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Pia GAMRADT

Tissue-resident memory T cells in eczema – Contribution and protective regulatory mechanisms.

Lymphocytes T mémoires résidants dans l'eczéma – Contribution et mécanismes de régulation.

Devant le jury composé de :

Prof. Julien SENESCHAL, Hôpital Saint-André Bordeaux – France	Président/Rapporteur
Prof. Iris GRATZ, Université de Salzburg – Autriche	Rapporteur
Dr. Olivier GAIDE, Université de Lausanne – Suisse	Examinateur
Dr. Jessica STRID, Imperial College London – Royaume-Uni	Examinatrice
Prof. Jean-François NICOLAS, Université Lyon 1 – France	Directeur de thèse
Dr. Marc VOCANSON, CIRI, Lyon – France	Co-directeur de thèse

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List of Abbreviations

A

ACD. *Allergic contact dermatitis*

ACK. *Ammonium-Chloride-Potassium*

AD. *Atopic dermatitis*

Ag. *Antigen*

AhR. *Aryl hydrocarbon receptor*

AMP. *Adenosine monophosphate*

AOO. *Acetone alcohol: Olive oil*

APC. *Ag-presenting cell*

APT. *Atopy Patch Test*

ATCC. *American Type Culture Collection*

ATP. *Adenosine triphosphate*

B

BATF. *Basic Leucine Zipper ATF-like TF*

Blimp. *B lymphocyte-induced maturation protein*

BM. *Bone marrow*

BMDC. *Bone marrow-derived DC*

BSA. *Bovine serum albumin*

C

C. albicans. *Candida albicans*

CDU. *Collagen disgestion unit*

CHS. *Contact hypersensitivity*

CLA. *Cutaneous Lymphocyte Antigen*

CMLE. *Classic maximum likelihood estimation*

CNS. *Central nervous system*

CTCL. *Cutaneous T-cell lymphoma*

CTL. *Cytotoxic T lymphocytes*

CTLA-4. *Cytotoxic T-lymphocyte-associated protein 4*

D

Da. *Dalton*

DAMP. *Danger associated molecular pattern*

DAPI. *4',6-Diamidino-2-phenylindole dichloride*

DC. *Dendritic cell*

Der f. *Dermatophagoides farina*

Der p. *Dermatophagoides pteronyssinus*

DETC. *Dendritic epidermal T cell*

dLN. *Draining LN*

DMSO. *Dimethyl sulfoxide*

DNCB. *2,4 Dinitrochlorobenzene*

DNFB. *2,4-Dinitrofluorobenzene*

DT. *Diphteria Toxin*, *Diphteria Toxin*

DTH. *Delayed-type hypersensitivity*

E

EA. *Eczéma atopique*

EAC. *Eczéma allergique de contact*

EASI. *Eczema Area and Severity Index*

EFS. *Etablissement Français du Sang*

ELISpot. *Enzyme-linked immuno spot*

Eomes. *Eomesodermin*

EU. *European Union*

EUG. *Eugenol*

F

FACS. *Fluorescence-activated cell sorting*

FB. *FACS buffer*

FDE. *Fixed drug eruption*

FFA. *Free fatty acid*

FITC. *Fluorescein isothiocyanate*

FLG. *Filaggrin*

FRT. *Female reproductive tract*

G

GM-CSF. *Granulocyte-macrophage colony-stimulating factor*, *Siehe*

Gzm. *Granzyme*

H

HCA. α-*Hexylcinnamaldehyde*

HDCL. *Hydroxzcitronellal*

HDM. *House dust mite*

Hobit. *Homolog of Blimp-1 in T cells*

HRP. *Horseradish peroxidase*

HSV. *Herpes simplex virus*

I

i.p.. *Intraperitoneal, Intraperitoneal*

i.v.. *intravenously*

ICR. *Inhibitory checkpoint receptor*

iDTR. *Inducible diphtheria toxin receptor*

IEL. *Intraepithelial lymphocyte*

IF. *Immunofluorescence*

IFN. *Interferon*

Ig. *Immunoglobulin*

IL. *Interleukin*

ILC. *Innate lymphoid cells*

Iono. *Ionomycin*

J

JAK. *Janus kinase*

K

KLF. *Kruppel-like factor*

KLRG1. *Killer cell lectin-like receptor subfamily G member 1*

KU. *Kunitz unit*

