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PhD Thesis

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Immune mechanisms behind local skin reactions to contact allergens

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I. **Funch AB**, Mraz V, Gadsbøll AØ, Jee MH, Weber JF, Ødum N, Woetmann A, Johansen JD, Geisler C, Bonefeld CM. $CD8^+$ tissue-resident memory T cells recruit neutrophils that are essential for flare-ups in contact dermatitis. *Allergy* 2022;**77**:513–524. DOI:10.1111/all.14986.

II. **Funch AB**, Weber JF, Lohmann RD, Mraz V, Yeung K, Jee MH, Ødum N, Woetmann A, Johansen JD, Geisler C, Bonefeld CM. $CD4^+$ T cells inhibit the generation of $CD8^+$ epidermal-resident memory T cells directed against clinically relevant contact allergens. *(Manuscript in review)*

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Preface

The studies within this thesis were carried out from the 1st of November 2018 to the 1st of January 2023. The experimental work was performed in Professor Carsten Geisler's and Professor Charlotte Menné Bonefeld's group affiliated to the Leo Foundation Skin Immunology Research Center, Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark. The thesis was conducted under principal supervision by Professor, PhD Charlotte Menné Bonefeld and co-supervised by Clinical Professor, MD, PhD Jeanne Duus Johansen head of the National Allergy Research Centre, Gentofte Hospital. Department of Clinical Medicine, University of Copenhagen, Denmark.

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Abbreviations

ACD	Allergic contact dermatitis
AhR	Aryl hydrocarbon receptor
AMP	Antimicrobial peptide
AP-1	Activator protein 1
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD)
ATP	Adenosine 5´triphosphate
Bcl	B cell lymphoma
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CG	Cathepsin G
Chal.	Challenge
CHS	Contact hypersensitivity
CT	Cholera toxin
CXCL	C-X-C motif ligand
CXCR	C-X-C motif receptor
Da	Dalton
DAMP	Damage-associated molecular pattern
dDC	Dermal dendritic cell
DETC	Dendritic epidermal T cell
dLN	Draining lymph node
DNCB	2,4-dinitrochlorobenzene
DNFB	1-fluoro-2,4-dinitrobenzene
DNP	2,4-dinitrophenol
DNTB	2,4-dinitrithiocyanobenzene
DTH	Delayed-type hypersensitivity
ECM	Extra cellular matrix
ELISA	Enzyme-linked immunosorbent assay
EUG	Eugenol
FABP	Fatty acid-binding protein
FasL	Fas ligand
FFA	Free fatty acid
FITC	Fluorescein isothiocyanate
FMI	Fragrance mixture
FoxP3	Forkhead box protein P3
HCA	α-hexylcinnamaldehyde
HDCL	Hydroxycitronellal
HLA	Human leukocyte antigen
HMGB1	High-mobility group protein B1
HSV	Herpes simplex virus
i.p.	Intraperitoneal
i.v.	Intravenous
ICD	Irritant contact dermatitis
ICE	IL-1β converting enzyme
ICOS	Inducible T-cell co-stimulator
ICR	Inhibitory co-receptor
IFN	Interferon
IL	Interleukin
IL-1R	IL-1 receptor
ILC	Innate lymphoid cell
iNOS	Inducible nitric oxide synthase
IRAK	IL-1R-associated kinases
iSALT	Inducible skin-associated lymphoid tissues
KC	Keratinocyte
kDa	Kilodalton
KO	Knock out
LC	Langerhans cell
LLNA	Local lymph node assay
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MI	2-Methyl-4-isothiazolin-3-one
MIP-2	macrophage-inhibitory protein 2
MVA	Modified vaccinia ankara
MW	Molecular weight
MYD88	Myeloid differentiation factor 88
NET	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKT	Natural killer T
NLR	NOD-like receptor
NLRP3	NOD-like receptor family pyrin domain containing 3
NMF	Natural moisturizing factor
OVA	Ovalbumin
Oxazolone	4-ethoxylmethylene-2-phenyloxazol-5-one
P2X7R	P2X purinoceptor 7 receptor
PAMP	Pathogen-associated molecular pattern
PD	Programmed cell death protein
PPD	*para*-Phenylenediamine
PPR	Pattern recognition receptor
ROS	Reactive oxygen species
s.c.	Subcutaneous
SC	Stratum corneum
SCD	Systemic contact dermatitis
SLS	Sodium lauryl (dodecyl) sulphate
Tc	Cytotoxic T
T_{CM}	Central memory T
TCR	T cell receptor
T_{EM}	Effector memory T
Tfh	T follicular helper
TGF	Tumor growth factor
Th	T helper
TIM3	T-cell immunoglobulin and mucin-domain containing-3
TIR	Toll-like/IL-1R
TLR	Toll-like receptor
TNCB	2,4,6-trinitrochlorobenzene
TNF	Tumor necrosis factor
TRAF6	TNF receptor–associated factor 6
Treg	Regulatory T
T_{RM}	Resident memory T
WB	Western blot

Summary

Our skin is a large and multifunctional organ that is anatomically divided into an inner dermal and an outer epidermal layer. By harbouring multiple immune cells, our skin provides an immunological barrier capable of recognizing and eliminating external treats on the site of entry. Similar to skin infections, skin penetration of contact allergens can result in the development of skin-resident memory T (T_{RM}) cells in the allergen-exposed skin, mediating allergic contact dermatitis (ACD) responses upon local re-exposure. ACD is classically characterized as a delayed type-IV hypersensitivity reaction where symptoms appear days after re-exposure. However, many patients experience ACD symptoms within hours after re-exposure, if exposed on an allergen-experience skin area. These enhanced responses seem primarily mediated by the epidermal-resident allergen-specific $CD8^+$ T_{RM} cells. However, the mechanism behind $CD8^+$ T_{RM} cell mediated ACD and how they survive in the skin over time is unknown.

The overall aim of this thesis was to investigate how $CD8^+$ T_{RM} cells mediate ACD and whether constitutive antigen presentation is required for $CD8^+$ T_{RM} cell survival. In **study I**, using a contact hypersensitivity (CHS) model in mice, we aimed to investigate the mechanism behind $CD8^+$ T_{RM} cells mediated ACD using the experimental contact allergen 1-fluoro-2,4-dinitrobenzene (DNFB). We found that $CD8^+$ T_{RM} cells mediate local ACD reactions by inducing rapid recruitment of neutrophils in a C- X- C motif chemokine ligand (CXCL) 1 and CXCL2 dependent manner. In **study II** we wanted to elucidate the clinical relevance of the results found in study I, by exposing mice to common contact allergens (cinnamal, PPD and MI). We further aimed to study the role of $CD4^+$ T cells in the response. We found that formation of allergen-specific $CD8^+$ T_{RM} cells were highly allergen-dependent, as $CD4^+$ T cells inhibited epidermal $CD8^+$ T_{RM} cell development to MI (partially), cinnamal and PPD (completely). However, we found that the magnitude of the response correlated with the number of epidermal $CD8^+$ T_{RM} cells, CXCL1/CXCL2 release and recruitment of neutrophils. In **study III** we wanted to investigate whether long-term survival of allergen-specific $CD8^+$ T_{RM} cells in the epidermis require constitutive T cell receptor (TCR) triggering. We found that DNFB lead to permanent epidermal deposition of chemical adducts, facilitating survival of the allergen-specific $CD8^+$ T_{RM} cells from local TCR activation and proliferation. Taken together this thesis provides new insight on the immunological mechanisms behind local skin reactions to contact allergens.

Dansk résumé

Huden udgør et stort og multifunktionelt organ som anatomisk er opdelt i et indre dermalt- og et ydre epidermalt hudlag. Huden indeholder mange forskellige immunceller, som tilsammen former en immunologisk barrierer, der er i stand til at genkende og eliminere udefrakommende trusler. På samme måde som efter hudinfektioner, kan eksponering til kontaktallergener føre til lokal udvikling af hud-iboende-hukommelses T (T_{RM})-celler som er i stand til at i gangsætte et kontakt allergisk respons ved re-eksponering for den sensibiliserende kontaktallergen. Kontakt dermatitis er normalt anerkendt som en forsinket type IV reaktion, hvor symptomer typisk opstår dage efter re-eksponering. Dog oplever mange patienter symptomer allerede inden for få timer når de re-eksponeres på det specifikke hudområde som tidligere er eksponeret for kontakt allergenet. Disse hurtige kontaktallergiske reaktioner kan blandt andet relateres til reaktivering af $CD8^+$ T_{RM}-celler. Den præcise mekanisme bag $CD8^+$ T_{RM}-celle medieret kontakt dermatitis, eller hvordan disse celler overlever i huden over længere tid er dog ukendt.

Det overordnede formål med denne afhandling var at undersøge hvordan $CD8^+$ T_{RM}-celler medierer kontakt dermatitis og om lokal antigen-præsentering er nødvendig for deres overlevelse lokalt i huden. I **studie I** anvendte vi en kontakt allergi model i mus, samt det eksperimentelle kontakt allergen 1-fluoro-2,4-dinitrobenzene (DNFB), til at undersøge mekanismen bag $CD8^+$ T_{RM}-celle medieret kontakt dermatitis. Vi fandt at $CD8^+$ T_{RM}-celler hurtigt rekruttere neutrophile celler via C- X- C motif chemokine ligand (CXCL) 1 and CXCL2 ved re-eksponering. I **studie II** ønskede vi at undersøge klinisk relevans af resultaterne fra studie I ved anvendelse af klinisk relevante kontaktallergener (cinnamal, PPD and MI), samt at undersøge $CD4^+$ T-cellers rolle i responset. Vi så at lokal udvikling at allergen-specifikke $CD8^+$ T_{RM}-celler i huden var allergenafhængigt, idet $CD4^+$ T-celler delvist hæmmede udviklingen ved eksponering for MI og fuldstændigt ved cinnamal og PPD. Størrelsen på responset korrelerede dog med antallet af $CD8^+$ T_{RM}-celler, CXCL1/CXCL2 produktion og rekruttering af neutrophile celler. I **studie III** ønskede vi at undersøge om overlevelse af allergen-specifikke $CD8^+$ T_{RM}-celler i er afhængig T-celle receptor (TCR) stimulering. Vi fandt en permanent deponiring DNFB addukter i epidermis og at disse faciliterede overlevelsen af $CD8^+$ T_{RM}-celler via lokal TCR aktivering og proliferation. Samlet set giver denne afhandling ny viden om de immunologiske mekanismer bag lokale hud reaktioner til kontakt allergener.

Introduction

1.1 The Skin

The skin is the largest organ of the human organism, and it upholds multiple functions vital for our survival and general homeostasis. By forming a physical, chemical and immunological barrier the skin effectively prevents excessive water loss, entry of harmful substances and protection against pathogens (1–6). Anatomically, the skin is divided into the dermis (the inner layer) and the epidermis (the outer layer) separated by the basal membrane (Figure 1). The dermis is subdivided into the lower stratum reticulare and the upper stratum papilare. Structurally, the dermis primarily consists of fibroblasts that forms a complex network of collagen and elastin fibers providing a scaffold for nerves, lymphatic- and blood vessels that enables easy access of nutrients and immune cells (1,2,4,6). From the inside-out the epidermis is subdivided into the stratum basale, stratum spinosum, stratum granulosum and the stratum corneum (SC). The layers within the epidermis primarily consist of keratin-rich cells called keratinocytes (KC). In addition, melanocytes that produce the skin pigmentation and nerve-ending cells (Merkel cells), which are important for tactile sensing, are found in the epidermis. The KCs continuously develop from progenitor cells within the stratum basale from where they travel outward through the epidermal layers. Once in the SC they are dead and referred to as corneocytes (enucleated cells covered by a lipid envelope). Eventually the corneocytes shed of the skin in a process called desquamation which ensures that the epidermis is continuously renewed (1–8). The physical barrier is established by the SC and by cell-cell adhesion molecules in the lower layers of the epidermis i.e. tight junctions, adherens junctions and desmosomes (6–8). As highlighted by deficiencies in the *FLG* gene, another key element in the barrier function is the protein filaggrin (6–10). Filaggrin is synthesized in a pro-form by KCs within the stratum granulosum and during KC differentiation filaggrin is cleaved into protein monomers found abundantly inside the corneocytes (6–9). The breakdown products of filaggrin contributes to the pool of natural moisturizing factors (NMFs) that is found topically on the skin. In addition to NMFs the KCs also produce antimicrobial peptides (AMPs) i.e. β-defensins and cathelicidins. Thus, the chemical barrier is formed on the skin surface from extracellular lipids, NMFs and AMPs that collectively form a nutrient-poor and acidic microenvironment (1–9). Nonetheless, numerous commensal microorganisms, referred to as the skin microbiota, thrive in this environment and colonize the skin surface and by battling non-commensal microorganisms they also contribute to skin homeostasis (4,11).

1.1.1 The immunological barrier

The physical and chemical barriers are not perfect as hair follicles, sweat glands and mechanical injuries enable entry of pathogens and foreign substances into the epidermal end dermal layers (4,6,11–13). Therefore, to protect against infections, the cells within the skin are well equipped with a variety of immunological defence mechanisms enabling them to recognize and initiate both innate- and adaptive immune responses (Figure 1.). KCs play a central role in recognizing danger and initiating innate skin immunity. They express a variety of stress molecules and pattern recognition receptors (PRRs) i.e., NOD-like receptors (NLRs) and Toll-like receptors (TLRs) that recognize conserved microbial structures referred to as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Upon activation, KCs can release a large array of pro- and anti-inflammatory cytokines and chemokines that leads to local skin inflammation and subsequent healing (1–7). In addition to KCs, a subpopulation of antigen presenting cells (APCs) called Langerhans cells (LCs) along with $CD4^+$ and $CD8^+$ T cells are also found in the epidermis of adult humans (1–6,14–16). The dermis is a more diverse immunological structure where a wide range of immunological cells are located. During skin homeostasis, natural killer (NK) cells, dermal dendritic cells (dDCs), mast cells, macrophages, innate lymphoid cells (ILCs), γδ T cells and $CD4^+$ and $CD8^+$ T cells are found in the dermis (3–5). By their dendritic shape, the LCs collectively form a structured network within the epidermis and together with dDC, they establish an important link between the innate and adaptive immune system (17–19). Upon pathogen recognition, they become activated which initiates maturation and migration towards the local draining lymph nodes (dLNs). Here they present pathogen-specific peptides or lipid antigens in the context of major histocompatibility complex (MHC) class I/II- or CD1 molecules to antigen-specific T cells (4,17–23). This leads to activation, differentiation and expansion of naïve antigen-specific T cells into effector T cells. Within few days the effector T cells begin to express skin homing molecules that enable migration to the infected skin area, where they mediate a variety of effector functions depending on the pathogen (1,3,23–26). Once the infection is cleared, a fraction of the effector T cells survive as memory T cells that provide enhanced immunity against re-infections. Initially, two memory T cell subsets were described as C-C chemokine receptor 7 ($CCR7^+$)$CD62L^+$ central memory T (T_{CM}) T cells found in the dLNs and circulation and $CCR7^-CD62L^-$ effector memory T (T_{EM}) cells found in the circulation and peripheral tissues including the skin (27). More recently a subset known as tissue-resident memory T (T_{RM}) cells recognized by their surface expression of the E-cadherin binding receptor integrin αEβ7 (CD103) and CD69 were identified in the skin following infection with

herpes simplex virus (HSV) (28). Once the infection is cleared, the skin-resident T_{RM} cells stay dormant in the skin and as the skin is continuously exposed to a variety of harmful substances they accumulate in the skin reflecting the antigen-exposure over time (29,30). In relation, the number of T cells in adult human skin have been described as twice the number of T cells found in the circulation (31). The majority of skin-resident T_{RM} cells are found in the epidermis with a very limited migratory capacity (32). Because they stay at the site of pathogen entry they establish a protective barrier of rapid adaptive immunity against secondary infections (15,16,33–39). In relation, skin-resident $CD8^+$ T_{RM} cells can induce skin recruitment of both innate and adaptive immune cells upon re-infections, including $CD8^+$ T_{EM} cells from the circulation (34,35,40). In healed skin following antigen exposure, skin-resident ($CD103^-CD69^+$) $CD4^+$ T_{RM} cells are primarily found in the dermis, whereas the majority of skin-resident ($CD103^+CD69^+$) $CD8^+$ T_{RM} cells are located within the stratum basale of the epidermis (14–16,32). Furthermore, most of the dermal-resident $CD4^+$ T_{RM} cells are located in near proximity to the hair follicles from where many have been found capable of re-entering the circulation (16,30,32,41–43). Despite playing a key role in skin immunity, skin-resident T_{RM} cells have also been linked to several autoimmune and allergic skin diseases when wrongly activated towards self-proteins in the skin (44,45), including psoriasis (16,46,47), vitiligo (16,48,49), alopecia areata (50–52) and recently in allergic contact dermatitis (ACD) (53–58).

1.1.2 Anatomical and immunological differences between murine and human skin

The anatomy of human and murine skin is generally similar (Figure 1), although the number and density of hair follicles are much greater in mice, resulting in a synchronized hair growth which is not seen in humans. The thickness of the epidermis is about four times greater in human skin. However, the shallow epidermal thickness together with a higher epidermal cell turnover, results in faster wound healing in mice (1,2,5). Differences in epidermal T cell compartmentalization also exist. In mice and not in humans, a subset of γδ T cells known as dendritic epidermal T cells (DETCs) are found with immunological roles in both wound healing, clearance of cancers as well as allergic responses (59–62). Also, the skin of laboratory mice simply do not reflect a similar reservoir of memory T cells as found in adult human skin due to lack of antigen-exposure (63,64). Finally, the distribution of circulating neutrophils and lymphocytes are known differ between mice (75–90% lymphocytes, 10–25% neutrophils) and humans (50–70% neutrophils, 30–50% lymphocytes) (65,66). These are all factors that may challenge the ability to translate results found in murine models directly into the human disease condition.

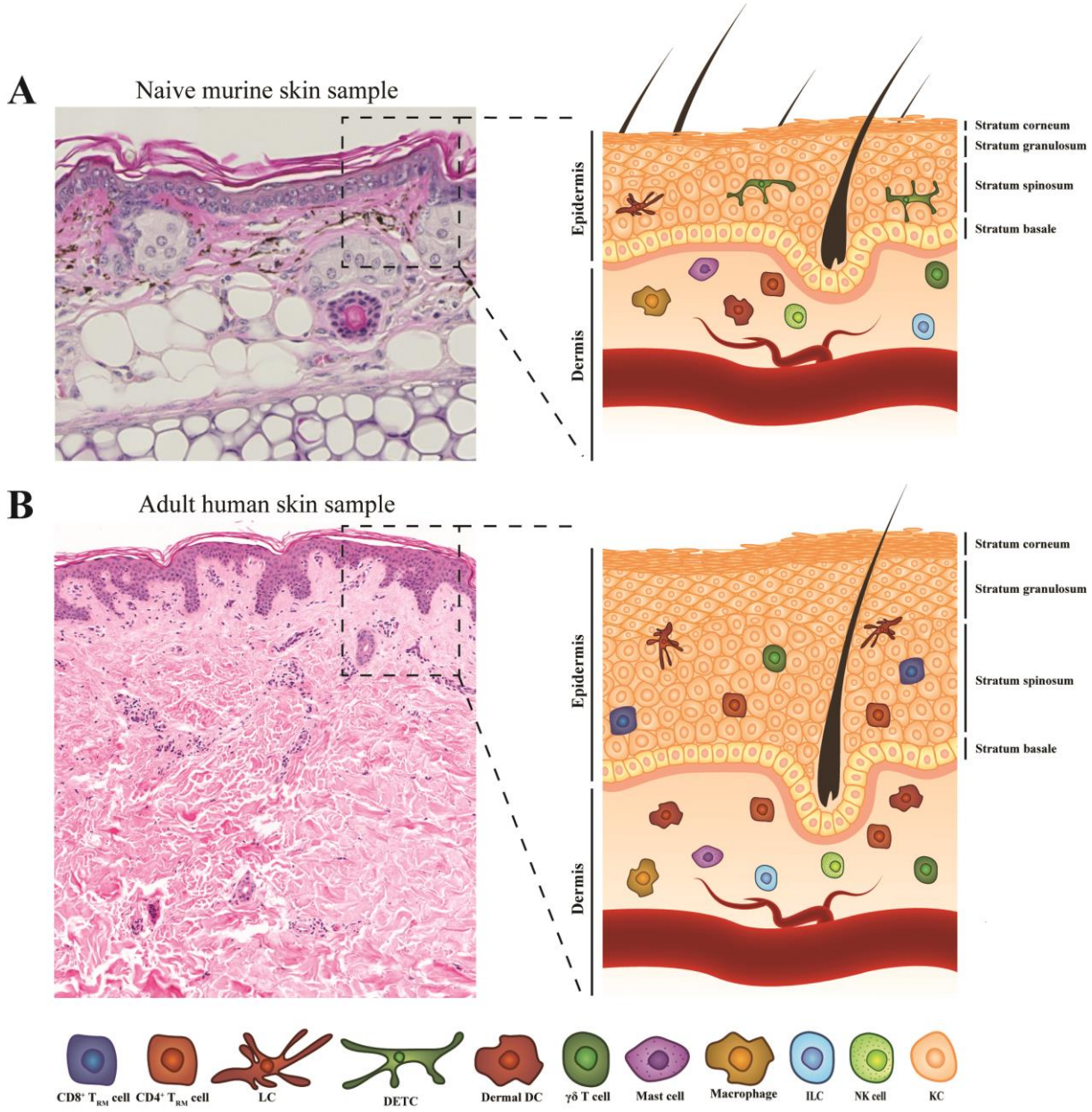

Figure 1. Model of the immune cells located in murine vs. human skin during homeostasis. ***(A)*** *Left side: Hematoxylin- and eosin-stained skin from an ear cross-section obtained from a naïve (untreated) mouse. Right side: cartoon of the anatomical and immunological composition of naïve mouse skin at steady state.* ***(B)*** *Left side: Hematoxylin- and eosin-stained skin biopsy from an adult human during homeostasis. Right side: cartoon of the anatomical and immunological composition of adult human skin during steady state. For both species, the dermis and epidermis (Stratum corneum, stratum granulosom, stratum spinosum and stratum basale) are illustrated by different keratinocyte (KC) constructed layers. Murine skin is much thinner and contains numerous hair follicles compared to human skin. Dendritic epidermal T cells (DETCs) are only found within murine epidermis, whereas Langerhans cells (LCs) are found in both species. $CD4^+$ and $CD8^+$ epidermal-resident T_{RM} cells are found in the epidermis of adult human skin as they reflect local antigen exposure over time. The dermis of both naïve mouse and adult human harbour numerous immune cells during homeostasis. These include $CD4^+$ T_{RM} cells, dermal dendritic cells (dDCs), γδ T cells, mast cells, macrophages, innate lymphoid cells (ILCs) and natural killer (NK) cells.*

1.2 Allergic Contact Dermatitis

Allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) are the two main forms of contact dermatitis. Both ACD and ICD are visible as acute inflammatory skin reactions after topical exposure to specific agents, however the immunological mechanisms behind ACD and ICD are not the same. ICD is mediated by exposure to skin irritants e.g. detergents/soaps and solvents/oils that induce cellular toxicity leading to innate immune responses (67–69). In contrast, ACD is characterized as a type IV- or delayed-type hypersensitivity (DTH) reaction, as clinical symptoms appear when memory T cells become reactivated, infiltrate the skin, and mediate their effector functions (Figure 2.) (70). Thus, the adaptive immune system plays an important role in ACD (67,68,70–75). Furthermore, ACD reactions typically occur locally at the skin area re-exposed to the contact allergen, although few cases of systemic skin reactions referred to as systemic contact dermatitis (SCD), have been described in allergic individuals following re-exposure via ingestion or inhalation (67,68,76). Also, local flare-up reactions at healed skin areas previously exposed to the contact allergen have been described upon exposure at distal skin sites (77–79). Such responses indicate that development of local allergen-specific memory in the skin may be re-activated by circulatory mediators.

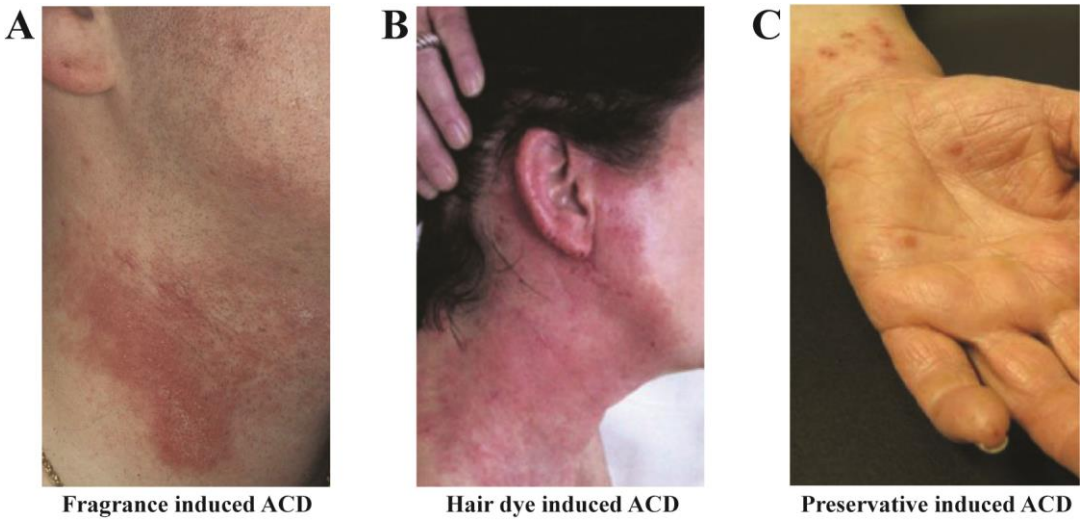

Figure 2. Clinical examples of ACD reactions to contact allergens.
(A) *Clinical example of an ACD reaction to fragrance exposure (e.g. cinnamal).* ***(B)*** *Clinical example of an ACD reaction to hair dye exposure (e.g. PPD).* ***(C)*** *Clinical example of an ACD reaction to paint-preservative exposure (e.g. MI). The observable clinical symptoms often includes erythema, oedema and vesicle formation, although it may differ between contact allergens as seen in the figure. Images are provided by the National Allergy Research Centre, Denmark.*

The most recent data suggests that the prevalence of contact allergy in the general population is about 20%, albeit not all individuals diagnosed with contact allergy develop ACD (80–82). In Europe, ACD is characterized as the third most common skin disease affecting about 15% of the adult population (83). Furthermore, it is well described that occupational related exposures to contact allergens are currently the biggest risk factor, with a high prevalence of ACD seen among hairdressers, health-care workers and people working in industries where exposure to reactive chemicals is common (67,68,70,84,85). The clinical symptoms of ADC may vary depending on the contact allergen, dose and the area of skin (Figure 2) (86–88). However, ACD is generally described by acute and local pruritic eczematous skin lesions with formation of erythema, oedema, vesicles or even bullae at the exposed skin area (68,70,89,90). The clinical manifestation of ACD can be quite severe and invalidating for the affected individuals and because current treatment for ACD is limited to use of allergen free emollients and topical or systemic application of immunosuppressant drugs (e.g. corticosteroids), avoidance strategies to reduce allergen exposure are often necessary (68,91). The later can be difficult or even impossible in occupational related ACD, thus ACD is known to have large socio-economic consequences for both affected individuals and for society in general (92,93).

The current diagnostic procedures for contact allergy is the patch test, where positive patch test reactions are measured 3, 5 and/or 7 days after exposure to a panel of suspected contact allergens (primarily on the upper back) (67,68,70). Even though patch testing is often a successful diagnostic tool, it can be inconvenient for patients. Further, clinicians are not able to give an adequate diagnosis if the causing agent is not included in the test panel (67,68,70). Thus, the need to identify a general biomarker for ACD and for better therapeutic options is evident (68)

1.2.1 Contact Allergens

The pioneering work by Landsteiner and Jacobs published in 1935, led to the discovery that contact allergens or haptens are small molecules that become antigenic when reacting with self-proteins in the skin (94). Today we know that most contact allergens have specific chemical properties that enable skin entry and activation of the immune system leading to sensitization of the exposed individual. First, lipophilic properties and a low molecular weight (MW), most < 500 Daltons (Da) are common traits for contact allergens that enables penetration of the lipid-filled skin barrier at the SC (95,96). Secondly, contact allergens are protein-reactive as most bear

electrophilic (partially positively charged) centers, or induce formation of free radicals, that can react chemically with nucleophilic (partially negatively charged) centers on side chains, such as thiols (-SH) and primary amines (-NH_2) (97,98). Most contact allergens are capable of forming strong covalent bonds with skin proteins leading to formation of contact allergen/self-protein complexes referred to as adducts. The most common protein positions for covalent bond interactions between a contact allergen and skin protein are primarily with cysteine and lysine amino acid residues. Even though covalent bond formation most frequently occurs between an electrophilic contact allergen and a nucleophilic skin protein, this is not always the case. For example metal salts (i.e. nickel, cobalt and chromium) differ from most allergens as they form co-ordination bonds with amino acid residues (e.g. histidine) (95,96,98–102). Both covalent and co-ordination bonds are strong chemical interactions, thus the formation of contact allergen/self-protein adducts leading to contact allergy are almost irreversible once developed (96,101)

Currently, more than 4000 different contact allergens have been described of which many are present in our daily-life environment and the number is increasing as novel allergens are identified in relation to new industrial- and consumer products (67,100). Although the chemical structure of contact allergens is highly diverse, contact allergens can be characterized by the pathway by which they become chemically reactive (electrophilic), as either pro- or pre-haptens (103). More specifically, pro-haptens such as poison ivy derived urushiol, are chemically inert and thus harmless molecules before penetrating the skin barrier. However, they become reactive by enzymatic processes in the skin, as reactive intermediates that are not easily removed by the metabolic detoxification system (i.e. by the cytochromes P450 enzyme family). In contrast, pre-haptens, often found in fragrance mixtures (e.g. limonene and fragrance terpenes), are not activated by enzymatic processes in the skin. Instead, they become chemically reactive via environmental triggers such as oxidization, light or heat. Pre-haptens can therefore modify skin proteins directly upon penetration of the skin (96,99,103,104).

Because memory T cells are only re-activated through their T cell receptor (TCR), contact allergic individuals almost exclusively develop ACD lesions when re-exposed to the specific sensitizing agent. However, in some cases cross-reactions may occur after exposure to a novel contact allergen, when the modified allergen-specific molecules presented to memory T cells, are highly similar in chemical- and spatial structure (96). In relation, human $CD4^+$ T cells have recently been shown with overlapping TCR repertoires, following exposures to different metal-

salt allergens, explaining why cross-reactivity reactions may occur between these contact allergens (105).

Potency, or the dose of a given contact allergen needed to induce sensitization, is another important factor in the development of contact allergy. The potency of a contact allergen was initially proposed as proportional to the formation of contact allergen/self-protein adducts in the skin (106). However, more recent studies clearly suggest that irritancy or the ability to induce nonspecific pro-inflammatory signalling in the skin when exposed to a contact allergen is pivotal for sensitization and determining the severity of ACD (69,96,99,107). The potency of contact allergens can be assessed by quantifying the cellular proliferation in the dLN via local lymph node assays (LLNA) in mice (100,108). By conducting a LLNA an EC3 value is calculated by extrapolating the dose needed to provoke a threefold proliferative cellular increase in the dLNs by a given contact allergen, when compared to a vehicle control five days after topical exposure (109). As the proliferation and number of allergen-specific effector T cells tend to peak in the dLN five days after exposure, the EC3 value/potency of a contact allergen is directly related to activation of allergen-specific T cells (108).

1.2.2 Chemical characteristics of DNFB, cinnamal, PPD and MI

In the context of this thesis a description of the experimental contact allergen 1-Fluoro-2,4-dinitrobenzene (DNFB, MW = 186.10 Da) and a description of three clinical relevant contact allergens; Cinnamic aldehyde (cinnamal, MW = 132.16 Da), *para*-Phenylenediamine (PPD, MW = 108.14 Da) and 2-Methyl-4-isothiazolin-3-one (MI, MW = 115.16 Da) (110,111) will be provided. All off the clinically relevant allergens are currently part of the baseline series used for diagnosing contact allergy (allergic skin reactions with these allergens can be seen in Figure 2).

1-Fluoro-2,4-dinitrobenzene (DNFB)

DNFB is an extremely potent contact sensitizer that is not present in our daily environment and therefore a commonly used contact allergen in experimental settings. On a chemical level, when applied topically on the skin, DNFB rapidly induces enzymatic reactions resulting in contact allergen/self-protein adducts between 2,4-dinitrophenol (DNP) moieties and lysine (Figure 3.) or cysteine residues (112). Furthermore, using electron microscopy DNP-immunogold labelling technique 0-96 h after challenge with DNFB, it has been shown that DNP-moieties are distributed

throughout the epidermal sublayers, primarily forming adducts with cytoskeleton and keratin proteins within KCs or Golgi apparatus proteins within LCs (113,114). Finally, in a study detecting DNP moieties using anti-DNP specific antibodies, epidermal DNP-adducts have been demonstrated to decrease but to persist in the skin up to four weeks after DNFB challenge (56).

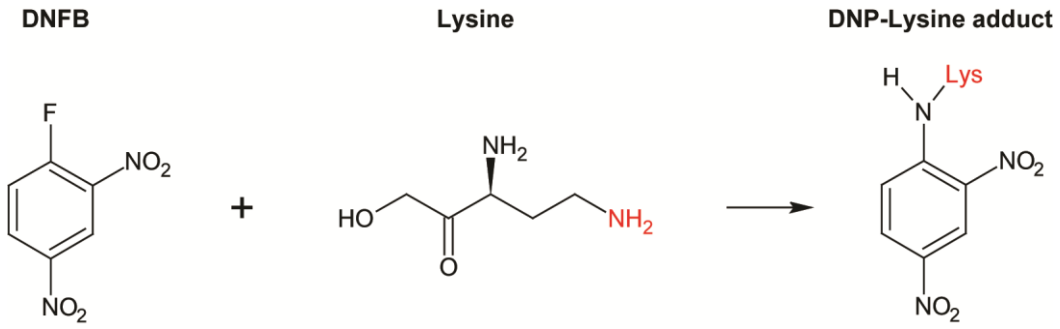

Figure 3. Example of a chemical reaction between DNFB and a lysine residue.
Chemical reaction between 1-fluoro-2,4-dinitrobenzene (DNFB) and lysine (Lys) residues present in skin proteins. DNFB contains a benzene ring with two nitrogen dioxide (NO_2) molecules and a fluorine (F) molecule that can react and share a hydrogen atom with the primary amine (NH_2^-) on lysine residues. In this example it results in the formation of 2,4-dinitrophenol (DNP)-lysine adducts. The NH_2 reaction site on lysine is colored red. Oxygen atom (O), hydrogen atom (H), nitrogen atom (N).

Cinnamaldehyde

Cinnamaldehyde (cinnamal) is a moderate contact sensitizer and a common part of fragrance products as it provides a cinnamon odour (84,111). Cinnamal is currently among the most frequent mediators of ACD in the European population and thus part of one of fragrance mixtures I (FMI) that is currently a common part of diagnostic patch test panels. In relation, 6.8% of tested European patients have positive reactions towards FMI I and 20% of those have positive reactions towards cinnamal (111,115). Chemically, cinnamal is an electrophilic molecule by itself and is thus able to react with nucleophilic skin proteins. Although, cinnamal may also develop in the skin as an intermediate by enzymatic degradation of cinamic alcohol (101,104,116,117). When present in the skin, cinnamal may be detoxified in two ways: by irreversible oxidation into cinamic acid or reversibly by binding to glutathione metabolites. However, cinnamal may also become pathogenic as it reacts with the cysteine or lysine residues in the skin leading to cinnamal/self-protein adducts (Figure 4.) (101,116,118).

