1, as we found that the PTD concentration played a significant role in the generation of immune responses to pure PTD. In addition, HD1 contains five and HD2 contains four other hair dye substances classified as strong sensitizers (Table 1),[9] which most likely play an important role in the immune responses. In support, it has been shown that cross-reactions among hair dye-related chemicals exist.[10–12] According to a study making a quantitative structure–activity relationship analysis, hair dye substances were grouped in clusters according to chemical topology.[9] Both hair dyes used in the present study contain substances belonging to three different clusters [cluster 5: N,N-bis(2-hydroxyethyl)-*p*-phenylenediamine sulfate; *m*-aminophenol; cluster 6: resorcinol; cluster 8: PTD; 2,4-diaminophenoxyethanol-HCl; 1,3-bis-(2,4-diaminophenoxy) propane].[9] Substances belonging to the same cluster are theoretically expected to cross-react.[9] Strikingly, whereas HD1 contains two chemicals belonging to cluster 5 and two to cluster 8, HD2 contains only one chemical belonging to cluster 5 and two to cluster 8 (Table 1). Taken together, this argues for an increased cross-reactivity of the chemicals in HD1 leading to a stronger immune response. Furthermore, the concentration of other hair dye substances and fragrances, which probably also contributes to the immune response, is unknown in HD1 and HD2.

HD1 contains PTD base whereas HD2 contains PTD sulfate, according to the manufacturer. We investigated the sensitization potential of PTD sulfate using the modified version of LLNA and aqua : acetone : olive oil as vehicle but were unable to detect any response, which we ascribe to insolubility of the PTD sulfate salt (data not shown). However, it may be suggested that PTD sulfate is less reactive than PTD base, which could also add to the difference in sensitization potency of the two hair dyes. The solubility of PTD sulfate varies up to 10-fold depending on the vehicle, and thus seems to reflect the sensitizing potency. However, there is no literature on concomitant testing of PTD and its sulfate salt in human patch testing.[13]

We have recently shown that mixing the fragrance ingredients cinnamal with hydroxyisohexyl 3-cyclohexene carboxaldehyde and isoeugenol during sensitization increases the challenge response to cinnamal as measured by ear swelling and T-cell proliferation compared with sensitization with cinnamal alone.[14] This cocktail effect might also play a role in the potent immune response to HD1, as according to the product labelling HD1 contains one more fragrance allergen than HD2 (Table 1).

It has recently been suggested that Treg cells can regulate the response to contact allergens in several ways: (i) inter-individual differences in Treg-cell function may be important for susceptibility to developing contact allergy; (ii) Treg cells are likely to dampen the response to strong allergens to avoid immunopathology; (iii) for weak allergens, Treg cells might limit immunological priming and thereby avoid sensitization; (iv) Treg cells may inhibit the immune response following repeated exposure to the same allergen.[15] In this study we found that exposure to HD1 induced recruitment of Treg cells to the draining lymph nodes in accordance with our previous observations on PPD-containing hair dye.[6] In contrast, HD2 did not induce recruitment of Treg cells to the draining lymph nodes. Vocanson *et al.*[16] have shown that depletion of $CD4^+$ T cells was required for the induction of an allergic response to weak allergens using a mouse model. In future studies it would be interesting to examine how depletion of $CD4^+$ T cells affects the response to HD1 and

© 2013 British Association of Dermatologists

Table 1 List of chemical contents in hair dye 1 and hair dye 2 as declared on the product label. High-performance liquid chromatography analysis of toluene-2,5-diamine (PTD) content in the colour gel of hair dye 1 and hair dye 2, respectively. Allergens classified as strong as reported by Søsted *et al.*[9] are in **bold**. References to Scientific Committee on Consumer Products reports are included in the table.[13,18–21]

	Hair dye 1 L'Oréal – Féria – Préférence	Hair dye 2 Schwarzkopf – Brillance
% PTD (w/w)[a]	1·60	0·48
Colouring component	**Toluene-2,5-diamine**[9,13]	**Toluene-2,5-diamine sulfate**[9,13]
	2,4-Diaminophenoxyethanol-HCl[9]	**1,3-bis-(2,4-Diaminophenoxy)propane**[9,13]
	Hydroxypropyl bis (N-hydroxyethyl-p-phenylenediamine)-HCl[18]	**m-Aminophenol**[20]
	N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate[19]	6-methoxy-2-2-methylamino-3-aminopyridine-HCl
	m-Aminophenol[20]	**Resorcinol**[21]
	Resorcinol[21]	Cetearyl alcohol
	Trideceth-2 carboxamide mea	Ammonium hydroxide
	Propylene glycol	Coconut alcohol
	Hexylene glycol	Sodium laureth sulfate
	PEG-2 oleamine	Isostearic acid
	Oleyl alcohol	Sodium lauryl glucose carboxylate
	Alcohol denat.	Lauryl glucoside
	Polyglyceryl-2-oleyl ether	Potassium hydroxide
	Ammonium hydroxide	Myristic acid
	Oleic acid	Sodium sulfite
	Sodium diethylaminopropyl cocoaspartamide	Ascorbic acid
		Ceteareth-12
	Eugenol	Ceteareth-20
	Ammonium acetate	1,3-Bis-(2,4-diaminophenoxy)propane HCl
	Ammonium thiolactate	Acrylamidopropyltrimonium chloride/ acrylates copolymer
	Pentasodium pentetate	Sodium chloride
	Linalool	Sodium silicate
	Erythorbic acid	Etidronic acid
	α-Isomethyl ionone	Sorbic acid
	Citronellol	Linalool
	Eugenol	Citronellol
	Parfum (undisclosed)	Parfum (undisclosed)
	Water	Water
Developing component	Hydrogen peroxide	Hydrogen peroxide
	Cetearyl alcohol	Acrylates copolymer
	Sodium stannate	Etidronic acid
	Trideceth-2 carboxamide mea	Sodium laureth sulfate
	Pentasodium pentetateoleamine	2,6-dicarboxypyridine
	Phosphoric acid	Disodiumpyrophosphate
	Ceteareth-25	Dimethicone
	Tetrasodium pyrophosphate	Water
	Glycerin	
	Water	

[a]Measured in the colour component.

HD2, as $CD4^+$ T cells seem to play both pro- and anti-inflammatory roles in the response to hair dyes.[6] Repeated exposure to allergens has previously been shown to dampen ear inflammations and cellular proliferation in the draining lymph nodes, which is in good agreement with the present study.[17] Thus, both the allergen dose and the way of exposure seem to be important for the induction of Treg cells. We find it likely that exposure to potent mixtures of cosmetic allergens like HD1 induces a balance between pro- and anti-inflammatory mechanisms, and that this might explain why consumers can use these potent mixtures of allergens without getting severe reactions. However, to understand how the induction of this balance affects the immune system over time needs further investigation.

In conclusion, consumer-available PTD-containing permanent hair dyes can be potent immune activators inducing both pro- and anti-inflammatory responses. The scale of the immune responses depends on the PTD concentration and possibly on the number of other PTD-related and unrelated allergens in the hair dyes as well as on the exposure regime.

© 2013 British Association of Dermatologists

References

1 Aeby P, Wyss C, Beck H *et al.* Characterization of the sensitizing potential of chemicals by *in vitro* analysis of dendritic cell activation and skin penetration. *J Invest Dermatol* 2004; **122**:1154–64.

2 Søsted H, Rastogi SC, Andersen KE *et al.* Hair dye contact allergy: quantitative exposure assessment of selected products and clinical cases. *Contact Dermatitis* 2004; **50**:344–8.

3 Yazar K, Boman A, Liden C. Potent skin sensitizers in oxidative hair dye products on the Swedish market. *Contact Dermatitis* 2009; **61**:269–75.

4 Yazar K, Boman A, Liden C. p-Phenylenediamine and other hair dye sensitizers in Spain. *Contact Dermatitis* 2012; **66**:27–32.

5 Bonefeld CM, Larsen JM, Dabelsteen S *et al.* Consumer available permanent hair dye products cause major allergic immune activation in an animal model. *Br J Dermatol* 2010; **162**:102–7.

6 Rubin IM, Dabelsteen S, Nielsen MM *et al.* Repeated exposure to hair dye induces regulatory T cells in mice. *Br J Dermatol* 2010; **163**:992–8.

7 Søsted H, Hesse U, Menné T *et al.* Contact dermatitis to hair dyes in a Danish adult population: an interview-based study. *Br J Dermatol* 2005; **153**:132–5.

8 Larsen JM, Geisler C, Nielsen MW *et al.* Cellular dynamics in the draining lymph nodes during sensitization and elicitation phases of contact hypersensitivity. *Contact Dermatitis* 2007; **57**:300–8.

9 Søsted H, Basketter DA, Estrada E *et al.* Ranking of hair dye substances according to predicted sensitization potency: quantitative structure-activity relationships. *Contact Dermatitis* 2004; **51**:241–54.

10 Basketter DA, English J. Cross-reactions among hair dye allergens. *Cutan Ocul Toxicol* 2009; **28**:104–6.

11 Laberge L, Pratt M, Fong B *et al.* A 10-year review of p-phenylenediamine allergy and related para-amino compounds at the Ottawa Patch Test Clinic. *Dermatitis* 2011; **22**:332–4.

12 Xie Z, Hayakawa R, Sugiura M *et al.* Experimental study on skin sensitization potencies and cross-reactivities of hair-dye-related chemicals in guinea pigs. *Contact Dermatitis* 2000; **42**:270–5.

13 Scientific Committee on Consumer Products. *Opinion on toluene-2,5-diamine*. SCCP/1084/07. 2007. Available at: http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_108.pdf (last accessed 13 December 2013).

14 Bonefeld CM, Nielsen MM, Rubin IM *et al.* Enhanced sensitization and elicitation responses caused by mixtures of common fragrance allergens. *Contact Dermatitis* 2011; **65**:336–42.

15 Kimber I, Travis MA, Martin SF *et al.* Immunoregulation of skin sensitization and regulatory T cells. *Contact Dermatitis* 2012; **67**:179–83.

16 Vocanson M, Hennino A, Cluzel-Tailhardat M *et al.* CD8+ T cells are effector cells of contact dermatitis to common skin allergens in mice. *J Invest Dermatol* 2006; **126**:815–20.

17 Kimber I, Shepherd CJ, Mitchell JA *et al.* Regulation of lymphocyte proliferation in contact sensitivity: homeostatic mechanisms and a possible explanation of antigenic competition. *Immunology* 1989; **66**:577–82.

18 Scientific Committee on Consumer Products. *Opinion on hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine)HCl*. SCCP/1051/06. 2006. Available at: http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_085.pdf (last accessed 13 December 2013).

19 Scientific Committee on Consumer Products. *Opinion on N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulfate*. SCCP/0983/06. 2006. Available at: http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_065.pdf (last accessed 13 December 2013).

20 Scientific Committee on Consumer Products. *Opinion on m-aminophenol*. SCCP/0978/06. 2006. Available at: http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_088.pdf (last accessed 13 December 2013).

21 Scientific Committee on Consumer Products. *Opinion on Resorcinol*. SCCP/1117/07. 2008. Available at: http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_124.pdf (last accessed 13 December 2013).

© 2013 British Association of Dermatologists

slide trays should be disposed of, marked as hazardous or sterilized after use.[1]

Royal Free Hospital, Royal Free London NHS Foundation Trust, Pond Street, London NW3 2QG, U.K.
B.S. McDonald
E.D. Seaton
E-mail: bryanmcd@doctors.org.uk

References

1 LoPiccolo MC, Balle M, Kouba DJ. Safety precautions in Mohs micrographic surgery for patients with known blood-borne infections: a survey-based study. *Dermatol Surg* 2012; **38**:1059–65.
2 National Audit Office. *A Safer Place to Work – Improving and Management of Health and Safety Risks in NHS Trusts*. London: The Stationery Office, 2003.
3 Tokars JI, Marcus R, Culver DH *et al.* Surveillance of HIV infection and zidovudine use among health care workers after occupational exposure to HIV-infected blood: the CDC Cooperative Needlestick Surveillance Group. *Ann Intern Med* 1993; **118**:913–19.
4 Legislation.gov.uk. Equality Act 2010, chapter 15. Available at: http://www.legislation.gov.uk/ukpga/2010/15 (last accessed 16 October 2014).