L

LAG-3. *Lymphocytes activation gene 3*

LC. *Langerhans cell*

LCMV. *Lymphocytic choriomeningitis virus*

LELP1. *Late Cornified Envelope-like Proline-1 rich gene*

LN. *Lymph node*

LTcm. *Lymphocyte mémoire centrale*

LTeff. *Lymphocyte T effecteur*

LTem. *Lymphocyte mémoire effectrice*

LTi. *Lymphoid tissue inducer cell*

LTm. *Lymphocyte T mémoire*

LTrm. *Lymphocyte T mémoire résidant*

LTT. *Lymph Node Transformation Test*

LYREC. *Lyon Recherche Clinique*

M

mAb. *Monoclonal antibody*

MAdCAM. *mucosal vascular addressin cell adhesion molecule*

MEST. *Mouse ear swelling test*

MF. *Mycosis fungoides*

MFI. *Mean fluorescence intensity*

MHC. *Major histocompatibility complex*

mTOR. *Mammalian target of rapamycin*

N

NF-AT. *Nuclear factor of activated T cells*

NK. *Natural killer cell*

NKT. *Natural killer T cell*

Nrf2. *Nuclear factor erythroid 2-related factor 2*

O

O/N. *overnight*

OVA. *Ovalbumin*

Oxa. *Oxazolone*

P

PAMP. *Pathogen associated molecular patterns*

PBES. *Plateau de Biologie Expérimentale de la Souris*

PBL. *Peripheral blood lymphocyte*

PBMC. *Peripheral blood mononuclear cell*

PBS. *Phosphate-buffered saline*

PD-1. *Programmed cell death protein 1*

PDE. *Phosphodiesterase*

PD-L1. *Programmed death ligand-1*

PFA. *Paraformaldehyde*

PMA. *Phorbol 12-myristate 13-acteate*

PO-SCORAD. *Patient-oriented SCORAD*

PPD. *Para-phenylenediamine*

PRR. *Pattern recognition receptor*

PSF. *Point spread function*

R

RBC. *Red blood cell*

ROAT. *Repeat Open Application Test*

ROS. *Reactive oxgen species*

RPMI. *Roswell Park Memorial Institute 1640*

RPMI-c. *RPMI-complete*

RT. *Room temperature*

S

S. aureus. *Staphyolococcus. aureus*

S. epidermidis. *Staphylococcus epidermidis*

s.c.. *Subcutaneously*

S1PR1. *Sphingosine 1 phosphate receptor 1*

SAB. *human AB sera*

SCORAD. *Score in Atopic Dermatitis*

SFC. *Spot-forming cell*

SLO. *Secondary lymphoid organ*

SPF. *Specific pathogen free*

STAT. *Signal transducer and activator of transcription*

T

T-bet. *T-box protein expressed in T cells*

Tcm. *Central memory T cell*

TCR. *T cell receptor*

Teff. *T effector*

Tem. *Effector memory T cell*

TEWL. *Transepidermal water loss*

TF. *Transcription factor*

Tfh. *T follicular helper cell*

TGF. *Transforming growth factor*

Th. *T helper cell*

TIM-3. *T-cell Ig and mucin-domain containing protein 3*

TLR. *Toll-like receptor*

Tm. *Memory T cell*

TNCB. *2,4,6-Trinitrochlorobenzene*

TNF. *Tumor necrosis factor*

Trcm. *Recirculating memory T cell*, *Recirculating memory T cell*

Treg. *Regulatory T cell*

Trm. *Tissue-resident memory T cells*

TSLP. *Thymic stromal lymphopoietin*

U

USA. *United States of America*

UV. *Ultraviolet*

V

VLA. *Very Late Ag*

Résumé: Lymphocytes T mémoire résidants dans l'eczéma – Contribution et mécanismes de régulation

Les eczémas [eczéma allergique de contact (EAC) et eczéma atopique (EA)] sont des dermatoses inflammatoires fréquentes des pays industrialisés. Elles sont induites suite au recrutement et à l'activation dans la peau de lymphocytes T spécifiques d'allergènes, qui sont présents dans notre environnement, et qui sont habituellement très bien tolérés par la majorité des individus exposés.