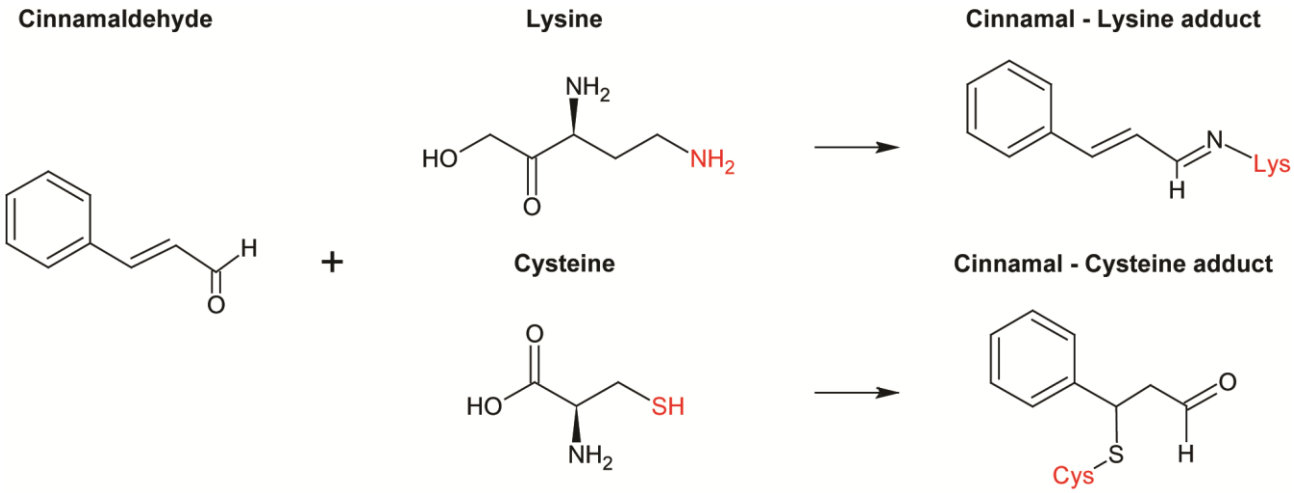

Figure 4. Example of chemical reactions between cinnamaldehyde, lysine or cysteine residues. *Chemical reaction between cinnamaldehyde (cinnamal) and a lysine residue (top) or cysteine residue (bottom) both present in skin proteins. Cinnamal contains a benzene ring and an aldehyde group to form 3-phenylprop-2-enal. The aldehyde group can react with nucleophilic groups: primary ammines (-NH_2) or thiol groups (-SH) present on lysine and cysteine residues. This can lead to the formation of cinnamal-lysine and cinnamal-cysteine adducts. The NH_2 reaction site on lysine and SH reaction site on cysteine is depicted in red. Oxygen atom (O), hydrogen atom (H), sulphur atom (S), nitrogen atom (N), lysine residue (Lys), cysteine residue (Cys).*

***Para*-Phenylenediamine (PPD)**

PPD is a strong contact sensitizer and a common component in black hair dyes often causing ACD when the skin gets exposed (84,89,119–121). Like cinnamal, PPD is highly relevant clinically as it is among the most frequent mediators of ACD in the European population. In relation, 3.6% of tested European patients develop positive reactions towards PPD (111). From a chemical perspective, PPD readily induce formation of electrophilic reactive intermediates (p-benzoquinonediimine, p-benzoquinones and Bandrowski's base) following enzymatic processes in the skin. PPD itself, or these intermediates, primarily form covalent bonds with cysteine (Figure 5.) and lysine residues. Reactions with histidine, arginine and tryptophan are also described in the formation of PPD/self-protein adduct formation in the skin (103,104,120,122).

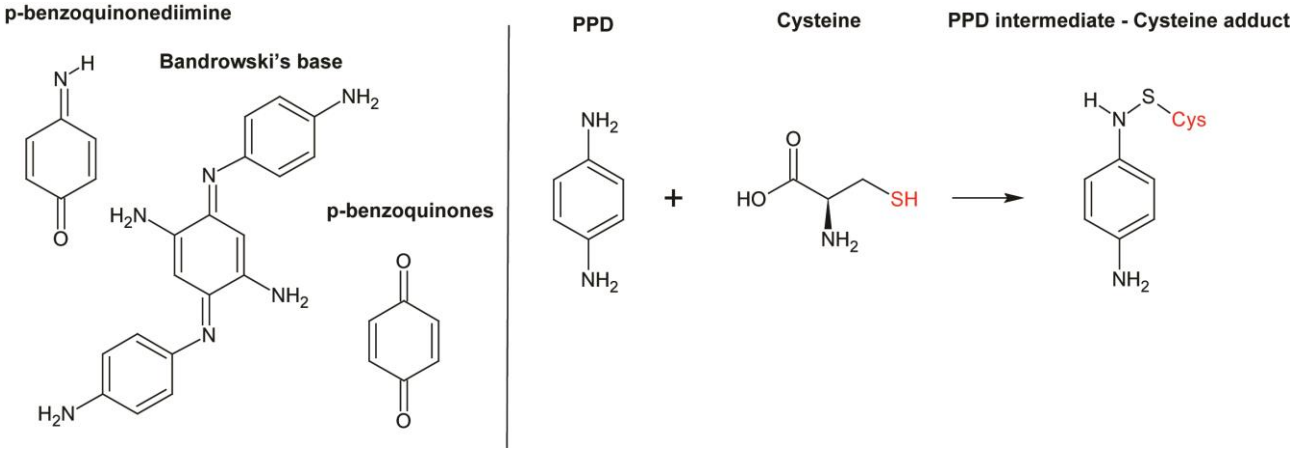

Figure 5. Example of chemical reactions between PPD and cysteine residues.
Chemical reaction between para-Phenylenediamine (PPD) and cysteine residues present in skin proteins. The enzymatically induced intermediates (p-benzoquinonediimine, p-benzoquinones and Bandrowski's base) are also illustrated as these can react accordingly, forming PPD/intermediate-cysteine adducts. The -SH reaction site on cysteine is depicted in red. Oxygen atom (O), nitrogen atom (N), hydrogen atom (H), sulphur atom (S), nitrogen atom (N), cysteine residue (Cys).

2-Methyl-4-isothiazolin-3-one (MI)

MI is a strong contact sensitizer and a preservative that has been widely used in cosmetic-, household- and industrial products including wet wipes and paints (84,123,124). In relation, 7.5% of tested European patients develop positive reactions towards MI (111). The use of MI was suddenly introduced in cosmetic products in 2005 resulting in an ACD epidemic across Europe until regulations were implemented in 2017 by the European Union (67,125). Because MI is chemically reactive by nature, topical skin exposure with MI induce a rapid reaction with primarily cysteine residues within skin proteins (Figure 6.) (126).

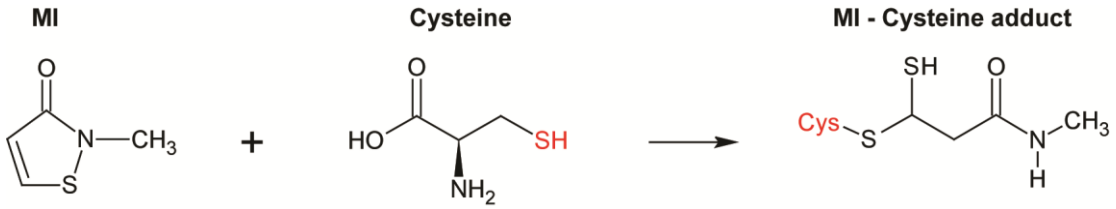

Figure 6. Example of chemical reactions between MI and cysteine residues.
Chemical reaction between 2-Methyl-4-isothiazolin-3-one (MI) and cysteine residues present in skin proteins. MI contains and oxygen atom (O) and methyl group (CH_3) attached to a cyclopentane ring containing sulphur (S) and nitrogen (N) atoms. These can e.g. react with thiol groups (SH^-) present on cysteine residues, leading to the formation of MI-cysteine adducts. The SH reaction site on cysteine is depicted in red. Carbon atom (C), hydrogen atom (H), cysteine residue (Cys).

1.2.3 The immune response to contact allergens

The immune responses behind contact allergy and ACD is signified by two phases. First, the sensitization phase where a contact allergen crosses the skin barrier, induces local danger, and reacts with self-proteins leading to the activation and differentiation of allergen-specific naïve T cells, resulting in the development of contact allergy towards the allergen. Secondly, the elicitation phase where the memory T cells are activated upon allergen re-exposure leading to ACD (68,72,127).

During the sensitization phase (Figure 7.), exposure of the skin to a contact allergen leads to formation of contact allergen/self-protein adducts and local danger signalling that activates APCs (LCs and dDCs) (18,19,87). The APCs mature and migrate to the dLNs, where they activate allergen-specific naïve T cells by promoting three signals. (I) APC expression of contact allergen-modified peptides by MHC class I/II complexes that are recognized by TCRs (128,129). (II) APC presentation of co-stimulatory receptors CD80 (B7) and CD86 (B70) to CD28 receptors expressed by T cells (130). (III) Release of T cell polarizing cytokines that guide differentiation of specific T cell subsets (121). The TCR present on allergen-specific naïve $CD4^+$ T cells recognize peptide bound MHC class-II molecules and the TCR on allergen-specific naïve $CD8^+$ T cells recognize peptide bound MHC class-I molecules. When all three signals are supplied, the naïve T cells become activated leading to proliferation and differentiation into effector T cells (68,69,131–133). Within a few days (5-7 days in mice and 10-15 days in humans), effector T cell numbers peak and acquire the capacity to produce effector cytokines (68,72,108). In addition, by expressing a variety of skin homing surface molecules, including C-X-C motif receptor 3 (CXCR3), the effector T cells leave the dLN and egress towards skin following a gradient of specific skin-homing chemokines, i.e. C-X-C motif ligand (CXCL) 9, CXCL10 and CXCL11, that are released by skin resident cells at the allergen exposed skin area (26,121,134–136). By binding vascular integrins expressed by endothelial cells, the effector T cells cross the endothelial wall into the skin (68,134,137,138). Eventually, most of the allergen-induced/allergen-specific T cells die off, leaving behind a subpopulation of surviving $CD44^+$ (mouse) or $CD45RO^+$ (human) allergen-specific memory T cells. These are located in the circulation and secondary lymphoid organs as $CCR7^+CD62L^+$ T_{CM} cells and $CCR7^-CD62L^-$ T_{EM} cells or locally in the skin as $CD69^+CD103^+$ skin-resident T_{RM} cells (15,54,57,139).

The elicitation or challenge phase (Figure 7), is characterized by re-activation of the allergen-specific memory T cells leading to ACD locally at the re-exposed skin area (68,72,127). The inflammatory response in ACD is potentiated by 3 effector mechanisms: 1) Local release of pro-inflammatory cytokines and chemokines (134,135,140–142); 2) Direct cytotoxic killing of skin cells that present contact allergen specific peptides by memory $CD8^+$ T cell Fas-Fas Ligand (FasL) binding-induced apoptosis and by targeted release of perforin (143,144); 3) Activation and recruitment of other immune cells (68). A variety of different immune cells including neutrophils, macrophages, eosinophils, mast cells, γδ T cells, natural killer T (NKT) cells and ILCs accumulate in ACD lesions and have been linked to different roles in ACD (68). Eventually, within a few days without continued allergen exposure the ACD lesions resolve (72). In human skin, the control and resolution of ACD is mainly ensured by LCs and $CD4^+$ $CD25^+$ forkhead box protein P3 $(FoxP3)^+$ regulatory T (Treg) cells, by their release of the anti-inflammatory mediators IL-10 and tumor growth factor β (TGFβ) (145). Once the skin heal, the number of local epidermal $CD8^+CD69^+CD103^+$ T_{RM} cells have increased, resulting in even further enhanced ACD responses with future exposures (57).

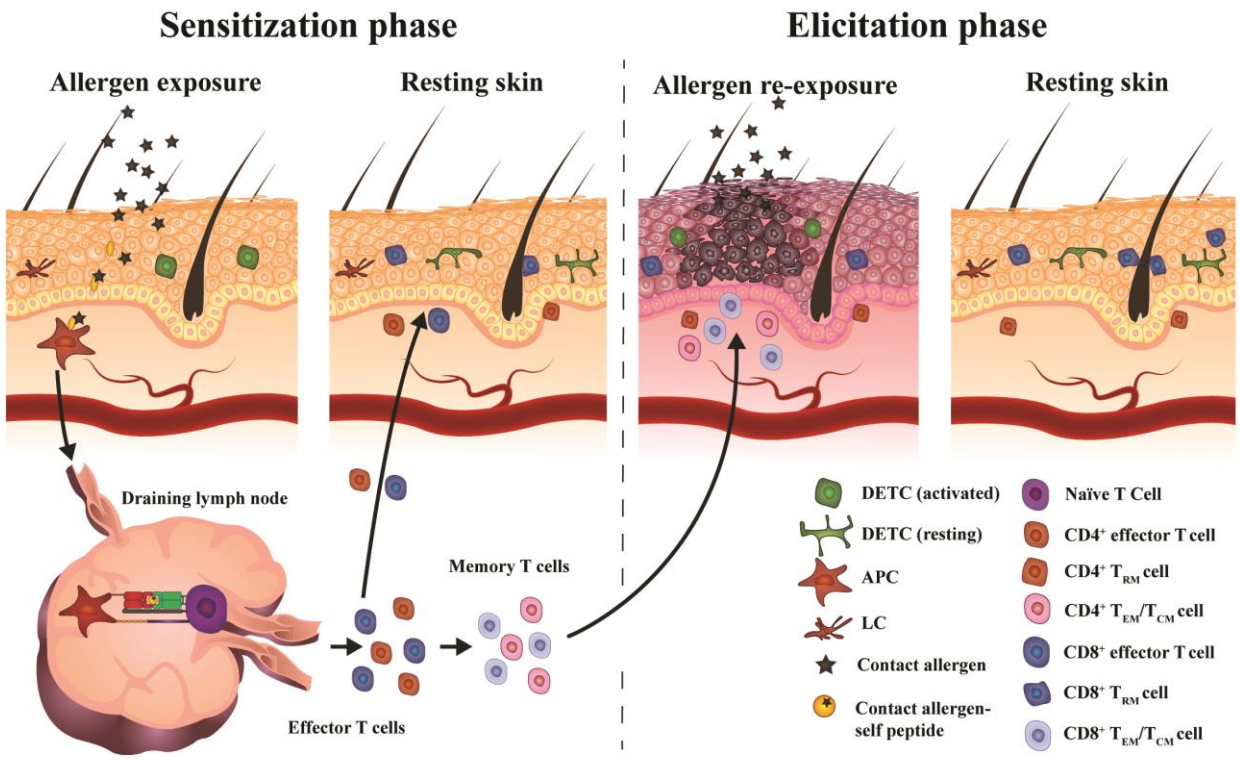

Figure 7. T cell responses in the skin during the sensitization and elicitation phase.
The sensitization phase is initiated by skin exposure to contact allergen and formation of contact allergen-self protein adducts that are recognized by antigen presenting cells (APCs). Once activated, the APCs maturate and migrate towards the draining lymph nodes (dLNs) to activate

naïve allergen-specific $CD4^+$ and $CD8^+$ T cells. Naïve T cell activation induces the formation of $CD4^+$ and $CD8^+$effector T cells that migrate back to the allergen exposed skin site. Within a few days the effector $CD4^+$ and $CD8^+$ T cells die off leaving behind subpopulations of long-lived $CD4^+$ and $CD8^+$ memory T cells including effector memory T (T_{EM}) cells and central memory T (T_{CM}) cells in the circulation and secondary lymphoid organs, and skin-resident memory T (T_{RM}) cells in the skin. $CD4^+$ T_{RM} cells are primarily found in the dermis while $CD8^+$ T_{RM} cells are primarily found in the epidermis. The elicitation phase is initiated after the skin is re-exposed to the contact allergen leading to skin inflammation. This involves recruitment of T_{EM} and T_{CM} cells from the circulation. Rapid inflammatory responses occur following re-exposure on an allergen-experienced skin site by re-activation of $CD8^+$ T_{RM} cells. Once the skin heals, the number of local $CD8^+$ T_{RM} cells increase. Dendritic epidermal T cell (DETC), Langerhans cell (LC)

1.2.4 Contact hypersensitivity (CHS) – modelling the pathogenesis of ACD in mice

In the context of research, use of the contact hypersensitivity (CHS) mouse model is still the preferred approach when aiming to study different immunological aspects of ACD *in vivo* (72,74,108,146,147). CHS is induced experimentally by topical application of contact allergens directly to the skin of the mice, leading to immune activation that is easily translated into human ACD. Historically, use of extremely potent experimental contact allergens like 2,4-dinitrochlorobenzene (DNCB), 1-fluoro-2,4-dinitrobencene (DNFB), 2,4,6-trinitrochlorobenzene (TNCB) or 4-ethoxylmethylene-2-phenyloxazol-5-one (oxazolone), have often been preferred to study immunological aspects of ACD. This because these contact allergen all induce strong immune responses and are not considered a health risk for researches, as they are not found in our daily-life environment (72,84). Furthermore, sensitization with allergen in the conventional short-term CHS model is typically induced on the abdomen followed by a resting period of 5-7 days to allow proper T cell activation. After 5-7 days, challenge with contact allergen is then typically performed on a different (allergen-naïve) skin area, most often the ears. This approach results in an acute inflammatory skin reaction within the ears 24-48 hours after challenge, mediated by effector T cells recruited to the skin. The response is typically measured *in vivo* by changes in ear thickness over approximately one week followed by different *ex vivo* assays depending in the research question (72). To study the isolated role of memory T cells in the studies conducted for this thesis, we increased the resting period between sensitization and challenge to 21 days or more. Using this approach, the primary immune response (i.e. effector T cells) wanes of before the challenge response is induced. Thus enabling us to focus on the response mediated by the surviving memory T cell subsets. Similar approaches aiming to study the role memory T cells in the response to contact allergens have been performed successfully by others (54–58).

1.2.5 Innate recognition and danger signalling in response to contact allergens

Danger signalling by resident skin cells in response to contact allergens is pivotal for APC maturation and subsequent activation of naïve T cells leading to contact allergy (Figure 8.) (68,71). The mechanism is emphasized by studies where exposure to low doses of contact allergens or chemical compounds (tolerogens) that do not sufficiently induce danger signalling, instead leads to immunological tolerance towards the exposed chemical (148–151). Conversely, by enforcing danger signalling experimentally, contact allergy towards tolerogens or weak allergens has been performed (152–157). In mice, co-application with a 10% solution of the irritant sodium lauryl (dodecyl) sulphate (SLS) together with a low dose (0.1%) of the contact allergen DNCB, led to increased APC migrating to the dLN and further to a 3-fold amplification of proliferated lymphocytes when compared to DNCB exposure alone (152). Contact allergy towards the tolerogen 2,4-dinitrothiocyanobenzene (DNTB) was experimentally induced in mice by concomitant danger signalling using SLS or by intraperitoneal (i.p.) injections with pro-inflammatory recombinant cytokines interleukin (IL)-1β or IL-12) (153,154). Furthermore, contact allergy has been induced from an additive effect using mixtures of weak fragrance allergens (155), by concomitant lipopolysaccharide (LPS) injections (indicative for a bacterial infection) (157) and even by norepinephrine induced psychological stress during allergen exposure (156). Importantly, most studies investigating the significance of danger signalling in contact allergy has been performed in mice, although similar mechanisms have been suggested in humans (158,159).

Danger signalling by the NLRP3-inflammasome IL-1β signalling pathway

The cellular mechanism to which a contact allergen induce local danger is related to activation of innate immune receptors. Specifically, danger signalling is promoted by direct or indirect PPR activation (primarily through the TLR and NLR families) upon contact allergen exposure. TLRs and NLRs are expressed by many skin resident cells both on the cell surface and in the endosomes (68,154,160–168). Direct activation of TLR4 (bacterial LPS receptor), has been described for metals such as nickel and cobalt (161,162), whereas indirect TLR activation occurs upon contact allergen induced formation of DAMPs. The DAMPs form in the extracellular space upon cellular damage and include molecules such as adenosine 5´triphosphate (ATP) (recognized by the P2X purinoceptor 7 receptor (P2X7R)), high-mobility group protein B1 (HMGB1) (recognized by TLR4) and release of extra cellular RNA (recognized by TLR3) (163,164). In addition, contact allergen-induced formation of reactive oxygen species (ROS) enforce damage of

the extracellular matrix (ECM), leading to release of low molecular-weight hyaluronic acids (HAs) (recognized by TLR2 and TLR4) (165–167). The importance of TLR signalling in order to develop contact allergy has been demonstrated in TLR3 knock out (KO) mice and $TLR2^{-/-}$ and $TLR4^{-/-}$ deficient mice (163,164,168). Generally, induction of TLR signals leads to genetic transcription by the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) protein complex, further leading to the synthesis of a variety of inflammatory cytokines such as IL-1α, pro-IL-1β, IL-6, IL-12, pro-IL-18, IL-23, tumor necrosis factor (TNF) and the NOD-like receptor family pyrin domain containing 3 (NLRP3) (68,131). However, TLR activation alone does not mediate sufficient danger signalling to induce sensitization. In addition, enzymatic activation of pro-IL-1β and pro-IL-18 by the NLRP3-inflammasome pathway is essential (68,169). The cytosolic assembly of the NLRP3-inflammasome is dependent on ATP activation of the ligand-gated ion channel P2X7R, where activation cause a shift in the intracellular potassium gradient by $P2X_7R$ mediated potassium efflux (170,171). When activated NLRP3 rapidly recruits the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD)), that enables NLRP3 linkage with pro-caspase-1 (169,172). Activation of the NLRP3-inflammasome induce cleavage of pro-caspase-1 into caspase-1, also known as IL-1β converting enzyme (ICE). When activated, ICE readily cleaves pro-IL-1β and pro-IL-18 into their biologically active forms, which are then released by the cell. In relation, IL-1β has been detected in murine skin already 15 min after TNCB exposure (131). Once in the extracellular space IL-1β binds and activates receptors of the interleukin-1 receptor (IL-1R) family leading to activation and formation of cytoplasmic Toll-like/IL-1R (TIR) domains. Activated TIR domains initiate an intracellular signalling cascade by recruitment of adaptor proteins including myeloid differentiation factor 88 (MYD88), IL-1R-associated kinases (IRAK) and TNF receptor–associated factor 6 (TRAF6) (169,172). Eventually, following several phosphorylation and ubiquitination steps, activation of pro-inflammatory transcription factors including NF-κB, activator protein 1 (AP-1) and mitogen-activated protein kinase (MAPK) leads to transcription and a vast amplification of several inflammatory cytokines including IL-6, IL-8, IL-12, IL-23 and interferon gamma (IFNγ) (160,169,172,173). The crucial role of IL-1β activation by the NLRP3-inflammasome, specifically in contact allergy, has been thoroughly demonstrated in studies using $P2X7^{-/-}$, $ASC^{-/-}$, $CARD9^{-/-}$, $NALP3^{-/-}$, caspase-$1^{-/-}$, $IL\text{-}1R^{-/-}$ or $MyD88^{-/-}$ deficient mice and in mice concomitantly treated with IL-1 receptor antagonist (Anakinra) or caspase-1 inhibitors (zYVAD or Ac-YVAD-cmk) (154,160,170,173–177).

1.2.6 Cytokines involved in the sensitization response to contact allergens

The importance of IL-1β danger signalling in the skin in response to irritants and contact allergens has been emphasized by several studies and IL-1β has been suggested as a master switch determining the fate of a chemical to function either as a sensitizer or a tolerogen (131,154,178,179). Several skin resident cells including KCs are capable of producing IL-1β when activated, although epidermal LCs seems to be the main source of IL-1β upon exposure with contact allergen (131,154,179–181). Despite the central role of IL-1β in contact allergy, other upstream cytokines have also been described with different roles in the response to contact allergens. In this regard, the importance of IL-18, that is also activated by the NLRP3-inflammasome, has been demonstrated during sensitization (176,182,183). Furthermore TNFα seems to work in synergy with IL-1β by providing an essential second signal that enables the mobilization of LCs to the dLN (183,184). Finally, the roles IL-12 and IL-23 in driving the formation of allergen-specific $CD4^+$ effector T cell subsets (Th1 and Th17) has been described in both murine and human studies (153,164,185,186)

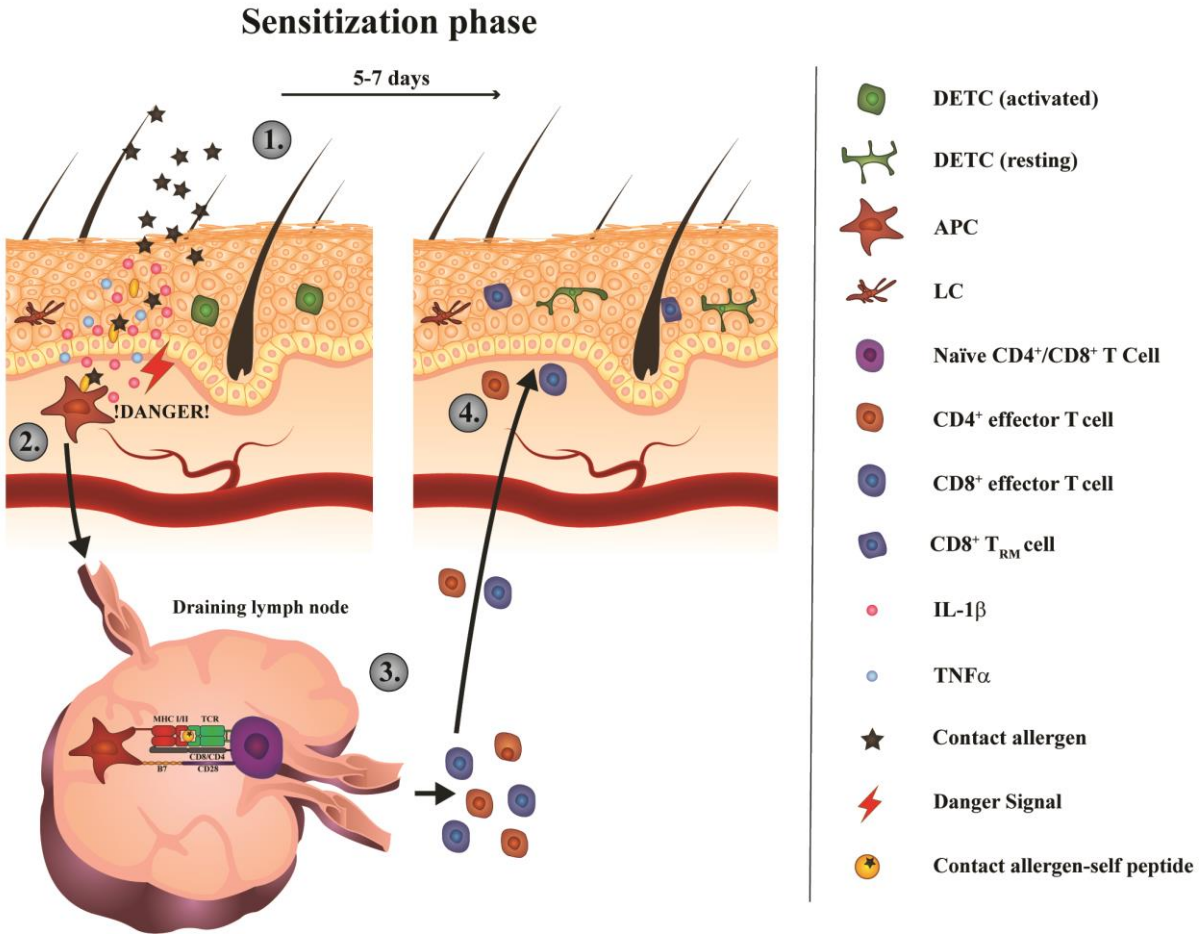

Figure 8. Model of the immunological skin responses during sensitization to contact allergens. *Activation of naïve-T cells following exposure to a contact allergen in murine skin.* ***(1)*** *The contact allergen penetrates the skin and activates skin-resident cells leading to local danger signalling by local IL-1β and TNFα expression.* ***(2)*** *Formation of contact allergen-self proteins which are recognized by APCs, leading to APC maturation and mobilization towards the draining lymph nodes (dLNs), while processing the contact allergen-self proteins into peptides expressed by MHC class-I/II molecules.* ***(3)*** *Activation of naïve $CD4^+$/$CD8^+$ T cells in the dLN leading to differentiation and proliferation of $CD4^+$/$CD8^+$ effector T cells.* ***(4)*** *Egress of allergen specific $CD4^+$/$CD8^+$ effector T cells back into the allergen exposed skin site 5-7 days later with subsequent formation of local $CD8^+$ T_{RM} cells in the epidermis. Dendritic epidermal T cell (DETC), antigen presenting cell (APC), langerhans cell (LC), major histocompatibility complex (MHC), interleukin (IL), tumor necrosis factor (TNF)*

1.2.7 T cells involved in ACD

T cells are recognized as central in the pathogenesis of ACD, however, different T cell subsets exist with different and sometimes contrasting roles in the response to contact allergens. Upon development in the thymus, two main T cell lineages are defined by their heterodimeric TCR composition, consisting of either a γ and a δ chain (γδ T cells) or an α and a β chain (αβ T cells) (59,62). In both humans and mice, the majority of γδ T cells are fully differentiated as they leave the thymus and many localize permanently in the skin early during embryonic development (187). Functionally, γδ T cells have been suggested to work as innate sentinel cells, by recognizing of variety of stress-related molecules leading to release of several pro-inflammatory mediators (62,188–190). In mice but not humans, the primary γδ T cell subset is the DETCs which are found in the epidermal layer of the skin. DETCs are reported to have both effector and regulatory roles upon exposure to contact allergens (59,62). The abundancy of DETCs can be altered by repeated local antigen exposure, as they are gradually displaced by $CD8^+$ T_{RM} cells. This was shown both by repeated viral-antigen exposures and repeated DNFB exposures (57,191). As the focus in this thesis is on the role αβ T cells, the role of DETCs and other γδ T cells in response to contact allergens will not be further described.

αβ T cells are a crucial part of the classical adaptive immune system. Following positive and negative selection in the thymus, the pool of αβ T cells collectively express a highly diverse TCR repertoire and is thus capable of recognizing a near infinite number of antigens (192). In contrast to γδ T cells, naïve αβ T cells are primarily found in the circulation and secondary lymphoid organs, as αβ T cells need TCR activation by their cognate antigen to egress and reside in peripheral tissues (15,16). Two main αβ T cell subsets are distinguished by their expression of

either the CD4 or the CD8 co-receptors. $CD4^+$ T cells are activated by APCs presenting peptide in the groove of MHC class-II and $CD8^+$ T cells are activated by APCs presenting peptide in the groove of MHC class-I molecules (17). Furthermore, the local cytokine environment in the dLNs during T cell activation tailor the differentiation of $CD4^+$ and $CD8^+$ T cell into subsets specialized in combating the ongoing external threat (136,193).

1.2.8 $CD4^+$ T cells – effectors and/or regulators of ACD?

$CD4^+$ T cells primarily function by stimulating other immune cells through the expression of surface receptors and secretion of cytokines. Thus, in response to cytokine stimulation in the dLNs, the $CD4^+$ T cells differentiate and expand into specific subtypes: IFNγ, TNFα and IL-2 producing T helper (Th) 1 cells identified by the T-box transcription factor (T-bet); IL-4, IL-5 and IL-13 producing Th2 cells identified by the transcription factor GATA-3; IL-17 (IL-17A/IL-17F) and IL-22 producing Th17 cells identified by the transcription factor RORγt; IL-4 and IL-21 producing T follicular helper (Tfh) cells often defined by a combination of markers such as CXCR5, inducible T-cell co-stimulator (ICOS), programmed cell death protein (PD)-1, and B cell lymphoma (Bcl)-6 and by their follicular localization in the dLN; and IL-10 and TGF-β producing Treg cells identified by the transcription factor forkhead box P3 (FoxP3) often in combination with the surface expression of the IL-2 receptor (CD25) (193). All $CD4^+$ T cell subtypes have been described with different roles in response to contact allergens (194–197).

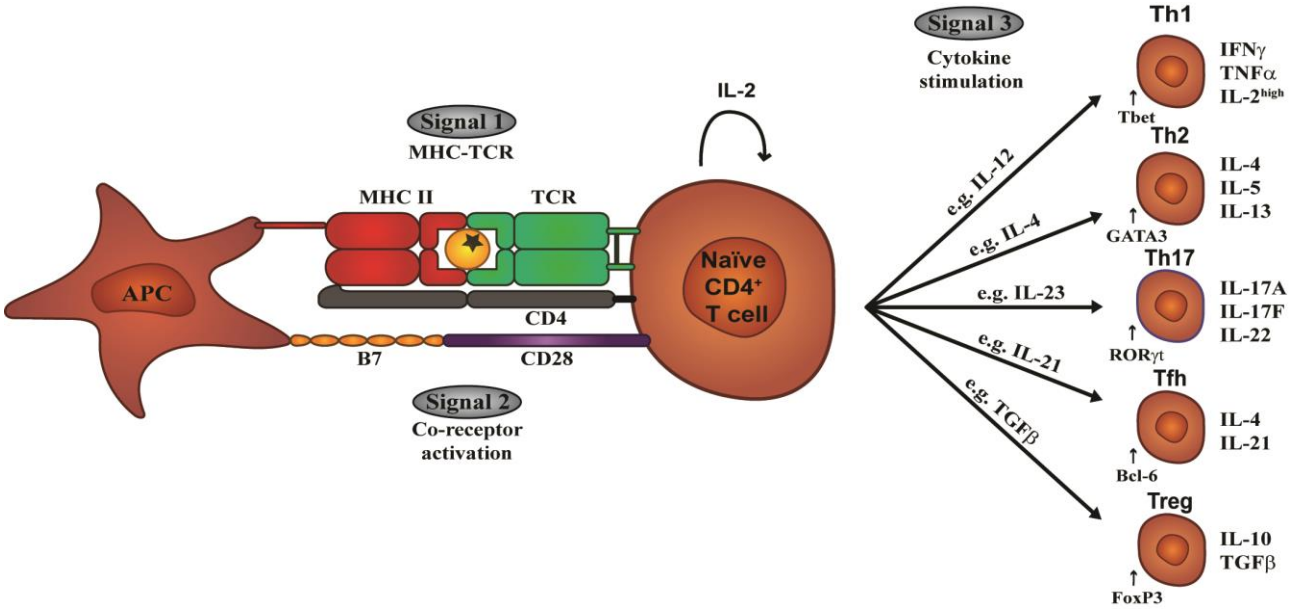

Figure 9. Model of the signalling events during activation and differentiation of CD4$^+$ T cells. *Activation and differentiation of both naïve CD4$^+$ and CD8$^+$ T cells depend on three essential signals occurring in the draining lymph node (dLN). This figure illustrates activation and differentiation of CD4$^+$T cell subsets.* ***Signal 1.*** *Antigen presenting cells (APCs) present peptide in a major histocompatibility complex (MHC), which ligate with T cell receptors (TCRs) and the CD4 co-receptor expressed by naïve CD4$^+$ T cells.* ***Signal 2.*** *Co-stimulatory signalling through ligation between APC expressed B7-molecules and T cell expressed CD28 co-receptors that initiate autocrine signalling through IL-2 binding the IL-2 receptors (CD25) on the activated CD4$^+$ T cell.* ***Signal 3.*** *Formation of a local cytokine signalling milieu in the dLN promotes differentiation and proliferation of specific T helper (Th) subsets. The main cytokines involved to induce differentiation of a specific CD4$^+$ T cell subset is indicated. Key transcription factors and effector cytokines are highlighted. Interleukin (IL), T follicular helper (Tfh), B cell lymphoma (Bcl), forkhead box protein P3 (FoxP3), interferon (IFN), tumor necrosis factor (TNF), tumor growth factor (TGF)*

Whether CD4$^+$ T cells are primarily effector or regulatory cells in the response to contact allergens, have been heavily debated by conflicting evidence reported using the conventional short-term CHS mouse model. Specifically, two early studies both using an antibody depletion regiment, either reported CD4$^+$ T cells as the main effector cells in the challenge response to DNFB (198), or as down-regulating the response (199). The later study did, however, indicate that some CD4$^+$ T cells were functioning as effector cells, because the CHS response to DNFB in anti-CD8 depleted mice was not completely abrogated (199). The conflicting evidence is likely due to differences in antibody dosage, as the first study used relatively small amounts of anti-CD4 depleting antibody and did not quantify the number of CD4$^+$ T cells upon challenge (198). However, other studies using CD4KO mice confirmed that CD4$^+$ T cells have effector roles in the challenge response to DNFB (194–196). The contradicting results were further investigated by a more recent study, suggesting that the decreased challenge response to DNFB observed in CD4$^{-/-}$ deficient mice, are caused by impaired MHC class-II dependent CD8$^+$ T effector cells and not loss of CD4$^+$ T cells (200). The suggestion was based on comparisons between a decreased DNFB challenge response in CD4$^{-/-}$ deficient mice and an increased DNFB challenge response in MHC class II$^{-/-}$ deficient mice (200). Accordingly, the later observation was in line with other studies (201,202) and differentiation of some naïve CD8$^+$ T cells were later shown to depend on MHC class II expression in response to DNFB (203). These data collectively imply that CD4$^+$ T cells are indeed regulatory in the challenge response to DNFB.