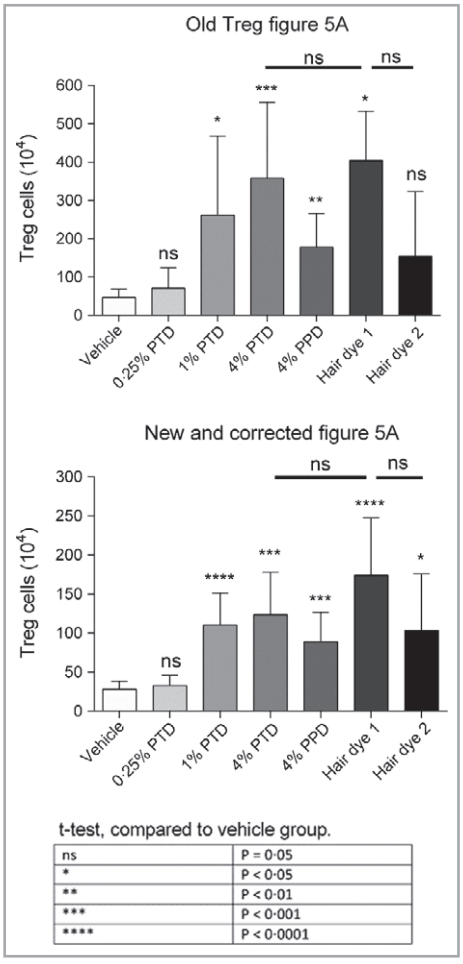
Funding sources: none.

Conflicts of interest: none declared.

Corrigenda

DOI: 10.1111/bjd.13242

In the article by Schmidt et al[1], there was a minor miscalculation in Figure 5a. The data analyses have been redone and a revised figure appears below:



This correction does not conflict with the conclusions of the publication or affect the text in any way. The authors apologize for the error.

© 2014 British Association of Dermatologists

Reference

1 Schmidt JD, Johansen JD, Nielsen MM *et al.* Immune responses to hair dyes containing toluene-2,5-diamine. *Br J Dermatol* 2014; **170**:352–9.

DOI: 10.1111/bjd.13281

In the article by Schnuch *et al.*[1], there were errors in the footnotes of Tables 1 and 3. The revised footnotes appear below:

In Table 1, wrong information on patch test vehicles was given. It should read as "concentration in petrolatum, except MI, MCI/MI, formaldehyde and DMDM hydantoin (in aqua)".

In Table 3, it should read as "MCI/MI (4:1) 100 p.p.m. in aqua; MI 500 p.p.m. in aqua".

The authors apologize for the errors.

Reference

1 Schnuch A, Lessmann H, Geier J, Uter W. Contact allergy to preservatives. Analysis of IVDK data 1996–2009. *Br J Dermatol* 2011; **164**:1316–25.

DOI: 10.1111/bjd.13329

In the article by Kayabasoglu et al[1], there should be an additional author. It is Prof Dr Fatma Hüsniye Dilek from İzmir Katip Çelebi University, Pathology Department.

The authors apologize for the error.

Reference

1 Kayabasoglu G, Kaymaz R, Yilmaz MS, Altundag A. Rhinophyma-like venous malformation of the nose. *Br J Dermatol* 2014; **171**:195–7.

DOI: 10.1111/bjd.13526

In the article by Colmenero and Hoeger,[1] an incorrect medical term was used erroneously. The expression 'intermediate dignity' is an unintended neologism derived from Spanish and German expressions but not used in the English literature. It should be replaced by the term 'intermediate malignancy'.

The authors apologize for the error.

Reference

1 Colmenero I, Hoeger PH. Vascular tumours in infants. Part II: vascular tumours of intermediate dignity and malignant tumours. *Br J Dermatol* 2014; **171**:474–84.

© 2014 British Association of Dermatologists

Repeated exposures to low concentration of *p*-phenylenediamine induce local tolerance

Running head: Tolerance induction by repeated exposure to low concentrations of PPD

J. D. Schmidt[1,2], C. M. Bonefeld[1], C. Agerbeck[1], Elsa Nielsen[3], C. Geisler[1] & J. D. Johansen[2]

[1]Department of Immunology and Microbiology. [2]National Allergy Research Centre, Department of Dermato-Allergology, Copenhagen University Hospital Gentofte, Denmark. [3]Division for Risk Assessment and Nutrition, National Food Institute, Technical University of Denmark, Denmark.

Corresponding author: Jeanne Duus Johansen, National Allergy Research Centre, Department of Dermato-Allergology, Copenhagen University Hospital Gentofte, Denmark. Phone: + 45 38677301,

E-mail: Jeanne.Duus.Johansen@regionh.dk

Acknowledgement of funding: The Danish Environmental Protection Agency, Kgl. Hofbuntmager Aage Bangs Fond.

Abstract

Background. Contact allergy to hair dye is a widely known phenomenon and many consumer-available hair dyes contain strongly sensitizing chemicals like *p*-phenylenediamine (PPD). Although hair dyes are widely used, only 1% of the general population is responsive to PPD in patch testing. Repetitive exposure to hair dyes can induce tolerance in mice. Occupational professions like hairdressers are in high risk of exposure to low doses of hair dye.

Objectives. To investigate whether long-term exposure to low doses of PPD induces tolerance or hypersensitivity in a mouse model.

Methods. C57BL/6 mice were painted on the ear dorsum for 5 consecutive days per week for 4 weeks. Ear thickness was monitored weekly, and lymphocyte infiltration and proliferation (BrdU) were assessed after 1 and 4 weeks. Challenge with 2.000% PPD was done 3 week after stopping repeated PPD-exposure.

Results. We found significant ear swelling after 1 week of exposure to PPD in a dose-dependent manner in parallel with increased lymphocyte infiltration and proliferation in the draining lymph nodes. After 4 weeks of exposure to PPD, the ear swelling had waned whereas an increased lymphocyte infiltration in the draining lymph nodes was still found. The local tolerance in the skin was intact after further three weeks.

Conclusion. Our findings demonstrate that development of local tolerance to PPD takes place after repeated exposures to low doses of PPD.

Introduction

Contact allergies to oxidative hair dyes are well known and can cause severe inflammatory skin reactions with itching and vesicle formation[1,2]. Hair dying is widely used in the general population and cross-sectional studies have estimated that 50.9% of the population have dyed hair at least once in a life-time, with the median age for first-time-of-use at 16 years[3]. Although hair dyes can contain strong contact sensitizers as *p*-phenylenediamine (PPD), toluene-2,5-diamine (PTD), resorcinol and *m*-aminophenol[4–6], only about 1% of the general European population respond to *p*-phenylenediamine (PPD) in diagnostic patch testing, although ~6% have reported adverse skin reactions[3].

Recent data from our group have demonstrated that two consumer-available hair dyes containing PPD and PTD, respectively, induce strong immune responses in a mouse model for sensitization[7,8]. Conversely, weekly exposures to the hair dyes, induced a regulatory T (Treg) cell subpopulation ($CD4^+CD25^+FoxP3^+$) in the dLN accompanied by a plateau in ear thickening, suggesting the development of tolerance[8,9]. In accordance with these findings, peripheral blood derived monocyte cells (PBMC) from PPD tolerant individuals display a pre-dominant Th2 cytokine profile when re-stimulated in vitro with PPD[10].

Whereas the frequency of hair dye use for consumers is approximately 1.5-2 times per month[1], occupational hairdressers are in high risk of PPD exposure during a typical working week, as they have been estimated to perform full-head hair coloring 9.3 times per week[11].

Repeated skin exposure to strong contact allergens e.g. trinitrochlorobenzene, in low dose is known to induce low zone tolerance (LZT)[12]. In mice, LZT has been shown to involve IL-10 producing $CD4^+$ and $CD8^+$ Treg cells, and depletion of activated T cells using anti-CD25 abrogate LZT, which also holds true for ablation of $FoxP3^+$ cells[13,14]. Following induction of LZT, a distinct $CCR7^+CD4^+CD25^{high}FoxP3^+$ Treg cell phenotype is up-regulated, whereas no change is observed when analyzing for the classical $CD4^+CD25^{high}FoxP3^+$ Treg cell phenotype[13].

In the present study we investigate the long-term effect of topical PPD exposure ranging over sub-sensitization and sensitization concentration levels, in a murine model for contact hypersensitivity.

Materials and methods

Animals

Female C57BL/6 mice were obtained from Taconic (Glostrup, Denmark) and fed *ad libitum* with standard chow. Mice were 7-8 weeks old upon experiment start and housed 4 animals in individually ventilated cages in a pathogen specific free facility. Experiments were conducted according to license no.: 2012-2934-00663 from the national animal inspectorate.

Chemicals and in vivo application

Dilutions of p-phenylenediamine (PPD; CAS no.: 106-50-3) were prepared fresh from frozen (-20°C) aliquots, in olive oil : acetone (OOA, ratio 1:4). PPD dilutions were applied topically on the dorsum of the ears, 25 µl on each ear. To assess cell proliferation in the draining lymph nodes, 5-Bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich, USA) was administered through the drinking water (80µg/ml) 48 hours prior to experiment termination. Ear thickness measurements were obtained using an engineer's micrometer (mitutoyo, Illinois, USA).

Flow cytometric analyses

After experiment termination, the lymph nodes were ablated from the mice and single-cell suspensions were prepared by disintegration through a nylon mesh (cell strainer, 70 µm). Following, single-cell suspensions were washed in medium RPMI-1640 containing fetal bovine serum, L-glutamine, streptavidin and penicillin. Cell suspensions were adjusted to 1x10^7 cells per ml and washed in PBS containing 2% serum and 0.1% azide. Cells were seeded in a 96-well plate in 1x10^6 cells per well and stained for proliferating cells according to the manufacturers protocol

(APC BrdU Flow Kit, BD Pharmingen™). Cells were analyzed on a Fortessa cytometer instrument (BD) at the integrated FACS facility. Data analysis was performed using FlowJo.

Software

Data was visualized with Graphpad prism (version 6, La Jolla, USA), which was also used for statistical analyses.

Statistics

Statistical testing of inter-column differences was calculated using a two-way student's t-test, CI=95%, assuming equal variance among experimental groups.

Results

Repeated long term exposure to low amounts of PPD suppress skin inflammation and T cell proliferation

To explore the response to repeated exposure of low dose *p*-phenylenediamine (PPD), groups of mice were topically painted on the ear dorsum with PPD concentrations (0.001%, 0.010%, 0.100% (w/v)) or vehicle for control for 5 days per week. Ear thickness measurements were recorded at the end of each week. Following 1 week of exposure, the 0.100% PPD group resulted in the most pronounced ear swelling, with the lower dosages producing gradually decreasing responses, indicating a dose-response relationship (Figure 1A). In accordance, lymphocyte infiltration and proliferation in the draining lymph nodes (dLN) reflected the ear thickness response by a dose-response relationship with 0.100% treatment inducing the strongest response (Figure 1A, B, and C). Interestingly, when observing ear-swelling measurements over a 4-week period, treatment with 0.100% PPD elicited a maximum response after 2 weeks followed by a complete remission after 4weeks of PPD exposure (Figure 1A). Following 4 weeks of exposure, lymphocyte infiltration in the dLNs remained highly significant (Figure 1D), although lymphocyte proliferation was not statistical significant (Figure 1E). Altogether, these data suggest that tolerance is induced following 4 weeks of repeated PPD exposure.