Ce travail de thèse porte sur un aspect novateur de la physiopathologie des eczémas, à savoir : la contribution des lymphocytes T mémoires résidants (LTrm) dans la peau à la chronicité et à la sévérité de ces maladies.

Capitalisant sur des modèles précliniques pertinents ainsi que sur des échantillons cliniques prélevés chez les patients, ce travail a permis d'acquérir de nouvelles connaissances : (i) de nombreux LTrm CD8+ spécifiques colonisent les lésions d'eczéma (ii) ils s'accumulent avec la persistance de l'allergène dans la peau, (iii) ils jouent un rôle majeur dans les récidives de la maladie, mais (iv) ils expriment à leur surface divers récepteurs inhibiteurs, tels que PD-1 ou TIM-3, qui empêchent la survenue de réponses allergiques excessives.

Ces travaux apportent donc des informations majeures sur la nature unique des LTrm CD8+ spécifiques d'allergènes et des mécanismes qui contrôlent leur réactivation, afin de préserver l'intégrité de la peau et la survenue de réactions chroniques sévères. Le développement des nouvelles stratégies thérapeutiques ciblant la réactivation des LTrm via leurs récepteurs inhibiteurs pourrait permettre de restaurer la tolérance chez les individus allergiques.

Abstract: Tissue resident memory T cells in eczema – Contribution and protective regulatory mechanisms

Allergic contact dermatitis (ACD) and atopic dermatitis (AD), also referred to contact or atopic eczema, are frequent skin inflammatory diseases with increasing prevalence and high socio-economic impact in Western countries. Eczemas are the prototype of skin delayed-type hypersensitivity reactions. Skin lesions are induced by the recruitment and activation in the skin of effector/memory T cells specific for environmental antigens that are innocuous to healthy non-allergic individuals.

The aim of this work was to better understand the pathophysiology of eczemas by a comprehensive analysis of the contribution of skin resident memory T cells (Trm) to the chronicity and severity of these diseases.

Capitalizing on relevant pre-clinical eczema models and on clinical samples collected from allergic patients, this work showed that: (i) numerous allergen-specific CD8+Trm colonize the eczema lesion, (ii) they accumulate in the epidermis in response to the long-term persistence of the allergen in the skin, (iii) they are instrumental for the recurrence of eczema, but (iv) they express several inhibitory check point receptors (ICRs, such as PD-1, TIM-3) at their surface, which keep them in check to prevent the development of severe immunopathology.

Thus, our work provides important information for considering the unique nature of hapten-induced CD8+ Trm and the mechanisms that prevent their unwanted re-activation and subsequent development of chronic or severe skin allergy. The development of therapeutic strategies targeting the re-activation of skin Trm *in situ* via their ICRs should open new avenues to restore tolerance in allergic individuals.

Les mots clés

Eczéma allergique de contact, Eczéma atopique, Lymphocytes T mémoires résidants, Immunopathologie, Récepteurs inhibiteurs *checkpoint*.

Key words

Allergic contact dermatitis, Atopic dermatitis, Tissue-resident memory T cells, Immunopathology, Inhibitory checkpoint receptors.

The name and address of the laboratory where the thesis was prepared

Centre International de Recherche en Infectiologie – CIRI

INSERM U1111

Équipe Immunologie de l'Allergie Cutanée et Vaccination

Professor Jean-François Nicolas et Marc Vocanson, PhD

21 Avenue Tony Garnier

69365 Lyon Cedex 07 - France

Résumé substantiel

Les eczémas, l'eczéma allergique de contact (EAC) et l'eczéma atopique (EA), sont des dermatoses inflammatoires fréquentes, présentant une forte prévalence et un impact socio-économique notable dans l'ensemble des pays industrialisés. Ces deux formes d'eczéma se définissent comme des réactions d'hypersensibilité retardée. Les lésions sont induites suite au recrutement et à l'activation dans la peau de lymphocytes T effecteurs/mémoires, spécifiques d'allergènes qui sont présents dans notre environnement, et qui sont habituellement très bien tolérés par la majorité des individus exposés. Ces allergènes sont soit des produits chimiques, également appelés haptènes, dans le cadre de l'EAC, soit des protéines dans l'EA. Chez de nombreux patients, l'eczéma est chronique et se développe sous forme de poussées de plus en plus sévères, entrecoupées de rémission. En outre, à l'instar d'autres maladies inflammatoires telles que le psoriasis ou les érythèmes pigmentés fixes, les poussées se situent préférentiellement sur les sites de lésions antérieures.