Still, observations on varying CD4$^+$ versus CD8$^+$ T cell responses have also been reported after exposure to other contact allergens. In one of the aforementioned studies, depletion of CD4$^+$ T

cells potentiated the response to both DNFB and oxazolone, whereas depletion of $CD8^+$ T cells completely abrogated the challenge response to DNFB, but only partially to oxazolone (199). Interestingly, depletion of both $CD4^+$ and $CD8^+$ T cells was required to abrogate the challenge response to oxazolone (199). Other studies have demonstrated that depletion of $CD4^+$ T cells is required to induce CHS responses to the weak fragrance allergens α-hexylcinnamaldehyde (HCA), eugenol (EUG), and hydroxycitronellal (HDCL) (204), and to induce DTH responses to topical amoxicillin exposure in mice (205). Furthermore, varying $CD4^+$ T cells responses to different contact allergens, were further emphasized by a study comparing T cell responses in mice after challenge with either DNFB or fluorescein isothiocyanate (FITC) (206). Using $CD4^{-/-}$ and $CD8^{-/-}$ deficient mice the authors show that CHS responses to FITC are highly dependent on $CD4^+$ T cells compared to DNFB responses. FITC responses were further shown to be primarily IL-4/Th2 cell driven (206). This suggests that $CD4^+$ T cells may behave as effector cells to some contact allergens (e.g. to FITC), as both effector and regulatory cells (e.g. to oxazolone), or solely as regulatory cells to (e.g. to DNFB and fragrance allergens).

T cell responses to oxazolone challenge were further investigated in a study using an adoptive transfer mouse model where either $CD4^+$ or $CD8^+$ effector T cells from oxazolone sensitized mice were transferred into T- and B cell deficient ($RAG^{-/-}$) recipient mice before challenge with oxazolone (207). The results from this model suggested that allergen-specific $CD4^+$ T cells in the dLNs orchestrate skin infiltration of $CD8^+$ effector T cells upon oxazolone challenge, as co-transfer of $CD4^+$ T cells was required to induce skin mobilization of transferred $CD8^+$ T cells (207). Another study showed that i.v. injections with recombinant IL-12, that induce activation of IFNγ-producing Th1 cells during sensitization, partially rescued the DNFB challenge response in anti-CD8 treated mice (208). However, IL-12 treatment only prolonged the challenge response to oxazolone (208). These results underpins that CHS responses to oxazolone are highly driven by $CD4^+$ T cells, whereas DNFB responses seem almost exclusively driven by $CD8^+$ T cells. In accordance, human studies have also demonstrated varying roles of $CD4^+$ and $CD8^+$ T in ACD with exposure to different contact allergens (144,209–213). $CD4^+$ T cell were described as the main infiltrating T cells in response to nickel (212), whereas infiltrating $CD8^+$ T cells were more prevalent in response to urushiol (213).

Although $CD4^+$ T cell responses in ACD seem highly allergen-dependent, most murine studies have focused on $CD4^+$ T cells as primarily regulatory cells that limit the inflammatory reaction to

contact allergens (197,202,204,214,215). In relation, immune tolerance towards a contact allergen mediated by $CD4^+$ T cells, has been demonstrated following systemic application with nickel and low doses of TNCB (216,217). This study demonstrated that the tolerogenic response was mediated specifically by IL-10 producing $CD4^+$ Treg cells (217). This is in line with others showing that depletion of $CD4^+CD25^+FoxP3^+$ Treg cells, restores the challenge response to TNCB in TNCB tolerant mice and that transfer of allergen-specific Treg cells or injections with anti-IL10 antibodies, significantly reduce the response to TNCB (148,218). In accordance, skin infiltrating $CD4^+$ T cells have been shown to correlate with the resolution of the inflammatory response to contact allergens and to the release of IL-10 (215,219). Thus, the potentiated and for some allergens prolonged CHS response observed in anti-CD4 depleted mice (199,202,204,215), is likely due to loss of IL-10 producing $CD4^+$ Treg cells, that develop simultaneously with allergen-specific effector T cells in the dLNs. Importantly, $CD4^+$ Tregs have also been shown to suppress the response to contact allergens by direct cell-cell induced anergy of allergen-specific $CD8^+$ T cells independently of IL-10 (151,220). Furthermore, upon DNFB exposure, direct Fas-FasL induced killing of allergen-specific effector $CD8^+$ T cells by $CD4^+$ Tregs in the dLNs, was demonstrated using anti-CD4 depleted and *gld*$^{-/-}$ (FAS-ligand deficient) mice (220). In conclusion, it seems that $CD4^+$ Treg cells, both through the expression of IL-10 and independently of IL-10, are pivotal in preventing excessive tissue damage upon contact allergen exposure and in subsequent healing of the skin.

1.2.9 $CD8^+$ T cells – effectors of ACD

Since the involvement of $CD8^+$ T cells was reported in a murine CHS model (199), several studies have focused on $CD8^+$ T cells as the primary effector cells in the challenge response to DNFB (197,200–202,215,221), DNCB (222), TNCB (214,223) oxazolone (197,224), fragrance allergens (204) and to palladium (a metal used in dental restorations and jewellery) (225). Using the conventional short-term CHS model $CD8^+$ T cells have been described to infiltrate murine skin early upon challenge, correlating with the initiation of the CHS response (215). Once recruited to peripheral tissues, $CD8^+$ T cells are known to induce FasL mediated apoptosis of Fas expressing skin cells, or to induce targeted killing of damaged skin cells by secretion of cytotoxic granules containing perforin and granzymes (226). In the context of CHS, $CD8^+$ T cells have been described to kill DNFB-modified skin cells using both Fas-FasL and perforin release in a redundant manner (143,144). Another important effector mechanism mediated by allergen-

specific $CD8^+$ T cells, is the release of pro-inflammatory cytokines i.e. IFNγ by $CD8^+$ T (Tc1) cells and IL-17 by $CD8^+$ T (Tc17) cells.

The central role of IFNγ-producing $CD8^+$ Tc1 cells in the challenge response to contact allergens has been demonstrated by several studies (140,196,197,221,224,225,227). The mechanism to which IFNγ induce CHS responses, are seemingly linked to promoting surface expression of MHC class II molecules by contact allergen-modified KCs, that thereby become susceptible for Th1 induced killing (228). After challenge with DNFB or DNCB, IFNγ release in the skin has also been associated with promoting local production of TNFα and ROS, specifically by release of inducible nitric oxide synthase (iNOS) (140,222).

The role of IL-17-producing $CD8^+$ Tc17 cells in the CHS response has been emphasized by multiple studies (140,221,222,229–231). Both IFNγ and IL-17 expressed by $CD8^+$ effector T cells was required in order to mount a proper CHS response to DNFB (140). However the key role of IL-17 in CHS reactions was highlighted in a study where antibody mediated neutralization of IL-17 was more efficient in supressing the challenge response to DNFB compared to IFNγ neutralization (230). Functionally, IL-17 produced by effector $CD8^+$ T cells can potentiate challenge responses to DNCB by inducing skin resident cells to release additional inflammatory cytokines and chemokines, resulting in the recruitment of circulating ($Ly\text{-}6C^+CD11b^+$) monocytes and ($Ly\text{-}6G^+CD11b^+$) neutrophils (222). In relation, adoptive transfer of DNFB primed $CD8^+$ T cells into $IL\text{-}17R^{-/-}$ deficient recipient mice, resulted in a significantly lowered mRNA expression of keratinocyte-derived chemokine (KC/CXCL1), IL-6, IL-1β and in an abrogated infiltration of $Gr\text{-}1^+CD11b^+$ cells (monocytes and neutrophils), when compared to wild type and $IFN\gamma R^{-/-}$ deficient recipient mice (140). Furthermore, subcutaneous (s.c.) injection with recombinant IL-17 prior to DNFB challenge, lead to CHS responses accompanied by leukocyte infiltration, whereas s.c. injections with recombinant IFNγ resulted in CHS responses signified by higher production of ROS (140). However, two other studies that measured the cytokine release on a protein level, suggested that IFNγ potentiate the expression of leukocyte recruiting chemokines CXCL1 and macrophage-inhibitory protein 2 (MIP-2/CXCL2) in the skin, indicating that IL-17 and IFNγ may work in synergy (221,231). These results were conducted using both $IFN\gamma^{-/-}$, $IL\text{-}17^{-/-}$ mice and mice treated with anti-IFNγ or anti-IL-17 depleting antibodies (221,231). A role for allergen-specific effector $CD8^+$ T cells in recruitment of circulating T cells has also been suggested, as a decreased CXCL10 mRNA expression was found in anti-CD8

depleted mice after challenge with DNFB (232). These data suggest that effector $CD8^+$ T cells are also involved in recruitment of additional CXCR3 expressing effector T cells into challenged skin.

1.2.10 The role of skin-resident memory T cells in ACD

Until recently, our understanding of ACD responses was primarily based on murine studies using of the conventional short-term CHS model. The use of this model reflects the view of ACD as being solely mediated by infiltrating T cells from the circulation. This view is also reflected in the classical description of ACD as a delayed type IV hypersensitivity response, where skin symptoms appear days after allergen re-exposure and in line with the observed inflammatory response measurable following patch testing of human allergic patients on day 3, 5 and 7 (70). However, recent discoveries of skin-resident memory T (T_{RM}) cells within allergen-experienced skin, suggests that patch testing and the short-term CHS model where challenge typically is performed on allergen-naïve skin, does not portray the full picture of ACD responses (53). In accordance, human ACD patients have long been described to experience immediate ACD reactions (< 24 hours), when re-exposed with contact allergen on a previously exposed skin area (Figure 10.) (55,233,234). Obviously, such reactions do not correspond to a classical type IV hypersensitivity reaction (70). The accelerated and enhanced contact allergic reactions was first described in animals by Arnason and Waksman already in 1963, who initially referred to the phenomenon as the so-called ´retest reaction´ (235). Arnason and Waksman's observations lead to a general debate the following years on possible retention of allergen-specific memory T cells in ACD healed skin sites (236–238). Yet, the memory T cell subset capable of surviving over time in the skin, now known as skin-resident memory T_{RM} cells, was first identified in the skin in 2009 following herpes simplex virus (HSV) infections (28). Evidence of the development of allergen-specific T_{RM} cells, were provided in an even more recent study by Gaide et al. from 2015 (54). Using different antigen exposure regiments including ovalbumin (OVA), adjuvant cholera toxin (CT), poxvirus modified Vaccinia Ankara (MVA) and DNFB, Gaide et al. defined a common clonal origin between central memory T (T_{CM}) cells in the dLN and T_{RM} cells in the skin (54). In addition, it was also shown that mice challenged with DNFB, following parabiotic surgery between naïve and DNFB sensitized mice, developed enhanced reactions to DNFB challenge when challenged on DNFB-experienced skin (peaking at ~ 24 hours). Furthermore, they showed that naïve parabiotic mice obtained circulating DNFB-specific memory T cells from

the DNFB sensitized parabiotic counterpart and thus developed typical type IV hypersensitivity responses to DNFB challenge (peaking at ~ 120 hours) (54). T_{RM} cells have now been demonstrated to mediate accelerated protection against re-infections, by rapid release of IFNγ, IL-17, TNFα and by secretion of granzymes and perforin (15,34,35,239,240).

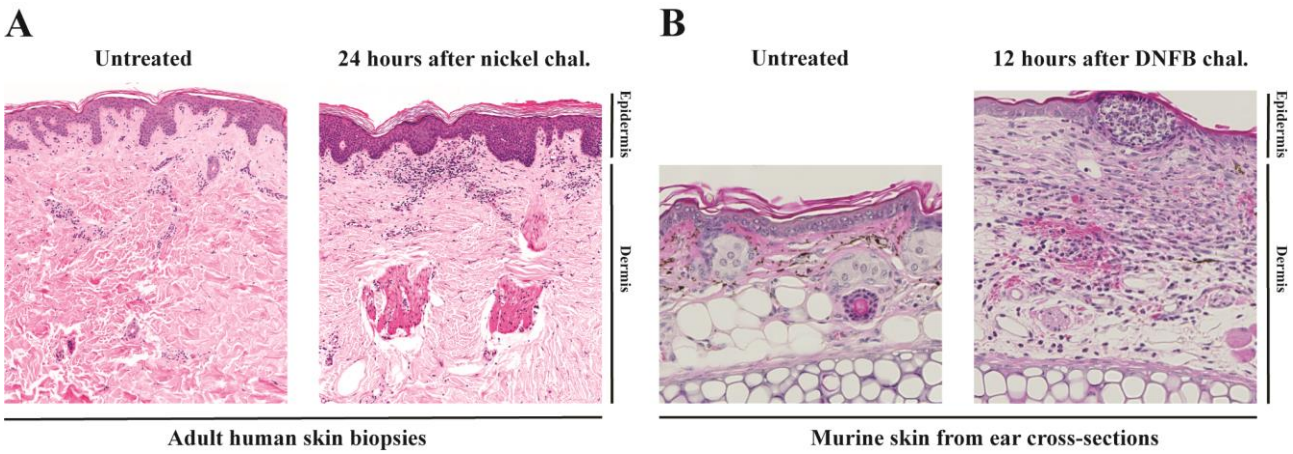

Figure 10. Examples of rapid local skin reactions in human and murine skin
(A) *hematoxylin- and eosin-stained images of;* ***(A)*** *human skin (epidermis and dermis), untreated (left) and 24 hours after nickel challenge directly on a nickel-experienced skin site (right); and* ***(B)*** *murine skin from ear cross-sections (epidermis and dermis), untreated (left) and 12 hours after challenge with 0.15% DNFB directly on DNFB-experienced ear skin (right). Skin swelling and both dermal and epidermal immune infiltrates are observable early (24 hours in human skin and 12 hours in murine skin) at allergen-experienced skin sites after challenge with contact allergen in both humans and mice. Challenge (chal.)*

Presence of epidermal-resident $CD8^+$ T_{RM} cells in DNFB-experienced skin of mice and in nickel-experienced skin of human nickel-allergic patients has been established in a recent study (55). Additionally, development of the epidermal-resident $CD8^+$ T_{RM} cells correlated with rapid challenge responses when re-exposure was performed at the same skin site, but not after challenge on an allergen-naïve skin area (55). Following *ex vivo* re-stimulation, the study further showed that the majority of the $CD8^+$ T_{RM} cells were IFNγ-producing Tc1 cells and some, but fewer, were IL-17 producing Tc17 cells (55). Yet another recent study demonstrated that DNFB-specific $CD69^+CD103^+CD8^+$ T_{RM} cells develop and reside in DNFB healed epidermis for at least a year after sensitization, although the number of $CD8^+$ T_{RM} cells decreased significantly without continued DNFB exposure (56). The study also demonstrated that repeated challenges with low doses of 0.05% DNFB, intensified the allergic response compared to a single challenge dose with 0.13% DNFB (56). By blocking inhibitory co-receptors (ICR); PD1, T-cell immunoglobulin and mucin-domain containing-3 (TIM3) and 2B4 expressed by $CD8^+$ T_{RM} cells, they showed a

massively intensified challenge response to the low (0.05%) DNFB dose (56). These results suggest that $CD8^+$ T_{RM} cells do not only induce local allergic inflammation, but also tolerate low doses of contact allergen to a certain threshold maintained by expression of ICRs. Finally, repeated (0.05%) DNFB exposure every other day on the same skin area intensifies the inflammatory response with every challenge, indicating that the allergen accumulates in the skin (56). Another study found that the magnitude of the allergic reaction to DNFB correlates with the number of $CD8^+$ T_{RM} cells and with the DNFB dosage applied to the skin (57). The same study also showed that the increase in the $CD8^+$ T_{RM} cell number after DNFB challenge was partially derived from local proliferation in the skin and partially from $CD8^+$ T cells recruited from the circulation (57). In cohesion, all of the mentioned studies only detected a few DNFB-specific $CD4^+$ T_{RM} cells of which all were located in the dermis, thus focusing on $CD8^+$ T_{RM} cells as the main effector cells behind rapid challenge responses to DNFB (55–57).

1.2.11 Mediators of local T_{RM} cell survival in the skin

Survival of T_{RM} cells in the skin is key in maintaining local long-term protection against infections, but it also preserves the chronic or reoccurring pathogenesis of autoimmune and allergic skin diseases (16,46–53). Consequently, prevention of T_{RM} cell re-activation and survival have been suggested as novel therapeutic strategies against ACD (53).

Long-term survival of skin-resident T_{RM} cells is linked to several mediators, including local cytokines signalling through IL-7, IL-15 and TGFβ (39,241). One study demonstrated that signalling through IL-15 and TGFβ was required to develop HSV-specific T_{RM} cells in the skin (39). In accordance, anti-TGFβ treatment was shown to significantly inhibit development of $CD4^+$ T_{RM} cells in a human engrafted skin model (15). Moreover, IL-15 was shown to be constitutively produced by KCs located in the isthmus and infundibulum regions in hair follicles and important for survival of epidermal-resident $CD8^+$ T_{RM} cells (241). The same study demonstrated that IL-7 was constitutively expressed by KCs located in the infundibulum and that IL-7 was primarily important for survival of skin-resident $CD4^+$ T_{RM} cells (241). In addition, using an adoptive transfer model of either DNFB-specific $CD4^+$ or $CD8^+$ T cells into $RAG^{-/-}$ deficient wild type, IL-7KO or IL-15KO mice they demonstrated that lack of either IL-7 (partially) or IL-15 (completely) abrogated the DNFB challenge response in recipient mice (241). The role of IL-15 has further been linked to $CD8^+$ T_{RM} cell cytotoxicity (release of granzyme B

and perforin) in human skin (16). Thus, the importance of IL-15 signalling for $CD8^+$ T_{RM} cell cytotoxicity and long-term survival has led to the suggestion of blocking the IL-15 receptor as a possible treatment targeting autoreactive $CD8^+$ T_{RM} cells in vitiligo (48) and in alopecia areata (50).

In addition to cytokine signalling, other intracellular mechanisms including local upregulation of apoptosis regulator Bcl-2 and the transcription factor aryl hydrocarbon receptor (AhR), have also been linked to $CD8^+$ T_{RM} cell survival in the skin (57,191,242). Interestingly, epidermal-resident $CD8^+$ T_{RM} cells were found with a superior metabolic fitness compared to DETCs, as they had increased glycolytic ATP production (57). Furthermore, two molecules that induce exogenous uptake of free fatty acids (FFA), namely fatty acid-binding protein 4 (FABP4) and 5 (FABP5), have further been linked to long-term survival of $CD8^+$ T_{RM} cells in both virally infected mice and human psoriatic patients (243).

Another debated aspect of T_{RM} cell survival is the possible need for constitutive antigen presentation. Some studies using HSV immunized mice, suggested that skin-resident $CD8^+$ T_{RM} cells do not need antigen presentation to survive (37,38). Moreover, using DNFB as a non-specific inflammatory stimulus to induce skin recruitment of HSV-primed (gBT-1) T cells, one study suggested that the surviving $CD8^+$ T_{RM} cells found in the skin one year after DNFB exposure, only developed an stayed as a result of non-specific inflammatory signalling and not antigen-presentation (37). Accordingly, another study used topical CXCL9 and CXCL10 treatment to ´pull´ transferred HSV-specific $CD8^+$ T cell into the genital tract inducing local development of HSV-specific $CD8^+$ T_{RM} cell (38). Significantly elevated numbers of gBT-1-specific $CD8^+$ T cells were found in the genital tract four weeks after treatment when compared to controls and these were demonstrated to mediate enhanced protection against HSV infections (38). Contrasting results have emerged recently on $CD8^+$ T_{RM} cell survival in the lungs, where antigen-presentation was shown to be required to facilitate long-term survival of adeno-based vector expressing influenza nucleoprotein (AdNP)-specific $CD8^+$ T cells (244). However, T_{RM} cell-mediated viral protection in the lungs and respiratory tract are known to decline faster compared to the skin (245).

T_{RM} cell survival in the skin in response to contact allergen exposures has only been investigated in a few studies with contradicting results (55,56). Using anti-DNP fluorescent microscopy and

western blot (WB), retention of DNP-moieties were detected in the epidermis 24 hours, but not 21 days after DNFB exposure (55). In contrast, another study was able to detect significant levels of DNP-moieties in the epidermis one month after DNFB exposure (56). It was further shown that the $CD8^+$ T_{RM} cells were located in near proximity to DNP-modified skin protein adducts and that the number of $CD8^+$ T_{RM} cells decreased in parallel with a decreased presence of DNP-moieties (56). From this it was suggested that survival of epidermal allergen-specific $CD8^+$ T_{RM} cells is dependent on cognate antigen/allergen stimulation (56). The reason for the conflicting results is unknown, however, methodical aspects such as incubation- and WB exposure times, may explain the inconsistent data.

1.2.12 The role of neutrophils in ACD

Neutrophils are classically categorized as short-lived innate immune cells that play a crucial part in the first line of defence against bacterial and fungal pathogens (189,190). At steady-state, neutrophils are found in the bone marrow and circulation from where they are rapidly recruited to peripheral tissues. Once in the skin, they mediate rapid pathogenic killing and tissue damage by release of cytotoxic granules containing proteases, AMPs and perforin, ROS production, expression of FasL, phagocytosis and extracellular release of DNA by neutrophil extracellular traps (NET) (68,246,247). Neutrophils are directed to infected, damaged or allergen exposed skin, by several pro-inflammatory mediators and by endothelial integrins expressed when activated (189,190). A well-known mechanism involved in neutrophil recruitment is chemokines capable of binding CXCR1 and CXCR2 expressed on the surface of circulating neutrophils. In relation, the role of CXCR2 in response to different contact allergens have been emphasised in $CXCR2^{-/-}$ deficient mice (248–250). The key skin chemoattractant mediators acting on CXCR1 and CXCR2 are IL-8/CXCL8 (only in humans), CXCL1 and CXCL2 (246,251–253). An early (2-4 h) increase in CXCL2 mRNA levels was detected in TNCB exposed skin of non-sensitized mice (131). Chemokine expression of both CXCL1 and CXCL2 has been detected in the skin of both sensitized and untreated mice early after DNFB exposure, although the chemokine levels were significantly higher in sensitized animals (221). Interestingly, CXCL1 and CXCL2 release was further demonstrated to be partially abrogated in anti-CD8 depleted mice and completely abrogated in $CD8^{-/-}$ deficient mice, whereas anti-$CD4^+$ and $CD4^{-/-}$ deficient mice showed increased levels of CXCL1 and CXCL2, indicating that $CD8^+$ T cells are highly involved in neutrophil recruitment after DNFB exposure (221). In addition, release of pro-inflammatory

cytokines (IL-1β, IL-6 and TNFα) and chemokines (CXCL1, CXCL2 and CXCL5), followed by recruitment of neutrophils to the skin, is potentiated by mechanical damage to the skin such as scratching after exposure to TNCB (254). Release of leukotriene B4 (LTB4) by neutrophils in response to skin disruption by scratching or tape stripping, has been demonstrated to augment neutrophil infiltration by a positive feedback loop (255). Furthermore, dermal γδ T cells, $CD4^+$ T (Th17) cells and $CD8^+$ T (Tc17) cells, are all likely involved in neutrophil recruitment in response to different contact allergens by their release of IL-17 (140,190,212,222,230). In synergy with IFNγ and TNFα, several studies have demonstrated IL-17 to promote expression of several pro-inflammatory molecules including CXCL1, CXCL2 and IL-8 (in human) expressed by KCs and endothelial expression integrins (140,230,256–259). In the challenge response to DNFB, IL-$17^{-/-}$ deficient mice had significantly reduced CXCL1, CXCL2 and CHS responses (229), depletion of IL-17 resulted in decreased CHS and neutrophil infiltration (190), and mice injected subcutaneously with recombinant IL-17 before challenge, showed a potentiated CHS response by the recruitment of neutrophils to the skin (140).

The role of neutrophils in the development of contact allergy and in ACD has been investigated by several studies using the conventional short-term CHS model. In this model, depletion of neutrophils or neutrophil recruiting chemokines have collectively been demonstrated to inhibit both sensitization and challenge responses (221,231,260–263). In the sensitization phase, along with most pro-inflammatory mediators, recruitment of neutrophils and release of CXCL1 and CXCL2, has been shown to depend on prior IL-1R activation (177,264). In relation, early recruitment of neutrophils to allergen exposed skin during the sensitization phase is regulated by dermal-resident macrophages and mast cells expressing IL-1R (260,265). Neutrophils have further been shown to potentiate IL-1β and APC mobilisation as both were decreased in Mcl-$1^{-/-}$ (neutrophil deficient) or anti-Ly-6G (neutrophil depleted) mice following sensitization with TNCB or FITC (260). Interestingly, a recent study demonstrated that neutrophils by their release of protease cathepsin G (CG), inhibit IL-12 production by APCs during sensitization with DNFB and loss of this mechanism skewed $CD4^+$ T cell responses from regulatory into Th1 and Th17 cell responses (249). The study further demonstrated that both anti-Ly-6G and anti-Gr-1 mediated depletion of neutrophils, rescued the challenge response in $CD8^+$ T cell depleted mice (249). These data indicate that neutrophil released CG inhibit IL-12 and thus drives the sensitization response towards a $CD8^+$ effector T cell response, whereas absence of this mechanism induce compensatory effector responses by $CD4^+$ T cells in an IL-12-dependent manner (266). This may

explain previous studies showing that IL-12Rβ2 deficiency alone did not affect the challenge response to TNCB (164), while treatment with recombinant IL-12 could break tolerance to DNTB (153).

The role of neutrophils in the elicitation phase (when challenged on an allergen-naïve skin site), has also been investigated experimentally. Adoptive transfer models and anti-Ly-6G depletion of neutrophils between sensitization and challenge with TNCB, suggested that neutrophils are promoting the challenge response (260). In accordance, *in vivo* and *in vitro* experiments have shown that challenge with DNFB on allergen-naïve skin promote CXCL1 release by KCs, resulting in neutrophil recruitment and that both CXCL1 and neutrophil infiltration are required to augment the DNFB challenge response (261). The severity of inflammation was also shown to correlate with the DNFB dosage applied during challenge and with the number of recruited neutrophils into the challenged skin site (262). An amplified challenge response was also observed when neutrophils or recombinant CXCL1 were injected directly into the skin concomitantly with low (0.04 % and 0.008 %) DNFB challenge dosages (262). In addition, a recent study showed that once in the skin, neutrophils are capable of NETosis formation in the challenge response to DNFB (267).

A role for neutrophils in recruitment of allergen-specific effector $CD8^+$ T cells into contact allergen exposed skin has also been suggested (263,268,269). Neutrophil induced recruitment of allergen-specific $CD8^+$ effector T cells following expression of perforin and FasL, was found in *gld*/perforin$^{-/-}$ deficient mice after DNFB challenge (263). Furthermore, neutrophils have been demonstrated to produce T cell recruiting chemokines including CXCL9 and CXCL10, in delayed type hypersensitivity responses in mice treated with Herpes simplex virus type-1 antigen or LPS (268,269). However, allergen-specific $CD8^+$ T cells have also been shown important for recruitment of neutrophils in to allergen exposed skin (221). Using ab-depletion models, adoptive transfer models, RAG-1$^{-/-}$, CD8$^{-/-}$, IFNγ$^{-/-}$ and IL-17$^{-/-}$ deficient mice, a study suggested that the expression of CXCL1, CXCL2 and the recruitment of neutrophils into the challenged skin site depended on activation of IFNγ- and IL-17-producing allergen-specific $CD8^+$ T cells (221). This raises the question on which cell type that infiltrate the allergen exposed skin first to signal the other. This has not been addressed directly, however, studies have shown that allergen-specific effector T cells are recruited into the dermal layer of allergen-naïve skin early after challenge independently of neutrophils (264,270). The mechanism behind was suggested to be IL-1R

activation of dermal-resident macrophages, leading to formation of dermal perivascular immune cell clusters or so-called inducible skin-associated lymphoid tissues (iSALT), consisting of dDCs, monocytes and effector T cells (264,270). In accordance, local release of IFNγ and IL-17, likely by the effector T cells located in these iSALT, can stimulate CXCL1 and CXCL2 expression by endothelial cells, leading to recruitment of neutrophils and subsequently potentiate a massive infiltration of allergen-specific $CD8^+$ T cells (221,231,261–263). Taken together, these data suggest that allergen-specific $CD8^+$ T cells initiate the response by recruitment of neutrophils which then amplify infiltration of additional allergen-specific T cells and vice versa. However, this mechanism leading to immune cell recruitment into allergen-naïve skin upon challenge needs further investigation.

Objectives

In our general pursuit towards discovering new and improved therapeutic targets for ACD, the overall aim of this thesis was to investigate the immunological mechanisms behind local T cell mediated skin reactions to contact allergens. More specifically, the studies of the thesis collectively aimed to improve our understanding on how allergen-specific resident memory T_{RM} cells develop, survive and induce inflammation in the skin after contact allergen exposure.

Study I: Formation of epidermal-resident memory $CD8^+$ T_{RM} cells occurs locally within allergen-experienced skin and upon re-exposure they induce both accelerated and enhanced ACD reactions. The aim of this study was to investigate the mechanism behind $CD8^+$ T_{RM} cell mediated local inflammation using the experimental contact allergen DNFB and further to investigate if such a mechanism could be inhibited by molecules targeting the $CD8^+$ T_{RM} cell induced inflammatory pathway.

Study II: Based on the results described in study I, the aim of study II was first to investigate whether similar local $CD8^+$ T_{RM} cell induced ACD flare-ups developed after exposure to clinically relevant contact allergens (cinnamal, PPD and MI) that are known to be among the most prevalent contact allergens causing ACD in the general population. In this study, we further aimed to study the role of $CD4^+$ T cells in the local response to contact allergens as the role of $CD4^+$ T cells in ACD has been heavily debated in the literature.

Study III: The first two studies highlighted the pathogenic role of epidermal-resident $CD8^+$ T_{RM} cells in local ACD flare-ups. The third and final study of this thesis aimed to investigate how allergen-specific $CD8^+$ T_{RM} cells are maintained over time in the epidermis. To do this, we investigated the survival rate, proliferative capacity and TCR specific activation of the epidermal-resident $CD8^+$ T_{RM} cells over a year. In addition, we studied whether contact allergen-induced modifications of skin proteins are permanently altered.

Study I

$CD8^+$ tissue-resident memory T cells recruit neutrophils that are essential for flare-ups in contact dermatitis

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ORIGINAL ARTICLE

Basic and Translational Allergy Immunology

CD8$^+$ tissue-resident memory T cells recruit neutrophils that are essential for flare-ups in contact dermatitis

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Abstract

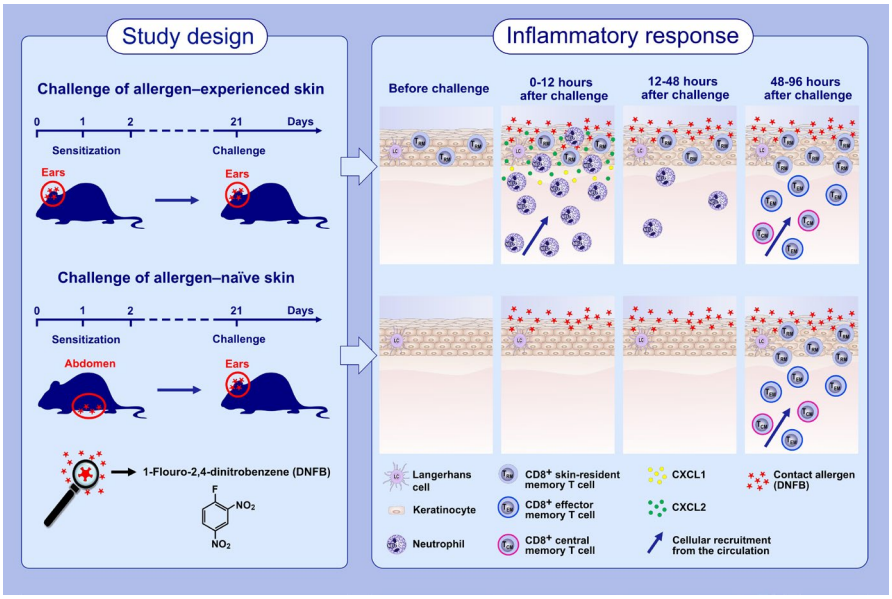
Background: Allergic contact dermatitis (ACD) is classically described as a delayed-type hypersensitivity reaction. However, patients often experience flare-ups characterized by itching erythema, edema, and often vesicles occurring within hours after re-exposure of previously sensitized skin to the specific contact allergen. Recent studies have indicated that skin-resident memory T (T_{RM}) cells play a central role in ACD. However, the pathogenic role of T_{RM} cells in allergen-induced flare-ups is not known.

Methods: By the use of various mouse models and cell depletion protocols, we investigated the role of epidermal T_{RM} cells in flare-up reactions to the experimental contact allergen 1-fluoro-2,4-dinitrobenzene. The inflammatory response was measured by changes in ear thickness, and the cellular composition in epidermis was determined by flow cytometry and confocal microscopy. Finally, adaptive transfer and inhibitors were used to determine the role of T_{RM} cells, neutrophils, and CXCL1/CXCL2 in the response.

Results: We show that CD8$^+$ T_{RM} cells initiate massive infiltration of neutrophils in the epidermis within 12 h after re-exposure to the contact allergen. Depletion of neutrophils before re-exposure to the allergen abrogated the flare-up reactions. Furthermore, we demonstrate that CD8$^+$ T_{RM} cells mediate neutrophil recruitment by inducing CXCL1 and CXCL2 production in the skin, and that blockage of the C-X-C chemokine receptor type 1 and 2 inhibits flare-up reactions and neutrophil infiltration.

Conclusion: As the first, we show that epidermal CD8$^+$ T_{RM} cells cause ACD flare-ups by rapid recruitment of neutrophils to the epidermis.