Reduced CHS response is intact following challenge with PPD.

Next, we investigated whether PPD tolerance persisted following a period of allergen relief. Mice were exposed to 0.01% PPD or vehicle for 4 weeks and left untreated for 3 weeks. After 3 weeks, a high-dose challenge of 2 % PPD or vehicle was administered by epicutaneous painting on the ears and the immune responses were analyzed after 24 hours (Figure 2). No change in ear thickness was found after PPD challenge (Figure 2A). However, PPD-tolerized mice responded significantly to

PPD challenge as measured by lymphocyte infiltration and proliferation in the dLNs (Figure 2B and 2C).

Discussion

In the present study we demonstrate that long-term (4-weeks) exposure to low doses of PPD induces an immune suppressive mechanism as measured by change in ear thickness and lymphocyte proliferation in the dLNs (Figure 1). The local immune suppressive mechanisms in the skin was long-lasting as challenged with a high dose of PPD 3 weeks after the last PPD exposure did not induce local inflammation as measured by ear thickness, whereas it induced significant lymphocyte infiltration and proliferation in the dLNs (Figure 2).

PPD is known as a strong contact sensitizer that can sensitize in concentrations as low as 0.09-0.20% (w/v)[6]. Interestingly, we find that repeated exposure for 4 weeks to PPD in the concentration range 0.001-0.100% induces tolerance with suppressed ear swelling and lymphocyte proliferation in the dLN. In a study by De Jong *et al.* (2007), mice were exposed to two formaldehyde releasers in sub-sensitization doses that induced sensitization in a prolonged exposure regimen[15]. The differences might be found in the different exposure sequence, as De Jong *et al.* used an initial three consecutive days: 0-1-2 followed by one weekly application for 7 weeks and a three day treatment 56-57-58 and analysis at day 61[15].

PPD- and PTD-containing hair dyes may contain up to 2%, 4% (w/v) for PPD and PTD in the on-head concentration, respectively, which is 10-15 fold above their minimal sensitization concentrations[16,17]. Data from our group have demonstrated that repeated exposures to PPD- and PTD-containing hair dyes may induce tolerance, possibly through the up-regulation of $CD4^+CD25^+FoxP3^+$ Treg cells[8,9]. The persistent or repeated exposure to high amounts of PPD and PTD may resemble a chronic virus infection. From studies in virus infection models, $CD8^+$ T cells have been shown to attain a state of antigen unresponsiveness known as exhaustion[18]. By studies of

lymphocytic choriomeningitis virus (LMCV) infections in mice, it was found that $CD8^+$ T cells can become un-responsive after LMCV infection lasting for 2-4 weeks[19]. This time frame fits with our study (Figure 1 and Figure 2) in which suppression of ear thickness can be seen after 2 weeks with full effect after 4 weeks for all tested PPD concentrations. Interestingly, in a study by Zinzelmeyer *et al.* they found that LCMV infection in mice causes arrestment of motility in $CD8^+$ T cell residing in the marginal/red pulp area of the spleen[20]. In accordance, the stromal cells of the spleen red zone, showed significant up-regulation of programmed death cell protein 1 ligand (PD-1L)[20]. $CD8^+$ T cell motility could be restored along with regained capability of IFNγ production by *i.v.* administration of antibodies specific for PD-1 or PD-1L[20].

The induction of LZT has been shown to occur independently of the route of exposure when comparing *i.v.*, oral gavage and epicutaneous administration of trinitrochlorobenzene (TNCB) as reported by Seidel-Guyenot *et al.*[21]. Furthermore, they showed that adoptive transfer of $CD8^+$ T cells from tolerized mice were required for generating allergen specific tolerance in sensitized animals[21]. Whether $CD8^+$ T cells is responsible for the peripheral LZT after prolonged PPD exposure could be revealed in future experiments by adoptive transfer of $CD8^+$ T cells from the LNs of tolerized mice into naïve mice with subsequent monitoring of the clinical reaction following PPD challenge. Likewise, it would be interesting to determine the presence of exhausted $CD8^+PD\text{-}1^+$ T cells and to determine the effect of antibodies toward PD-1 in LZT induction.

In the recent years, attention has called upon skin resident memory T cells, which are characterized as antigen-specific sentinels that can respond with fast production of cytokines following e.g. virus re-infection[22]. Interestingly, Seneschal *et al.* identified skin resident epidermal $CD3^+FoxP3^+$ Treg cells[23] that proliferate *in vitro* and *in vivo* when interacting with epidermal Langerhans cells (LCs). Upon introducing of *candida albicans* to *in vitro* co-culture with LCs, an effector memory T cell subset ($CD3^+CD25^+CD127^-$) vigorously proliferated[23]. Thus, local immune suppression can

probably occur in the skin via specific Treg cells, and this could provide an explanation for the local tolerance we observed (Figure 2).

Altogether, our results suggest that long-term exposure to the clinical relevant contact allergen PPD can induce tolerance to PPD. Although the results indicate local immunological tolerance as the underlying mechanism, setting safe-to-use concentrations for professional workers like hairdressers should be assessed with care, as low concentration contact allergens may greatly modulate the immune system although no local effects can be seen.

Figure legends

Figure 1

Repeated long-term exposure to low amounts of PPD suppresses the CHS response.

Mice (n=16) were treated with four concentrations of PPD, 0%, 0.001%, 0.01% and 0.1%, for 5 days per week and (A) ear thickness was measured after each week for 4 weeks. Lymphocyte infiltration in dLNs was assessed after (B) 1 week and (D) 4 weeks. Proliferation of dLN cells measured by $BrdU^+$ cells by flow cytometry, quantified after (C) 1 week and after (E) 4 weeks. Error bars show SEM. ****P<0.0001, ***P<0.001, **P<0.005, *P<0.05, not significant (ns).

Figure 2

Lymphocyte proliferation regains in the CHS response following relief-period of PPD load.

Mice (n=8) were treated with 0.01% or 0% PPD for 4 weeks. Following a 3-week recovery period without PPD exposure, mice received a challenge application 2% or 0% PPD 24 hour prior to analysis of (A) ear thickness and (B) lymphocyte infiltration and (C) lymphocyte proliferation in the dLNs. Error bars show SEM. **P<0.005, *P<0.05, not significant (ns).

Figure 1 Schmidt et al 2016

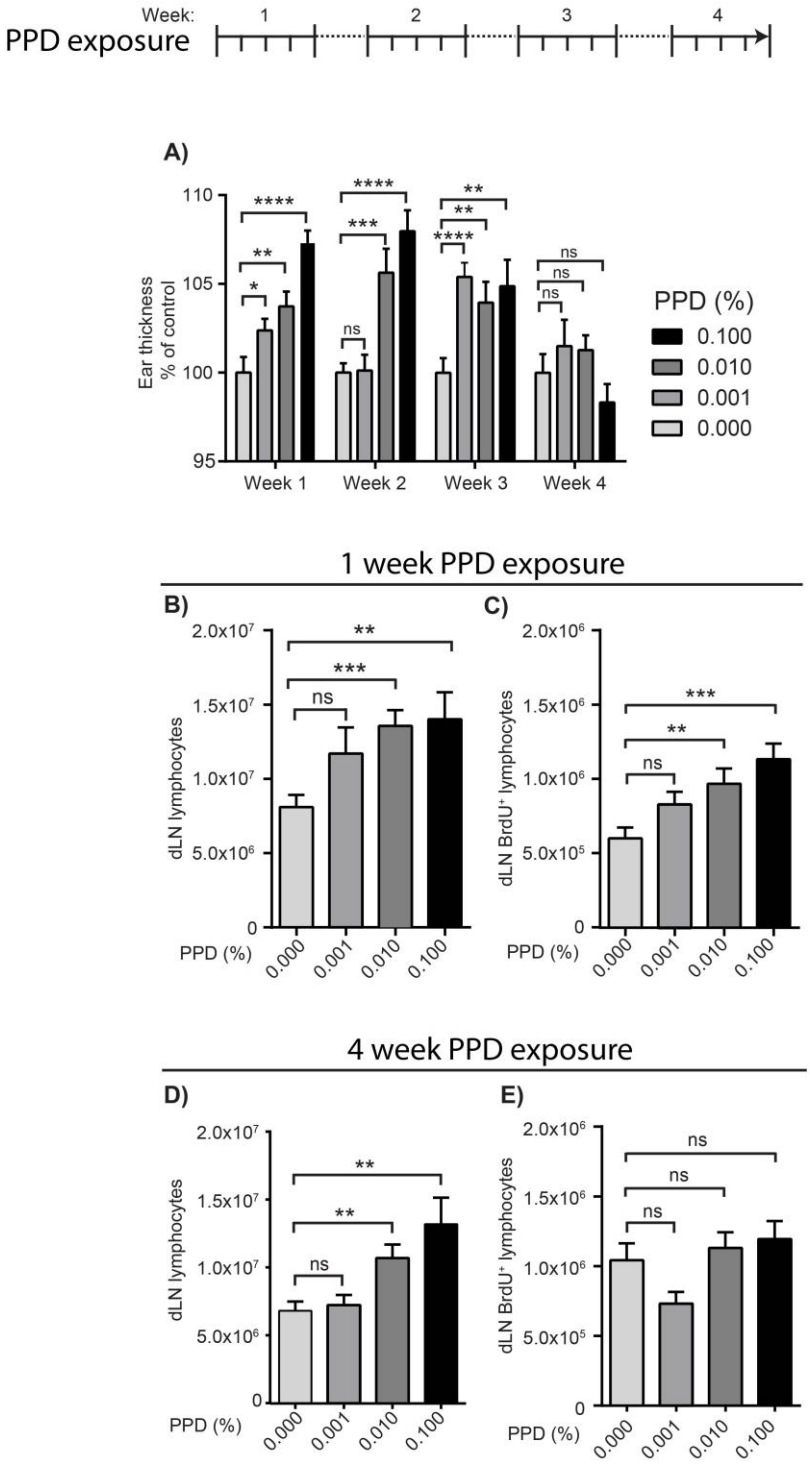
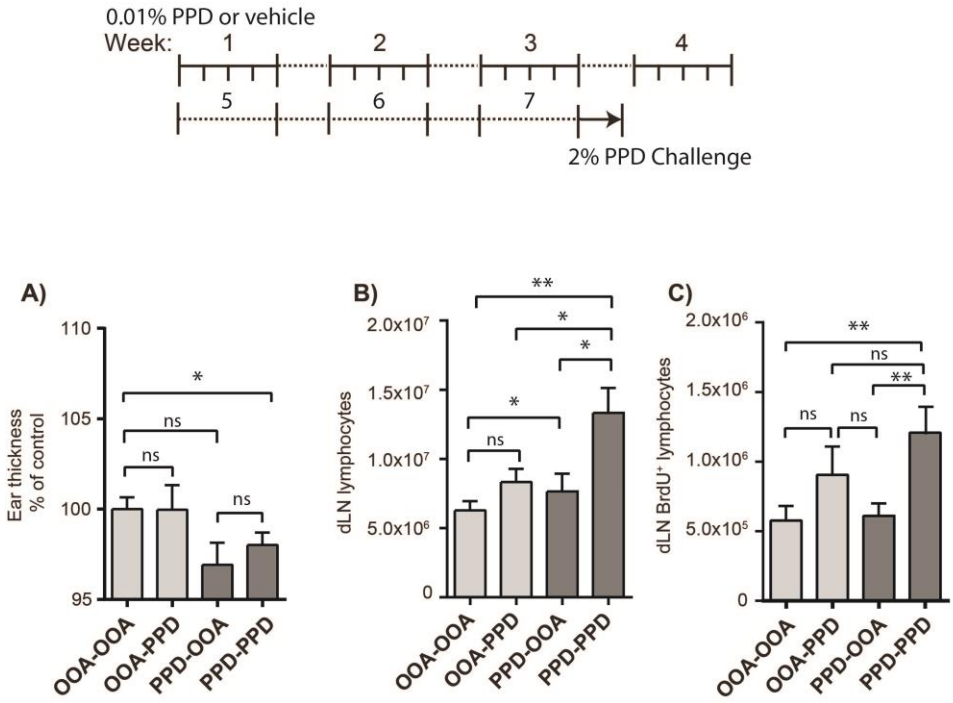