Les lymphocytes T spécifiques responsables des lésions d'eczéma se différentient à partir de précurseurs naïfs présents dans les ganglions drainants la peau des individus exposés, suite à la présentation de peptides dérivés de ces allergènes par des cellules dendritiques cutanées. Ces précurseurs se différencient alors en lymphocytes T effecteurs (LTeff) puis en lymphocytes T mémoires (LTm), qui sont capables de survivre et de répondre plus efficacement à une nouvelle exposition à l'allergène.

Selon leurs fonctions et leur capacité migratoire, les LTm qui sont induits chez les individus sensibilisés sont hétérogènes. Ils étaient jusqu'alors classés en 2 grandes catégories : i- les lymphocytes de la mémoire centrale (LTcm) et ii- les lymphocytes de la mémoire effectrice (LTem). Les LTcm circulent dans les organes lymphoïdes, alors que les LTem circulent du sang vers les tissus périphériques tels que la peau, les intestins, le cerveau ou encore les poumons. Après reconnaissance de l'antigène, les LTem acquièrent rapidement des fonctions effectrices telles que la production de cytokines inflammatoires (IFN-g, IL-17a...) et des fonctions cytotoxiques (production de protéases granzyme B, perforine...), mais ils prolifèrent peu. Les LTcm qui circulent du sang aux organes lymphoïdes secondaires, sans entrer dans les tissus périphériques, sont pourvus de fonctions effectrices limitées, mais prolifèrent fortement suite à une réactivation par l'antigène. Ils servent ainsi de réservoir pour renforcer le pool de LTem, lors des nouvelles expositions à l'antigène.

Récemment, une 3ème population de LTm a été décrite : les lymphocytes T résidents mémoires (LTrm). Ces derniers survivent plusieurs mois au sein des tissus périphériques

(notamment les barrières épithéliales) de l'organisme, sans re-circuler. Parfaitement positionné à l'interface avec l'environnement, ils jouent un rôle clé dans la protection des individus en réagissant rapidement à toute nouvelle invasion d'agents pathogènes, et en orchestrant le recrutement des LTem spécifiques circulant à distance dans l'organisme.

Cependant, au delà de leur rôle protecteur, certains Trm qui s'accumulent dans les tissus seraient pathogéniques pour l'organisme. Ils participeraient grandement au développement des pathologies inflammatoires/auto-immunes, ainsi que des maladies allergiques.

Nous avons donc fait l'hypothèse, dans ce projet de thèse, que des LTrm qui persistent sur les sites de lésions antérieures d'eczéma jouent un rôle clé dans les récidives et la sévérité des ces maladies.

Capitalisant sur l'utilisation de modèles précliniques pertinents [modèles d'EAC/d'EA induits par l'application répétée d'un haptène 2,4-Dinitrofluorobenzène (DNFB) ou d'un extrait protéique d'acarien *Dermatophagoïdes farinae* (*Der f*)], d'échantillons cliniques prélevés chez les patients, ainsi que sur un large panel de technologies (cytométrie de flux, microscopie confocale sur coupes de tissu, RT-PCR quantitative, expérimentation animale sur des souris transgéniques, transferts adoptifs...), j'ai cherché au cours de ma thèse à (i) caractériser de façon extensive le phénotype, la localisation, la spécificité et les propriétés fonctionnelles des LTrm présents dans les lésions antérieures d'eczéma, (ii) prouver leur rôle dans les récidives de ces maladies, ou encore (iii) déterminer les mécanismes qui gouvernent leur persistance et leur réactivation.