KEYWORDS
allergic contact dermatitis, CXCL1, CXCL2, epidermal-resident T cells, neutrophils



GRAPHICAL ABSTRACT

Abbreviations: CXCL, (C-X-C motif) ligand; TCM, central memory T cell; TEM, effector memory T cell; TRM, tissue-resident memory T cell

1 | INTRODUCTION

Allergic contact dermatitis (ACD) is a common T cell–mediated skin disease affecting about 10% of the adult population.[1] ACD is induced by exposure of the skin to low-molecular weight chemicals called haptens or contact allergens that have the ability to react with proteins in the skin.[2] Hapten-modified proteins are picked up by Langerhans and dendritic cells that subsequently migrate to the skin-draining lymph nodes where they activate specific T cells that recognize the hapten-modified self-protein in the context of major histocompatibility complex molecules. ACD is characterized by rapid flare-ups/exacerbations with intensely itching erythema, edema, and often vesicles occurring within hours after re-exposure to the specific contact allergen. Interestingly, these rapid flare-ups only develop at skin sites previously exposed to the contact allergen.[3] Recent studies have indicated that skin-resident memory T (T_{RM}) cells play a central role in the flare-up reactions in ACD.[4-7] Recently, Gaide et al elegantly demonstrated that T_{RM} cells in the skin mediate rapid, intense flare-up reactions whereas circulating memory T cells mediate delayed, attenuated reactions in an experimental model of ACD.[4] These observations were confirmed and extended in both mouse and man by Schmidt et al who found that sensitization to a specific contact allergen induces a strong, long-lasting local memory that is mediated by allergen-specific IL-17A- and IFNγ-producing $CD8^+$ T_{RM} cells in the epidermis.[5] In line with these studies, Gamradt et al demonstrated that long-lived $CD8^+$ T_{RM} cells accumulated in the epidermis of skin exposed to contact allergens and that these $CD8^+$ T_{RM} cells mediated the flare-up reactions following allergen challenge,[6] and Gadsbøll et al demonstrated that the intensity of the ACD flare-up reactions correlated with the number of $CD8^+$ epidermal T_{RM} cells.[7]

In healthy individuals, T_{RM} cells are crucial mediators of local infection control.[8,9] Thus, recognition of pathogens by T_{RM} cells leads to rapid cytokine production that augment the ability of neighboring cells to resist and combat infection, and activates the endothelium in local blood vessels to recruit neutrophils and other leucocytes to the site of infection.[10-15]

Although it has been shown that allergen-specific epidermal T_{RM} cells produce IL-17A and IFNγ within 4 h after challenge with contact allergens,[5] the pathogenic mechanisms induced by T_{RM} cells that lead to ACD flare-ups are not known. Here, we show that $CD8^+$ T_{RM} cells initiate infiltration of high numbers of neutrophils in the epidermis within 12 h after re-exposure to contact allergen. Depletion of neutrophils before re-exposure to the contact allergen abrogated the rapid flare-up reaction showing that neutrophils are essential for flare-up reactions. We demonstrate that $CD8^+$ T_{RM} cells mediate neutrophil recruitment by inducing CXCL1 and CXCL2 chemokine production in the skin, and in accordance with the pathogenic role of the neutrophils, we found that blockage of the C-X-C chemokine receptor type 1 and 2 (CXCR1 and CXCR2) inhibited the flare-up reactions and neutrophil infiltration in parallel. In conclusion, this study shows that epidermal $CD8^+$ T_{RM} cells cause ACD flare-ups by recruiting neutrophils to the epidermis.

2 | METHODS

2.1 | Mice

Six to 8 weeks old C57Bl/6J mice were purchased from Janvier Labs and used in all experiment of this study. All mice were housed in a specific pathogen-free animal facility at the Department of Experimental Medicine, University of Copenhagen in accordance with the national animal protection guidelines (license number 2018-15-0201-01409).

2.2 | Sensitization and challenge of contact hypersensitivity (CHS)

Female mice were sensitized on three consecutive days (day 0–2) by epicutaneous painting with 0.15% 1-Fluoro-2,4-dinitrobenzene (DNFB) (Sigma-Aldrich) diluted in a 1:4 solution of olive oil:acetone (OOA). Mice were painted either with 25 μl on the dorsum of both ears or with 50 μl on a similar sized area (~2 cm) on the shaved abdomen. Control groups were painted with OOA during sensitization. Mice were challenged after a minimum of 21 days by applying 25 μl DNFB on the dorsum of both ears. Ear thickness was measured using an electronic digital micrometer (Mitutoyo Corporation, Model: PK-1012CPX) and values presented were normalized to mean ear thickness within each group measured at 0 h. Depending on the intended ex vivo analysis, mice were euthanized and the ears, spleen and the submandibular and cervical draining lymph nodes were isolated for further analyses. All experiments were repeated on at least two separate occasions and data were pooled from both experiments resulting in a minimum of eight mice per group ($n = 8$).

2.3 | In vivo cellular depletion and receptor blocking and adoptive transfer

Please see Data S1.

2.4 | Ex vivo cellular phenotyping by flow cytometry (FC)

Please see Data S1.

2.5 | Ex vivo ImageStream analysis of epidermal neutrophils

Please see Data S1.

2.6 | Ex vivo fluorescent microscopy

Please see Data S1.

2.7 | Quantification of chemokines in skin

Please see Data S1.

2.8 | Statistics

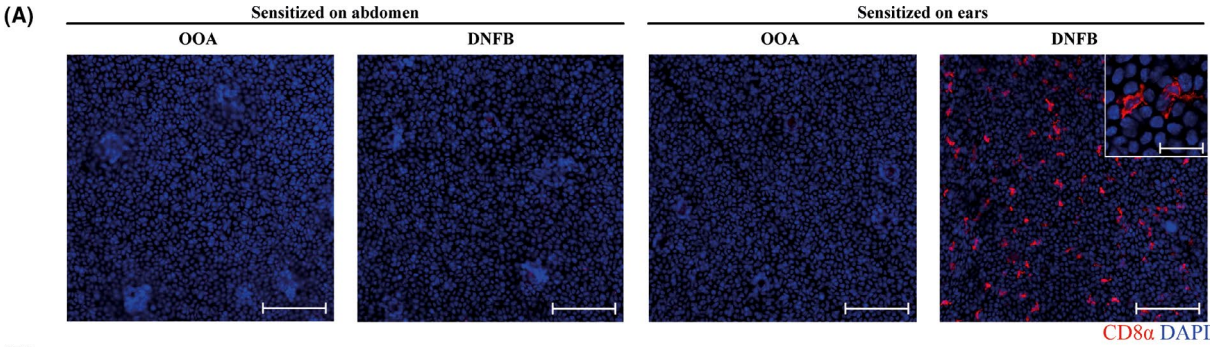
Statistical comparisons were performed using GraphPad Prism version 7.0. Gaussian distributions were tested using Shapiro-Wilk's normality test, and statistical significance was tested using one-way ANOVA, two-way ANOVA repeated measures or Students unpaired *t*-tests as indicated in the figures. For conditions where Gaussian distribution was not found a non-parametric Mann-Whitney *U* test was performed. Multiple comparisons were adjusted post hoc using Bonferroni's multiple comparisons test. Significance levels are illustrated in all figures as; Not significant (ns) = $p > .05$, *$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ****$p \leq .0001$.

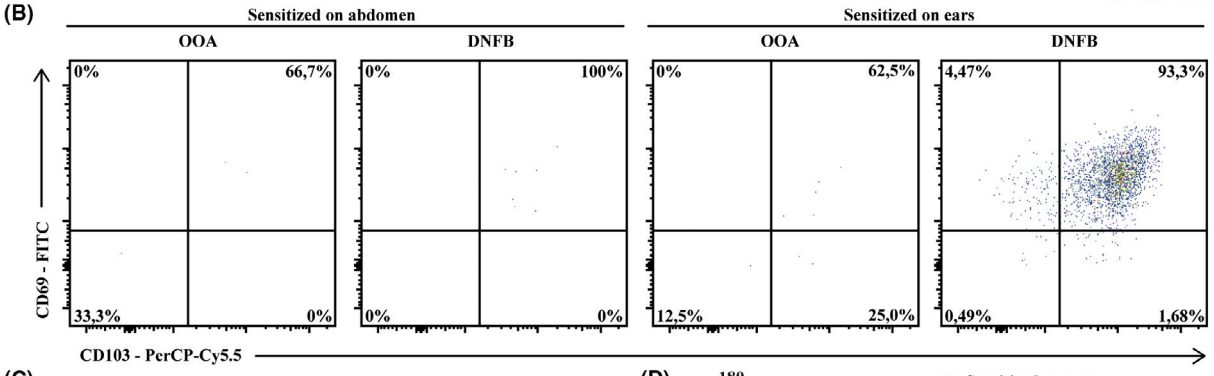
3 | RESULTS

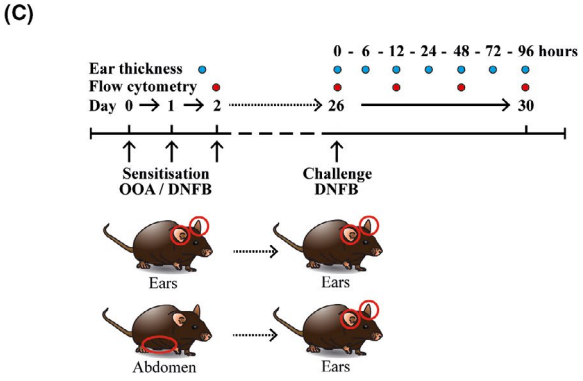
3.1 | Allergen-experienced skin harbors epidermal $CD8^+$ T_{RM} cells and mounts intense and rapid flare-ups upon allergen re-exposure

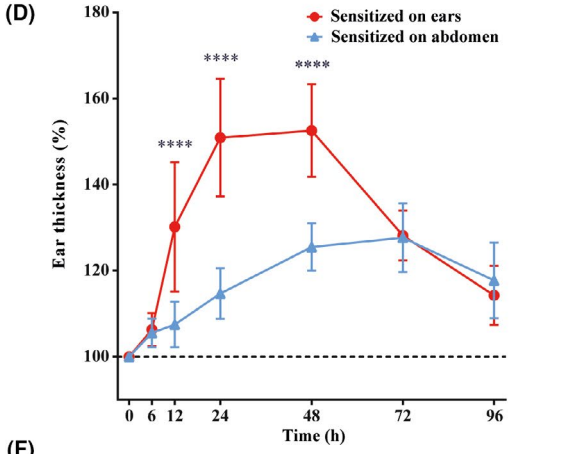
The vast majority of previous studies on immune mechanisms involved in ACD have used the acute CHS assay in mice. Importantly, in the acute CHS assay, sensitization and challenge with the contact allergen are separated by only 5 days, which is not enough time for adaptive immune memory to develop.[4,16] Thus, the acute CHS assay does not truly reflect the conditions in human ACD where individuals repeatedly are exposed to the contact allergen resulting in the generation of memory T cells in the skin and the characteristic flare-ups. To study the role of T_{RM} cells versus circulating effector/memory T cells in ACD, we developed a modified CHS assay in which we sensitized mice on their ears or on the abdomen for three consecutive days with 2,4-dinitro-1-fluorobenzene (DNFB) or with the vehicle control (OOA). Twenty-four days after sensitization, we determined the presence of $CD8^+$ T_{RM} cells in the epidermis isolated from ears of the mice by immunohistochemistry and FC. In accordance with previous studies, dendritic-shaped $CD69^+CD103^+CD44^+CD62L^-CD8^+$ T_{RM} cells were present in the epidermis of allergen-experienced

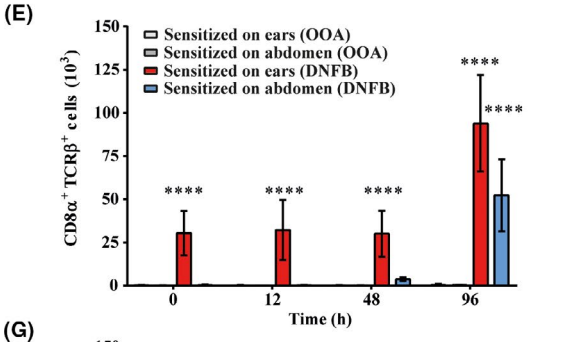
FIGURE 1 Allergen-experienced skin harbors epidermal $CD8^+$ T_{RM} cells and mounts intense and rapid flare-ups upon allergen re-exposure. (A) CD8α (red) and DAPI (blue) stained fluorescent microscopy images of non-challenged epidermal ear sheets isolated 24 days after sensitization with OOA or DNFB on the ears or abdomen. Scale bars; 100 μm (20× images) and 20 μm (63× image) ($n = 2$). (B) Representative dot plots of CD69 and CD103 expression in $CD8\alpha^+TCR\beta^+CD44^+CD62L^-$ epidermal cells isolated from non-challenged ears 24 days after sensitization with OOA or DNFB on the ears or abdomen ($n = 8$). (C) Experimental setup: Mice were exposed to DNFB or OOA at day 0–2 on the ears or abdomen. Mice were euthanized or challenged with DNFB on both ears on day 26. (D) Ear thickness was measured before challenge at 0 h and 6, 12, 24, 48, 72, and 96 h after challenge. Values are normalized to mean ear thickness at 0 h ($n = 8$). (E–H) Mean number of live epidermal $CD8^+$ T-cell subsets in ear sheets isolated at 0, 12, 48, and 96 h after the challenge ($n = 8$). (E) $CD8^+$ T cells ($CD8\alpha^+TCR\beta^+$ cells); (F) $CD8^+$ T_{RM} cells ($CD103^+CD69^+CD44^+CD8\alpha^+TCR\beta^+$ cells); (G) $CD8^+$ T_{EM} cells ($CD44^+CD8\alpha^+TCR\beta^+$ and either CD103 or CD69 single positive or double negative cells); (H) $CD8^+$ T_{CM} cells ($CD44^+CD62L^+CD8\alpha^+TCR\beta^+$). Statistical comparisons; two-way ANOVA. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > .05$, *$p \leq .05$, **$p \leq .01$, ****$p \leq .0001$

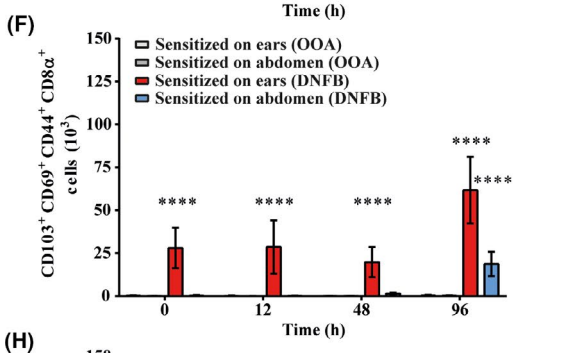

(A)
Sensitized on abdomen
Sensitized on ears
OOA
DNFB
OOA
DNFB
CD8α DAPI

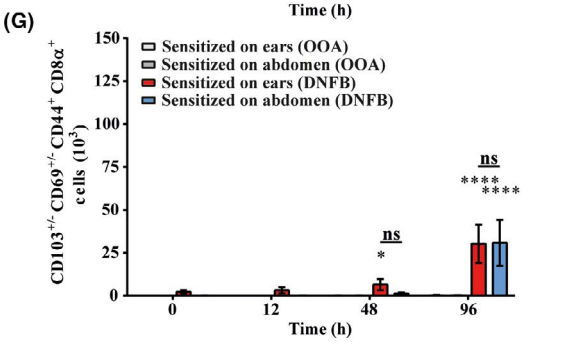

(B)
Sensitized on abdomen
Sensitized on ears
OOA
DNFB
OOA
DNFB
0%
66,7%
33,3%
0%
0%
100%
0%
0%
0%
62,5%
12,5%
25,0%
4,47%
93,3%
0,49%
1,68%
CD69 - FITC
CD103 - PerCP-Cy5.5

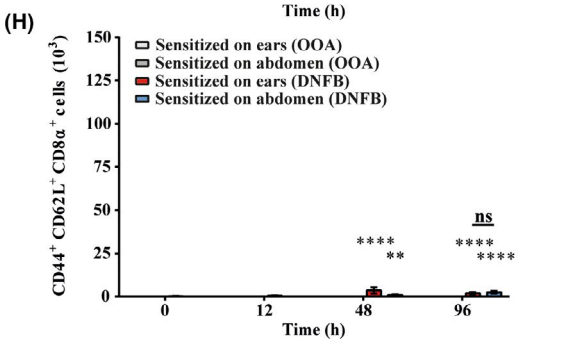

(C)
0 - 6 - 12 - 24 - 48 - 72 - 96 hours
Ear thickness
Flow cytometry
Day 0 → 1 → 2 → 26 → 30
Sensitisation
OOA / DNFB
Challenge
DNFB
Ears
Ears
Abdomen
Ears


(D)
Sensitized on ears
Sensitized on abdomen
Ear thickness (%)
Time (h)


(E)
Sensitized on ears (OOA)
Sensitized on abdomen (OOA)
Sensitized on ears (DNFB)
Sensitized on abdomen (DNFB)
CD8α+ TCRβ+ cells (10³)
Time (h)


(F)
Sensitized on ears (OOA)
Sensitized on abdomen (OOA)
Sensitized on ears (DNFB)
Sensitized on abdomen (DNFB)
CD103+ CD69+ CD44+ CD8α+ cells (10³)
Time (h)


(G)
Sensitized on ears (OOA)
Sensitized on abdomen (OOA)
Sensitized on ears (DNFB)
Sensitized on abdomen (DNFB)
CD103+/- CD69+/- CD44+ CD8α+ cells (10³)
Time (h)


(H)
Sensitized on ears (OOA)
Sensitized on abdomen (OOA)
Sensitized on ears (DNFB)
Sensitized on abdomen (DNFB)
CD44+ CD62L- CD8α+ cells (10³)
Time (h)

skin from mice sensitized on the ears but not in allergen-naïve skin of mice sensitized on the abdomen (Figure 1A,B and Figure S1 for gating strategy).[5,6,8] Having established the model, we studied the nature of the challenge response in allergen-experienced skin versus allergen-naive skin in sensitized mice as outlined in Figure 1C. Twenty-four days after sensitization on the ears or the abdomen, the mice were challenged on the ears and the inflammatory response and T-cell infiltration were measured by changes in ear thickness and by FC, respectively, at zero to 96 h after the challenge. In allergen-experienced skin that harbors epidermal $CD8^+$ T_{RM} cells, we found an intense and rapid flare-up reaction peaking already 24 h after allergen re-exposure (Figure 1D). In contrast, we found that the ACD response in allergen-naïve skin in sensitized mice was less intense and furthermore delayed compared to the response in allergen-experienced skin. Thus, the ACD response in the allergen-naïve skin peaked at 72 h in line with a classical delayed type IV allergic reaction and with the optimal time point for patch test readings in the clinical setting.[17] We next determined the presence and recruitment of various T-cell subsets to the epidermis during the challenge response in allergen-experienced and allergen-naïve skin. In accordance with the experiment described above, the epidermis of allergen-experienced skin contained $CD8^+$ T_{RM} cells already at the time of challenge, whereas allergen-naïve skin did not (Figure 1E,F and Figure S1 for gating strategy). Interestingly, the number of $CD8^+$ T_{RM} cells in the epidermis of allergen-experienced skin did not increase for the first 48 h after challenge. At 96 h, the number of $CD8^+$ T_{RM} cells had increased in the allergen-experienced skin, and $CD8^+$ T_{RM} cells were also found in allergen-naïve skin at this time point. $CD8^+$ T effector memory (T_{EM}) cells infiltrated the epidermis of allergen-experienced and allergen-naïve skin with similar kinetics and were clearly detected 96 h after the challenge (Figure 1G). The number of epidermal $CD8^+$ central memory (T_{CM}) cells was generally low or absent in all mice at all time points tested (Figure 1H). Epidermal $CD4^+$ T_{RM} cells were not generated during sensitization in our model (Figure S2). However, an increase in $CD4^+$ T_{RM} cells was seen 48 h after the challenge (Figure S2).

3.2 | ACD flare-up reactions are dependent on neutrophils

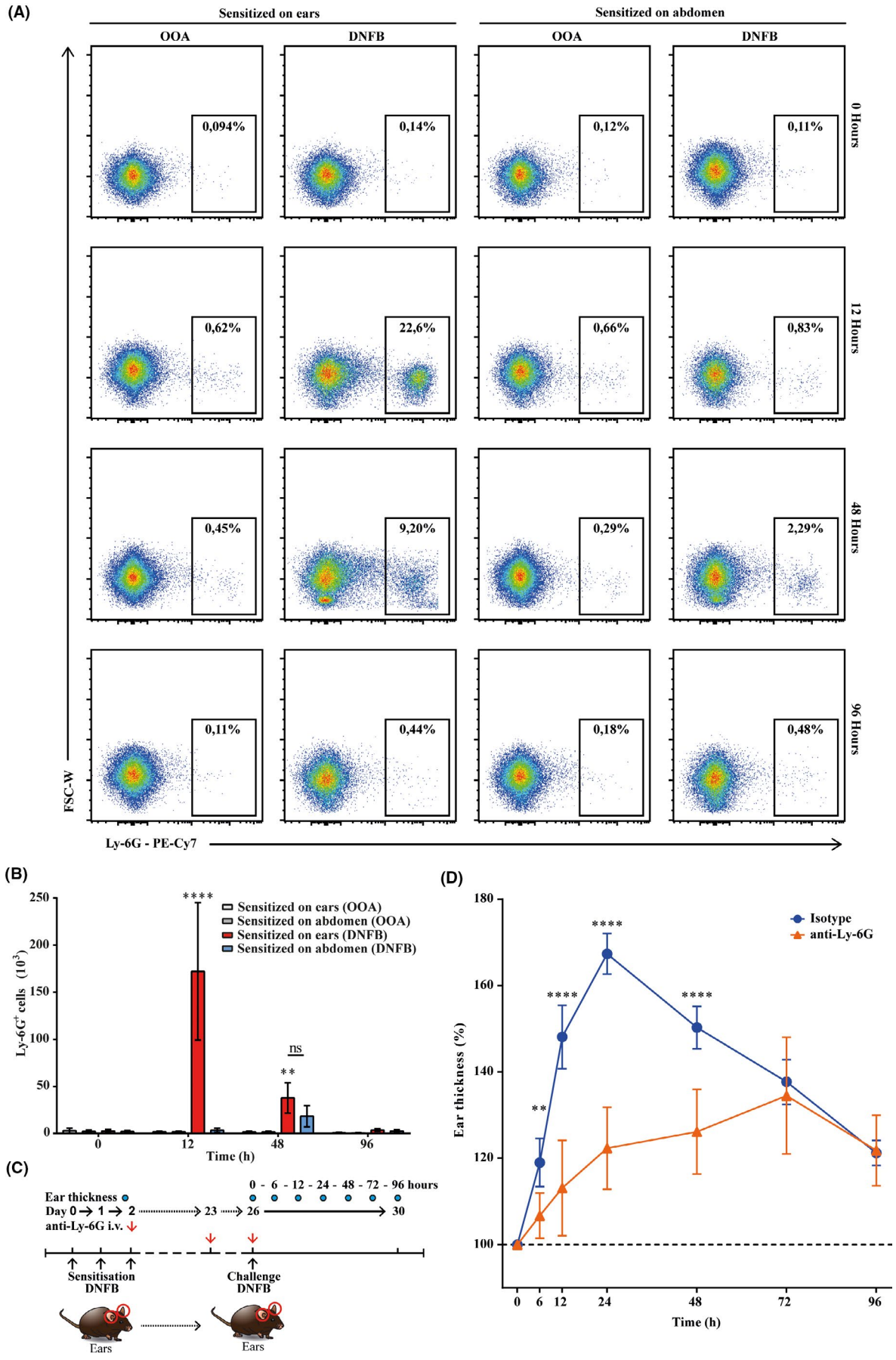
As the flare-up reactions were not explained by recruitment of new T cells to the epidermis, we next investigated neutrophil recruitment following re-exposure to the contact allergen. Neutrophils are the major pathogen-fighting cells in the organism and central to this function is their ability to be recruited to sites of infection. Furthermore, neutrophils play key roles in acute inflammation leading to tissue injuries.[18] Twenty-four days after sensitization on the abdomen or the ears, we challenged the mice on their ears and measured the neutrophil infiltration in the epidermis by FC at 0–96 h after the challenge. We found a clear difference in the number of infiltrating neutrophils between the epidermis of allergen-experienced and allergen-naïve skin (Figure 2A,B, Figure S2G). Already 12 h after re-exposure to the contact allergen, the number of epidermal neutrophils had strongly increased in allergen-experience skin, whereas neutrophils were not detected in allergen-naïve skin. At this early time point, the neutrophils actually outnumbered the $CD8^+$ T_{RM} cells in the epidermis of allergen-experienced skin 6–8 times (Figures 1F and 2B). After the swift increase, the number of neutrophils in the epidermis rapidly declined and nearly reached pre-challenge levels 96 h after challenge of the allergen-experienced skin (Figure 2A,B). Thus, a clear correlation was seen between neutrophil infiltration in the epidermis and the flare-up reaction in allergen-experienced skin after re-exposure to the allergen. In contrast to the rapid and intense neutrophil recruitment seen in allergen-experienced skin, only a minor increase in neutrophils was seen in allergen-naïve skin 48 h after allergen challenge (Figure 2A,B).

To determine whether the neutrophils were directly causative of the flare-up reactions seen in allergen-experienced skin, we sensitized mice on the ears for three consecutive days and injected them i.v. at 3 days and just before the challenge with either neutrophil-depleting anti-Ly-6G monoclonal antibodies (mAb) or isotype control mAb as depicted in Figure 2C. In contrast to mice treated with the isotype control mAb, the flare-up reaction was severely inhibited in mice depleted for neutrophils (Figure 2D). Thus, the ACD response in allergen-experienced skin in mice depleted for neutrophils resembled the attenuated, delayed response seen in allergen-naïve skin in mice not depleted for neutrophils (Figures 1D and 2D), showing that ACD flare-up reactions are dependent on neutrophils.

3.3 | $CD8^+$ T_{RM} cells are indispensable for ACD flare-ups and the rapid recruitment of neutrophils to the epidermis following re-exposure to allergen

As the ACD flare-up reactions and the rapid and intense recruitment of neutrophils were only seen after allergen re-exposure of allergen-experienced skin, and as allergen-experienced skin in contrast to allergen-naïve skin harbored $CD8^+$ T_{RM} cells, we next investigated whether $CD8^+$ T cells were directly required for neutrophil recruitment and flare-ups. We treated mice with anti-CD8α mAb to deplete $CD8^+$ T cells or with IgG isotype control mAb four times before and

FIGURE 2 ACD flare-up reactions are dependent on neutrophils. (A) Representative dot plots of Ly-6G expression in epidermal cells isolated at 0, 12, 48, and 96 h after the challenge ($n = 8$). (B) Number of live epidermal (Ly-$6G^+$) neutrophils in ear sheets isolated at 0, 12, 48, and 96 h after challenge ($n = 8$). (C) Experimental setup: Mice were sensitized with DNFB at day 0–2 on both ears and injected with anti-Ly-6G mAbs or IgG isotype control at day 23 and day 26 just before the challenge. Mice were challenged with DNFB on both ears at day 26. (D) Ear thickness was measured before challenge at 0 h and 6, 12, 24, 48, 72, and 96 h after challenge. Values are normalized to mean ear thickness at 0 h. Each dot/triangle represents the mean ear thickness of each group at each time point ($n = 8$). Statistical comparisons; two-way ANOVA. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > .05$, $^{**}p \leq .01$, $^{****}p \leq .0001$


(A)
Sensitized on ears
Sensitized on abdomen
OOA
DNFB
OOA
DNFB
0,094%
0,14%
0,12%
0,11%
0 Hours
0,62%
22,6%
0,66%
0,83%
12 Hours
0,45%
9,20%
0,29%
2,29%
48 Hours
0,11%
0,44%
0,18%
0,48%
96 Hours
FSC-W
Ly-6G - PE-Cy7
(B)
Ly-6G+ cells (10^3)
Sensitized on ears (OOA)
Sensitized on abdomen (OOA)
Sensitized on ears (DNFB)
Sensitized on abdomen (DNFB)
****
**
ns
Time (h)
(C)
Ear thickness
Day 0 → 1 → 2 ····· 23 ····· 26 → 30
0 - 6 - 12 - 24 - 48 - 72 - 96 hours
anti-Ly-6G i.v.
Sensitisation DNFB
Challenge DNFB
Ears
Ears
(D)
Ear thickness (%)
Isotype
anti-Ly-6G
**
****
****
****
Time (h)

after sensitization of the mice on their ears as depicted in Figure 3A. This treatment specifically depleted $CD8^+$ T cells (Figure S4). The mice were challenged on day 21 and the ear thickness was measured just before and 12 h after the challenge. In contrast to control mice treated with the IgG isotype control mAb, the allergen-induced flare-up reaction was almost completely abrogated in mice treated with anti-CD8α mAb (Figure 3B). To analyze the effect of the mAb treatment on the cellular composition in the epidermis, we prepared epidermal single-cell suspensions 12 h after the allergen challenge and determined the number of $CD8^+$ T cells and neutrophils by FC. We found that treatment with anti-CD8α mAb completely inhibited accumulation of $CD8^+$ T cells in the epidermis (Figure 3C) and profoundly reduced the recruitment of neutrophils to the epidermis (Figure 3E,F).

To substantiate that the flare-up reactions were dependent on $CD8^+$ T_{RM} cells, we next used a sex-match/sex-mismatch adoptive transfer model.[11,19] As depicted in Figure 3F, we transferred lymphocytes from female or male donor mice sensitized with DNFB or OOA to naïve female recipient mice. The recipient mice were treated on the ears with the irritant sodium lauryl sulfate for 3 days following the cell transfer to ensure recruitment of activated donor T cells into the epidermis of the recipient's ears in the absence of the allergen (Figure S3A–C). In this sex-match/sex-mismatch model both circulating memory T cells and T_{RM} cells are present in recipient mice that received cells from female donors, whereas only T_{RM} cells are left in recipients that received cells from male donors at the time of challenge.[11,19] All the recipient mice were challenged with DNFB 21 days after the adoptive transfer. We found a similar increase in ear thickness 12 h after the challenge in mice that had received cells from female and male donors (Figure 3G). Taken together, these results indicated that $CD8^+$ T_{RM} cells are indispensable for ACD flare-ups and the rapid recruitment of neutrophils to the epidermis following re-exposure of allergen-experienced skin to contact allergens.

3.4 | $CD8^+$ T_{RM} cells induce rapid release of CXCL1 and CXCL2 in the skin following re-exposure to allergen

CXCR1 and CXCR2 are the major chemokine receptors on neutrophils involved in neutrophil recruitment.[20-27] The primary chemokines binding to CXCR1/2 in mice are CXCL1 (KC) and CXCL2 (MIP-2).[21,22,28] To determine whether CXCL1 and CXCL2 might be involved in the rapid recruitment of neutrophils to allergen-experienced skin following allergen challenge, we sensitized mice on the ears with either DNFB or OOA. Twenty-one days later, we challenged the mice with DNFB on the ears and measured the expression of CXCL1 and CXCL2 at 0, 3, 6, and 12 h after the challenge. CXCL1 was up-regulated at 6 and 12 h and CXCL2 was strongly up-regulated at 12 h in allergen-experienced skin (Figure 4A,B). Furthermore, we found that DNFB-induced up-regulation of CXCL1 in allergen-naïve skin 6 h after the challenge (Figure 4A). By splitting the skin in epidermis and dermis, we found that whereas CXCL1 was mainly expressed in the dermis, CXCL2 was most intensely expressed in the epidermis of allergen-experienced skin (Figure 4C,D). This was confirmed by immunohistochemical analyses, where we found strong expression of CXCL2 in the epidermis of allergen-experienced skin 12 h after allergen challenge (Figure 4E,F).

To determine the role of $CD8^+$ T_{RM} cells in the production of CXCL1 and CXCL2, we treated mice with anti-CD8α mAb to deplete $CD8^+$ T cells or with IgG isotype control mAb four times before and after sensitization of the mice on their ears as depicted in Figure 3A. The mice were challenged on day 21 and the concentration of CXCL1 and CXCL2 was measured in parallel with the number of $CD8^+$ T_{RM} cells and neutrophils in the epidermis 12 h after the challenge. We found that CXCL1 and CXCL2 expression and the number of neutrophils in the epidermis strongly correlated with the presence of epidermal $CD8^+$ T_{RM} cells (Figures 3C,D and 4G,H).

3.5 | CXCR1/2 antagonism inhibits ACD flare-up reactions and neutrophil recruitment

To further investigate whether CXCR1/2 were involved in the ACD flare-up reactions and the rapid recruitment of neutrophils to the epidermis after re-exposure to allergens of allergen-experienced skin, we sensitized mice with DNFB on the ears and treated them with the CXCR1/2 allosteric inhibitor reparixin or PBS just prior to and 8 h after challenge as depicted in Figure 5A. Ear thickness was measured 0, 6, 12, 24, 48, 72, and 96 h after the challenge and recruitment of neutrophils to the epidermis was measured 12 h after the challenge. We found that reparixin strongly inhibited both the flare-up reaction and the neutrophil recruitment normally seen in allergen-experienced skin (Figure 5B,C).

4 | DISCUSSION

In the present study, we demonstrate that ACD flare-up reactions in allergen-experienced skin are caused by neutrophils recruited to the site of allergen challenge by chemokines induced by local $CD8^+$ T_{RM} cells.