Figure 2 Schmidt et al 2016



References

1. Søsted H, Agner T, Andersen KE, Menné T. 55 Cases of Allergic Reactions To Hair Dye: a Descriptive, Consumer Complaint-Based Study. Contact Dermatitis 2002;47:299–303.
2. Søsted H, Rustemeyer T, Gonçalo M, Bruze M, Goossens A, Giménez-Arnau AM, et al. Contact allergy to common ingredients in hair dyes. Contact Dermatitis 2013;69:32–9.
3. Diepgen TL, Naldi L, Bruze M, Cazzaniga S, Schuttelaar M-L, Elsner P, et al. Prevalence of Contact Allergy to p-Phenylenediamine in the European General Population. J Invest Dermatol [Internet] 2016;136:409–15. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022202X15000652
4. Yazar K, Boman A, Lidén C. Potent skin sensitizers in oxidative hair dye products on the Swedish market. Contact Dermatitis [Internet] 2009 [cited 2012 Nov 29];61:269–75. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19878241
5. Hamann D, Yazar K, Hamann CR, Thyssen JP, Lid??n C. P-Phenylenediamine and other allergens in hair dye products in the United States: A consumer exposure study. Contact Dermatitis 2014;70:213–8.
6. Warbrick E V., Dearman RJ, Lea LJ, Basketter DA, Kimber I. Local lymph node assay responses to paraphenylenediamine: intra- and inter-laboratory evaluations. J Appl Toxicol [Internet] 1999;19:255–60. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10439339
7. Bonefeld CM, Larsen JM, Dabelsteen S, Geisler C, White IR, Menné T, et al. Consumer available permanent hair dye products cause major allergic immune activation in an animal model. Br J Dermatol [Internet] 2010 [cited 2012 Oct 26];162:102–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19785606
8. Schmidt JD, Johansen JD, Nielsen MM, Zimersson E, Svedman C, Bruze M, et al. Immune responses to hair dyes containing toluene-2,5-diamine. Br J Dermatol [Internet] 2014 [cited 2014 Feb 20];170:352–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24124869
9. Rubin IMC, Dabelsteen S, Nielsen MM, White IR, Johansen JD, Geisler C, et al. Repeated exposure to hair dye induces regulatory T cells in mice. Br J Dermatol [Internet] 2010 [cited 2012 Oct 22];163:992–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20716223
10. Coulter EM, Jenkinson C, Farrell J, Lavergne SN, Pease C, White A, et al. Measurement of CD4+ and CD8+ T-lymphocyte cytokine secretion and gene expression changes in p-phenylenediamine allergic patients and tolerant individuals. J Invest Dermatol [Internet] 2010 [cited 2012 Oct 22];130:161–74. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19657353
11. Lysdal SH, Johansen JD, Flyvholm MA, S??sted H. A quantification of occupational skin exposures and the use of protective gloves among hairdressers in Denmark. Contact Dermatitis 2012;66:323–34.
12. Steinbrink K, Sorg C, Macher E. Low zone tolerance to contact allergens in mice: a functional role for CD8+ T helper type 2 cells. J Exp Med [Internet] 1996;183:759–68. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8642280
13. Luckey U, Schmidt T, Pfender N, Romer M, Lorenz N, Martin SF, et al. Crosstalk of regulatory T cells and tolerogenic dendritic cells prevents contact allergy in subjects with low zone tolerance. J Allergy Clin Immunol [Internet] 2012;130:781–97.e11. Available from: http://dx.doi.org/10.1016/j.jaci.2012.06.022

14. Maurer M, Seidel-Guyenot W, Metz M, Knop J, Steinbrink K. Critical role of IL-10 in the induction of low zone tolerance to contact allergens. J Clin Invest 2003;112:432–9.

15. De Jong WH, De Klerk A, Beek M Ter, Veenman C, Van Loveren H. Effect of Prolonged Repeated Exposure to Formaldehyde Donors with Doses Below the EC3 Value on Draining Lymph Node Responses. J Immunotoxicol 2007;4:239–46.

16. Committee S, Sccp CP. Opinion of the SCCP on toluene-2,5-diamine. 2007;

17. SCCS (Scientific Committee on Consumer Safety). Opinion on p-phenylenediamine. 2012;

18. Wherry EJ. T cell exhaustion. Nat Immunol [Internet] 2011;131:492–9. Available from: http://www.nature.com/doifinder/10.1038/ni.2035

19. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, et al. Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med [Internet] 1998;188:2205–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9858507

20. Zinselmeyer BH, Heydari S, Sacristán C, Nayak D, Cammer M, Herz J, et al. PD-1 promotes immune exhaustion by inducing antiviral T cell motility paralysis. J Exp Med [Internet] 2013;210:757–74. Available from: http://jem.rupress.org/content/210/4/757

21. Seidel-Guyenot W, Perschon S, Dechant N, Alt R, Knop J, Steinbrink K. Low zone tolerance induced by systemic application of allergens inhibits T C1-mediated skin inflammation. J Allergy Clin Immunol 2006;117:1170–7.

22. Carbone FR. Tissue-Resident Memory T Cells and Fixed Immune Surveillance in Nonlymphoid Organs. J Immunol [Internet] 2015;195:17–22. Available from: http://www.jimmunol.org/content/195/1/17.full.pdf

23. Seneschal J, Clark RA, Gehad A, Baecher-Allan CM, Kupper TS. Human epidermal Langerhans cells maintain immune homeostasis in skin by activating skin resident regulatory T cells. Immunity [Internet] 2012;36:873–84. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3716276&tool=pmcentrez&rendertype=abstract

9 Concluding remarks and perspectives

9.1 Study I

Based on the findings in study I, a mechanistic model for T_{RM} cells in local skin memory is proposed. In addition to study I, the model below includes a summary of published research that fits within the cellular and molecular interplay of local skin memory.

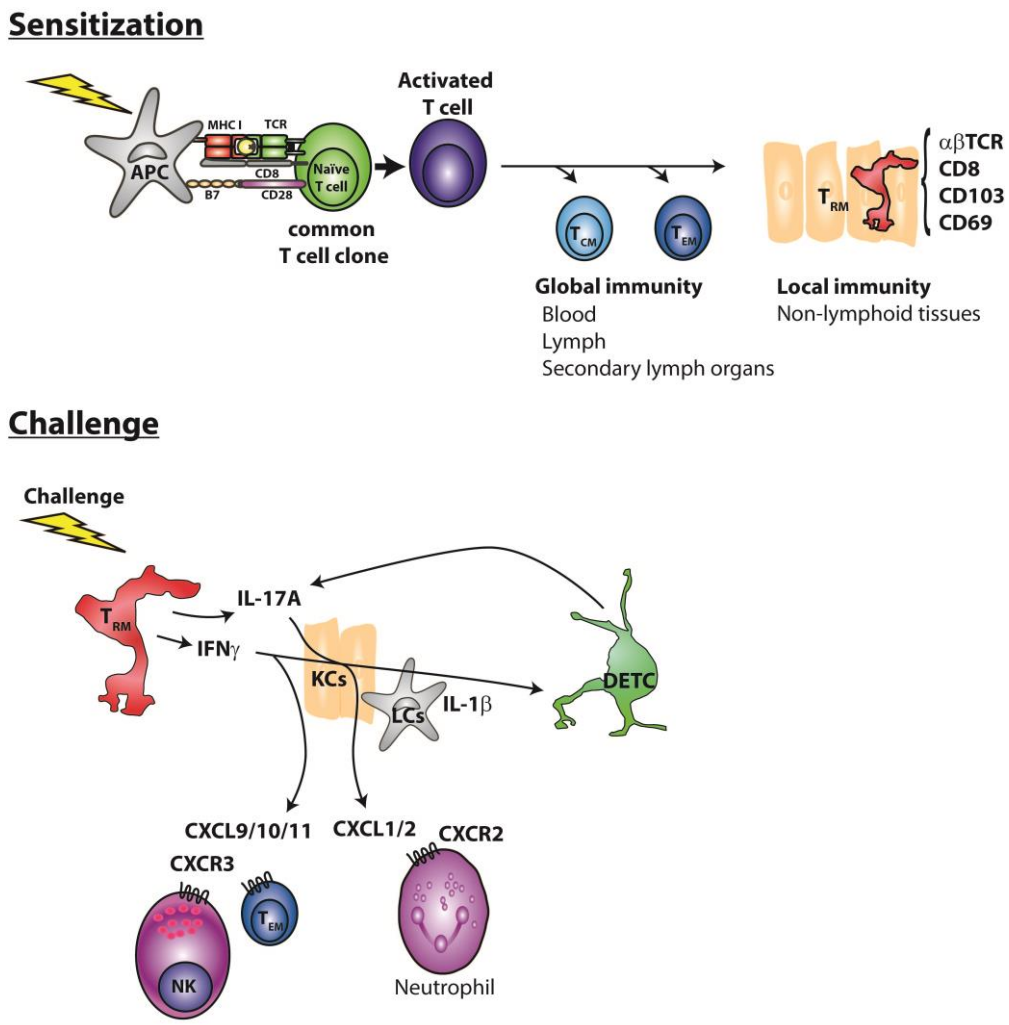


Figure 4: Proposed model for T_{RM} cells in the CHS response.
After sensitization, naïve T cells differentiate into central memory (TCM), effector memory CCR7-CD62L- (TEM) and tissue resident memory T cells (TRM). CCR7+CD62L+ TCM and CCR7-CD62L- TEM cells provide global immune protection in the blood, lymph and secondary lymphoid organs, whereas CCR7-CD62L- TEM cells and CD4/8+CD69+CD103+ TRM cells are found in non-lymphoid tissues[132,184]. Upon hapten challenge, CD8+ TRM cells can respond with rapid production of IFN-γ and IL-17A and induce IL-1β in LCs and KCs, which engage DETCs into a positive feed-back loop via IL-17A production (study I). IFN-γ and IL-17A induce mediates attraction of CXCR3+ effector leukocytes and monocytes by induction of CXCL9/10/11 and CXCL1/2[105,107,185,186].

The involvement of Tc1 and Tc17 cells in CHS has been described by Kish *et al*[103]. Here, both IL-17 and IFN-γ were shown important in the elicitation response as Ab mediated depletion and using knockout mice displayed suppressed responses measured by CXCL1/2 and ear thickness. Moreover they showed that CD8 T cells are essential to CHS, and that $CD8^+$ T cells were the main source of IL-17 and IFN-γ as compared to $CD4^+$ T cells[103]. In contrast to study I, Kish *et al* employ a CHS model in which the challenge response is induced in distal skin from the sensitization sites after a memory period of 5 days and thus differs from our setup (study I) by two parameters: (I) timing of sensitization to challenge (II) and skin re-exposure *vs.* distal exposure. Data from our group has demonstrated that T cell numbers in the dLNs peak 5 days after sensitization and decline to steady state levels after 3 weeks[96]. Based on these findings, Kish *et al* examines the T cell responses by challenging on top of the primary adaptive immune response, and leave out the true memory response[103]. Our findings demonstrate that the global memory (abdomen sensitization/ear challenge) is not reactive within the timeframe of 24 hours in the elicitation response. Future experiments may elucidate the kinetics of T_{EM} and T_{CM} cell recruitment to challenge site at later time points.