Mes principaux résultats, obtenus à partir des modèles précliniques, montrent (i) que de nombreux LTrm CD8+ (mais aussi LTrm CD4+ dans le modèle d'EA) colonisent les sites de lésions antérieures d'eczéma (ii) qu'un pourcentage notable de ces cellules sont spécifiques des allergènes appliqués, (iii) qu'ils s'accumulent avec la persistance à long terme des allergènes dans la peau, (iv) qu'ils jouent un rôle majeur dans les récidives de la maladie, ceci de façon indépendante à leur homologues circulants, mais (v) qu'ils expriment à leur surface divers récepteurs inhibiteurs, tels que PD-1 ou TIM-3, qui empêchent la survenue de réponses d'eczéma excessives. Enfin, vi) l'expression de ces récepteurs inhibiteurs seraient directement liée à la persistance des allergènes dans la peau.

Une étude clinique conduite sur des patients atteints d'EA, toujours en cours, servira à valider l'ensemble de nos observations obtenues dans les modèles. Les premiers résultats ont permis de confirmer que des LTrm spécifiques d'allergènes persistent sur les sites de lésions antérieurs.

Mes résultats de thèse apportent donc des informations essentielles pour mieux comprendre

l'histoire naturelle des eczémas, notamment quels sont les facteurs qui favorisent les poussées, la chronicité et la sévérité des ces maladies. Ces résultats ouvrent, en outre, de nouvelles perspectives sur la caractérisation des Trm cutanés. A terme, ils pourraient permettre de développer de nouvelles stratégies thérapeutiques, dîtes de maintenance, ciblant ces Trm via l'expression de leurs récepteurs inhibiteurs. Ces stratégies offriraient une alternative aux traitements actuels à base de corticostéroïdes, et pourquoi pas ainsi ré-induire un état de tolérance chez les patients allergiques. Une meilleure stratification des patients atteints d'eczéma basée sur une compréhension avancée de la biologie du Trm tirée de nos résultats aiderait également à améliorer la prise en charge clinique des eczémas, et améliorer ainsi la qualité de vie des patients.

Introduction

Allergic contact dermatitis (ACD) and atopic dermatitis (AD) are chronic T cell-mediated inflammatory skin diseases with increasing prevalence in industrialized countries, which are associated with cutaneous hyperreactivity to environmental antigens (Ags)/allergens [1], [2], [3]. ACD and AD, also referred to as eczema, share common mechanisms in disease development as well as symptoms. In AD, the causative allergens are proteins, such as proteins derived from house dust mites (HDM), and in ACD, small chemical compounds also referred to as haptens. While most individuals tolerate those allergens, in some patients they can penetrate the skin and sensitize the organism. The mechanisms underlyingthe difference between tolerant and non-tolerant individuals are not completely elucidated but they include for example genetic defects affecting the skin barrier of the patients or the immune response [4], [5], [6]. However, after sufficient sensitization occurred, re-exposure to the Ag leads to the development of eczematous lesion and recurrence of skin inflammation also referred to as flare-ups. Notably, a major hallmark of ACD and AD as well as other inflammatory skin pathologies such as psoriasis and fixed drug eruption (FDE), is the preferential localization of flare-ups at the sites of previously affected healed skin [7], [8], [9].

In order to explain this characteristic hallmark of skin inflammatory diseases, we have to understand which immunological events occur during the sensitization of an individual to a given Ag. After passing an epithelial barrier, the Ag is presented to naïve T cells within the draining lymph nodes, which then develop into effector T (Teff) cells mediating an immune response and the elimination of the Ag [10], [11], [12]. When the Teff cells have fulfilled their effector functions, most of them undergo cell death. However, some of them persist as long-living Ag-specific memory T (Tm) cells which can induce a more rapid and more efficient response upon pathogen re-encounter [13], [14], [15].

Tm cells can be further distinguished into three different populations, depending on their location, migration pattern, phenotype and function: effector memory T (Tem) cells, central memory T (Tcm) cells, and tissue-resident memory T (Trm) cells [13].

Tem cells patrol the blood and peripheral tissues such as skin, gut, brain or lung. After Ag-recognition, Tem develop strong effector functions, e.g. cytokine production and cytotoxicity, but they do not undergo strong proliferation. In contrast, Tcm cells circulate between the blood and secondary lymphoid organs (SLOs) but they do not enter peripheral tissues. Their effector functions are limited; instead they are capable of strong proliferation after Ag-recognition. Therefore, Tem cells are thought to mediate most of the effector functions upon

pathogen re-encounter, while Tcm cells generate a second wave of Teff cells due to their proliferative capacities. In contrast to Tem and Tcm cells, Trm cells do not recirculate through the organism but instead they establish a permanent residence in peripheral tissues. Here, they are concentrated at previous sites of infection where they are perfectly positioned to detect Ags and to provide local immune memory upon Ag-recognition [13], [16].