Mouse models for ACD have been widely used to study immune responses to contact allergens. Up to now, the vast majority of these studies have used the acute CHS assay, where mice are sensitized on the abdomen or flank once or twice and then challenged on allergen-naïve skin on the ears only 5–7 days after the sensitization.[16,29-34] In the acute CHS assay, the inflammatory response as measured as changes in ear thickness peaks 24 h after the challenge.[35,36] In contrast, in our model, the challenge response seen in allergen-naïve skin peaked 72 h after the challenge in accordance with a classical delayed type IV allergic reaction mediated by memory T cells, and in line with the optimal time for reading patch test responses in the clinic.[17] Importantly, whereas our model allows time for the development of allergen-specific memory T cells, including epidermal $CD8^+$ T_{RM} cells, the acute CHS assay where sensitization and challenge are

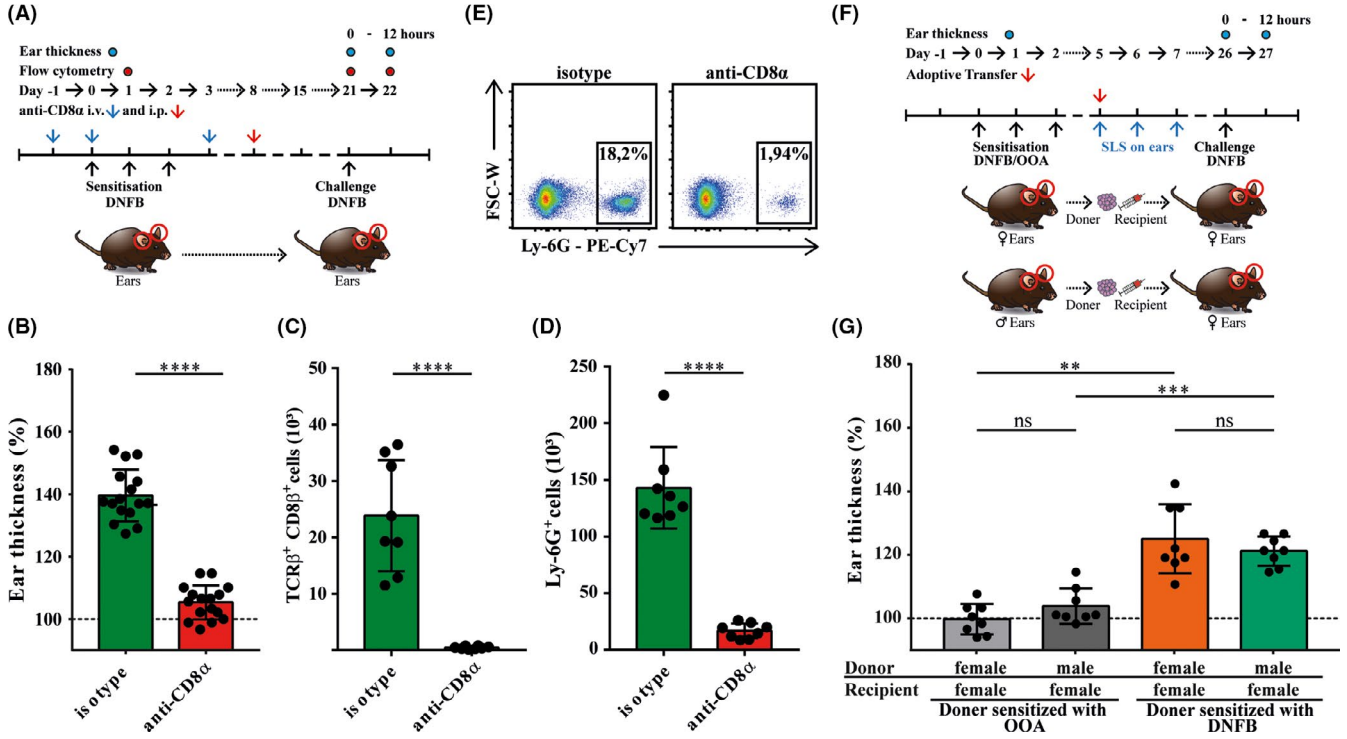


FIGURE 3 $CD8^+$ T_{RM} cells are indispensable for ACD flare-ups and the rapid recruitment of neutrophils to the epidermis following re-exposure to allergen. (A) Experimental setup. Mice were sensitized with DNFB at day 0–2 on both ears and challenged with DNFB on both ears at day 21. Depletion of $CD8^+$ cells was done by i.v. injection at day −1, 0, 3 and by one i.p. injection at day 8 with anti-CD8α mAbs or IgG isotype control mAb. (B) Ear thickness 12 h after challenge. Each dot represents ear thickness from one mouse normalized to the mean ear thickness measured before challenge at 0 h ($n = 16$). (C–D) Flow cytometry data on livings cells from epidermal ear sheets isolated 12 h after challenge. Each dot represents the number of isolated cells from one mouse ($n = 8$). Number of (C) $CD8^+$ T cells ($CD8\alpha^+TCR\beta^+$ cells), (D) neutrophils ($Ly\text{-}6G^+$ cells). (E) Representative dot plots of Ly-6G expression in epidermal cells isolated 12 after challenge. (F) Experimental setup: Female and male donor mice were sensitized with DNFB or OOA at day 0–2 on both ears. Lymphocytes were isolated at day 5 and transferred i.v. into female recipient mice. Recipient mice were treated on both ears with SLS at day 5–7 and challenged with DNFB on both ears at day 26. (G) Ear thickness 12 h after challenge. Each dot represents ear thickness from one mouse normalized to the mean ear thickness measured before challenge at 0 h ($n = 8$). Donor treatment during sensitization and donor/recipient combinations are illustrated below the bars. Statistical comparisons; Students unpaired *t*-test (B–E) and one-way ANOVA (H). Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > .05$, $^{**}p \leq .01$, $^{***}p \leq .001$, $^{****}p \leq .0001$

separated by only 5–7 days does not allow for the development of memory T cells.[4,16] Thus, as the acute CHS assay is analyzing boost-activation of effector T cells during the primary response and not memory responses, it does not reflect the underlying chronic conditions of ACD with allergen-specific memory T cells, where individuals repeatedly are exposed to contact allergens resulting in the characteristic flare-up reactions.

In accordance with recent studies, we found that $CD8^+$ T_{RM} cells primarily localized in allergen-experienced skin and that ACD flare-up reactions correlated with the presence of $CD8^+$ T_{RM} cells.[4-7] The link between $CD8^+$ T_{RM} cells and flare-up reactions seen in these studies likely reflects the flare-up reactions often observed in ACD patients after re-exposure of allergen-experienced skin to the contact allergen.[3] We found that the rapid and intense flare-up reactions correlated with a massive recruitment of neutrophils to the epidermis of allergen-experienced skin following allergen re-exposure. In contrast, re-exposure to allergen did not result in T-cell recruitment in neither allergen-experienced nor—naïve skin for the first 48 h. Neither did re-exposure to allergen result in significant recruitment of neutrophils to allergen-naïve skin. Depletion of neutrophils abrogated the flare-up reactions and transformed the response in allergen-experienced skin into a delayed-type response normally seen in allergen-naïve skin, strongly indicating that neutrophils caused the flare-up reactions.

Previous studies using the acute CHS assay have indicated that neutrophils play a role during both the sensitization and challenge phase.[16,29-33,37,38] One study found that sensitization by just one exposure of the skin to DNFB resulted in local inflammation and a moderate infiltration of neutrophils 24 h after the exposure.[39] Others found that neutrophils were required to activate dendritic cells during the sensitization phase[32] and that neutrophils were essential for recruitment of effector T cells to the site of allergen challenge during the elicitation phase.[30] Thus, the challenge response seen in the acute CHS assay is likely elicited by local inflammation directly caused by the contact allergen. This inflammatory response results in recruitment of neutrophils that subsequently recruit effector T

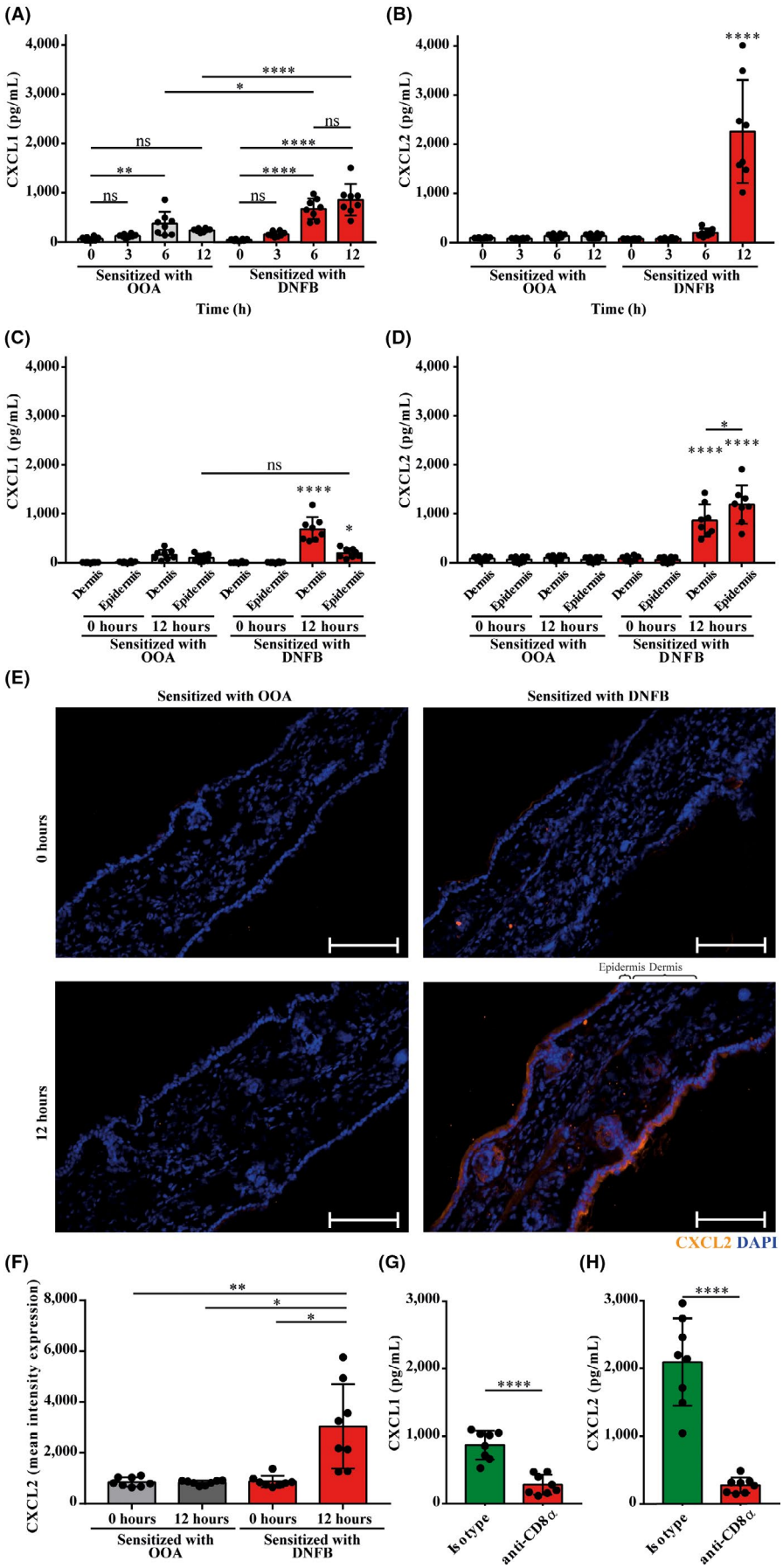


FIGURE 4 $CD8^+$ T_{RM} cells induce rapid release of CXCL1 and CXCL2 in the skin following re-exposure to allergen. (A–H) Mice were sensitized with DNFB at day 0–2 and challenged with DNFB on both ears at day 21. (A) CXCL1 and (B) CXCL2 chemokine concentrations from full skin samples obtained just before the challenge at 0 h and at 3, 6, and 12 h after the challenge. Each dot represents the chemokine concentration from one mouse ($n = 8$). (C) CXCL1 and (D) CXCL2 concentration in the dermis and epidermis obtained just before the challenge at 0 and 12 h after challenge. Each dot represents the chemokine concentration from one mouse ($n = 8$). (E) Fluorescent microscopy images of cross-sections of ears from mice 0 and 12 h (rows) after challenge with DNFB. The mice were previously sensitized on the ears with OOA or DNFB (columns); CXCL2 (orange) and DAPI (blue). Scale bars; 100 μm ($n = 2$). (F) Quantification of CXCL2 mean fluorescence intensity in epidermis ($n = 8$). (G) CXCL1 and (H) CXCL2 concentrations from full skin samples 12 h after challenge of mice treated with anti-CD8α mAbs or IgG isotype control mAb as depicted in Figure 3A. Each dot represents the chemokine concentration from one mouse ($n = 8$). Statistical comparisons; one-way ANOVA (A–F) and Students unpaired *t*-test (G–H). Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > .05$, $^*p \leq .05$, $^{**}p \leq .01$, $^{****}p \leq .0001$

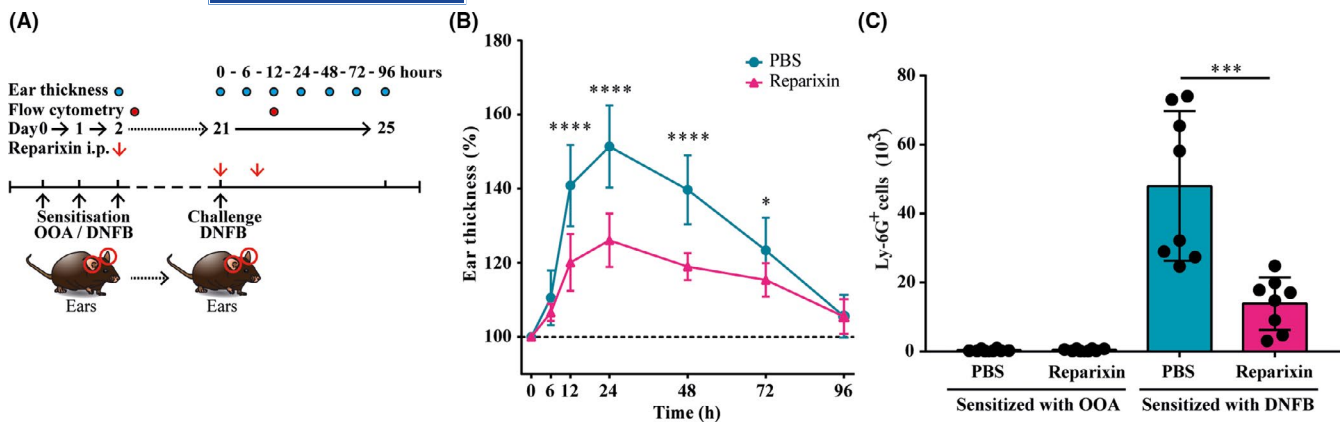


FIGURE 5 CXCR1/2 antagonism inhibits ACD flare-up reactions and neutrophil recruitment. (A) Experimental setup: Mice were sensitized with DNFB or OOA at day 0–2 and challenged with DNFB at day 21 on both ears. The mice were treated i.p. with reparixin or PBS 15 min before and 8 h after the challenge. (B) Ear thickness was measured before challenge at 0 h and 6, 12, 24, 48, 72, and 96 h after challenge. Values are normalized to mean ear thickness at 0 h. Each dot/triangle represents the mean ear thickness of each group at each time point ($n = 8$). (C) Number of neutrophils in epidermal ear sheets 12 h after the challenge. Each dot represent ear thickness from one mouse normalized to the mean ear thickness measured before challenge at 0 h ($n = 8$). Statistical comparisons; two-way ANOVA (B) and one-way ANOVA (C). Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > .05$, *$p \leq .05$, ***$p \leq .001$, ****$p \leq .0001$

cells. Importantly, it should be stressed that allergen-specific memory T cells, and in particular local T_{RM} cells, are not found in the acute CHS assay in contrast to the conditions in patients with ACD and in our model. Diametrically opposed to the acute CHS assay, we demonstrate that $CD8^+$ T_{RM} cells recruit neutrophils to the site of allergen re-exposure. Thus, we found that $CD8^+$ T_{RM} cells rapidly induced production of CXCL1 in the dermis and in particular CXCL2 in the epidermis leading to massive recruitment of neutrophils to the epidermis. The important role of CXCL1 and CXCL2 in the recruitment of neutrophils and the pathogenesis of ACD was supported by the observation that treatment with a CXCR1/2 antagonist inhibited both neutrophil recruitment and the intensity of the allergic reaction following allergen re-exposure. The role of neutrophils in human ACD is less clear. The majority of studies investigating the cellular infiltration in the skin of humans with ACD have investigated punch biopsies obtained after exposure of previously allergen naïve skin sites to allergen.[40-44] As we only observe the rapid, massive neutrophil recruitment in allergen-experienced skin harboring $CD8^+$ T_{RM} cells, we believe that neutrophils might play a role in flare-up reactions seen in human but that these cells are not found due to the predominant technique used up to now where previously allergen naïve skin sites have been investigated. However, this needs further investigation.

It has recently been shown that dermal $CD4^+$ T_{RM} cells play an important role for long-term local memory to contact allergens in BALB/c mice.[38] In accordance with our study, a rapid infiltration of $Gr\text{-}1^+$ cells (ie, neutrophils) into previous sensitized skin sites was seen following re-exposure with contact allergen. Furthermore, epidermal $CD8^+$ T_{RM} cells outnumbered epidermal $CD4^+$ T_{RM} cells more than 10 times.[38] As we did not investigate the role of dermal-resident memory T cells, we cannot exclude that they play a role in our model as well. However, as we found that treatment with anti-CD8α mAb strongly inhibited production CXCL1 and CXCL2, recruitment of neutrophils and the flare-up response, we believe that $CD8^+$ T_{RM} cells and not dermal $CD4^+$ T_{RM} cells are responsible for these responses. Whether local immunological memory against allergens in human skin is solely maintained by $CD4^+$ T_{RM} or $CD8^+$ T_{RM} cells is still not known. However, as dermal $CD4^+$ T_{RM} cells seem to survive for a prolonged period of time compared to epidermal $CD8^+$ T_{RM} cells, these may be more important over time.[6,38] Furthermore, the role of epidermal $CD8^+$ T_{RM} cells in human ACD is still a matter of debate. A study investigating the retention of T cells in the skin following patch testing found very few $CD8^+$ T cells in the skin 21 days after allergen exposure.[45] In contrast, we have previously shown that epidermal $CD8^+$ T_{RM} cells are generated in the skin following patch testing with nickel of nickel allergic individuals.[5] The reason for this discrepancy is not clear, but might be due to difference in allergen exposure in the two studies, as Moed et al remove the patch test after 24 h, whereas the patch test was first removed after 48 h in our study.[5,45] In support of this, we have recently shown that allergen-induced generation of $CD8^+$ T_{RM} cells directly correlates with the allergen doses.[7] Furthermore, the generation of $CD8^+$ T_{RM} cells might also differ between allergens. Clearly, more human studies are needed to clarify this important issue.

In conclusion, this is the first study demonstrating that ACD flare-up reactions are caused by neutrophils recruited to the site of allergen exposure by chemokines induced by local $CD8^+$ T_{RM} cells. The flare-up reactions were significantly reduced by CXCR1/2 antagonism suggesting CXC chemokines and receptors as future targets in treatment of ACD.

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CONFLICT OF INTEREST

The authors declare no competing financial interests. The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization of this study was led by ABF and CMB supported by JDJ, CG, NØ, and AW. Formal analysis was led by ABF supported by VM. Funding acquisition was done by CMB and JDJ. Investigation was led by ABF supported by VM, AG, MHJ, and JFW. Methodology was led by ABF and CMB supported by CG. Project administration was done by ABF. Resources was obtain by CMB and CG. Supervision was done by CMB supported by JDJ. Validation was led by ABF supported by VM, AG, MHJ, and JFW. Visualization was done by ABF. Writing of the original draft was done by ABF, CMB, and CG. Review and editing was done by all authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Study II

$CD4^+$ T cells inhibit the generation of $CD8^+$ epidermal-resident memory T cells directed against clinically relevant contact allergens

$CD4^+$ T cells inhibit the generation of $CD8^+$ epidermal-resident memory T cells directed against clinically relevant contact allergens

Short title: $CD4^+$ T cells regulate local memory to contact allergens

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Abstract

Background: $CD8^+$ epidermal-resident memory T (T_{RM}) cells play central roles in local flare-up responses to experimental contact allergens by inducing massive influx of neutrophils to the epidermis upon allergen challenge. Whether a similar immunopathogenic mechanism is involved in the responses to clinically relevant contact allergens is unknown.

Methods: The immune response to cinnamal, ρ-phenylenediamine (PPD) and methylisothiazolinone (MI) was studied in a well-establish mouse model for allergic contact dermatitis, which includes formation of T_{RM} cells, using cell depletion protocols combined with ELISA, flow cytometry and fluorescent microscopy.

Results: We show that the formation of $CD4^+$ and $CD8^+$ epidermal T_{RM} cells and the inflammatory response are highly allergen-dependent. However, the magnitude of the flare-up response correlated with the number of epidermal $CD8^+$ T_{RM} cells, CXCL1/CXCL2 release and recruitment of neutrophils into the epidermis. Finally, depletion of $CD4^+$ T cells strongly enhanced the number of epidermal $CD8^+$ T_{RM} cells, the flare-up response and the infiltration of neutrophils for all allergens.

Conclusion: As the first, this study demonstrates that clinically relevant contact allergens have the ability to generate pathogenic, epidermal $CD8^+$ T_{RM} cells but that this is normally counteracted by the simultaneous induction of anti-inflammatory $CD4^+$ T cells.

Key words

Allergic contact dermatitis, epidermal-resident memory T cells, neutrophils, CXCL2

Introduction

Allergic contact dermatitis (ACD) is a common T cell-mediated inflammatory skin disease induced by exposure of the skin to contact allergens[1]. In most previous studies, the conventional contact hypersensitivity (CHS) model, in which mice are sensitized with allergen on the abdomen and then challenged on the ears five days later, have been used to determine the immunopathogenic mechanisms involved in ACD. However, the different location used during sensitization and challenge and the short interval between sensitization and challenge used in this model does not allow for generation of resident memory T (T_{RM}) cells. Recent studies using more clinically relevant models that allow for the generation of memory T cells have pointed to a central role of various types of memory T cells in ACD. Particularly, the role of epidermal $CD8^+$ T_{RM} cells in the response to the experimental contact allergens dinitrofluorobenzene (DNFB) has been investigated[2–6]. These studies have established the crucial role of epidermal $CD8^+$ T_{RM} cells in ACD to DNFB. Recently, it was shown that $CD8^+$ T_{RM} cells mediate the rapid flare-up response after allergen re-exposure by inducing local production of chemokine (C-X-C motif) ligand 1 (CXCL1) and CXCL2 leading to a massive infiltration of neutrophils to the epidermis of the allergen-exposed skin[6]. The experimental contact allergen DNFB is classified as an extreme allergen that induces a strong immune responses[7]. The immune responses to weaker and more clinically relevant allergens have been more complex to study in mouse models, e.g. induction of responses to three common fragrance allergens, all classified as weak allergens, required the depletion of $CD4^+$ T cells in the classical short-term CHS model[8].

It is still debated whether $CD4^+$ or $CD8^+$ T cells are the major pathogenic cells mediating the inflammatory response during ACD[1,9]. Early studies suggested that $CD4^+$ T cells are the major effector cells as depletion of $CD4^+$ but not $CD8^+$ T cells strongly reduced the response to the experimental contact allergens dinitrochlorobenzene (DNCB) and trinitrochlorobenzene

(TNCB)[10]. Later studies found that both $CD4^+$ and $CD8^+$ T cells were effector cells in the response to the experimental contact allergen DNFB[11]. A role of $CD4^+$ T cells as effector cells in the response to DNFB was subsequently confirmed in some studies[12–14], whereas other studies indicated that $CD4^+$ T cells mainly played an anti-inflammatory role[8,15–22]. Together, these studies did not provide a clear role of $CD4^+$ T cells in ACD.

The aim of the present study was to determine the immunopathogenic mechanisms elicited by three clinically relevant contact allergens using a model for ACD that allows for the generation of memory T cells. We selected the allergens cinnamal (fragrance allergen, classified as a moderate allergen), ρ-phenylenediamine (PPD, black dye often used in permanent hair dyes, classified as a strong allergen) and methylisothiazolinone (MI, preservative, classified as a strong allergen)[7]. These allergens are all part of the baseline series used for diagnosing contact allergy and produce a positive response in 7.5% (MI) and 3.6% (PPD), while cinnamal is part of a fragrance mixture (FMI), which gives positive test reactions in 6.8% of European patients [23]. Cinnamal accounts for 20% of the reactions to the FMI[24]. We found that a local immune response was induced by all of these contact allergens, but that the magnitude and kinetic of the responses were highly allergen dependent. Interestingly, the magnitude of the flare-up response, seen in allergen-experienced skin after re-exposure to the allergen, correlated with the number of local epidermal $CD8^+$ T_{RM} cells, CXCL1/CXCL2 release and recruitment of neutrophils to the epidermis. Furthermore, depletion of $CD4^+$ T cells resulted in a strongly enhanced inflammatory response with augmented generation of epidermal $CD8^+$ T_{RM} cells and influx of neutrophils to the epidermis for all the allergens. This demonstrated that, as for DNFB, epidermal $CD8^+$ T_{RM} cells are the major pathogenic cells in ACD to the clinically relevant contact allergens tested. However, in contrast to DNFB, the clinically relevant allergens elicited a $CD4^+$ T cell-mediated anti-inflammatory response that reduced the generation of the pathogenic epidermal $CD8^+$ T_{RM} cells.

Methods

Mice

All *in vivo* experiments were performed using 6-8 weeks old female C57Bl/6J mice purchased from Janvier Labs (Le Genest-Saint-Isle, France). Mice were housed in a specific pathogen-free animal facility at the Department of Experimental Medicine, University of Copenhagen in accordance with the national animal protection guidelines (license number 2018-15- 0201-01409).

Contact hypersensitivity model

Mice were treated by epicutaneous painting with 3 x EC3 values of cinnamal = 6% (Sigma-aldrich, W228613), ρ-Phenylenediamine (PPD) = 0.48% (Sigma-Aldrich, P6001), methylisothiazolinone (MI) = 1.2% (Sigma-Aldrich, 725765) diluted in 1:4 vehicle solution of olive oil:acetone (OOA) or with OOA alone in control groups [7]. Sensitization was done over three consecutive days (day 0-2) with 25 µl allergen on the dorsum of both ears or with 50 µl allergen solution on the shaved abdomen. Mice were challenged with allergen or with OOA on the ears or the abdomen twice with a twenty-one-day interval. In experiments investigating the challenge response mice were challenged a third time on the ears only. Ear swelling was measured using an electronic digital micrometer (Mitutoyo Corporation, Model: PK-1012CPX). The capacity of each contact allergen to induce skin inflammation was measured by ear thickness measurements on day 6 normalized mean ear thickness before sensitization (day 0). The inflammatory challenge response was measured by ear thickness measurements at 24 h, 48 h, 72 h and 96 h after challenge normalized to mean ear thickness before challenge (0 h). Mice were euthanized and the ears were isolated for further analyses. All experiments were repeated twice.

$CD4^+$ T cell and $CD8^+$ T cell depletion

Cellular depletion was performed using InVivoPlus IgG2b-κ rat anti-mouse CD8α (clone YTS169.4) and InVivoPlus IgG2b-κ rat anti-mouse CD4 (clone GK1.5) (Nordic BioSite ApS, Copenhagen, Denmark, BP0117, BP0003-1). IgG2b-κ anti-KLH rat anti-mouse (LTF-2) (Nordic BioSite ApS, Copenhagen, Denmark, BP0090) was used as isotype control mAB. 200 µg antibody diluted in 100 µl phosphate-buffered saline (PBS; 137 mM NaCl, 2.6 mM KCl, 1.1 mM $Na_2HPO_4 \cdot H_2O$, 5 mM KH_2PO_4, pH 7.4) was given by intravenous (i.v.) injections through the lateral tail vein during sensitization and as intraperitoneal (i.p.) injections just before first and second challenge (day 21 and 42).

Flow cytometry (FC) analysis

Ear skin (dorsal/treated side) was separated from each ear and incubated for 1 hour at 37°C in a 0.3% trypsin (Sigma-Aldrich, St. Louis, T9201) Milli-Q water solution supplemented with 149 mM sodium chloride, 5 mM potassium chloride and 5 mM dextrose, pH 7.6 to enable epidermal peeling. Epidermis was digested into single cell suspension by 10 min incubation at 37°C in 0.3 % trypsin Milli-Q water solution supplemented with 149 mM sodium chloride, 5 mM potassium chloride, 5 mM dextrose, pH 7.6 and 0.1 % DNase (Sigma-Aldrich, St. Louis, D5025) while shaking. The suspension was washed through 70 µm falcon cell strainers (Corning Inc. NY USA, 352350) in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, 11965-092) supplemented with 15.5% Fetal Bovine Serum (Biological Industies, 04-007-1A) and 1.4% DNase (Sigma-Aldrich, St. Louis, D5025). The epidermal cells were then incubated overnight at 37°C in RPMI-1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 1% L-glutamine, 1% penicillin-streptomycin, 50 µM 2-Mercaptoethanol, 25 mM HEPES, 100 mM Na Pyruvate and 100 µM nonessential amino acids to allow re-expression of surface receptors. On day two the

number of cells were counted using a Neubauer hemocytometer and 1 x 10^6 cells were used for flow cytometry. Fc-receptors were blocked with anti-CD16/CD32 (clone 2.4.G2) and surface markers were stained with anti–CD8α (BV421, clone 53-6.7) or anti–CD8β (BV421, clone 53-6.7), anti-CD44 (BV605, clone IM7), anti–CD69 (FITC, clone H1.2F3) (BD Biosciences, Franklin Lakes, NJ), anti–TCRβ (BV711, clone H57-597), anti-Ly-6G (PE/Cy7, clone 1A8) , anti-CD11b (BUV395, clone M1/70), anti-CD11c (PE, clone HL3) and anti-NK-1.1 (BV480, clone PK136) were all purchased from BD Biosciences, Franklin Lakes, NJ. Anti-CD62L (AF700, clone MEL-14), anti-TCRγδ (AF488, clone GL3) and anti–CD103 (PerCP/Cy5.5, clone 2E7) were purchased from BioLegend, San Diego, CA. All antibodies were diluted in Brilliant Stain Buffer (BD Biosciences, Franklin Lakes, NJ). Fixable Viability Dye (eFluor 780) (eBioscience, San Diego, CA) was used to assess the viability of the cells. 1 x 10^6 cells from each samples were analyzed on a BD LSR Fortessa (5 laser) instrument. Data were processed with FlowJo (Treestar). The number of each cell type per mouse from two epidermal ear sheets was calculated based on the total number of live cells times the frequency of the gated population. tSNE plots were generated in FlowJo (Treestar) by concatenating data from four mice within each group.

Fluorescent microscopy

Hair was removed from the ears using Veet hair removal cream (Reckitt Benckiser, Slough, United Kingdom). Epidermal ear sheets were obtained following 13 minutes incubation at 37°C in PBS with 3.8 % ammonium thiocyanate. The epidermal sheets were then fixed in Zambonis fixative pH 7.4 with 2 % paraformaldehyde for 10 min at room temperature (RT) following three washing steps in PBS. Unspecific binding sites were blocked in blocking buffer containing 5% anti-goat serum (Sigma-Aldrich, S26) diluted in PBS shaking for 1.5 hours at RT. Primary Abs staining was done overnight at 4°C in blocking buffer; rat-anti-

mouse CD8α mAbs (clone 4SM15, eBioscience, Cat no. 15-0808-82). Secondary staining was done the following day with goat-anti-rat mAbs (AF555, Thermo Fisher Scientific, Cat no. 1987272) for 2 hours at RT. Finally, sheets were stained with DAPI for 10 minutes at RT and mounted on glass slides using Prolong glass anti-fade mounting media (Life Technologies, Eugene, USA, P36982). Secondary binding controls and single staining controls were done with each experiment to adjust the spectral view during image analysis. Fluorescent imaging was performed using a Carl Zeiss, LSM 710, Axio Imager 2 microscope (Carl Zeiss GmbH, Oberkochen, Germany). Images were obtained by a plan-Apochromat 20x air objective lens at 0.8 numerical aperature (NA). Samples were scanned at 405 nm (blue) and 561 nm (red). Images were analyzed in the Zen 3.0 blue edition software (Carl Zeiss GmbH, Oberkochen, Germany).

ELISA

Measurements of IL-1β/IL-1F2 (DY401), CXCL1/KC (DY453) and CXCL2/MIP-2 (DY452) in complete ear skin samples (dermis and epidermis) was determined using DuoSet enzyme-linked immunosorbent assays (ELISA), following the guidelines provided by the manufacturer (R&D Biotechne Ltd, Denver, America). Samples were snap-frozen in liquid nitrogen and homogenized in protease inhibitor cocktail (cOmplete, Roche Diagnostics GmbH, Germany) diluted in 600 µl lysis buffer (50 mM Trizma base, 250.2 mM NaCl, 6.4 mM EDTA and 17.6 mM Triton X-100 diluted in 1 L Milli-Q water pH adjusted to 7.4 pH) using a Precellys Evolution instrument (Bertin Technologies France) in hard tissue Precyllys lysing tubes (Bertin Technologies France). Protein concentrations were determined by Bradford assay. Prior to ELISA analysis the protein concentration was adjusted to 3 µg/µl. Analysis of ELISA targets was performed at 450 and 570 nm wavelengths by a CLARIOstar® plus instrument (BMG labtech Gmbh, Germany).

Statistics

Statistical comparisons were performed using GraphPad Prism version 7.0. Gaussian distributions were tested using D'Agostino and Pearson normality test and statistical significance was tested using One-way ANOVA and Two-way ANOVA as described in the figures. For conditions without Gaussian distribution a Mann-Whitney U test (for 2 comparisons) or a Kruskal-Wallis test (for > 2 comparisons) was performed. Multiple comparisons were adjusted post-hoc using Dunn's multiple comparisons test for data analyzed by One-way ANOVA and Bonferroni's multiple comparisons test for data analyzed by Two-way ANOVA. Simple linear regressions were used to test for correlation including 95% confidence intervals and goodness of fit shown as R squared values. Significance levels are illustrated in all figures as; Not significant (ns) = $p > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

Results

Cinnamal, PPD and MI have the capacity to induce local inflammation

To determine whether the clinically relevant allergens cinnamal, PPD and MI have the capacity to induce local inflammation in the skin, we sensitized mice on the ears with either cinnamal, PPD or MI for three consecutive days (Fig 1A). We measured the ear thickness at day zero before allergen application and at day 6 to determine the increase in ear thickness induced by each of the allergens. Compared to the vehicle olive oil acetone (OOA), all the allergens induced a significant increase in ear thickness indicating that they all have the capacity to induce local inflammation in the skin (Fig. 1B-D).

Cinnamal, PPD and MI induce local influx of epidermal T cells and an enhanced local memory response

Sensitization with DNFB for three consecutive days is sufficient to induce local $CD8^+$ T_{RM} cells at day 21[6]. To investigate whether cinnamal, PPD and MI had the capacity to induce influx of T cells to the epidermis, we sensitized mice on either the ears or the abdomen for 3 consecutive days and determined the number of $CD8^+$ and $CD4^+$ T cells in the epidermal ear sheets at day 21. We did not detect a significant accumulation of $CD8^+$ or $CD4^+$ T cells at this time point neither in mice sensitized at the abdomen nor the ears (data not shown). We have previously demonstrated that the number of epidermal $CD8^+$ T_{RM} cells in a specific skin area increases with the number of local exposures to DNFB[5]. Thus, to investigate whether re-exposure to cinnamal, PPD and MI after the initial sensitization could elicit recruitment of T cells to the epidermis of the ears, we sensitized mice for three consecutive days on the ears or the abdomen and challenged them twice with an interval of twenty-one days on the same sites they were sensitized. Twenty-one days after the second challenge, we determined the number

of $CD8^+$ and $CD4^+$ T cells in the epidermis of the ears by flow cytometry (Fig. 2A). Surprisingly, we only detected a significant accumulation of $CD8^+$ T cells in mice treated with MI on the ears, although a non-significant tendency towards $CD8^+$ T cell infiltration was observed in cinnamal-treated mice compared to OOA controls (Fig. 2B). Despite general higher numbers of epidermal $CD4^+$ T cells in mice sensitized and challenged at the ears, we did not find any significant accumulation of epidermal $CD4^+$ T cells when compared to the OOA control group. However, when compared to mice treated at the abdomen, a significant accumulation of epidermal $CD4^+$ T cells was seen in mice treated with cinnamal and MI at the ears (Fig. 2C). Interestingly, we did not detect accumulation of neither $CD8^+$ nor $CD4^+$ T cells in the ears of PPD-treated mice when compared to controls indicating that PPD do not induce T_{RM} accumulation in the skin (Fig. 2B and C). As previously shown for DNFB[6], neither of the contact allergens were able to induce T_{RM} cell accumulation at distal skin sites. Thus, mice treated at the abdomen did not generate T_{RM} cells in the ears (Fig. 2B and C). These observations indicated that only MI had the capacity to induce local generation of T_{RM} cells in the skin although and to a lesser degree than previously reported for DNFB[6].

We have recently shown that $CD8^+$ T_{RM} cells generate rapid flare-up responses in DNFB-experienced skin following re-exposure to DNFB[6]. To determine whether similar flare-up responses could be elicited by cinnamal, PPD and MI, we sensitized mice for three consecutive days on the ears or the abdomen and challenged them twice with an interval of twenty-one days on the same sites they were sensitized. Twenty-one days after the second challenge, we measured the ear thickness and challenged all groups of the mice on the ears. We subsequently measured the ear thickness every 24 h up to 96 h (Fig. 2A). We observed a clear flare-up response at 24 h in mice sensitized and challenged with MI on the ears (Fig. 2D and F), but not in mice that had been sensitized and challenged on the abdomen (Fig. 2G and I). For cinnamal and PPD, we also noticed increased responses in mice that had been sensitized

and challenged on the ears compared to mice that had been sensitized and challenged on the abdomen (Fig. 2E and H). This indicate that despite the lack of significant $CD8^+$ T cells accumulation in the epidermis, local memory was generated in mice treated at the ears with cinnamal and PPD.