In terms viral infection, T_{RM} cells act as antigen specific sentinels that negotiate fast antiviral responses. Besides, from antigen specificity, several studies have demonstrated that T cells are highly influenced by DAMP signals[187]. Thus, Treg $CD4^+$ T cells have enhanced expression of TLR2, 4, 5, 7/8 and 10[187]. Interestingly, TLR2 ligation has been shown to suppress Foxp3 expression in Treg cells and promote Th17 differentiation of naïve T cells[188]. Moreover, TLR4 stimulation by the High-mobility group box 1 (HMGB1) can promote Th1 over Treg cells by suppression of FoxP3, which may act through IL-6[189]. Indeed also, $CD8^+$ T cells express TLR2, 3 and 9[190]. Stimulation of TLR2 induce proliferation, and IFN-γ production in $CD8^+$ T cells, which furthermore promote cytolytic activity without the presence of TCR stimulation[188,191,192]. These studies suggest that T_{RM} cells not only may act as antigen specific sentinels, but also play a role in the innate immune responses.

In study I, OXA sensitized mice reacted with reduced, although significant ear swelling responses to DNFB challenge, thus indicating unspecific responses (study I, Figure 1). In part, OXA 0.5% (w/v) can cause unspecific inflammation owing to its irritancy, as ear swelling is induced in naïve mice[12]. However, the IL-1β levels within the OXA-DNFB reaction were no different from the DNFB-DNFB group when assessed by statistical testing (study I, data not

shown), thus indicating that sensitized skin have T_{RM} cells have a dual role in mediating both unspecific and antigen specific responses.

Following 21 day after sensitization we find a low, but significant up-regulation of *IL-1β* and *IFN-γ* in DNFB sensitized ear skin epidermis, compared to the non-sensitized control group (study I, Figure 4ab), suggesting that a low grade inflammation is induced after remission of the clinical symptoms. Together with enhanced, low-grade inflammation due to elevated cytokine transcription may cause a lowered threshold for elicitation and sensitization to new haptens in previously skin areas.

Patients with psoriasis can experience re-occurring skin lesions and flare-up reactions in previously affected skin areas e.g. by bacterial infections in the tonsils[193–195]. A recent study on psoriasis by Suárez-Fariñas *et al*, showed that previously affected skin areas retained transcriptional levels of TCR-β, IL-22, IL-17 and IFN-γ three months after treatment with etanercept[194]. In support of these findings, another group found that psoriatic lesional skin contained high numbers of epidermal Th22 $CD4^+$ and Tc17 $CD8^+$ T cells, whereas over the course of treatment, $CD103^+CD8^+$ T cells remained in the epidermis while $CD4^+$ T cells retracted from the epidermis while enriched the dermis[137]. The epidermal $CD103^+CD8^+$ T cells did respond *ex vivo* to re-stimulation with strong IL-17A production compared to healthy controls. Interestingly, $CD8^+$ T_{RM} cells were found to have Granzyme A & B and Perforin compared to their counterpart in lesional skin. found Dermal $CD4^+$ T cells responded no different from the healthy controls by IL-22 production[137]. Taken together, a possible mechanism for flare-up reactions may be explained by serum-borne PAMPs from bacterial infection that encounter a skin area which is enriched with T_{RM} cells, prone to respond with effector cytokines. In line with these findings, our murine model for local memory, show predominance of epidermal $CD8^+$ T cells over $CD4^+$ T cells in mice (study I, Figure 3).

In summary, two phenotypes of $CD8^+CD69^+CD103^+$ T_{RM} cells producing IL-17A or IFN-γ are found to convey early activation of CHS response in mice by induction of IL-1β. Our data suggest that this mechanism is likely translates to humans, as patients with high IL-1β and IL-17A to nickel re-exposure. Indeed $CD3^+$ cell were found in greater numbers compared to $CD8^+$ T cells when observing IHC from punch biopsies. Gaide et al also identified accumulation of $CD3^+$ cell T cells in the local skin following sensitization to DPCP[132]. This suggests that $CD4^+$ T_{RM} cells may also be involved in the nickel response. These findings align well with previous findings that implicate $CD4^+$ T cells in contact allergy to nickel[14,15]. Clearly,

mice and humans have differences in immune systems but also the nature of the hapten could explain the differences between $CD4^+$ and $CD8^+$ T cell responses.

9.2 Study II

In study II, overall HD1 induced by far the strongest lymphocyte proliferations measured by $CD4^+BrdU^+$ $CD8^+BrdU^+$ and $CD19^+BrdU^+$ cells, owing to its high content of PTD and inter-related substances as identified on the ingredients list. Although, HD2 did not induce strong lymphocyte proliferation, a significant increase of B cells was found in the dLN. Gerberick *et al* showed that increased B cell infiltration in dLNs by 1.25 fold could discriminate irritants from contact sensitizing chemicals, thus suggesting HD2 as a strong sensitizer[159]. The sum of potent contact sensitizers within complex mixtures e.g. perfumes and hair dyes, may add up and should in theory result in strong sensitization. As demonstrated in a recent publication from our group, the cocktail effect of sensitization to mixtures of contact allergens, can result in enhanced challenge responses to a single allergen[33]. Whether hair dyes induce cocktail to PTD and PPD remain to be examined in future experiments.

9.3 Study III

We found that repeated exposures to low concentrations of PPD induced significant ear swelling, which decreased gradually over the course 4 weeks of treatment (Study III, Figure 1). Interestingly, the peak response seemed to correlate with the PPD dose, thus the 0.001% PPD treated group peaked at week 3, whereas the 0.1% PPD treated group peaked after 2 weeks. From these results, it is intriguing that a 100-fold difference in PPD concentration resulted in similar ear swelling to the high PPD group. This suggests that PPD may accumulate in the skin, and when a threshold is reached, tolerance is induced. Moreover, the tolerance induced in mice treated with 0.01% PPD, persisted after three weeks of resting, as shown by absence of ear swelling response in the challenge to 2% PPD (study III, Figure 2). In contrast, significant dLN lymphocyte infiltration and proliferation accompanied the challenge, although the proliferation was not different from the vehicle sensitized - PPD challenged group the dLN infiltration was. In future experiments, the time-course development of the challenge response could reveal whether ear swelling is induced beyond the challenge time frame used in study III.

In conclusion to study II & III, sensitization to the PTD-containing hair dyes, and pure PTD and PPD displayed strong sensitization potency in a dose-responsive manner. When enrolled in a repetitive exposure regimen, a tolerance-like was induced in study II, and evidently for study III. The differences may rely on concentration and exposure frequency. Taking concentration and sensitization potencies into perspective, it is perplexing that the tissue necrosis can be induced in mouse ears to sensitization[181]. Although oxidative hair dyes are widely used in the general population, our findings may explain why the low clinical prevalence is of hair dye allergy.

It is clear that skin exposure to chemicals can modulate the skin beyond the acute inflammation that follows, as changed cytokine and chemokine expression can be detected after the inflammation has resolved, as demonstrated by local KC production of CCL27 as long as 3 weeks after sensitization, which retain dermal $CCR10^+$ T cells[118]. Moreover, repeated skin exposures to oxazolone in mice, has shown to develop a skin condition that resembles atopic dermatitis, by enhanced trans epidermal water loss, increased skin pH and up regulation of Th2 cells in the dermis[196]. In line with this, a murine disease model resembling psoriasis, can be induced by topical treatments of imiquimod, a TLR7/8 agonist[197]. Based on this, it seems plausible that our model for long-term PPD exposure may modulate expression of specific cytokines that may promote tolerance. Of notice, skin-resident regulatory T cells have been investigated. Thus, human epidermal $CD3^+FoxP3^+$ Treg cells from human skin, proliferated *in vitro* and *in vivo* upon by interacting with epidermal Langerhans cells (LCs)[60]. Upon introduction of *Candida albicans* to *in vitro* co-culture with LCs, an effector memory T cell subset ($CD3^+CD25^+CD127^-$) vigorously proliferated[60]. Thus demonstrating that LCs and skin resident Treg cells do interact. Whether inducible Treg cells can be generated to hapten treatment, remain to be elucidated.

The animal behavior of mice involves grooming of littermates, which makes it difficult to ensure that topical hapten treatment is only administered through skin absorption. Although in study II, hair dye was rinsed off, residual hair dye may deposit on the bedding etc. However, in terms of induction of LZT tolerance, a study has shown that systemic administration of experimental haptens induces tolerance, be it via oral gavage, i.v. injections or topical skin exposure[198]. Based on this, it seems likely that a systemic tolerance is induced to hair dye treatment in study II and III. To clarify whether the tolerance mechanism is local or systemic, challenging on a site distal from the sensitization skin could settle this argument. In addition,

adoptive transfer of lymphocytes from dLNs and/or spleen to naïve mice followed by a PPD challenge would indicate whether systemic tolerance is established.

10 References

1. Sandilands, A., Sutherland, C., Irvine, A. D. & McLean, W. H. I. Filaggrin in the frontline: role in skin barrier function and disease. *J. Cell Sci.* **122,** 1285–94 (2009).

2. Kubo, A., Nagao, K., Yokouchi, M., Sasaki, H. & Amagai, M. External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. *J. Exp. Med.* **206,** 2937–2946 (2009).

3. Heath, W. R. & Carbone, F. R. The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells. *Nat. Immunol.* **14,** 978–985 (2013).

4. Di Meglio, P., Perera, G. K. & Nestle, F. O. The Multitasking Organ: Recent Insights into Skin Immune Function. *Immunity* **35,** 857–869 (2011).

5. Clark, R. a *et al.* The vast majority of CLA+ T cells are resident in normal skin. *J. Immunol.* **176,** 4431–4439 (2006).

6. Nestle, F. O., Di Meglio, P., Qin, J.-Z. & Nickoloff, B. J. Skin immune sentinels in health and disease. *Nat. Rev. Immunol.* **9,** 679–91 (2009).

7. Thyssen, J. P., Linneberg, A., Menné, T. & Johansen, J. D. The epidemiology of contact allergy in the general population--prevalence and main findings. *Contact Dermatitis* **57,** 287–299 (2007).

8. Diepgen, T. L. *et al.* Prevalence of contact allergy in the general population in different European regions. *Br. J. Dermatol.* n/a–n/a (2015). doi:10.1111/bjd.14167

9. Peiser, M. *et al.* Allergic contact dermatitis: epidemiology, molecular mechanisms, in vitro methods and regulatory aspects. *Cell. Mol. Life Sci.* **69,** 763–781 (2012).

10. Sundhedsstyrelsen. Forebyggelse af kontakteksemer. at <https://sundhedsstyrelsen.dk/publ/publ1998/Kontakteksem/inde0004.htm>

11. *Contact Dermatitis*. (Springer Berlin Heidelberg, 2011). doi:10.1007/978-3-642-03827-3

12. Grabbe, S. *et al.* Dissection of antigenic and irritative effects of epicutaneously applied haptens in mice. Evidence that not the antigenic component but nonspecific proinflammatory effects of haptens determine the concentration-dependent elicitation of allergic contact de. *J. Clin. Invest.* **98,** 1158–64 (1996).

13. McLelland, J. & Shuster, S. Contact dermatitis with negative patch tests: the additive effect of allergens in combination. *Br. J. Dermatol.* **122,** 623–30 (1990).

14. Dyring-Andersen, B. *et al.* CD4(+) T cells producing interleukin (IL)-17, IL-22 and interferon-gamma are major effector T cells in nickel allergy. *Contact Dermatitis* **68,** 339–347 (2013).

15. Larsen, J. M., Bonefeld, C. M., Poulsen, S. S., Geisler, C. & Skov, L. IL-23 and T(H)17-mediated inflammation in human allergic contact dermatitis. *J. Allergy Clin. Immunol.* **123,** 486–92 (2009).

16. Dhingra, N. *et al.* Molecular profiling of contact dermatitis skin identifies allergen-dependent differences in immune response. *J. Allergy Clin. Immunol.* **134,** 362–372 (2014).

17. Bos, J. D. & Meinardi, M. M. The 500 Dalton rule for the skin penetration of chemical compounds and drugs. *Exp. Dermatol.* **9,** 165–169 (2000).