During the recent years Trm cells have been intensively studied to determine their phenotype and functions but also to decipher mechanisms of their maintenance inside peripheral tissues, their ontogeny, and their diversity. Several studies demonstrated a protective role of Trm cells against pathogens re-infection [17], [18], [19], [20]. Nevertheless, numerous issues regarding Trm cells remain to be addressed. For instance, since the re-encounter of a given pathogen might not necessarily occur at the same tissue site where Trm cells are usually concentrated [18], it remains to be completely elucidated what are the benefits of tissue-resident Trm cells versus circulating protective Tm cells. Also, to date it is not completely evaluated how long Trm cells really remain within certain tissues and which factors might influence their persistence. It remains to be clarified whether Trm cells underlie any space restriction regarding the seeding of their tissue niche or if tissue-renewal of certain organs (e.g. the skin) affects their longevity. Moreover, it has not been determined if, during the entrapment of protective Ag-specific Trm cells inside a tissue in response to a given Ag, Trm cells with other specificities e.g. against commensal microbiota are also accumulated. If this would be the case, it would be of interest to determine their contribution to any immunopathology. Although Trm cells are mainly thought to be beneficial for the host upon re-encounter of pathogens, it is known that pathogenic Trm cells mediating inflammatory diseases are generated using similar, though maybe not identical, pathways as their protective counterparts after exposure to allergens or auto-Ags [21]. Moreover, many clinical characteristics, e.g. the preferential recurrence of flare-ups at previously affected site in inflammatory skin diseases could be explained with the residential biology of Trm cells [22], [21], [9] [23], [24]. However, regulatory mechanisms must apply to both protective and pathogenic Trm cells, in order to avoid constant activation due to their sentinel position within barrier tissues.

Therefore, our relatively limited knowledge about Trm cells in ACD and AD, together with the noteworthy preferential occurrence of flare-ups at previous inflammation sites, led us to investigate the contribution of Trm cells to the chronicity of these pathologies. We hypothesize that in both ACD and AD Ag-specific Trm cells are not only key to mediate recurrent skin inflammation but that they are also highly susceptible to regulatory mechanisms which restrain the Trm activity and prevent exacerbated tissue-damage due to

un-regulated immune responses. Several recent studies confirmed the presence of skin Trm in skin immunopathologies and thereby already shed light on certain clinical characteristics [24], [25], [26]. Those major findings, however, demonstrated their Ag-specificity and contribution only indirectly. Therefore, in this project we aimed to directly demonstrate the contribution of skin Trm in the development of skin flare-ups upon allergen re-encounter by employing a new depletion approach but also to further untangle their developmental pathway during the establishment of skin allergy. Also, we sought to determine their Ag-specificity and to investigate which mechanisms are installed to keep them in check in order to prevent immunopathology either during the acute skin inflammation, during the resolution phase or in the homeostasis e.g. in the presence of low doses of Ag. In order to open new therapeutically approaches of skin inflammatory diseases, we think that those regulatory mechanisms of Trm cells can be considered as very interesting targets to increase their tissue intrinsic restrain and to prevent the recurrence of flare-up reactions.

Part 1: Allergic contact dermatitis and atopic dermatitis – Two skin inflammatory diseases

Allergic contact dermatitis (ACD) and atopic dermatitis (AD) also referred to as contact or atopic eczema, respectively, are chronic T cell-mediated inflammatory skin diseases with high and increasing prevalence in industrialized countries [1], [27], [3]. Often, ACD and AD are condensed under the term Eczema since they both are associated with cutaneous hyper-reactivity to environmental antigens (Ags)/allergens and they share mechanisms of disease development and symptoms. Therefore, in the following part, I will present clinical aspects, prevalence and social-economic aspects, causative agents, physiopathology, diagnostics, treatment, and pre-clinical models of ACD and AD side by side.