Allergen-induced influx of neutrophils correlates with the number of epidermal $CD8^+$ T_{RM} cells

The data above indicated that MI induces influx of $CD8^+$ T cells to the epidermis of the skin although the number of $CD8^+$ T cells was low compared to the number of $CD8^+$ T_{RM} cells generated by DNFB[6]. In mice treated with DNFB, more than 95% of the epidermal $CD8^+$ T cells are $CD8^+$ T_{RM} cells as defined by their co-expression of CD103, CD69 and CD44[6]. To characterize the nature of the T cells recruited to the epidermis and to determine whether an influx of neutrophils was induced by re-exposure to cinnamal, PPD and MI, we sensitized mice for three consecutive days on the ears and challenged them on the ears three times with an interval of twenty-one days. We determined the numbers of $CD8^+$ T_{RM} cells, $CD4^+$ T_{RM} cells and neutrophils in the epidermis just before (0 h) and 24 h and 72 h after the third challenge with cinnamal, PPD or MI (Fig. 3A). In accordance with Fig. 2B, we found low numbers of $CD8^+$ T_{RM} cells in mice treated with PPD (Fig. 3E), slightly higher numbers of $CD8^+$ T_{RM} cells in mice treated with cinnamal (Fig. 3B) and a clear accumulation of $CD8^+$ T_{RM} cells in the epidermis of mice treated with MI (Fig. 3H). In accordance with previous observations[6], the number of $CD8^+$ T_{RM} cells was not significantly affected up to 72 h after allergen exposure for either of the allergens. In contrast, the number of epidermal $CD4^+$ T_{RM} cells in mice treated with cinnamal and MI rapidly declined after allergen exposure (Fig. 3C and I). For PPD we noted an alternative pattern, where $CD4^+$ T_{RM} cells were recruited to the epidermis 24 h after exposure to PPD (Fig. 3F). The number of $CD8^+$ T_{RM} cells correlated with the

recruitment of neutrophils (Fig. 3D, G, J and K) and the magnitude of the inflammatory response (Fig. 2D-F) 24 h after the challenge. In contrast, the number of $CD4^+$ T_{RM} cells correlated neither with the influx of neutrophils (Fig. 3L) nor the magnitude of the inflammatory response.

CXCL1 and CXCL2 levels correlate with neutrophil recruitment to the epidermis

The levels of IL-1β, CXCL1 and CXCL2 quickly increase in allergen-experienced skin after re-exposure to DNFB[4,6]. To determine whether the levels of IL-1β, CXCL1 and CXCL2 also increase in allergen-experienced skin following challenge with clinically relevant allergens, we sensitized mice for three consecutive days with either cinnamal, PPD or MI on the ears and challenged them on the ears three times with an interval of twenty-one days. We determined the concentration of IL-1β, CXCL1 and CXCL2 in the ears by ELISA just before and 24 h after the third challenge (Fig. 4A). IL-1β and CXCL1 were significantly up-regulated in allergen-experienced skin compared to naïve skin upon challenge with either of the three allergens (Fig. 4 B, C, E, F, H, I). In contrast, CXCL2 was only significantly up-regulated after challenge with MI (Fig. 4J), whereas it was not up-regulated after challenge with cinnamal or PPD (Fig. 4D and G). For DNFB, we have demonstrated that $CD8^+$ T_{RM} cells mediate neutrophil recruitment by inducing CXCL1 and CXCL2 production in the skin[6]. In accordance, in the present study we found that the concentrations of CXCL1 and CXCL2 was upregulated after challenge with MI where accumulation of epidermal $CD8^+$ T_{RM} cells were present prior to the challenge (Fig. 3H), and the only condition where a massive epidermal influx of neutrophils was detected (Fig. 3J). Interestingly we also found a minor but significant upregulation of CXCL1 in skin treated with cinnamal and PPD (Fig. 4C and F) emphasizing some form of local memory that was not related to epidermal T_{RM} cell formation.

Depletion of CD4$^+$ T cells strongly enhances the number of CD8$^+$ T_{RM} cells and the flare-up response to cinnamal, PPD and MI

The number of CD8$^+$ T_{RM} cells, the flare-up responses and infiltration of neutrophils were significantly reduced after challenge with cinnamal, PPD and MI compared to challenge with DNFB. This suggests that cinnamal, PPD and MI are not as strong inducers of pathogenic CD8$^+$ T_{RM} cells as DNFB and/or that mechanisms that counteract the generation of CD8$^+$ T_{RM} cells are activated during sensitization and challenge with these allergens. To determine whether CD4$^+$ T cells might play an inhibitory role in the responses to cinnamal, PPD and MI, as previously indicated for fragrance allergens[8], we sensitized mice for three consecutive days on the ears and challenged them three times on the ears with an interval of twenty-one days. The mice were treated with anti-CD4 IgG monoclonal antibodies (mAb) at day -1, 0, 3, 21 and 42 to deplete the CD4$^+$ T cells (Fig. 5A). Control groups of mice were either treated with isotype control IgG mAb or anti-CD8α IgG mAb. Compared to the control groups, mice treated with anti-CD4 mAb had strongly enhanced flare-up responses for the three allergens tested (Fig. 5B – D). To analyze whether the enhanced responses correlated with increased numbers of epidermal CD8$^+$ T cells, we isolated epidermal ear-sheets just before the third challenge and analyzed them by immunohistochemistry. Following anti-CD4 mAb treatment, we found strongly increased numbers of CD8$^+$ T cells in the epidermis for all three allergens (Fig. 5E). This indicated that CD4$^+$ T cells inhibit the formation of CD8$^+$ T cells locally in the allergen-exposed epidermis.

Depletion of CD4$^+$ T cells strongly enhances recruitment of neutrophils in the flare-up response

To further investigate how depletion of CD4$^+$ T cells affected the challenge response to the three allergens, we treated mice as depicted in Fig. 5A and measured the numbers of CD8$^+$

T_{RM} cells, $CD4^+$ T_{RM} cells and neutrophils in the epidermis 24 h after allergen challenge. We found that depletion of $CD4^+$ T cells resulted in the massive generation of epidermal $CD8^+$ T_{RM} cells in mice sensitized and challenged with either of the allergens (Fig. 6A, D, G, J-L). Importantly, in parallel with the massive increase in $CD8^+$ T_{RM} cells, a strongly enhanced influx of neutrophils was seen in mice depleted for $CD4^+$ T cells (Fig. 6C, F, I, J-L). As expected, depletion of $CD4^+$ T cells inhibited the formation of $CD4^+$ T_{RM} cells (Fig. 6B, E, H, J-L). Despite differences in the responses to the three contact allergens, the depletion experiments confirmed the correlation between the number of $CD8^+$ T_{RM} cells, the influx of neutrophils to the epidermis and the magnitude of the flare-up response after re-exposure to the allergens.

Discussion

In this study, we show that the clinically relevant contact allergens cinnamal, PPD and MI induce a strong $CD4^+$ T cell-mediated anti-inflammatory response that impairs the generation of pathogenic epidermal $CD8^+$ T_{RM} cells. Previous studies have pointed towards an anti-inflammatory role of $CD4^+$ T cells in ACD[8,11,15–22]. However, this is the first study that demonstrates that $CD4^+$ T cells can inhibit the generation of pathogenic epidermal $CD8^+$ T_{RM} cells.

Although $CD4^+$ T cells might play a pathogenic role in ACD to some contact allergens[25], $CD8^+$ T cells are commonly considered the key driver of ACD inflammation[1,9]. Indeed, animals deficient in $CD8^+$ T cells are unable to develop ACD to a multitude of experimental contact allergens[26,27]. In accordance, recent studies have pointed to the central pathogenic role of epidermal $CD8^+$ T_{RM} cells in ACD[3–6,9]. These studies were mainly based on ACD models in mice using the strong experimental allergen DNFB. However, an important role of epidermal $CD8^+$ T_{RM} cells in ACD to nickel has also been shown in patients with nickel allergy[4]. The aim of the present study was to determine the immunopathogenic mechanisms elicited by the clinically relevant contact allergens cinnamal, PPD and MI. Compared to DNFB, cinnamal, PPD and MI elicited a much weaker inflammatory response and a strongly reduced generation of epidermal $CD8^+$ T_{RM} cells. Importantly, the magnitude of the response after re-exposure to the allergens correlated with the number of epidermal $CD8^+$ T_{RM} cells but not the number of epidermal $CD4^+$ T_{RM} cells. Furthermore, depletion of the $CD4^+$ T cells greatly enhanced the generation of epidermal $CD8^+$ T_{RM} cells, the inflammatory response and the influx of neutrophils in the response to either of the tested contact allergens. These data suggests that epidermal $CD8^+$ T_{RM} cells are the key drivers of the inflammatory responses elicited to clinically relevant allergens as previously found for DNFB[6]. This further indicated that $CD4^+$ T cells mainly play an anti-inflammatory roles in response to contact allergens in line with previous observations[8,11,15–22]. However, previous studies on the role of $CD4^+$ T cells in ACD

used the classical short-term CHS model excluding the possibility to investigate the effect of $CD4^+$ T cells in the generation of epidermal $CD8^+$ T_{RM} cells. Thus, the present study is the first to provide evidence that $CD4^+$ T cells regulate the formation of epidermal-resident $CD8^+$ T_{RM} cells. Our finding could seem in contrast with a recent study, which found that $CD4^+$ T_{RM} cells can induce the inflammatory response seen following re-exposure to TNCB or oxazolone[28]. This discrepancy in the role of $CD4^+$ T cells in this and our study could be due to many factors such as use of different allergens, protocols and mouse strains, e.g. BALB/c mice have a higher proportion of circulating $CD4^+$ T and reduced local pro-inflammatory signaling after exposure to contact allergen compared to C57Bl/6 mice[29–31].

In conclusion, our results show that the generation of epidermal $CD8^+$ T_{RM} cells in response to clinically relevant contact allergens is regulated by $CD4^+$ T cells and differs between the contact allergens. Specifically, we show that $CD4^+$ T cells down-regulate, and for some contact allergens near completely inhibit, the formation of epidermal $CD8^+$ T_{RM} cells thereby suppressing the local pathological memory response. Furthermore, we show that once $CD8^+$ T_{RM} cells have developed, re-exposure to the allergen rapidly leads to local CXCL1 and CXCL2 production, recruitment of neutrophils and inflammation. These results improve our understanding of how local T cell memory to contact allergens is regulated and why different allergens may give different inflammatory responses. Determining exactly how $CD4^+$ T cells inhibit the generation of the pathogenic epidermal $CD8^+$ T_{RM} cells and whether the inhibition is allergen specific will be important to elucidate in future studies.

Conflict of interest

The authors declare no competing financial interests or other conflicts of interest.

Author contributions

Conceptualization of this study was done by ABF and CMB, JDJ, CG, NØ, and AW. Data curation and formal analysis was done by ABF. Funding acquisition was done by CMB and JDJ. Investigation was done by ABF, JFW, RDL, VM, KY and MHJ. Methodology was done by ABF and CMB. Project administration was done by ABF. Resources were obtained by CMB and CG. Supervision was done by CMB and JDJ. Data curation was done by ABF, JFW, RDL, VM, KY and MHJ. Visualization was done by ABF. Writing of the original draft was done by ABF, CMB and CG. Review and editing were done by all authors.

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Figure Legends

Figure 1. Cinnamal, PPD and MI have the capacity to induce local inflammation.

(A) Experimental setup: Mice were exposed to OOA, cinnamal, PPD or MI on day 0-2 (sensitization) and ear thickness were measured before sensitization on day 0 and after allergen exposure on day 6. **(B)** Mice were exposed to OOA (white) or cinnamal (green). **(C)** Mice were exposed to OOA (white) or PPD (blue). **(D)** Mice were exposed to OOA (white) or MI (orange). **(B-D)** Each dot represents mean ear thickness normalized to mean ear thickness at 0 hours within each group (n = 8). Statistical comparisons; Students unpaired t-tests. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

Figure 2. Cinnamal, PPD and MI induce local influx of epidermal T cells and an enhanced local memory response

(A) Experimental setup: Mice were exposed to OOA, cinnamal, PPD or MI on day 0-2 (sensitization) and re-exposed at day 21 (1^{st} chal.) and day 42 (2^{nd} chal.), on the ears or abdomen. Mice were re-exposed (3^{rd} chal.) to cinnamal, PPD or MI on the ears only and ear thickness was measured at 0 hours (before challenge) 24, 48, 72 and 96 hours after challenge. Flow cytometry experiments were performed twenty-one days after chal. 2^{nd} chal. on day 63. **(B-C)** Mean number of live epidermal T cells from mice exposed to OOA (white), cinnamal (green), PPD (blue) and MI (orange). Each dot represents cells from one mouse (n = 8). **(B)** $CD8^+$ T cells ($TCR\beta^+CD8\alpha^+$ cells); **(C)** $CD4^+$ T cells ($TCR\beta^+CD4^+$ cells)**. (D-I)** Each dot represents mean ear thickness normalized to mean ear thickness at 0 hours within each group; cinnamal (green), PPD (blue), MI (orange) and OOA (Gray) controls are exposed to reference allergen at 3^{rd} chal. (n = 8). **(D-F)** Ear thickness of mice exposed on the ears during sen-

sitization and challenge. **(G-I)** Ear thickness of mice exposed on the abdomen during sensitization, 1st chal. and 2nd chal., but on the ears at 3rd chal. Statistical comparisons; **(B-C)** by One-way ANOVA and **(D-I)** by Two-way ANOVA. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

Figure 3. Allergen-induced influx of neutrophils correlates with the number of epidermal CD8$^+$ T_{RM} cells.

(A) Experimental setup: Mice were exposed to OOA, cinnamal, PPD or MI on day 0-2 (sensitization) and re-exposed at day 21 (1st chal.) and day 42 (2nd chal.) on the ears. Mice were euthanized on day 63 at 0 hours (before 3rd chal.) or re-exposed (3rd chal.) to cinnamal, PPD or MI on the ears where epidermal ears sheet were isolated for flow cytometry 24 and 72 hours after 3rd chal. **(B-J)** Mean number of live epidermal cells from mice exposed to OOA (white), cinnamal (green), PPD (blue) and MI (orange). Each dot represents cells from one mouse (n = 8). **(B, E, K)** CD8$^+$ T_{RM} cells (CD103$^+$CD69$^+$CD44$^+$TCRβ$^+$CD8α$^+$ cells); **(C, F, I)** CD4$^+$ T_{RM} cells (CD103$^+$CD69$^+$CD44$^+$TCRβ$^+$CD4$^+$cells); **(C, F, I)** Neutrophils (CD11b$^+$Ly-6G$^+$ cells). **(L-K)** Correlation plots between CD8$^+$ T_{RM} cells **(L)** or CD4$^+$ T_{RM} cells **(K)** and neutrophils for all mice treated with allergens. Full line shows a simple linear regression and dotted lines shows 95% confident intervals. Each dot cinnamal (green), PPD (blue) and MI (orange), represents one mouse. Statistical comparisons; One-way ANOVA. Error bars; standard deviation (SD). Goodness of Fit = R^2 value. Statistical significance levels; not significant (ns) = $p > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

Figure 4. CXCL1 and CXCL2 levels correlate with neutrophil recruitment to the epidermis.

(A) Experimental setup: Mice were exposed to OOA, cinnamal, PPD or MI on day 0-2 (sensitization) and re-exposed at day 21 (1st chal.) and day 42 (2nd chal.) on the ears. Mice were euthanized on day 63 at 0 hours (before 3rd chal.) or re-exposed (3rd chal.) to cinnamal, PPD or MI on the ears 24 hours later. Complete ear skin (dermis and epidermis) were isolated for ELISA. **(B-J)** OOA (white), cinnamal (green), PPD (blue) and MI (orange). Each dot represents cytokine/chemokine from one mouse (n = 8). **(B, E, H)** Il-1β, **(C, F, I)** CXCL1 and **(D, G, J)** CXCL2 concentrations in the dermis and epidermis obtained just before the challenge at 0 hours and 24 hours after challenge. Statistical comparisons; One-way ANOVA. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

Figure 5. Depletion of $CD4^+$ T cells strongly enhances the number of $CD8^+$ T_{RM} cells and the flare-up response to cinnamal, PPD and MI.

(A) Experimental setup: Mice were exposed to cinnamal, PPD or MI on day 0-2 (sensitization) and re-exposed on day 21 (1st chal.) and day 42 (2nd chal.) on the ears. Depletion of $CD8^+$ cells or $CD4^+$ T cells were performed by i.v. injection at day -1, 0, 3 and by one i.p. injection at day 21 before 1st chal. and day 42 before 2nd chal. with anti-CD8α mAbs or anti-CD4 mAbs or IgG isotype control mAb. Mice were euthanized on day 63 at 0 hours (before 3rd chal.) re-exposed (3rd chal.) to cinnamal, PPD or MI. **(B-D)** OOA cinnamal (green), PPD (blue), MI (orange), isotype (non-marked), anti-CD8α (striped) and anti-CD4 (dotted). Each dot represents mean ear thickness normalized to mean ear thickness at 0 hours within each group (n = 8). **(E)** CD8α (red) and DAPI (Gray) stained fluorescent microscopy images of epidermal ear sheets isolated twenty-one days after 2nd chal. Scale bars; 100 µm (20x images)

and 20 µm (63x image) (n = 2). Statistical comparisons; One-way ANOVA. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = p > 0.05, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001.

Figure 6. Depletion of CD4$^+$ T cells strongly enhances recruitment of neutrophils in the flare-up response.

(A-J) Mice were exposed to cinnamal, PPD or MI on day 0-2 (sensitization) and re-exposed at day 21 (1st chal.) and day 42 (2nd chal.) on the ears. Depletion of CD8$^+$ cells or CD4$^+$ T cells was performed by i.v. injection at day -1, 0, 3 and by one i.p. injection at day 21 before 1st chal. and day 42 before 2nd chal. with anti-CD8α mAbs or anti-CD4 mAbs or IgG isotype control mAb. Mice were euthanized on day 64 at 24 hours after 3rd chal. to cinnamal, PPD or MI and epidermal cells were isolated for flow cytometry. OOA cinnamal (green), PPD (blue), MI (orange), isotype (non-marked), anti-CD8α (striped) and anti-CD4 (dotted). (n = 8). **(A, D, G)** CD8$^+$ T_{RM} cells (CD103$^+$CD69$^+$CD44$^+$TCRβ$^+$CD8β$^+$ cells); **(B, E, H)** CD4$^+$ T_{RM} cells (CD103$^+$CD69$^+$CD44$^+$TCRβ$^+$CD4$^+$cells); **(C, F, I)** Neutrophils (CD11b$^+$Ly-6G$^+$ cells). **(J-L)** tSNE plots illustrating CD4$^+$ T_{RM} cells (blue), CD8$^+$ T_{RM} cells (red), neutrophils (green) and other live cells (grey) in isotype controls, anti-CD8 mAb and anti-CD4 mAb treated mice (n = 4). **(J)** Exposed to cinnamal, **(K)** exposed to PPD, **(L)** exposed to MI. Statistical comparisons; One-way ANOVA. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = p > 0.05, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001.

Supplementary Figure S1

Flow cytometry gating strategy for identification of neutrophils and T cell subsets.

(A) Each gating plot shows a representative example obtained from a mouse exposed to MI on the ears 24 hours after 3rd chal. To best illustrate all gated populations SSC-A/FSC-A, FSC-W/FSC-A, FSC-W/Viablity Dye, CD11b/Ly-6G, TCRβ/CD8α/β, CD62L/CD44, CDCD69/CD103 show epidermal cells from an anti-CD4 and MI treated mouse and TCRβ/CD4, CD62L/CD44, CDCD69/CD103 show epidermal cells from isotype and MI treated mouse. As illustrated with red arrows CD11b/Ly-6G, TCRβ/CD8α and TCRβ/CD4 plots are gated on live single cells. CD62L/CD44 plots are gated on TCRβ$^+$CD8α/β$^+$ cells or TCRβ$^+$CD4$^+$, and CD69/CD103 plots are gated on TCRβ$^+$CD8α/β$^+$CD44$^+$CD62L$^-$ cells or TCRβ$^+$CD4$^+$CD44$^+$CD62L$^-$ cells. Identification of each subset are indicated by transparent color of the target quadrant: Neutrophils (green), CD8$^+$ T cells (blue), CD4$^+$ T cells (red), CD8$^+$ T_{RM} (red) and CD4$^+$ T_{RM} (green). The percentage of cells within each gate or quadrant is shown.

Figure 1.

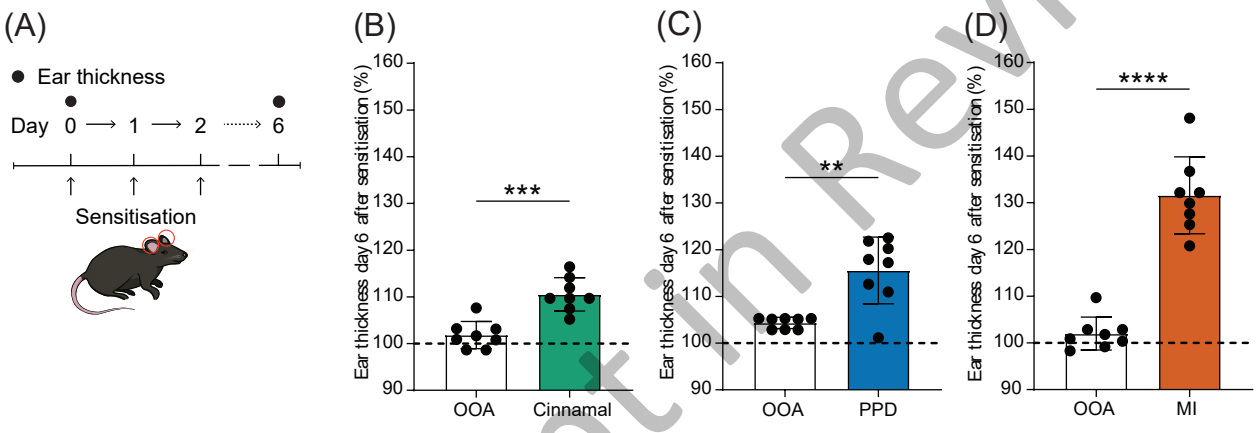
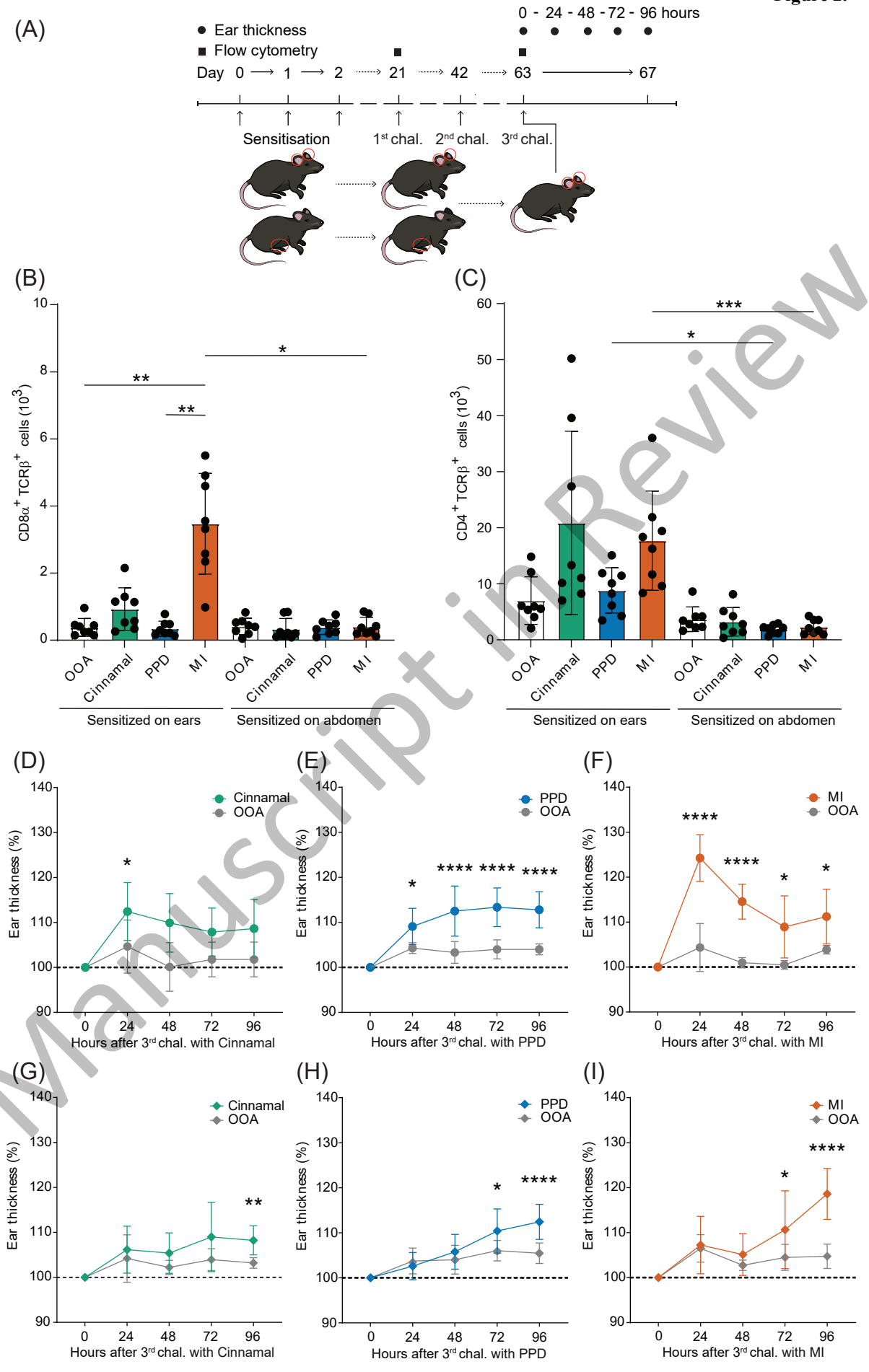


Figure 2.



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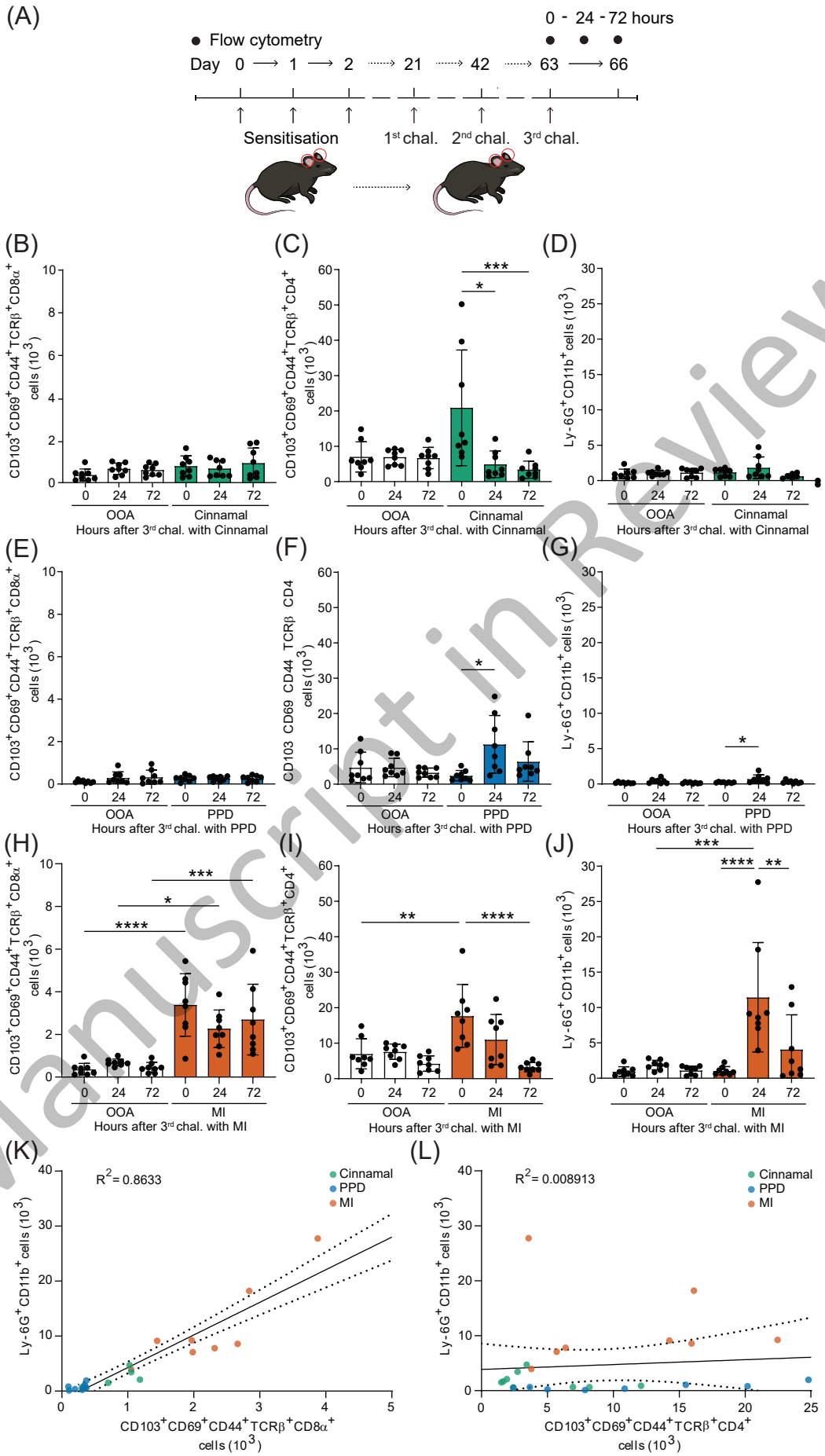

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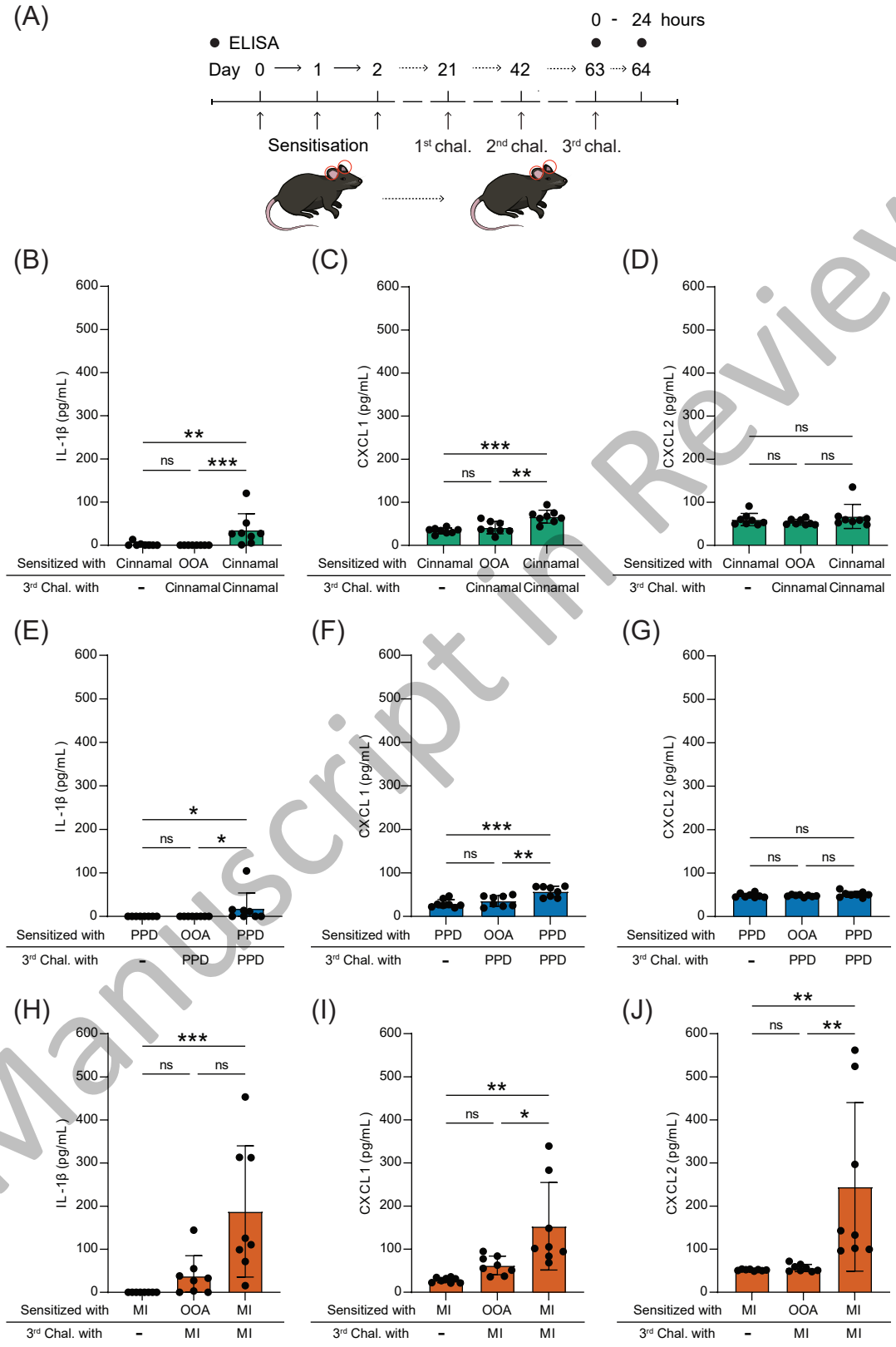


Figure 5.