18. Landsteiner, K. and & Jacobs, J. STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS. II. *J. Exp. Med.* 147–170 (1936).

19. Niklasson, I. B. *et al.* Cinnamyl alcohol oxidizes rapidly upon air exposure. *Contact Dermatitis* **68,** 129–38 (2013).

20. Kalergis, A. M. *et al.* Modulation of fatty acid oxidation alters contact hypersensitivity to urushiols: role of aliphatic chain beta-oxidation in processing and activation of urushiols. *J Invest Dermatol* **108,** 57–61 (1997).

21. Elahi, E. N. *et al.* Protein Binding and Metabolism Influence the Relative Skin Sensitization Potential of Cinnamic Compounds. *Chem. Res. Toxicol.* **17,** 301–310 (2004).

22. Raghavan, B., Martin, S. F., Esser, P. R., Goebeler, M. & Schmidt, M. Metal allergens nickel and cobalt facilitate TLR4 homodimerization independently of MD2. *EMBO Rep.* **13,** 1109–15 (2012).

23. Schmidt, M. *et al.* Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nat. Immunol.* **11,** 814–9 (2010).

24. Ross-Hansen, K. *et al.* Filaggrin Is a Predominant Member of the Denaturation-Resistant Nickel-Binding Proteome of Human Epidermis. *J. Invest. Dermatol.* **134,** 1164–1166 (2014).

25. Kligman, A. M. The SLS provocative patch test in allergic contact sensitization. *J. Invest. Dermatol.* **46,** 573–83 (1966).

26. Agner, T. *et al.* Combined effects of irritants and allergens. Synergistic effects of nickel and sodium lauryl sulfate in nickel- sensitized individuals. *Contact Dermatitis* **47,** 21–6 (2002).

27. Johansen, J. D., Skov, L., Volund, a., Andersen, K. & Menné, T. Allergens in combination have a synergistic effect on the elicitation response: A study of fragrance-sensitized individuals. *Br. J. Dermatol.* **139,** 264–270 (1998).

28. Oh, D.-R. *et al.* PMA induces vaccine adjuvant activity by the modulation of TLR

signaling pathway. *Mediators Inflamm.* **2014,** 406514 (2014).

29. Bonneville, M. *et al.* Skin contact irritation conditions the development and severity of allergic contact dermatitis. *J. Invest. Dermatol.* **127,** 1430–1435 (2007).

30. Watanabe, H. *et al.* Danger signaling through the inflammasome acts as a master switch between tolerance and sensitization. *J. Immunol.* **180,** 5826–32 (2008).

31. SCCP/00847/04. SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS SCCP Opinion on Atranol and Chloroatranol present in natural extracts (e.g. oak moss and tree moss extract). *Eur. Comm.* (2004). at <http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_006.pdf>

32. Guideline, O., The, F. O. R. & Of, T. *Test No. 429: Skin Sensitisation*. *OECD GUIDELINE FOR THE TESTING OF CHEMICALS Skin* (OECD Publishing, 2010). doi:10.1787/9789264071100-en

33. Bonefeld, C. M. *et al.* Enhanced sensitization and elicitation responses caused by mixtures of common fragrance allergens. *Contact Dermatitis* **65,** 336–42 (2011).

34. Bonefeld, C. M. *et al.* An immune response study of oakmoss absolute and its constituents atranol and chloroatranol. *Contact Dermatitis* **70,** 282–90 (2014).

35. Zarember, K. A. & Godowski, P. J. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J. Immunol.* **168,** 554–61 (2002).

36. Takeda, K. & Akira, S. Toll-like receptors in innate immunity. *Int. Immunol.* **17,** 1–14 (2005).

37. Akira, S. & Takeda, K. Toll-like receptor signalling. *Nat. Rev. Immunol.* **4,** 499–511 (2004).

38. Toshchakov, V. *et al.* TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. *Nat. Immunol.* **3,** 392–8 (2002).

39. Martin, S. F. *et al.* Toll-like receptor and IL-12 signaling control susceptibility to contact hypersensitivity. *J. Exp. Med.* **205,** 2151–2162 (2008).

40. Scheibner, K. a. *et al.* Hyaluronan Fragments Act as an Endogenous Danger Signal by Engaging TLR2. *J. Immunol.* **177,** 1272–1281 (2006).

41. Yu, L., Wang, L. & Chen, S. Endogenous toll-like receptor ligands and their biological significance. *J. Cell. Mol. Med.* **14,** 2592–603 (2010).

42. Esser, P. R. *et al.* Contact sensitizers induce skin inflammation via ROS production and hyaluronic acid degradation. *PLoS One* **7,** e41340 (2012).

43. Vennegaard, M. T. *et al.* Epicutaneous exposure to nickel induces nickel allergy in

mice via a MyD88-dependent and interleukin-1-dependent pathway. *Contact Dermatitis* **71,** 224–32 (2014).

44. Mortazavi, S. M. J., Mortazavi, G. & Paknahad, M. Dental metal-induced innate reactivity in keratinocytes. *Toxicol. Vitr.* **33,** 180–181 (2016).

45. Nakamura, N., Tamagawa-Mineoka, R., Ueta, M., Kinoshita, S. & Katoh, N. Toll-like receptor 3 increases allergic and irritant contact dermatitis. *J. Invest. Dermatol.* **135,** 411–7 (2015).

46. Enk, a H. & Katz, S. I. Early molecular events in the induction phase of contact sensitivity. *Proc. Natl. Acad. Sci. U. S. A.* **89,** 1398–402 (1992).

47. Enk, A. H., Angeloni, V. L., Udey, M. C. & Katz, S. I. An essential role for Langerhans cell-derived IL-1 beta in the initiation of primary immune responses in skin. *J. Immunol.* **150,** 3698–3704 (1993).

48. Watanabe, H. *et al.* Activation of the IL-1beta-processing inflammasome is involved in contact hypersensitivity. *J. Invest. Dermatol.* **127,** 1956–63 (2007).

49. Nielsen, M. M. *et al.* IL-1β-dependent activation of dendritic epidermal T cells in contact hypersensitivity. *J. Immunol.* **192,** 2975–83 (2014).

50. Antonopoulos, C. *et al.* IL-18 is a key proximal mediator of contact hypersensitivity and allergen-induced Langerhans cell migration in murine epidermis. *J. Leukoc. Biol.* **83,** 361–7 (2008).

51. Riemann, H. *et al.* IL-12 breaks dinitrothiocyanobenzene (DNTB)-mediated tolerance and converts the tolerogen DNTB into an immunogen. *J. Immunol.* **175,** 5866–74 (2005).

52. Gross, O., Thomas, C. J., Guarda, G. & Tschopp, J. The inflammasome: an integrated view. *Immunol. Rev.* **243,** 136–51 (2011).

53. Weber, F. C. *et al.* Lack of the purinergic receptor P2X(7) results in resistance to contact hypersensitivity. *J. Exp. Med.* **207,** 2609–19 (2010).

54. Kaplan, D. H. In vivo function of Langerhans cells and dermal dendritic cells. *Trends Immunol.* **31,** 446–51 (2010).

55. Bedoui, S. *et al.* Cross-presentation of viral and self antigens by skin-derived CD103+ dendritic cells. *Nat. Immunol.* **10,** 488–495 (2009).

56. Gomez de Agüero, M. *et al.* Langerhans cells protect from allergic contact dermatitis in mice by tolerizing CD8 (+) T cells and activating Foxp3 (+) regulatory T cells. *J. Clin. Invest.* **122,** 1700–1711 (2012).

57. Hoath, S. B. & Leahy, D. G. The organization of human epidermis: functional epidermal units and phi proportionality. *J. Invest. Dermatol.* **121,** 1440–6 (2003).

58. Valladeau, J. *et al.* Langerin, a Novel C-Type Lectin Specific to Langerhans Cells, Is an Endocytic Receptor that Induces the Formation of Birbeck Granules. *Immunity* **12,** 71–81 (2000).

59. Birbeck, M. S., Breathnach, A. S. & Everall, J. D. An Electron Microscope Study of Basal Melanocytes and High-Level Clear Cells (Langerhans Cells) in Vitiligo**From the Chester Beatty Research Institute, Royal Cancer Hospital, London, S.W. 3, and the Departments of Anatomy, and Dermatology, St. Mary's Hos. *J. Invest. Dermatol.* **37,** 51–64 (1961).

60. Seneschal, J., Clark, R. A., Gehad, A., Baecher-Allan, C. M. & Kupper, T. S. Human epidermal Langerhans cells maintain immune homeostasis in skin by activating skin resident regulatory T cells. *Immunity* **36,** 873–84 (2012).

61. Paglia, P., Chiodoni, C., Rodolfo, M. & Colombo, M. P. Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. *J. Exp. Med.* **183,** 317–22 (1996).

62. Igyártó, B. Z. & Kaplan, D. H. Antigen presentation by Langerhans cells. *Curr. Opin. Immunol.* **25,** 115–9 (2013).

63. Stoitzner, P. *et al.* Langerhans cells cross-present antigen derived from skin. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 7783–8 (2006).

64. Klechevsky, E. *et al.* Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells. *Immunity* **29,** 497–510 (2008).

65. Blauvelt, A., Katz, S. I. & Udey, M. C. Human Langerhans cells express E-cadherin. *J. Invest. Dermatol.* **104,** 293–6 (1995).

66. Saeki, H., Moore, A. M., Brown, M. J. & Hwang, S. T. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J. Immunol.* **162,** 2472–5 (1999).

67. Worbs, T., Mempel, T. R., Bölter, J., von Andrian, U. H. & Förster, R. CCR7 ligands stimulate the intranodal motility of T lymphocytes in vivo. *J. Exp. Med.* **204,** 489–95 (2007).

68. Villadangos, J. A. *et al.* MHC class II expression is regulated in dendritic cells independently of invariant chain degradation. *Immunity* **14,** 739–749 (2001).

69. Grabbe, S., Steinbrink, K., Steinert, M., Luger, T. A. & Schwarz, T. Removal of the majority of epidermal Langerhans cells by topical or systemic steroid application enhances the effector phase of murine contact hypersensitivity. *J. Immunol.* **155,** 4207–4217 (1995).

70. Bennett, C. L. *et al.* Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. *J. Cell Biol.* **169,** 569–576 (2005).

71. Bursch, L. S. *et al.* Identification of a novel population of Langerin+ dendritic cells. *J. Exp. Med.* **204,** 3147–56 (2007).

72. Wang, L. *et al.* Langerin Expressing Cells Promote Skin Immune Responses under Defined Conditions. *J. Immunol.* **180,** 4722–4727 (2008).

73. Edelson, B. T. *et al.* Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. *J. Exp. Med.* **207,** 823–36 (2010).

74. Kaplan, D. H. In vivo Function of Langerhans Cells and Dermal DC. *Trends Immunol.* **31,** 446–451 (2010).

75. Igyártó, B. Z. *et al.* Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses. *Immunity* **35,** 260–72 (2011).

76. Nielsen, M. M. *et al.* NKG2D-Dependent Activation of Dendritic Epidermal T Cells in Contact Hypersensitivity. (2015). doi:10.1038/jid.2015.23.

77. Martin, S., Ortmann, B., Pflugfelder, U., Birsner, U. & Weltzien, H. U. Role of hapten-anchoring peptides in defining hapten-epitopes for MHC-restricted cytotoxic T cells. Cross-reactive TNP-determinants on different peptides. *J. Immunol.* **149,** 2569–75 (1992).

78. Vollmer, J., Weltzien, H. U. & Moulon, C. TCR reactivity in human nickel allergy indicates contacts with complementarity-determining region 3 but excludes superantigen-like recognition. *J. Immunol.* **163,** 2723–31 (1999).

79. Vinay, D. S., Wolisi, G. O., Yu, K. Y., Choi, B. K. & Kwon, B. S. Immunity in the absence of CD28 and CD137 (4-1BB) molecules. *Immunol. Cell Biol.* **81,** 176–184 (2003).

80. Lanier, L. L. *et al.* CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. *J. Immunol.* **154,** 97–105 (1995).

81. Reiser, H. & Schneeberger, E. E. Expression and function of B7-1 and B7-2 in hapten-induced contact sensitivity. *Eur. J. Immunol.* **26,** 880–885 (1996).

82. Gorbachev, A. V, Heeger, P. S. & Fairchild, R. L. CD4+ and CD8+ T cell priming for contact hypersensitivity occurs independently of CD40-CD154 interactions. *J. Immunol.* **166,** 2323–32 (2001).

83. Chen, A. I. *et al.* Ox40-ligand has a critical costimulatory role in dendritic cell:T cell interactions. *Immunity* **11,** 689–98 (1999).

84. Chen, L. & Flies, D. B. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat. Rev. Immunol.* **13,** 227–42 (2013).

85. Schwarz, A. *et al.* Evidence for functional relevance of CTLA-4 in ultraviolet-

radiation-induced tolerance. *J.Immunol.* **165,** 1824–1831 (2000).

86. Tsushima, F. *et al.* Preferential contribution of B7-H1 to programmed death-1-mediated regulation of hapten-specific allergic inflammatory responses. *Eur. J. Immunol.* **33,** 2773–2782 (2003).

87. Leitner, J. *et al.* B7-H3 is a potent inhibitor of human T-cell activation: No evidence for B7-H3 and TREML2 interaction. *Eur. J. Immunol.* **39,** 1754–64 (2009).

88. Sica, G. L. *et al.* B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity* **18,** 849–61 (2003).

89. Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136,** 2348–57 (1986).

90. Dong, C. Genetic controls of Th17 cell differentiation and plasticity. *Exp. Mol. Med.* **43,** 1–6 (2011).

91. Dong, C. TH17 cells in development: an updated view of their molecular identity and genetic programming. *Nat. Rev. Immunol.* **8,** 337–348 (2008).

92. Szabo, S. J. *et al.* A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100,** 655–669 (2000).

93. Chakir, H., Wang, H., Lefebvre, D. E., Webb, J. & Scott, F. W. T-bet/GATA-3 ratio as a measure of the Th1/Th2 cytokine profile in mixed cell populations: Predominant role of GATA-3. *J. Immunol. Methods* **278,** 157–169 (2003).

94. Li, L., Sad, S., Kagi, D. & Mosmann, T. R. CD8Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo but induce similar inflammatory reactions. *J. Immunol.* **158,** 4152–4161 (1997).

95. Early, A. N. Biphasic Pattern of Ear- or Footpad-swelling Reactions after Challenge of Actively Immunized. **157,** 1604–1617 (1983).

96. Larsen, J. M. *et al.* Cellular dynamics in the draining lymph nodes during sensitization and elicitation phases of contact hypersensitivity. *Contact Dermatitis* **57,** 300–8 (2007).

97. Coulter, E. M. *et al.* Measurement of CD4+ and CD8+ T-lymphocyte cytokine secretion and gene expression changes in p-phenylenediamine allergic patients and tolerant individuals. *J. Invest. Dermatol.* **130,** 161–74 (2010).

98. Tang, A., Amagai, M., Granger, L. G., Stanley, J. R. & Udey, M. C. Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature* **361,** 82–5 (1993).

99. Hirata, T. *et al.* P-Selectin glycoprotein ligand 1 (PSGL-1) is a physiological ligand for E-selectin in mediating T helper 1 lymphocyte migration. *J. Exp. Med.* **192,** 1669–76

(2000).

100. Dilulio, N. A. *et al.* Groα-mediated recruitment of neutrophils is required for elicitation of contact hypersensitivity. *Eur. J. Immunol.* **29,** 3485–3495 (1999).

101. Weber, F. C. *et al.* Neutrophils are required for both the sensitization and elicitation phase of contact hypersensitivity. *J. Exp. Med.* **212,** 15–22 (2015).

102. Kish, D. D., Gorbachev, A. V, Parameswaran, N., Gupta, N. & Fairchild, R. L. Neutrophil Expression of Fas Ligand and Perforin Directs Effector CD8 T Cell Infiltration into Antigen-Challenged Skin. *J. Immunol.* **189,** 2191–2202 (2012).

103. Kish, D. D., Li, X. & Fairchild, R. L. CD8 T cells producing IL-17 and IFN-gamma initiate the innate immune response required for responses to antigen skin challenge. *J. Immunol.* **182,** 5949–59 (2009).

104. Engeman, T., Gorbachev, A. V, Kish, D. D. & Fairchild, R. L. The intensity of neutrophil infiltration controls the number of antigen-primed CD8 T cells recruited into cutaneous antigen challenge sites. *J. Leukoc. Biol.* **76,** 941–949 (2004).

105. Laan, M. *et al.* Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J. Immunol.* **162,** 2347–52 (1999).

106. Chen, J. P. *et al.* Reduced 2,4-dinitro-1-fluorobenzene-induced contact hypersensitivity response in IL-15 receptor α-deficient mice correlates with diminished CCL5/RANTES and CXCL10/IP-10 expression. *Eur. J. Immunol.* **35,** 690–698 (2005).

107. Rouzaire, P. *et al.* Natural killer cells and T cells induce different types of skin reactions during recall responses to haptens. *Eur. J. Immunol.* **42,** 80–8 (2012).

108. Xu, H., DiIulio, N. A. & Fairchild, R. L. T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon gamma-producing (Tc1) effector CD8+ T cells and interleukin (Il) 4/Il-10-producing (Th2) negative regulator. *J. Exp. Med.* **183,** 1001–12 (1996).

109. Kehren, J. *et al.* Cytotoxicity is mandatory for CD8(+) T cell-mediated contact hypersensitivity. *J. Exp. Med.* **189,** 779–786 (1999).

110. Akiba, H. *et al.* Skin inflammation during contact hypersensitivity is mediated by early recruitment of CD8+ T cytotoxic 1 cells inducing keratinocyte apoptosis. *J. Immunol.* **168,** 3079–87 (2002).

111. Vocanson, M. *et al.* CD8+ T cells are effector cells of contact dermatitis to common skin allergens in mice. *J. Invest. Dermatol.* **126,** 815–820 (2006).

112. Gocinski, B. L. & Tigelaar, R. E. Roles of CD4+ and CD8+ T cells in murine contact sensitivity revealed by in vivo monoclonal antibody depletion. *J. Immunol.* **144,** 4121–8 (1990).

113. Bour, H. *et al.* Major histocompatibility complex class I-restricted CD8+ T cells and

class II-restricted CD4+ T cells, respectively, mediate and regulate contact sensitivity to dinitrofluorobenzene. *Eur. J. Immunol.* **25,** 3006–3010 (1995).

114. Skog, E. Spontaneous flare-up reactions induced by different amounts of 1,3-dinitro-4-chlorobenzene. Frequency of reactions, time of appearance and histology. *Acta Derm. Venereol.* **46,** 386–95 (1966).

115. Grolnick, M. Studies in Contact-Dermatitis : IV . The Spontaneous Flare-up of Negative Test-Sites in Experimental Sensitization in man. *J. immunlogy* **41,** 127–142 (1941).

116. Möller, H., Ohlsson, K., Linder, C., Björkner, B. & Bruze, M. The flare-up reactions after systemic provocation in contact allergy to nickel and gold. *Contact Dermatitis* **40,** 200–4 (1999).

117. Scheper, R. J. *et al.* Induction of immunological memory in the skin. Role of local T cell retention. *Clin. Exp. Immunol.* **51,** 141–8 (1983).

118. Moed, H. *et al.* Increased CCL27–CCR10 expression in allergic contact dermatitis: implications for local skin memory. *J. Pathol.* **204,** 39–46 (2004).

119. Natsuaki, Y. *et al.* Perivascular leukocyte clusters are essential for efficient activation of effector T cells in the skin. *Nat Immunol* **15,** 1064–1069 (2014).

120. Sallusto, F., Lenig, D., Förster, R., Lipp, M. & Lanzavecchia, a. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401,** 708–12 (1999).

121. Masopust, D. Preferential Localization of Effector Memory Cells in Nonlymphoid Tissue. *Science (80-.).* **291,** 2413–2417 (2001).

122. Gebhardt, T. *et al.* Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat. Immunol.* **10,** 524–530 (2009).

123. Gebhardt, T. *et al.* Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. *Nature* **477,** 216–219 (2011).

124. Masopust, D. *et al.* Dynamic T cell migration program provides resident memory within intestinal epithelium. *J. Exp. Med.* **207,** 553–564 (2010).

125. Jiang, X. *et al.* Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature* **483,** 227–31 (2012).

126. Iijima, N. & Iwasaki, A. A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells. *Science* **93,** (2014).

127. Zhu, J. *et al.* Immune surveillance by CD8αα+ skin-resident T cells in human herpes virus infection. *Nature* **497,** 494–7 (2013).

128. Mackay, L. K. *et al.* Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 7037–42 (2012).

129. Mackay, L. K. *et al.* Cutting edge: CD69 interference with sphingosine-1-phosphate receptor function regulates peripheral T cell retention. *J. Immunol.* **194,** 2059–63 (2015).

130. Zaid, A. *et al.* Persistence of skin-resident memory T cells within an epidermal niche. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 5307–12 (2014).

131. Mackay, L. K. *et al.* T-box Transcription Factors Combine with the Cytokines TGF-β and IL-15 to Control Tissue-Resident Memory T Cell Fate. *Immunity* **43,** 1101–11 (2015).

132. Gaide, O. *et al.* Common clonal origin of central and resident memory T cells following skin immunization. *Nat. Med.* **21,** 647–653 (2015).

133. Watanabe, R. *et al.* Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. *Sci. Transl. Med.* **7,** 279ra39 (2015).

134. Ariotti, S. Skin-resident memory CD8+ T cells trigger a state of tissue-wide pathogen alert. *Science (80-.).* **346,** 101–105 (2014).

135. Schenkel, J. M., Fraser, K. a, Vezys, V. & Masopust, D. Sensing and alarm function of resident memory $CD8^+$ T cells. *Nat. Immunol.* **14,** 509–13 (2013).

136. Slütter, B. & Harty, J. T. Instructing the instructor: tissue-resident T cells activate innate immunity. *Cell Host Microbe* **16,** 421–3 (2014).

137. Cheuk, S. *et al.* Epidermal Th22 and Tc17 Cells Form a Localized Disease Memory in Clinically Healed Psoriasis. *J. Immunol.* **192,** 3111–3120 (2014).

138. Toda, A. & Piccirillo, C. A. Development and function of naturally occurring CD4+CD25+ regulatory T cells. *J. Leukoc. Biol.* **80,** 458–70 (2006).

139. Vignali, D. a a, Collison, L. W. & Workman, C. J. How regulatory T cells work. *Nat. Rev. Immunol.* **8,** 523–532 (2008).

140. Agostini, M. *et al.* The glucocorticoid-induced tumor necrosis factor receptor-related gene modulates the response to Candida albicans infection. *Infect. Immun.* **73,** 7502–7508 (2005).

141. Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y. & Sakaguchi, S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat. Immunol.* **3,** 135–42 (2002).

142. Lehtimäki, S. & Lahesmaa, R. Regulatory T cells control immune responses through their non-redundant tissue specific features. *Front. Immunol.* **4,** 1–10 (2013).

143. Deaglio, S. *et al.* Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* **204,** 1257–65 (2007).

144. Ring, S., Oliver, S. J., Cronstein, B. N., Enk, A. H. & Mahnke, K. CD4+CD25+ regulatory T cells suppress contact hypersensitivity reactions through a CD39, adenosine-dependent mechanism. *J. Allergy Clin. Immunol.* **123,** 1287–96.e2 (2009).