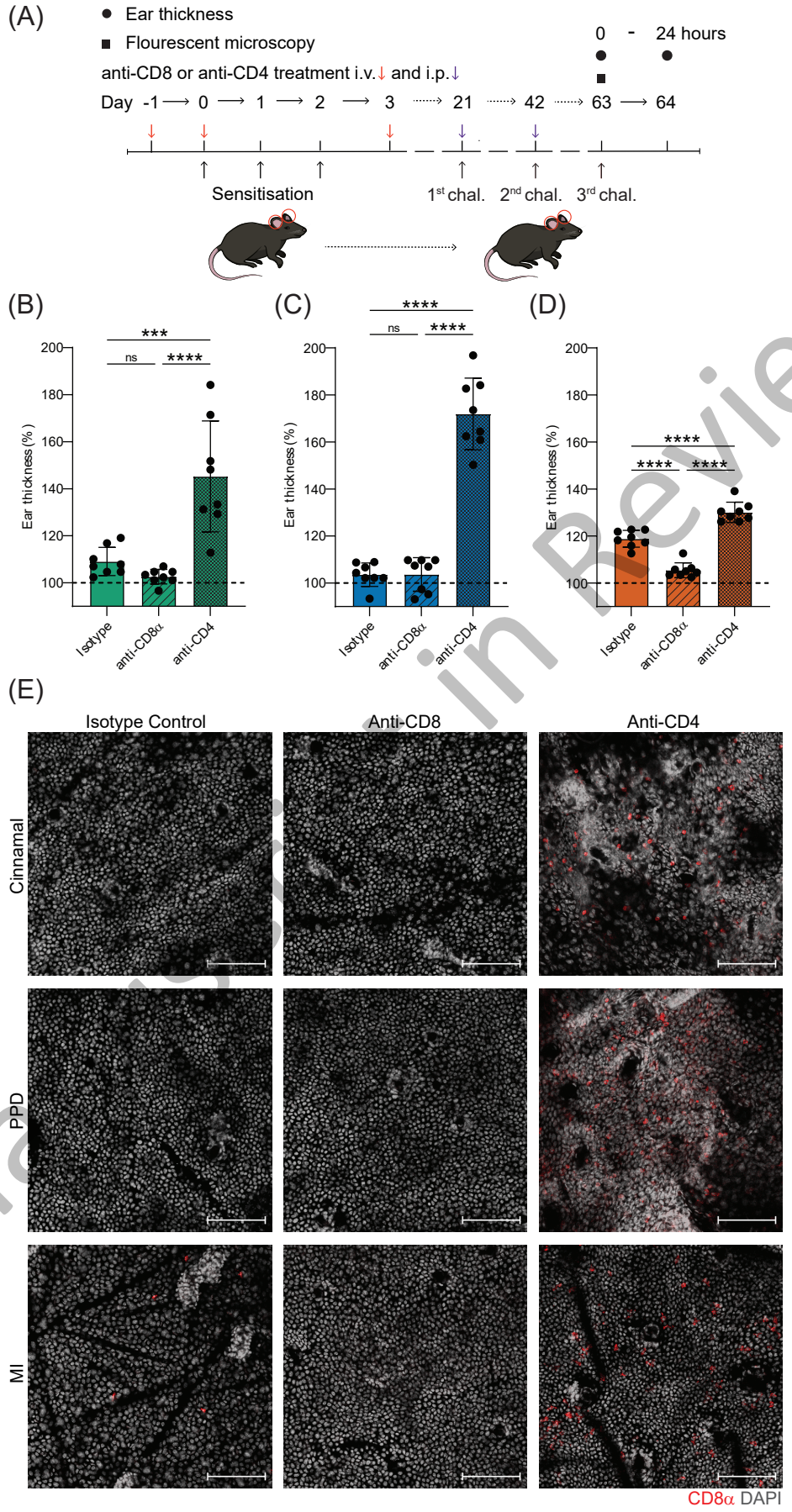
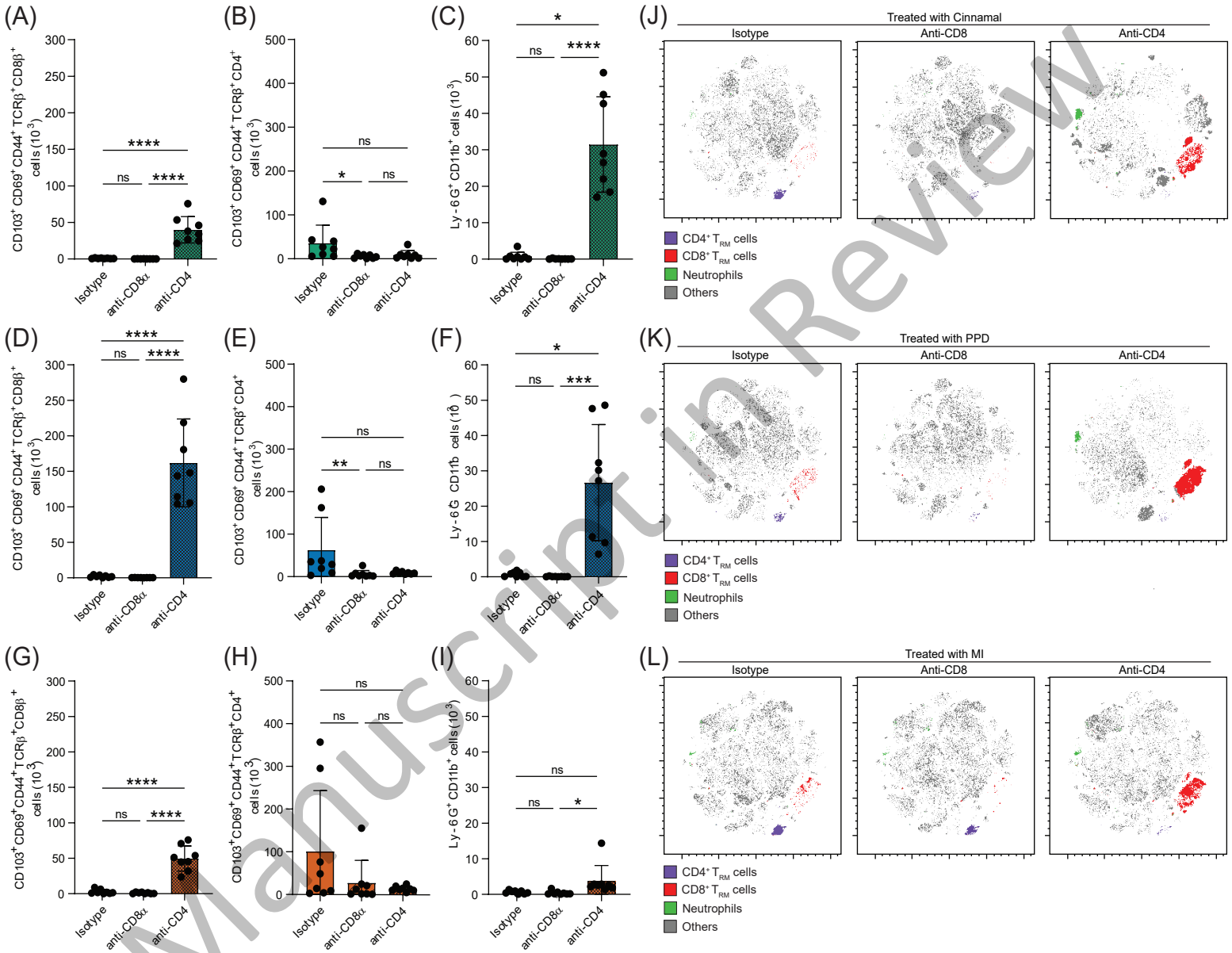
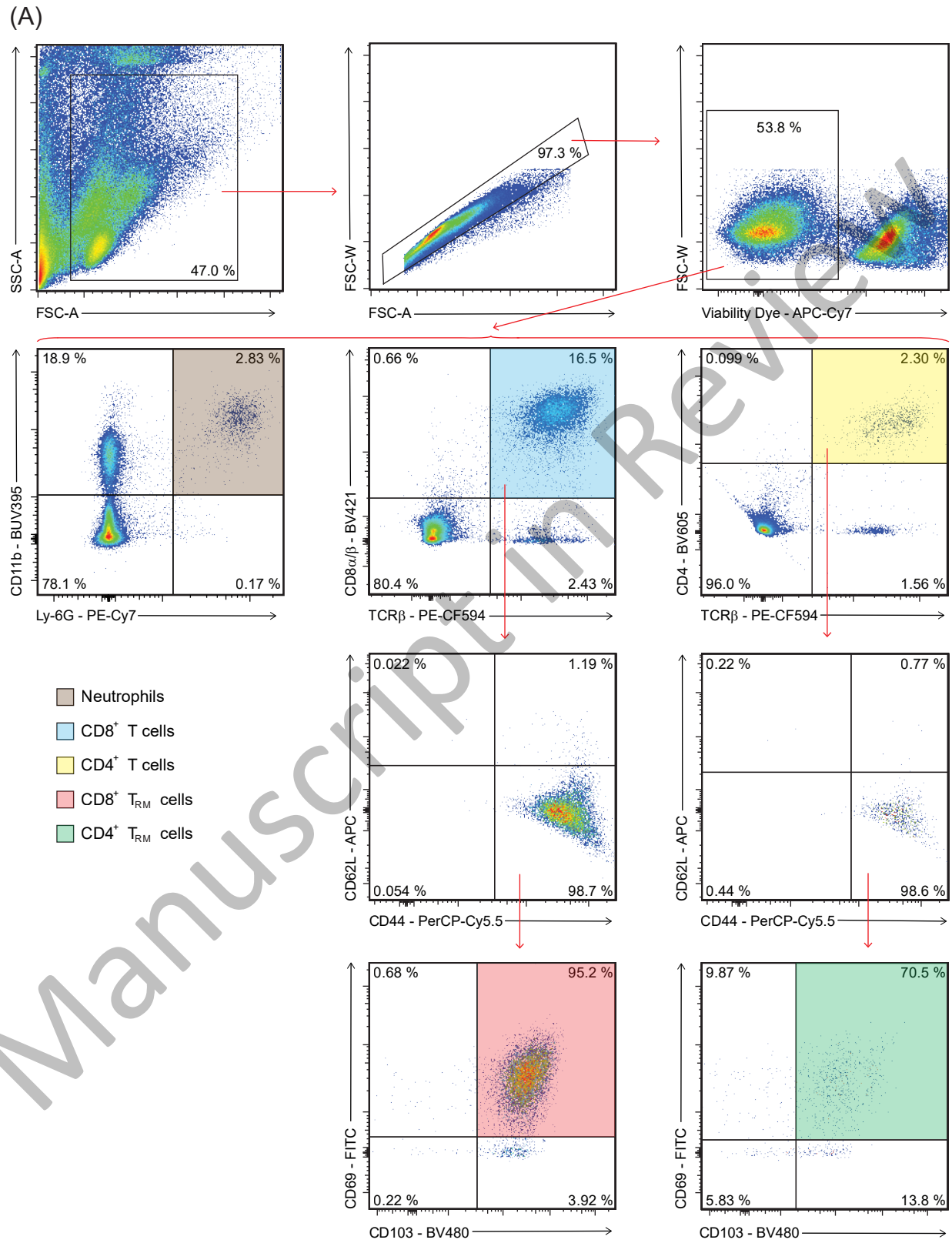


Figure 6.



Supplementary Figure 1.



Study III

Long-term survival of epidermal-resident $CD8^+$ T cells are mediated by permanent deposition of contact allergen in the epidermis

Long-term survival of epidermal-resident CD8$^+$ T cells is mediated by permanent deposition of contact allergen in the epidermis

Short title: Epidermal-resident CD8$^+$ T_{RM} cells survive by constitutive TCR activation

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Abstract

Background: A subset of $CD8^+$ memory T (T_{RM}) cells survive in epidermal skin long after exposure to contact allergen and thus maintaining the capacity to induce a rapid response upon local allergen re-exposure. Whether permanent allergen deposition is required for the survival of $CD8^+$ T_{RM} cells is unknown.

Methods: Using adoptive transfer and allergic contact dermatitis (ACD) mouse models and *ex vivo* skin analysis by flow cytometry and western blot, we investigated the presence of epidermal-resident $CD8^+$ T_{RM} cells, their status of activation (Ki67 and Nur77 expression) and the presence of 2,4-dinitrophenol (DNP)-self protein adducts in epidermis 1, 6 and 12 months after sensitization with 1-fluoro-2,4-dinitrobenzene (DNFB).

Results: We show that a significant number of $CD8^+$ T_{RM} cells survive in the epidermis for at least one year after DNFB exposure and that a fraction of the $CD8^+$ T_{RM} cells expressed Ki67 and Nur77. Furthermore, using an adoptive transfer model, we show that local exposure to DNFB is needed for the long-term survival of $CD8^+$ T_{RM} cells. Finally, we demonstrate that DNP-self protein adducts are still detectable in the epidermis one year after DNFB exposure.

Conclusion: This study demonstrates a permanent deposition of contact allergen in the epidermis one year after topical exposure, suggesting that this deposition facilitates local survival of allergen-specific $CD8^+$ T_{RM} cells in the epidermis by low-grade TCR activation.

Key words: Allergic contact dermatitis, epidermal-resident $CD8^+$ memory T cells, TCR activation, T_{RM} cell survival.

Introduction

Allergic contact dermatitis (ACD) is a common T cell mediated inflammatory skin disease that affects about 15% of the European population (1–3). Some patients are reported to experience both accelerated an enhanced ACD reactions when re-exposed on skin sites previously exposed to the sensitizing contact allergen (4–6). In relation, allergen-specific $CD69^+CD103^+CD8^+$ T (T_{RM}) cells form locally in allergen-experienced skin and re-activation of these cause rapid ACD reactions (7–11). More specifically, re-activation of allergen-specific $CD8^+$ T_{RM} cells were recently shown to induce a massive infiltration of neutrophils into the epidermis (8). The number of epidermal-resident $CD8^+$ T_{RM} cells increases in relation to allergen dosage and the number of allergen exposures, which determines the magnitude of the challenge response (9). In addition, the increase in epidermal-resident $CD8^+$ T_{RM} cell numbers upon challenge was shown to develop from both local proliferation and infiltration of circulating $CD8^+$ T cells (9). However, little is known about the mediators needed for survival and maintenance of allergen-specific $CD8^+$ T_{RM} cells in the skin over time.

In relation, survival of skin-resident pathogen-specific T_{RM} cells have been shown to depend on different factors, including local cytokine expression (i.e. IL-7, IL-15 and TGFβ) (12–14), expression of B-cell lymphoma 2 (Bcl2) (15), fatty acid-binding protein 4 (FABP4) and 5 (FABP5) (16), and signaling through the ligand-gated aryl hydrocarbon receptor (AhR) (17). Also, different studies using viral skin infections (Herpes simplex virus (HSV) and vaccinia virus (VV)), have shown that long-term survival of virus-specific $CD8^+$ T_{RM} cells in the skin do not require local antigen presentation (18–20). However, in ACD models, the number of epidermal-resident $CD8^+$ T_{RM} cells decreases significantly without continued contact-allergen exposure (10,11). Thus, the decrease in epidermal-resident $CD8^+$ T_{RM} cells was suggested to correlate with local epidermal persistence of the contact allergen, indicating that survival of

$CD8^+$ T_{RM} cells is dependent on continued presence of contact allergen in the skin (10). Furthermore, a subset of $CD8^+$ T_{RM} cells survive in the epidermis of allergen-exposed skin for least a year, where they maintain local reactivity to the contact allergen (10). Interestingly, the epidermal-resident $CD8^+$ T_{RM} cells also express different inhibitory checkpoint receptors (ICR), such as Programmed cell death protein 1 (PD1), T-cell immunoglobulin and mucin-domain containing-3 (TIM3), and Natural killer cell receptor (2B4) (10). These data suggest that $CD8^+$ T_{RM} cells have an inherent activation threshold that allow small amounts of allergen in the skin without inducing chronic skin inflammation. These important observations indicate that contact allergen or allergen-modified proteins are present in epidermal skin over time to facilitate local survival of allergen-specific $CD8^+$ T_{RM} cells.

In this study, we demonstrate that a significant number of allergen-specific $CD8^+$ T_{RM} cells survive locally in the epidermis at least one year after exposure to DNFB. Measuring Nur77 and Ki67 expression, we demonstrate that a fraction of the surviving $CD8^+$ T_{RM} cells are activated by cognate-antigen induced TCR activation and that some still proliferate one year after DNFB exposure. Furthermore, we show that survival and activation of DNFB-specific $CD8^+$ T_{RM} cells is completely abrogated within three months in an adoptive transfer model, where recipient mice are only exposed to the irritant sodium lauryl sulphate (SLS) and not to DNFB. Finally, by measuring presence of 2,4-dinitrophenol (DNP) conjugated protein by western blot, we demonstrate that DNFB permanently modifies epidermal proteins by the formation of DNP-self protein adducts. Taken together, we show that permanent epidermal deposition of contact allergen moieties facilitates local activation and survival of allergen-specific epidermal-resident $CD8^+$ T_{RM} cells.

Methods

Mice

Female C57Bl/6J mice (6-8 weeks old) were purchased from Janvier Labs (Le Genest-Saint-Isle, France). The mice were housed in a specific pathogen-free environment at the animal facility at the Department of Experimental Medicine, University of Copenhagen in accordance with the national animal protection guidelines (license number 2018-15- 0201-01409).

Contact Hypersensitivity (CHS) Model

Mice were sensitized for three consecutive days (day 0-2) by epicutaneous painting with 0.15% 1-fluoro-2,4-dinitrobenzene (DNFB) (Sigma-Aldrich, St. Louis, MO) diluted in a 1:4 solution of olive oil:acetone (OOA). Allergen exposure was performed with 25 µl on the dorsum of each ear. Mice were euthanized and both ears were harvested for further analysis 30, 180 or 365 days after sensitization. All experiments were repeated on at least two separate occasions.

Flow cytometry (FC) analysis

Following isolation of the ear skin, the allergen exposed side was separated from each ear and incubated for 1 hour at 37°C in a 0.3% trypsin (Sigma-Aldrich, St. Louis, T9201) Milli-Q water solution supplemented with 149 mM sodium chloride, 5 mM potassium chloride and 5 mM dextrose, pH 7.6 to enable epidermal peeling. The epidermis was incubated for 10 min (shaking) at 37°C in 0.3 % trypsin Milli-Q water solution supplemented with 149 mM sodium chloride, 5 mM potassium chloride, 5 mM dextrose, pH 7.6 and 0.1 % DNase (Sigma-Aldrich, St. Louis, D5025) to make single cell suspensions. The suspensions were washed through 70µm falcon cell strainers (Corning Inc. NY USA, 352350) in Dulbecco’s

Modified Eagle Medium (Sigma-Aldrich, St. Louis, 11965-092) supplemented with 15,5% Fetal Bovine Serum (Biological Industies, 04-007-1A) 1,4% DNase (Sigma-Aldrich, St. Louis, D5025) and incubated overnight at 37°C in RPMI-1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 1% L-glutamine, 1% penicillin-streptomycin, 50 μM 2-Mercaptoethanol, 25 mM HEPES, 100 mM Na Pyruvat and 100 μM nonessential amino acids. To prepare a positive control for TCR specific activation 1 x 10^6 cells was incubated overnight with 5 x 10^5 magnetic Dynabeads™ Mouse T-Activator CD3/CD28 (Gibco™, Thermo Fisher, 11453D). The following day, the number of cells was counted using a Neubauer hemocytometer and 1 x 10^6 cells from each mouse was used for flow cytometry. Before staining, Fc-receptors were blocked by 5 min incubation (RT) with anti-CD16/CD32 (2.4.G2) (BD Biosciences). Cell surface staining was performed for 30 min at 4°C incubation with anti–CD8α (BUV395, 53-6.7), anti–CD69 (FITC, H1.2F3) anti–CD103 (BV480, 2E7), anti–TCRβ (PE-CF594, H57-597) (BD Biosciences) and Fixable Viability Dye (eFluor 780) (eBioscience), diluted in Brilliant Stain Buffer (BD Biosciences). The cells were permeabilized and fixed for 45 min at room temperature (RT) using FOXP3/Transcription factor staining buffer set (eBioscience™, Cat no. 00-5523-00), followed by intracellular staining for 30 min (RT) with anti-Ki67 (BV421, B56) or IgG1κ isotype control (BD Biosciences, BV421, Cat no. 562438) and anti-Nur77 (AF647, 12.14) or IgG1κ isotype control (BD Biosciences, AF647, Cat no. 557732). Samples were analyzed on a BD LSR Fortessa (5 laser) instrument and data were processed in FlowJo (Treestar). Fluorescence minus one (FMO) stainings were used for all antibodies to optimize gating. The number of cells per mouse shown in the figures was calculated based on the total number of live cells.

Adoptive transfer

Submandibular and cervical draining lymph nodes were isolated on day 5 from sensitized female donor mice and single cell suspensions were prepared in Hanks' Balanced Salt Solution (HBSS) (Sigma Aldrich, H6648). 5 x 10^7 lymphocytes were transferred i.v. into recipient mice and they were treated topically directly after transfer and on the following two days (day 5-7) on the ears with 2.0 % solution of sodium lauryl sulphate (SLS), (Sigma Aldrich, 74255, CAS-no: 152-21-3) diluted with OOA. Recipient mice were euthanized and epidermal skin from the ears was isolated either 30 or 90 days after transfer for further analysis by flow cytometry.

Western blot

For Western blotting (WB) analysis, ears were lysed using the precellys 24 tissue homogenizer. One mouse ear was transferred to a precellys hard tissue grinding MK28-R tube and supplemented with ice cold WB lysis buffer (50 mM Tris base, pH 7.5, 150 mM NaCl and 1 mM Mg2Cl) supplemented with 1% (vol/vol) Triton X-100, 1 x Protease/phosphatase inhibition cocktail (5872S, Cell Signaling Technologies). Samples were homogenized using the soft tissue program twice with 10 minutes rest on ice in between runs. Subsequently, the lysates were transferred to new tubes and cleared by centrifugation at 10.000 G for 10 minutes at 4°C. For western blotting analysis, lysates were transferred to new tubes and supplemented with NuPAGE LDS sample buffer (4x) (NP0007, ThermoFisher Scientific) and NuPAGE sample reducing agent (10x) (NP0004, ThermoFisher Scientific) and treated for 5 minutes at 90°C. Next, the proteins were separated by electrophoresis through NuPAGE™ 10% BisTris gels (NP0301BOX, Life Technologies). Proteins were transferred to nitrocellulose membranes (LC2001, Life Technologies) and visualized with primary anti-dinitrophenol (DNP) antibodies (#D1D6, Cell signaling technology) and

secondary HRP-conjugated swine anti-rabbit Ig (P0399, DAKO) with ECL luminescence reagent (RPN2232, Sigma Aldrich) on a ChemiDoc^{TM} MP Imaging System (Bio Rad). The gels were exposed for 900 seconds and subsequently analyzed using ImageLab software.

Statistics

Statistical comparisons were performed using GraphPad Prism version 7.0. Gaussian distributions were tested using D'Agostino and Pearson normality test and statistical significance was tested using Students unpaired t-tests. For conditions without Gaussian distribution a Mann-Whitney U test was performed. Significance levels are illustrated in all figures as; Not significant (ns) = $p > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

Results

Long-term epidermal CD8$^+$ T_{RM} cell survival correlate with slow-rate TCR activation and proliferation

To investigate local survival, proliferation and TCR activation of CD8$^+$ T_{RM} cells in the epidermis, we exposed mice on the ears for three consecutive days with DNFB using olive oil:acetone (OOA) as a vehicle. We euthanized the mice on day 30, 180 or 365 after DNFB or OOA exposure to determine the number of CD69$^+$CD103$^+$CD8$^+$ T_{RM} cells in the epidermis over time by flow cytometry (Fig. 1A), using the gating strategy illustrated in (Sup Fig. 1A). As previously reported (8–10), a significant number of CD8$^+$ T_{RM} cells were present in the epidermis at all time-points in DNFB compared to OOA exposed mice (Fig. 1B-D). However, the number of CD8$^+$ T_{RM} cells decreased by ~50% between both 30-180 days after exposure (mean no. of cells ~30x10^3 versus mean no. of cells ~15x10^3 cells) and between 180-365 days after exposure (mean no. of cells ~15x10^3 versus mean no. of cells ~8x10^3 cells) (Fig. 1B-D). We investigated the proliferative capacity of the surviving epidermal-resident CD8$^+$ T_{RM} cells over time by measuring Ki67 expression (Fig. 1E-G, K). At all the measured time-points we found a significant number of proliferating epidermal-resident CD8$^+$ T_{RM} cells in DNFB compared to OOA exposed mice, although the number of proliferating CD8$^+$ T_{RM} cells significantly decreased from 30 to 180 days (from ~50% to ~13%) after DNFB exposure (Fig 1E-F). Interestingly, the number of Ki67$^+$ proliferating epidermal-resident CD8$^+$ T_{RM} cells was stabilized at ~2-3x10^3 cells between 180 and 365 days after exposure (Fig. 1F-G). Finally, we measured TCR specific activation of the epidermal-resident CD8$^+$ T_{RM} cells using an antibody against the endogenous nuclear receptor 4A1 (Nur77), which is expressed transiently in T cells following TCR specific activation and thus serve as a TCR specific activation reporter (21). Using FMO and isotype as negative controls and CD3-activating dynabeads as a positive control (Fig. 1L), we found

that ~3-5x10^3 $CD8^+$ T_{RM} cells expressed Nur77 in DNFB exposed mice at all the measured time-point and that this was significantly higher compared to OOA controls (Fig. 1I-J). These data show that a subset of epidermal-resident $CD8^+$ T_{RM} cells survive for at least a year after allergen exposure and that a fraction of these proliferate and receive a TCR-stimulating signal.

Contact allergen exposure to the skin is required for long-term $CD8^+$ T_{RM} cell survival

To investigate whether local presence of contact allergen is required for $CD8^+$ T_{RM} cell survival in the epidermis, we used an adoptive transfer model between female C57Bl/5 mice as previously performed (8). To induce epidermal T_{RM} cell formation in recipient mice without treating them with DNFB, donor mice were exposed to either DNFB or OOA and dLN cells were transferred into recipient mice that were subsequently exposed on the ears with irritant sodium lauryl sulphate (SLS). Following SLS exposure all recipient mice were left untreated until they were euthanized on day 30 or 90 where epidermal cells were analyzed by flow cytometry (Fig. 2A) and the gating strategy illustrated in Sup Fig. 1A and Fig. 1K-L was used. 30 days after transfer, we detected significant formation of $CD69^+CD103^+CD8^+$ T_{RM} cells in the epidermis of recipient mice that received DNFB-primed cells compared to OOA controls (Fig 2B). However, in recipient mice euthanized on day 90, the number of epidermal-resident $CD8^+$ T_{RM} cells decreased and there was no significant difference between OOA and DNFB-primed recipient mice at this time point. This clearly shows that direct DNFB exposure is required to maintain a pool of $CD8^+$ T_{RM} cells in the epidermis. Once more, we analyzed $CD8^+$ T_{RM} cell expression using the Ki67 proliferation marker (Fig. 2D-E) and TCR activation reporter (Nur77) (Fig. 2F-G). We detected a small difference in Ki67 expression in epidermal $CD8^+$ T_{RM} cells between OOA and DNFB transferred recipient mice 30 after transfer (Fig. 2D). However, this tendency was

undetectable 90 days after transfer (Fig. 2E), suggesting that proliferation of DNFB-specific epidermal-resident $CD8^+$ T_{RM} cells is dependent on epidermal presence of their cognate antigen. Finally, we did not detect any difference between Nur77 expression in epidermal $CD8^+$ T_{RM} cells between OOA and DNFB transferred recipient mice, neither at 30 nor 90 days after transfer (Fig. 2F-G), emphasizing that TCR specific activation of DNFB-primed epidermal-resident $CD8^+$ T_{RM} cells is mediated by presence of DNFB related antigens residing in the skin.

DNP-modified proteins persist in the epidermis over time after topical DNFB exposure

To determine if DNFB modified proteins persist in the epidermis over time, we examined epidermal homogenates by western blot (WB) analysis. DNFB modified proteins were detected by anti-DNP antibodies. Mice were euthanized 5, 30, 180 or 365 days after exposure to either OOA or DNFB (Fig. 3A). Each row in the WB gels illustrates DNP-conjugated proteins from one mouse. In epidermal skin from mice euthanized 5 days after DNFB exposure we detected high amounts of DNP-conjugated proteins as exposure for more than 1 second resulted in overexposure. The signal seemed very specific to the DNFB treated epidermal samples, as the OOA treated lysates did not yield a signal even following 60 seconds of exposure (besides an unspecific band around 25 kDa) (Fig. 3B). As illustrated (Fig. 3C), mice treated with OOA and euthanized 30, 180 and 365 days after treatment, showed minimal detection of DNP-conjugated proteins. However in epidermal skin harvested 30, 180 and 365 days after DNFB exposure we detected DNP-conjugated proteins compared to OOA treated mice (Fig. 3C) and variation in DNP-conjugated proteins between mice euthanized at day 365 were highly similar (Fig. 3D). Furthermore, images of DNFB exposed skin were not overexposed at 900 seconds of WB exposure, suggesting that significantly fewer DNP-conjugated proteins were present in the epidermis 30, 180 and 365 days after

DNFB exposure (Fig. 3C), when compared to epidermal skin harvested 5 days after DNFB exposure (Fig. 3B). Furthermore, the DNP-conjugated proteins were generally found to be of different sizes within the detection rate between 15 and 140 kilo Daltons (kDa). By visual observation the amount of epidermal DNP-conjugated proteins only seemed to decrease slightly from 30 to 365 days after DNFB exposure (Fig. 3C). However, this observation needs further quantitative investigation.

Discussion

As the first, this study demonstrates that presence of contact allergen modified proteins in the epidermis one year after allergen exposure, facilitates local survival of allergen-specific $CD8^+$ T_{RM} cells. This was shown by stable TCR specific activation evaluated by Nur77 expression in epidermal-resident $CD8^+$ T_{RM} cells, in DNFB exposed epidermis where DNP-conjugated proteins were present. These data suggest that a permanent formation of DNP-self protein adducts occur and they act as TCR specific epitopes mediating long-term survival and proliferation.

DNP-conjugated proteins in the epidermis have previously been detected by Gamradt et al. (2019), showing DNP-stained epidermis present at different time-points up to 30 days after exposure (10). Moreover, they demonstrated that the DNP-stained areas decreased significantly from day 2 to day 30 after DNFB exposure, indicating that the contact allergen gradually disappear from the epidermis (10). In relation, we observed high amounts of DNP-conjugated proteins in the epidermis on day 5 as a clear signal was detected already after 1 second of exposure time and further to be overexposed already after 60 seconds. Less DNP-conjugated proteins seemed present in the DNFB exposed epidermis after 30 days as overexposure were not reached within 900 seconds of exposure time. Compared to OOA controls, we did however detect a clear DNP signal in epidermis of DNFB sensitized mice at all the time points measured. Interestingly, we only detected a slight decrease in DNP-conjugated proteins from day 30 to 365, which leads us to suggest that the DNP-moieties that are not covalently bound to skin proteins, are removed within 30 days after exposure. However, further investigations are needed to quantify and directly compare the amount of DNP-conjugated proteins at different time points and to identify which epidermal proteins are permanently modified by DNFB. In relation, *para*-phenylenediamine (PPD) have been demonstrated to accumulate at least short-term in the skin of rats after repeated exposure to

radiolabeled PPD (21). In accordance, some studies have shown that clinical relevant contact allergens accumulate in the skin of contact allergic patients (22–25). However, none of the studies investigated the presence of contact allergen in the skin over time without continued exposure. Whether the findings of this study are relevant for ACD patients, should be further elucidated, e.g. by measuring Nur77 expression in $CD8^+$ T_{RM} cells from skin of allergic patients where local memory has formed.

A few studies have investigated DNP-conjugated proteins early (24 hours) after DNFB exposure and shown that DNP-conjugated proteins are preferentially distributed throughout the epidermal layer of the skin, and both intra and extracellular proteins are modified (6,26–28). Yet, a few studies have investigated the presence of DNP-conjugated proteins in the skin over time but with contradicting results (6,10). Specifically, Schmidt at al. (2017) did not find permanent deposition of DNP in the skin, as DNP-conjugated proteins was not detectable in the skin 21 days after exposure when measured by fluorescence microcopy and WB (6). In line with the results shown in this study, Gamradt et al. (2019) were able to detect DNP-conjugated proteins in the epidermis 30 days after DNFB exposure by fluorescence microscopy (10). The inconsistency between Schmidt et al. and our data using western blot could be explained by the experimental setup where Schmidt at al. measured the presence of DNP-conjugated proteins in skin isolated 24 hours and 21 days after DNFB exposure simultaneously (within the same gel). In relation, we observed a faster overexposure time, indicating a higher amount of DNP-conjugated proteins in the skin isolated early (day 5) after DNFB exposure when compared to later time point. Thus, unless the gel is overexposed, no signal would be detected in skin isolated 30 days after DNFB with skin isolated 5 days after exposure when analyzed within the same WB gel. Furthermore, in relation to this study, we also tried to measure DNP-conjugated protein in epidermal ear sheets by fluorescence microscopy using primary anti-DNP antibodies. However, in line with Schmidt et al., we

were not able to detect DNP-conjugated proteins in skin isolated more than three weeks after DNFB exposure (data not shown). This indicate that our protocol or the DNP-specific antibody we used, were not sensitive enough to detect lower amounts of DNP-conjugated protein present in the epidermis.

Another scientific conundrum is that Gamradt et al. (10) and our observations clearly suggest that DNFB-specific $CD8^+$ T_{RM} cells require constitutive antigen presentation to survive, whereas viral-specific $CD8^+$ T_{RM} cells have been demonstrated to survive without antigen presentation (18–20). The reason for this difference is unknown and need further investigations.

In summary, the results reported in this study show that allergen-specific $CD8^+$ T_{RM} cells require constitutive antigen-presentation in order to survive over time in the epidermis. Further investigations on clonal specificity of the surviving $CD8^+$ T_{RM} cells will elucidate if epidermal survival is mediated by few or many different T cell clones. This will be important to understand the mechanisms involved in local $CD8^+$ T_{RM} cells survival. Finally, quantification and identification of the different DNFB modified proteins using e.g. mass spectrometry could give further insight into local changes in the epidermis that facilitate long-term survival of the allergen-specific $CD8^+$ T_{RM} cells. Understanding the factors mediating local $CD8^+$ T_{RM} cell survival in ACD patients, will likely be an important step in future prevention of reoccurring flare-up.

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Conflict of interest

The authors declare no competing financial interests or other conflicts of interest.

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Figure Legends

Figure 1. Long-term epidermal CD8$^+$ T_{RM} cell survival correlate with slow-rate TCR activation and proliferation

(A) Experimental setup: Mice were exposed to OOA or DNFB on day 0-2 (sensitization). Mice were euthanized 1 month (day 30), 6 months (day 180) or 12 months (day 365) after sensitization and epidermal skin from both ears was isolated for flow cytometric analysis. **(B-D)** Flow cytometric analysis of CD69$^+$CD103$^+$TCRβ$^+$CD8α$^+$ cells in mice exposed to OOA (white bars) or DNFB (red bars) 1 month **(B)**, 6 month **(C)** and 12 month **(D)** after sensitization. **(E-G)** Flow cytometric analysis of Ki67$^+$CD69$^+$CD103$^+$TCRβ$^+$CD8α$^+$ cells in mice exposed to OOA (white bars) or DNFB (green bars) 1 month **(E)**, 6 month **(F)** and 12 month **(G)** after sensitization. **(H-J)** Flow cytometric analysis of Nur77$^+$CD69$^+$CD103$^+$TCRβ$^+$CD8α$^+$ cells in mice exposed to OOA (white bars) or DNFB (blue bars) 1 month **(H)**, 6 month **(I)** and 12 month **(J)** after sensitization. **(K)** Histogram illustrates gating of Ki67$^+$CD69$^+$CD103$^+$TCRβ$^+$CD8α$^+$ cells using isotype control (green) and fluorescence minus one (FMO) control (orange). **(L)** Histogram illustrates gating of Nur77$^+$CD69$^+$CD103$^+$TCRβ$^+$CD8α$^+$ cells using isotype control (green), fluorescence minus one (FMO) control (orange), TCR/CD3 activating beads positive control (blue) and TCR/CD3 activating beads + isotype negative control (purple). **(A-J)** Each dot represents number of cells from one mouse (n = 8). Statistical comparisons; Students unpaired t-tests. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

Figure 2. Contact allergen exposure to the skin is required for long-term $CD8^+$ T_{RM} cell survival (A) Recipient mice were exposed to OOA or DNFB at day 0-2 on the ears. On day 5, 5 x 10^7 lymphocytes were isolated from the draining lymph nodes of recipient mice and transferred i.v. into donor mice. On day 5-7, donor mice were treated on the ears with irritant SLS to induce recruitment of epidermal T_{RM} cells. Donor mice were euthanized 1 month (day 30), 6 months (day 180) or 12 months (day 365) after exposure and epidermal skin from both ears was isolated for flow cytometric analysis. **(B-C)** Flow cytometric analysis of $CD69^+CD103^+TCR\beta^+CD8\alpha^+$ cells in donor mice following transfer from OOA exposed recipient (white bars) or DNFB exposed recipients (red bars) after 1 month **(B)** or 3 months **(C). (D-E)** Flow cytometric analysis of $Ki67^+CD69^+CD103^+TCR\beta^+CD8\alpha^+$ cells in donor mice following transfer from OOA exposed recipients (white bars) or DNFB exposed recipients (green bars) after 1 month **(D)** or 3 months **(E)**. **(F-G)** Flow cytometric analysis of $Nur77^+CD69^+CD103^+TCR\beta^+CD8\alpha^+$ cells in donor mice following transfer from OOA exposed recipients (white bars) or DNFB exposed recipients (blue bars) after 1 month **(F)** or 3 months **(G)**. **(B-G)** Each dot represents number of cells from one donor mouse (n = 8). Statistical comparisons; Students unpaired t-tests. Error bars; standard deviation (SD). Statistical significance levels; not significant (ns) = $p > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.