145. Van Hoogstraten, I. M. *et al.* Reduced frequency of nickel allergy upon oral nickel contact at an early age. *Clin. Exp. Immunol.* **85,** 441–5 (1991).

146. Todd, D. J. & Burrows, D. Nickel allergy in relationship to previous oral and cutaneous nickel contact. *Ulster Med. J.* **58,** 168–171 (1989).

147. Vreeburg, K. J. J., de Groot, K., von Blomberg, M. & Scheper, R. J. Induction of immunological tolerance by oral administration of nickel and chromium. *J. Dent. Res.* **63,** 124–128 (1984).

148. Artik, S., Haarhuis, K., Wu, X., Begerow, J. & Gleichmann, E. Tolerance to nickel: oral nickel administration induces a high frequency of anergic T cells with persistent suppressor activity. *J. Immunol.* **167,** 6794–803 (2001).

149. Wu, X. *et al.* Dose dependence of oral tolerance to nickel. *Int. Immunol.* **19,** 965–975 (2007).

150. Dubois, B. *et al.* Sequential Role of Plasmacytoid Dendritic Cells and Regulatory T Cells in Oral Tolerance. *Gastroenterology* **137,** 1019–1028 (2009).

151. Steinbrink, K., Sorg, C. & Macher, E. Low zone tolerance to contact allergens in mice: a functional role for CD8+ T helper type 2 cells. *J. Exp. Med.* **183,** 759–68 (1996).

152. Maurer, M., Seidel-Guyenot, W., Metz, M., Knop, J. & Steinbrink, K. Critical role of IL-10 in the induction of low zone tolerance to contact allergens. *J. Clin. Invest.* **112,** 432–439 (2003).

153. Luckey, U. *et al.* Crosstalk of regulatory T cells and tolerogenic dendritic cells prevents contact allergy in subjects with low zone tolerance. *J. Allergy Clin. Immunol.* **130,** 781–797.e11 (2012).

154. Luckey, U. *et al.* T cell killing by tolerogenic dendritic cells protects mice from allergy. *J. Clin. Invest.* **121,** 3860–71 (2011).

155. Magnusson, B. & Kligman, A. M. The Identification of Contact Allergens by Animal Assay. the Guinea Pig Maximization Test**From the Department of Dermatology, University of Gothenburg, Sahlgrenska Sjukhuset, Gothenburg, Sweden and the Department of Dermatology, University of Pennsylvani. *J. Invest. Dermatol.* **52,** 268–276 (1969).

156. Frank Gerberick, G., Ryan, C. A., Dearman, R. J. & Kimber, I. Local lymph node assay (LLNA) for detection of sensitization capacity of chemicals. *Methods* **41,** 54–60

(2007).

157. Haneke, K. E., Tice, R. R., Carson, B. L., Margolin, B. H. & Stokes, W. S. ICCVAM evaluation of the murine local lymph node assay. Data analyses completed by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods. *Regul. Toxicol. Pharmacol.* **34,** 274–86 (2001).

158. Loveless, S. E. *et al.* Further evaluation of the local lymph node assay in the final phase of an international collaborative trial. *Toxicology* **108,** 141–152 (1996).

159. Gerberick, G. F. *et al.* Use of a B cell marker (B220) to discriminate between allergens and irritants in the local lymph node assay. *Toxicol. Sci.* **68,** 420–428 (2002).

160. Betts, C. J. *et al.* B220 analysis with the local lymph node assay: proposal for a more flexible prediction model. *J. Appl. Toxicol.* **27,** 506–10

161. Ahmed, S. S. *et al.* An in vitro human skin test for assessing sensitization potential. *J. Appl. Toxicol.* 669–684 (2015). doi:10.1002/jat.3197

162. Gerberick, G. F. *et al.* Development of a peptide reactivity assay for screening contact allergens. *Toxicol. Sci.* **81,** 332–343 (2004).

163. Patlewicz, G. *et al.* An evaluation of selected global (Q)SARs/expert systems for the prediction of skin sensitisation potential. *SAR QSAR Environ. Res.* **18,** 515–41

164. Søsted, H., Agner, T., Andersen, K. E. & Menné, T. 55 Cases of Allergic Reactions To Hair Dye: a Descriptive, Consumer Complaint-Based Study. *Contact Dermatitis* **47,** 299–303 (2002).

165. Søsted, H. *et al.* Contact allergy to common ingredients in hair dyes. *Contact Dermatitis* **69,** 32–39 (2013).

166. Yazar, K., Boman, A. & Lidén, C. Potent skin sensitizers in oxidative hair dye products on the Swedish market. *Contact Dermatitis* **61,** 269–75 (2009).

167. Søsted, H., Basketter, D. a, Estrada, E., Johansen, J. D. & Patlewicz, G. Y. Ranking of hair dye substances according to predicted sensitization potency: quantitative structure-activity relationships. *Contact Dermatitis* **51,** 241–54 (2004).

168. Søsted, H., Rastogi, S. C., Andersen, K. E., Johansen, J. D. & Menné, T. Hair dye contact allergy: quantitative exposure assessment of selected products and clinical cases. *Contact Dermatitis* **50,** 344–8 (2004).

169. McFadden, J. P. *et al.* Allergy to hair dye. *BMJ* **334,** 220–220 (2007).

170. Warbrick, E. V., Dearman, R. J., Lea, L. J., Basketter, D. A. & Kimber, I. Local lymph node assay responses to paraphenylenediamine: intra- and inter-laboratory evaluations. *J. Appl. Toxicol.* **19,** 255–60 (1999).

171. Jenkinson, C. *et al.* Characterization of p-phenylenediamine-albumin binding sites and

T-cell responses to hapten-modified protein. *J. Invest. Dermatol.* **130,** 732–42 (2010).

172. Galbiati, V., Papale, A., Galli, C. L., Marinovich, M. & Corsini, E. Role of ROS and HMGB1 in contact allergen-induced IL-18 production in human keratinocytes. *J. Invest. Dermatol.* **134,** 2719–27 (2014).

173. White, J. M. L. *et al.* p-Phenylenediamine allergy: the role of Bandrowski's base. *Clin. Exp. Allergy* **36,** 1289–93 (2006).

174. Coulter, E. M. *et al.* Activation of T-cells from allergic patients and volunteers by p-phenylenediamine and Bandrowski's base. *J. Invest. Dermatol.* **128,** 897–905 (2008).

175. Goebel, C. *et al.* Introduction of a methoxymethyl side chain into p-phenylenediamine attenuates its sensitizing potency and reduces the risk of allergy induction. *Toxicol. Appl. Pharmacol.* **274,** 480–7 (2014).

176. Kock, M., Coenraads, P.-J., Blömeke, B. & Goebel, C. Continuous usage of a hair dye product containing 2-methoxymethyl-para-phenylenediamine by hair-dye-allergic individuals. *Br. J. Dermatol.* **174,** 1042–50 (2016).

177. Diepgen, T. L. *et al.* Prevalence of Contact Allergy to p-Phenylenediamine in the European General Population. *J. Invest. Dermatol.* **136,** 409–415 (2016).

178. Sosted, H., Hesse, U., Menne, T., Andersen, K. E. & Johansen, J. D. Contact dermatitis to hair dyes in a Danish adult population: an interview-based study. *Br. J. Dermatol.* **153,** 132–135 (2005).

179. Aeby, P. *et al.* Characterization of the sensitizing potential of chemicals by in vitro analysis of dendritic cell activation and skin penetration. *J. Invest. Dermatol.* **122,** 1154–64 (2004).

180. Kligman, A. M. The Identification of Contact Allergens by Human Assay. *J. Invest. Dermatol.* **47,** 393–409 (1966).

181. Bonefeld, C. M. *et al.* Consumer available permanent hair dye products cause major allergic immune activation in an animal model. *Br. J. Dermatol.* **162,** 102–7 (2010).

182. Schmidt, J. D. *et al.* Immune responses to hair dyes containing toluene-2,5-diamine. *Br. J. Dermatol.* **170,** 352–9 (2014).

183. Rubin, I. M. C. *et al.* Repeated exposure to hair dye induces regulatory T cells in mice. *Br. J. Dermatol.* **163,** 992–8 (2010).

184. Mueller, S. N. & Mackay, L. K. Tissue-resident memory T cells: local specialists in immune defence. *Nat. Rev. Immunol.* **16,** 1–11 (2015).

185. Mori, T. *et al.* Cutaneous hypersensitivities to hapten are controlled by IFN-gamma-upregulated keratinocyte Th1 chemokines and IFN-gamma-downregulated langerhans cell Th2 chemokines. *J. Invest. Dermatol.* **128,** 1719–27 (2008).

186. Booth, V., Keizer, D. W., Kamphuis, M. B., Clark-Lewis, I. & Sykes, B. D. The CXCR3 binding chemokine IP-10/CXCL10: structure and receptor interactions. *Biochemistry* **41,** 10418–25 (2002).

187. Dai, J., Liu, B. & Li, Z. Regulatory T cells and Toll-like receptors: what is the missing link? *Int. Immunopharmacol.* **9,** 528–33 (2009).

188. Nyirenda, M. H. *et al.* TLR2 Stimulation Drives Human Naive and Effector Regulatory T Cells into a Th17-Like Phenotype with Reduced Suppressive Function. *J. Immunol.* **187,** 2278–2290 (2011).

189. Li, J. *et al.* Enhanced high-mobility group box 1 (HMGB1) modulates regulatory T cells (Treg)/T helper 17 (Th17) balance via toll-like receptor (TLR)-4-interleukin (IL)-6 pathway in patients with chronic hepatitis B. *J. Viral Hepat.* **21,** 129–140 (2014).

190. Dar, A. A., Patil, R. S. & Chiplunkar, S. V. Insights into the relationship between toll like receptors and gamma delta T cell responses. *Front. Immunol.* **5,** 1–12 (2014).

191. Mercier, B. C., Cottalorda, A., Coupet, C.-A., Marvel, J. & Bonnefoy-Bérard, N. TLR2 engagement on CD8 T cells enables generation of functional memory cells in response to a suboptimal TCR signal. *J. Immunol.* **182,** 1860–1867 (2009).

192. Cottalorda, A. *et al.* TLR2 engagement on memory CD8+ T cells improves their cytokine-mediated proliferation and IFN-γ secretion in the absence of Ag. *European Journal of Immunology* **39,** 2673–2681 (2009).

193. Suárez-Fariñas, M. *et al.* Nonlesional atopic dermatitis skin is characterized by broad terminal differentiation defects and variable immune abnormalities. *J. Allergy Clin. Immunol.* **127,** 954–64.e1–4 (2011).

194. Suárez-Fariñas, M., Fuentes-Duculan, J., Lowes, M. A. & Krueger, J. G. Resolved psoriasis lesions retain expression of a subset of disease-related genes. *J. Invest. Dermatol.* **131,** 391–400 (2011).

195. Vollmer, S., Menssen, A. & Prinz, J. C. Dominant lesional T cell receptor rearrangements persist in relapsing psoriasis but are absent from nonlesional skin: Evidence for a stable antigen-specific pathogenic T cell response in psoriasis vulgaris. *J. Invest. Dermatol.* **117,** 1296–1301 (2001).

196. Man, M.-Q. *et al.* Characterization of a hapten-induced, murine model with multiple features of atopic dermatitis: structural, immunologic, and biochemical changes following single versus multiple oxazolone challenges. *J. Invest. Dermatol.* **128,** 79–86 (2008).

197. van der Fits, L. *et al.* Imiquimod-Induced Psoriasis-Like Skin Inflammation in Mice Is Mediated via the IL-23/IL-17 Axis. *J. Immunol.* **182,** 5836–5845 (2009).

198. Seidel-Guyenot, W. *et al.* Low zone tolerance induced by systemic application of allergens inhibits T C1-mediated skin inflammation. *J. Allergy Clin. Immunol.* **117,**

1170–1177 (2006).