Figure 3. DNP-modified proteins persist in the epidermis over time after topical DNFB exposure

(A) Mice were exposed to OOA or DNFB on day 0-2 (sensitization). Mice were euthanized 5 days, 1 month (day 30), 6 months (day 180) or 12 months (day 365) after sensitization and epidermal skin from both ears were isolated for western blot. **(B)** Western blot analysis of epidermal homogenates harvested five days after DNFB exposure from two OOA and three

DNFB treated mice stained with anti-DNP antibody either exposed for 1 second (left) or overexposed or 60 seconds (right) (n = 2). **(C)** Western blot analysis of epidermal homogenates harvested on day 30, 180 and 365 after DNFB exposure from OOA and DNFB treated mice stained with anti-DNP antibody (n = 1). **(D)** Western blot analysis of epidermal homogenates harvested on day 365 from four OOA and four DNFB treated mice (n = 4) (D) Each row illustrates DNP-conjugated proteins from one mouse. Kilo Daltons (kDa).

Supplementary Figure S1

Gating strategy following flow cytometry analysis.

(A) Each plot shows a representative example obtained from one mouse 30 days after DNFB exposure. The gating strategy is illustrated with red arrows, SSC-A/FSC-A (gating cell of interest), FSC-W/FSC-A (gating single cells), FSC-W/Viablity Dye (gating viable cells), TCRβ/CD8α (gating $CD8^+$ αβ T cells), CD69/CD103 (gating $CD8^+$ αβ T_{RM} cells), Ki67 (gating proliferating $CD8^+$ αβ T cells) and Nur77 (gating TCR activated $CD8^+$ αβ T cells). Each subset is depicted by transparent color of the target quadrant: $CD8^+$ T cells (blue), $CD8^+$ T_{RM} cells (red), proliferating $Ki67^+CD8^+$ T_{RM} cells (green) and TCR activated $Nur77^+CD8^+$ T_{RM} cells (yellow). The percentage of cells within each gate or quadrant is shown.

Figure 1.

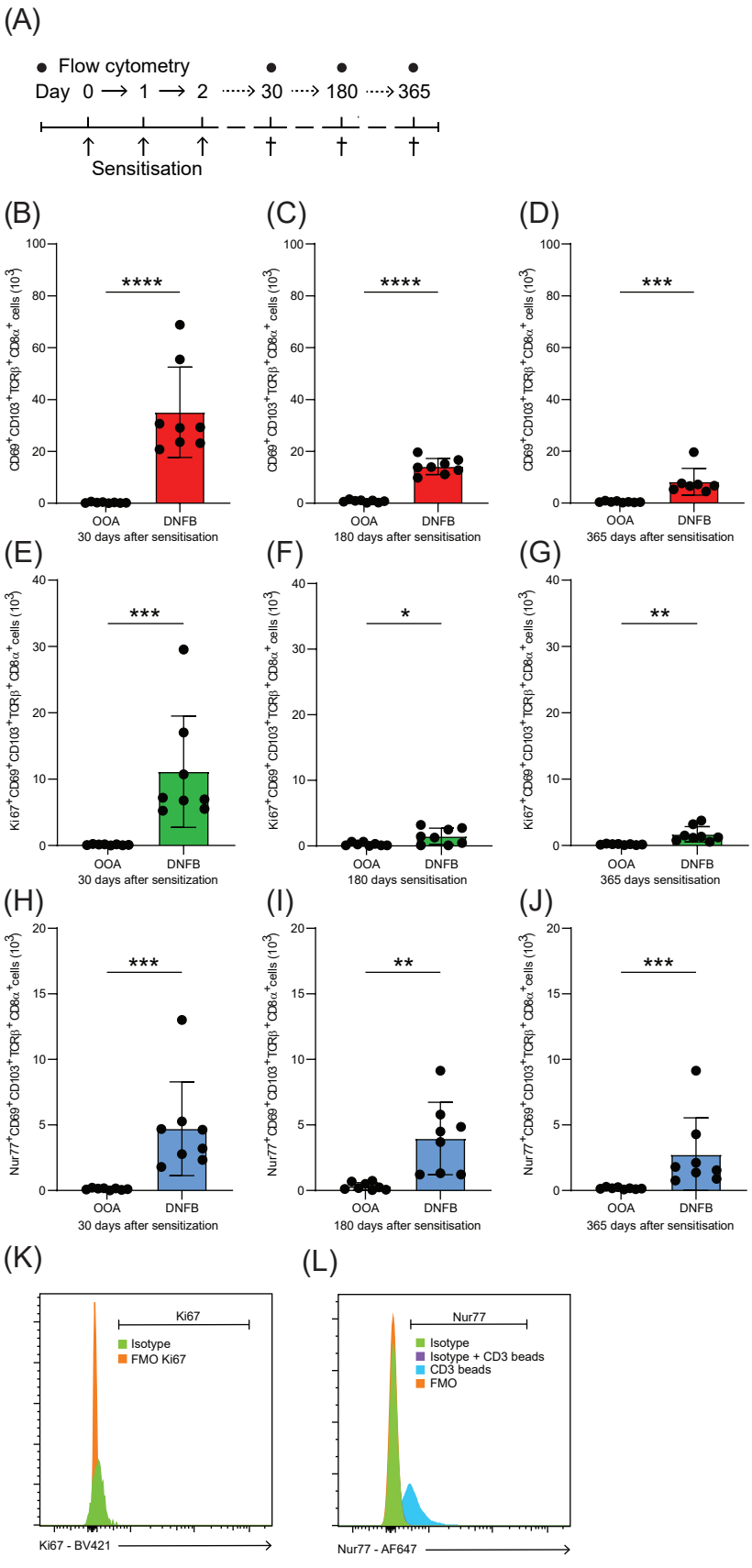

(A)
Flow cytometry
Day 0 → 1 → 2 ····→ 30 ····→ 180 ····→ 365
Sensitisation
(B)
(C)
(D)
CD69+CD103+TCRβ+CD8α+ cells (10^3)
****
****
***
OOA
DNFB
30 days after sensitisation
180 days after sensitisation
365 days after sensitisation
(E)
(F)
(G)
Ki67+CD69+CD103+TCRβ+CD8α+ cells (10^3)
***
*
**
30 days after sensitization
180 days sensitisation
365 days sensitisation
(H)
(I)
(J)
Nur77+CD69+CD103+TCRβ+CD8α+ cells (10^3)
***
**
***
30 days after sensitization
180 days after sensitisation
365 days after sensitisation
(K)
Ki67
Isotype
FMO Ki67
Ki67 - BV421
(L)
Nur77
Isotype
Isotype + CD3 beads
CD3 beads
FMO
Nur77 - AF647

Figure 2.

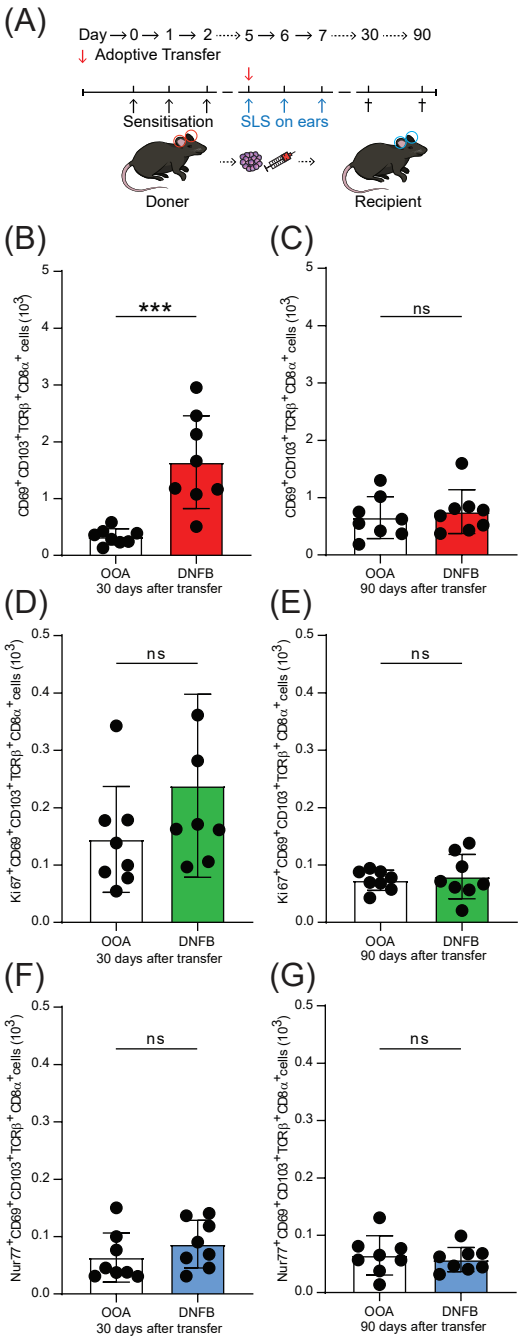
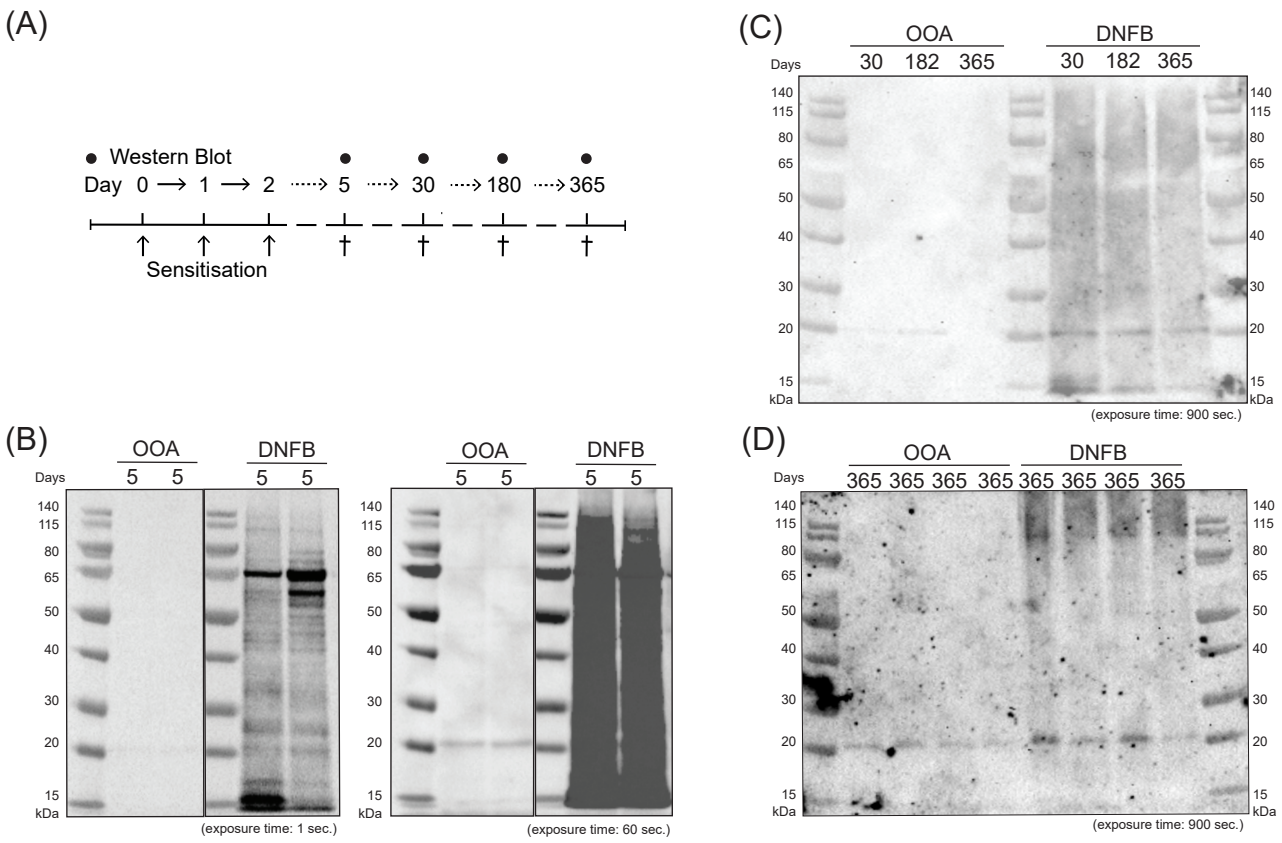
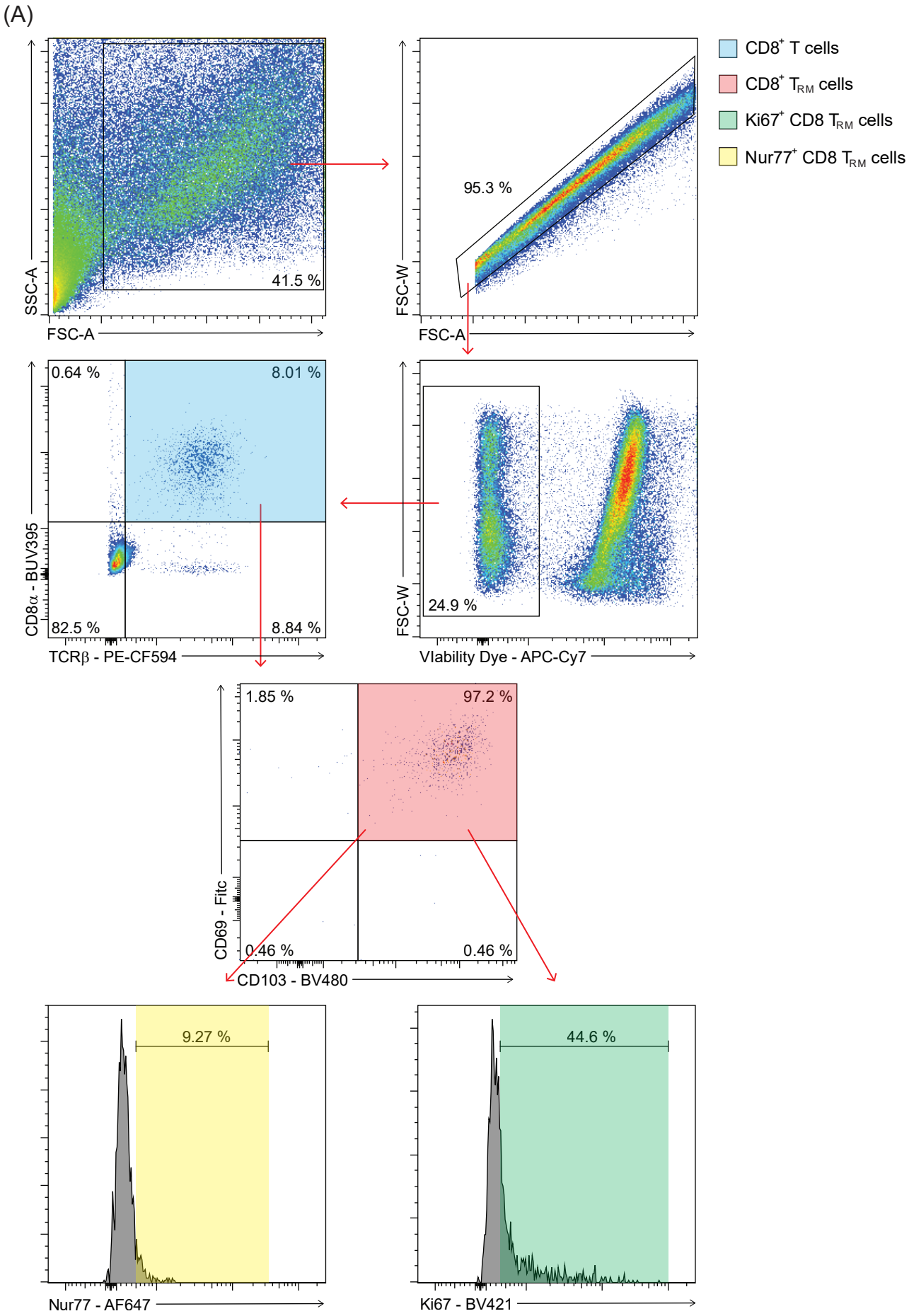

(A)
Day → 0 → 1 → 2 ····→ 5 → 6 → 7 ····→ 30 ····→ 90
↓ Adoptive Transfer
Sensitisation
SLS on ears
Doner
Recipient
(B)
***
CD69+CD103+TCRβ+CD8α+ cells (10^3)
OOA
DNFB
30 days after transfer
(C)
ns
CD69+CD103+TCRβ+CD8α+ cells (10^3)
OOA
DNFB
90 days after transfer
(D)
ns
Ki67+CD69+CD103+TCRβ+CD8α+ cells (10^3)
OOA
DNFB
30 days after transfer
(E)
ns
Ki67+CD69+CD103+TCRβ+CD8α+ cells (10^3)
OOA
DNFB
90 days after transfer
(F)
ns
Nur77+CD69+CD103+TCRβ+CD8α+ cells (10^3)
OOA
DNFB
30 days after transfer
(G)
ns
Nur77+CD69+CD103+TCRβ+CD8α+ cells (10^3)
OOA
DNFB
90 days after transfer

Figure 3.


(A)
Western Blot
Day 0 → 1 → 2 ····> 5 ····> 30 ····> 180 ····> 365
Sensitisation
(B)
OOA
DNFB
Days
5 5 5 5
140
115
80
65
50
40
30
20
15
kDa
(exposure time: 1 sec.)
(exposure time: 60 sec.)
(C)
OOA
DNFB
Days
30 182 365 30 182 365
(exposure time: 900 sec.)
(D)
OOA
DNFB
Days
365 365 365 365 365 365 365 365
(exposure time: 900 sec.)

Supplementary Figure 1.



Discussion and Perspectives

The studies of this thesis provides new insight on the immunological mechanism behind local skin responses to contact allergens. However, conducting basic research using animal models always leads to the question - what is the clinical relevance and perspectives of the results in a human disease setting? Also, answering one research question often cultivate formation of many new ones. Indeed, such questions should too be considered moving forward.

In this thesis I have focussed on local challenge responses formed within allergen-experienced skin sites in relation to re-activation of epidermal-resident $CD8^+$ T_{RM} cells as these are generally considered to be the key effector cells in local ACD flare-ups (53,68). In study I, we found that once epidermal-resident $CD8^+$ T_{RM} cells have developed, they mediate both accelerated and enhanced challenge responses by rapid CXCL1/2 dependent recruitment of neutrophils into the epidermis. In accordance, human contact allergic patients are described to experience accelerated ACD when challenged on an allergen-experienced skin site (55,233,234). Furthermore, $CD8^+$ T_{RM} cells have been demonstrated to reside locally in human skin exposed to e.g. nickel-, urushiol-, rubber- and fragrances (55,210,213,271,272). By patch-testing with nickel, Schmidt et al. (2017) demonstrated a correlation between $CD8^+$ T_{RM} cells and rapid (24 hour) memory responses and Cavani et al. (1998) showed that nickel exposed human skin harbored IFNγ-producing nickel-specific $CD8^+$ T_{RM} cells (55,211). However, others have demonstrated that development of $CD8^+$ T_{RM} cells in the skin vary depending on the sensitizing allergen (210,272). Collectively, these results suggest that for some allergens, $CD8^+$ T_{RM} cells form locally and orchestrate local flare-up reactions in human ACD, while for other allergens this do not occur to the same extend. These observations are generally in accordance with our murine data.

Whether skin-resident $CD8^+$ T_{RM} cells are capable of mediating massive infiltration of neutrophils into human allergen-experienced skin after challenge is still unknown. Generally, the role of neutrophils in human ACD has not gained much attention. This is likely because most human studies have analyzed the cellular composition within punch biopsies harvested from patch test sites and thus investigated challenge responses within allergen-naïve skin. At these skin sites only small numbers of skin-infiltrating neutrophils have been detected and these are first seen in the skin 48-96 hours after challenge (135,151,185,210,212,273). Again, these observations are generally in accordance with our murine data, where we also observed a small, although not

significant, number of neutrophils infiltrating the epidermis 48 hours after challenge of allergen-naïve skin sites. Interestingly, the neutrophils seemed to arrive together with circulating $CD8^+$ T_{EM} and T_{CM} cells. This observation is similar to others, using the conventional short-term CHS model showing that neutrophils and effector $CD8^+$ T cells interact and amplify the inflammatory response during the acute phase (221,231,260–263). Our data do, however, not provide new insight to the interactions between memory $CD8^+$ T cells and neutrophils in allergen-naïve skin. More research is therefore needed.

In study I we showed that depletion of $CD8^+$ T cells during sensitization inhibit epidermal $CD8^+$ T_{RM} cell formation and thus the rapid CXCL1/2-induced recruitment of neutrophils. We also showed that treatment with a CXCR1/2 antagonist abrogated the response. Nevertheless, we did not provide new data illustrating which skin-resident cells express these chemokines. We observed a high amount of CXCL2 expression in both dermis and epidermis 12 hours after DNFB challenge, we therefore do not believe that the $CD8^+$ T_{RM} cells are the main source of CXCL2, because they almost exclusively reside in the epidermis (56,58). Several other skin-resident cells can produce CXCL2 once activated, including macrophages, mast cells and KCs (228,264,274). In both man and mouse, others have shown that release of IL-17 in the skin induce local chemokine production leading to neutrophil recruitment (140,221,230,256–259,272). The IL-17-mediated response was amplified by IFNγ expression (140,258,259). Because $CD8^+$ T_{RM} cells are adept producers of both IL-17 and IFNγ early upon re-activation (55), I therefor propose that IL-17, in synergy with IFNγ, are rapidly released by $CD8^+$ T_{RM} cells upon local re-exposure and thus induce CXCL1/2 production by nearby skin-resident cells. However, the exact signalling pathway needs further investigation.

In study I and II we found that development of epidermal-resident $CD8^+$ T_{RM} cells drive the inflammatory response to the experimental contact allergen (DNFB) and to clinically relevant allergens (cinnamal, PPD and MI). This observation is generally in line with other studies looking at the memory T cell response to contact allergens in mice (55–57), and with many studies looking in the acute phase using the conventional short-term CHS mouse model (201,205,206,208,218–221,224,228). However, a recent study by Murata and Hayashi (2020), investigated the memory T cell response in BALB/c mice following TNCB exposure an found $CD4^+$ T_{RM} cells to be the main effector cells over time (58). They detected a 10-fold higher number of dermal-resident $CD4^+$ T_{RM} cells compared to the number of epidermal-resident $CD8^+$

T_{RM} cells in the skin 35 days after TNCB exposure (58). Unexpectedly, they further showed that the mice developed enhanced challenge responses to TNCB when the time between sensitization and challenge was increased from 15 to 56 weeks. This suggests that skin-resident $CD4^+$ T_{RM} effector responses are amplified over time or that local regulatory mechanisms decrease in this model (58). Additionally, Murata and Hayashi showed that the number of dermal-resident $CD4^+$ T_{RM} cells was stable over time, whereas the epidermal-resident $CD8^+$ T cells completely disappeared (58). The fact that epidermal-resident $CD8^+$ T cells decrease over time is in line with our results shown in study III and data published by Gamradt et al. (56). In accordance with Gamradt et al. we still found presence of a significantly large fraction of the epidermal-resident $CD8^+$ T cells one year after sensitization with DNFB. Based on the data published by Murata and Hayashi, they suggested that $CD4^+$ T_{RM} cells are the main effector cells behind local ACD responses over time. The contradicting results to those published by Gamradt et al. and our results may be due to use of different contact allergens or use of different mouse strains (BALB/c versus C57Bl/6 mice). This because BALB/c mice have been reported to be more Th2 prone (275), have higher numbers of naïve circulating $CD4^+$ T cells (276) and to have lower pro-inflammatory reactions to DNFB (107), while higher challenge responses to PPD (277) when compared to C57Bl/6 mice. Thus, understanding the immunological differences between different mouse strains may be essential when translating result to the human condition.

In study II, we showed that $CD4^+$ T cells regulate the development of epidermal-resident $CD8^+$ T cells and thus subsequent challenge responses in an allergen-dependent manner. Moreover, development of epidermal-resident $CD8^+$ T_{RM} cells was completely inhibited in response to cinnamal and PPD exposure, while not to MI. The T cell responses to MI were more similar to those following DNFB exposure in study I. Still, the response was significantly lower in MI- compared to DNFB exposed mice, even though the mice were challenged with MI on two additional occasions. The reason for these differences is unknown, but other studies have also described varying $CD4^+$ T cell responses depending on the sensitizing contact allergen. Specifically, $CD4^+$ T cells were the primary effector cells toward FITC (206), both effector and regulatory cells towards oxazolone (199,207,208), and solely regulatory cells towards DNFB and fragrance allergens (200,202,204,221). On the other hand, $CD8^+$ T cells were described as the primary effector cells in response to contact allergens including DNFB (197,200–202,215,221), DNCB (222), TNCB (214,223) oxazolone (197,224), fragrance allergens (204) and palladium (225). As of now we can only speculate on the mechanism behind the varying T cell responses to

different contact allergens, although activation of urushiol-specific $CD4^+$ have been shown to depend on endogenous processing, whereas urushiol-specific $CD8^+$ T cells were shown to rely on exogenous processing (278). This indicates that the capacity of a contact allergen to induce different innate signalling pathways may be key. In relation, several innate signalling factors may also affect subsequent T cell responses to contact allergens, including ROS production, IL-1β/danger signalling, TLR activation, and cytokine stimulation in the dLNs (169,193,279,280). Investigating different aspects of innate immune activation and signalling in response to different contact allergens, could therefor provide more answers.

To add further complexity to the question on allergen-dependent T cell responses, humans are known to develop contact allergy towards much lower doses of cinnamal and PPD than we exposed the mice to in study II (81,89,115,116,281,282). The reason for this is unknown but may be due to lack of danger signalling or faster enzymatic removal in the mice compared to humans. However, human studies have also described varying T cell responses to different contact allergens. In relation, allergen-specific $CD4^+$ T cells in human ACD have generally been described as effector cells when analyzing patch test biopsies. Infiltration of large numbers of allergen-specific $CD4^+$ T cell subsets have been demonstrated in response to e.g. fragrance-, rubber- and metal/nickel exposed skin (210,212,256). Also, recruitment of IL-17- and IFNγ-producing $CD4^+$ Th17 and Th1 cells, have been shown to exceed the number of $CD8^+$ T cells massively when analysing nickel patch test sites three days after challenge of nickel allergic patients (212). Other human studies have, however, demonstrated a more significant role of allergen-specific $CD8^+$ T cells in response to nickel (211,283,284) and urushiol (poison ivy) (213). Cavani et al. (1998) demonstrated that nickel exposed human skin, in addition to $CD8^+$ T cells, also harbored IL-10-producing nickel-specific $CD4^+$ T cell, thus emphasizing that allergen-specific $CD4^+$ T cells play regulatory roles in human ACD (285). Collectively, these studies emphasize that results from murine studies should be validated in humans, but also that challenge responses in allergen-naïve skin are probably not the same as challenge responses in allergen-experienced skin. Considering this, more human studies should be performed.

Another interesting observation from study II, was the formation of local memory responses by alterations in IL-1β, CXCL1 and ear swelling occurred independently of epidermal-resident $CD8^+$ T_{RM} cells when comparing OOA controls to cinnamal- and PPD-challenged mice 24 hours after challenge. Our data do not explain these responses, however, as we did not address the role of

dermal-resident $CD4^+$ T_{RM} cells, these may be responsible as suggested by Murata and Hayashi (58). Further investigations are needed to determine the origin of these responses

In study III, we showed that DNP-self protein adducts stayed in epidermal skin of mice for at least one year after exposure and that this facilitated long-term survival of epidermal-resident $CD8^+$ T_{RM} cells by constitutive low-grade TCR activation and local proliferation. In line with this, Gamradt et al. showed epidermal presence of DNP 30 days after DNFB exposure (56). In relation, radiolabelled-PPD have been demonstrated to accumulate in skin of rats following repeated exposures (286), and daily repeated exposures of human patients to different contact allergens led to a cumulative effect on the challenge responses (282,286–288). Whether inhibitory checkpoint receptors (ICRs) expressed by $CD8^+$ T_{RM} cells, as shown by Gamradt et al. (56), are activated alongside with the TCRs to avoid chronic skin inflammation is an assuring idea, although it needs further investigation. Early after DNFB exposure, DNP-modified proteins are distributed throughout the epidermis but not to the same extend in the dermis (55,112–114). How DNP continues to stay in the epidermis during the constant epidermal renewal is another unanswered question. One explanation could be that DNP-modified proteins are mostly present within large structural extracellular proteins that are perhaps not renewed to the same extend as proteins within the KCs. Another explanation could be that DNP-modified proteins are present in epidermal-resident immune cell such as LCs and DETCs (in mouse) and/or T_{RM} cells that survive longer than the KCs. Indeed, further investigations are needed to identify which skin proteins are permanently modified and whether similar results are found using more clinical relevant contact allergens.

Graphical illustration of main results

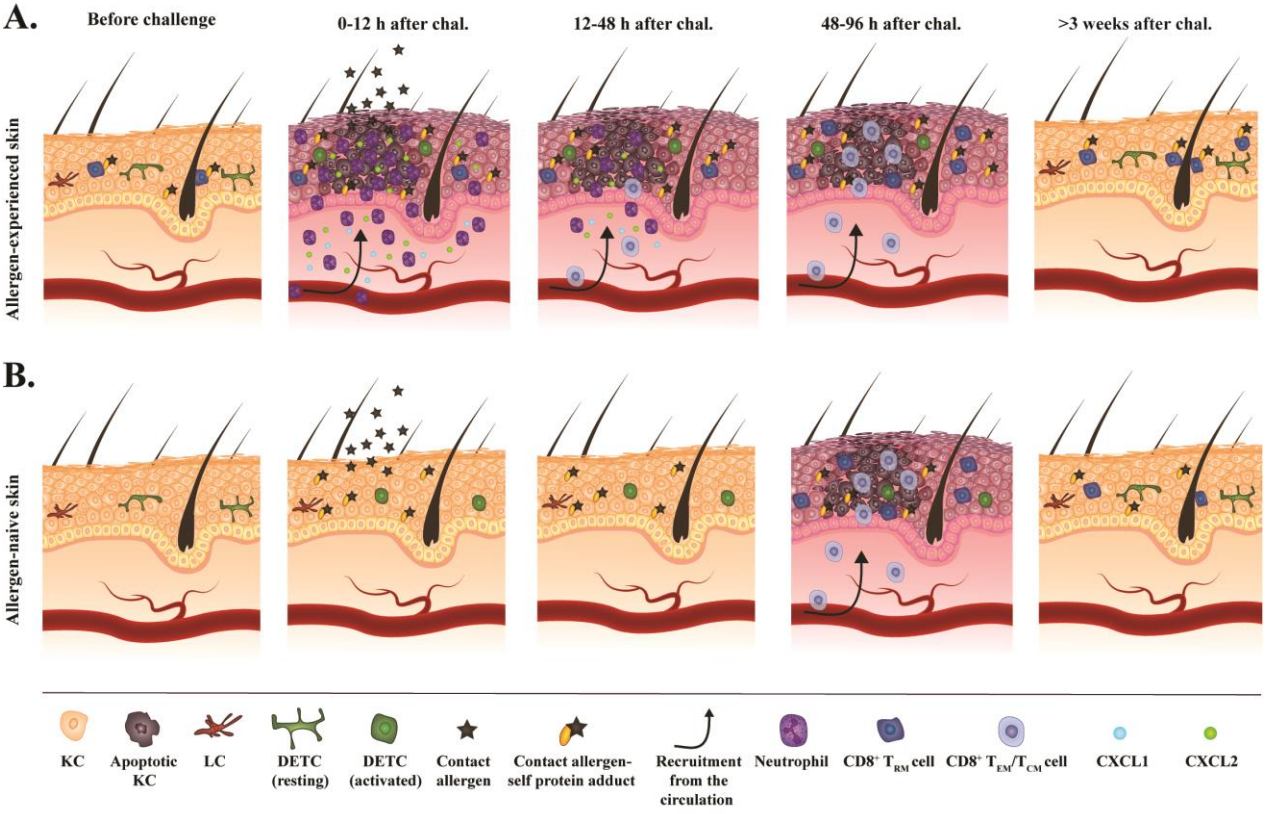


Figure 11. Graphical illustration of main results.
Using an allergic contact dermatitis (ACD) mouse model, we investigated the formation, function, and survival of allergen-specific epidermal-resident $CD8^+$ T (T_{RM}) cells. ***(A)*** *We found that epidermal-resident $CD8^+$ T_{RM} cells only develop locally in allergen-experienced skin, although for some allergens this development is highly regulated by $CD4^+$ T cells. Within 0-12 hours after allergen challenge the $CD8^+$ T_{RM} cells are re-activated and induce expression of C-X-C motif chemokine ligand (CXCL) 1 in the dermis and CXCL2 in both dermis and epidermis. Upon expression, these chemokines induce a massive epidermal infiltration of neutrophils leading to rapid dermatitis. Already 12-48 hours after allergen challenge most of the neutrophils disappear and epidermal infiltration of $CD8^+$ effector memory T (T_{EM}) cells and $CD8^+$ central memory T (T_{CM}) cells from the circulation starts. 48-96 hours after allergen challenge the neutrophils have disappeared and the number of infiltrating $CD8^+$ T_{EM}/T_{CM} cells increase while the inflammatory response resolves.* ***(B)*** *In contrast to allergen-experienced skin, epidermal-resident $CD8^+$ T_{RM} cells are not found in the epidermis of allergen-naïve skin and therefore ACD reactions do not develop within 0-48 hours after allergen challenge. Instead, classical type-IV ACD reactions are observed 48-96 hours after challenge by infiltrating $CD8^+$ T_{EM}/T_{CM} cells. These then facilitate formation of local $CD8^+$ T_{RM} cell memory. Generally, following skin exposure to contact allergens, permanent epidermal deposition of contact allergen-self protein adducts occurs as these ensure long-term survival of the epidermal-resident $CD8^+$ T_{RM} cells. Challenge (chal.), hours (h), dendritic epidermal T cell (DETC), langerhans cell (LC)*

Conclusion

Using a CHS mouse model that allow investigation of memory T cell subsets, I studied the role of epidermal-resident $CD8^+$ T_{RM} cells in local skin responses to contact allergens. Moreover, whether contact allergens can be permanently deposited in the epidermis was addressed to investigate the requirement of local antigen presentation for long-term survival of allergen-specific $CD8^+$ T_{RM} cells within the epidermal niche.

In **study I**, we demonstrated that challenge with 1-fluoro-2,4-dinitrobenzene (DNFB) on DNFB-experienced skin results in a massive epidermal influx of neutrophils by $CD8^+$ T_{RM} cell induced CXCL1/2 signalling within 12 hours after challenge and that recruitment of neutrophils was the main source behind rapid ACD flare-ups.

In **study II**, we investigated local responses following cinnamal, PPD and MI exposure. First, we demonstrated that formation of allergen-specific $CD8^+$ T_{RM} cells in the epidermis was highly allergen-dependent. We found that for all of the allergens tested, the number of $CD8^+$ T_{RM} cells correlated with epidermal neutrophil infiltration 24 hours after allergen exposure and that local expression of CXCL2 was only detected in skin where $CD8^+$ T_{RM} cells had developed. Finally, we showed that $CD4^+$ T cells counteracted local development $CD8^+$ T_{RM} cells in the epidermis during sensitization in an allergen-dependent manner.

In **study III**, we demonstrate that DNFB exposure led to permanent epidermal deposition of DNFB-self protein adducts as DNP moieties were detected in the epidermis at day 5, 30 180 and 365 after DNFB exposure. In parallel, we showed that a fraction of DNFB-specific $CD8^+$ T_{RM} cells only survive over time in epidermal skin directly exposed to DNFB from constitutive low-grade TCR activation and proliferation.

In conclusion, our results suggest that local epidermal-resident $CD8^+$ T_{RM} cells are pivotal in mediating rapid ACD flare-ups in allergen-experienced skin and that these survive by constitutive epidermal presence of cognate antigen leading to local proliferation. Further investigations in local development, inflammatory signalling pathways and long-term survival of allergen-specific $CD8^+$ T_{RM} cell may open avenues for future therapeutic treatments against ACD.

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