1 Clinical characteristics of ACD and AD

ACD results from exposure to chemical allergens to the skin. ACD is characterized by pruritic inflammation of the skin which usually appears in pre-sensitized individuals, within 24 hours in mice and 72 hours in humans after skin contact with an allergen [28]. The primary lesions are restricted to the site of contact with the respective Ag. They can be presented as vesicles or bulla with erythema and pustule formation which can rapidly develop into erythematous and edematous plaques [29] (see FIGURE 2). Without further contact of the Ag, the lesions usually remain for 7-10 before they start to heal spontaneously without leaving scares in most of the cases. However, if the contact to the causative Ag is not avoided, chronicity and dissemination of the lesions can be observed [30], [3].

AD is defined by the development of relapsing and pruritus dermatitis associated with typical anatomical sites, dry skin, and a tendency towards other atopic diseases (asthma, allergic rhinitis, and food allergy) in the patient or the patients' family members [31], [32]. The clinical presentation of AD changes with the age of the patients [33]. During infancy, erythematous papules and plaques can be observed in the face, mainly on the cheek but sparing the nose and on extensor surfaces of the extremities [34]. Usually by the age of 2 years, the cheeks are less involved and AD affects instead skin folds including the eyelids, the neck, the inner crook of the arm, the wrists and ankles as well as the buttock region [35]. Finally, in adulthood, AD patients develop mainly hand and head dermatitis. In addition, the use of daily personal care items can contribute to AD affecting more severely the eyelids [33] (see FIGURE 1).

Aside from these typical forms of AD, this inflammatory skin disease has many atypical

clinical appearances such as lichenoid AD, follicular AD, alopecia associated with AD, psoriasisform AD, and several more [33].

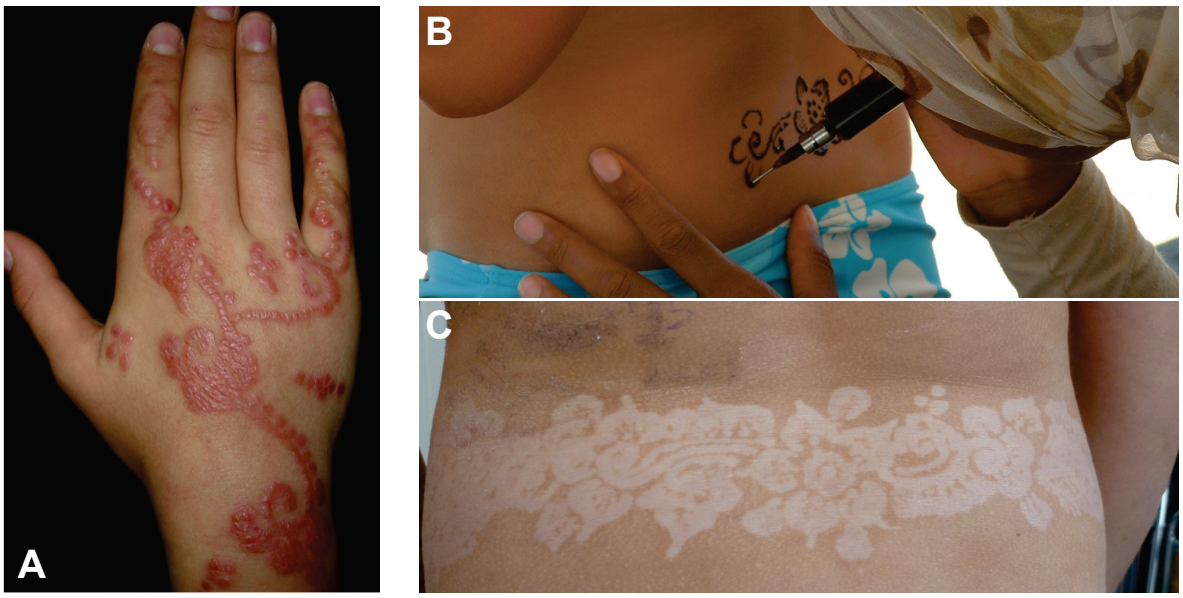


FIGURE 2: REPRESENTATIVE PHOTOGRAPHS OF ACD.
Acute (A) and healed (C) skin lesions after the application of henna tattoos (B). Clinical images courtesy of Audrey Nosbaum, M.D., Ph.D.

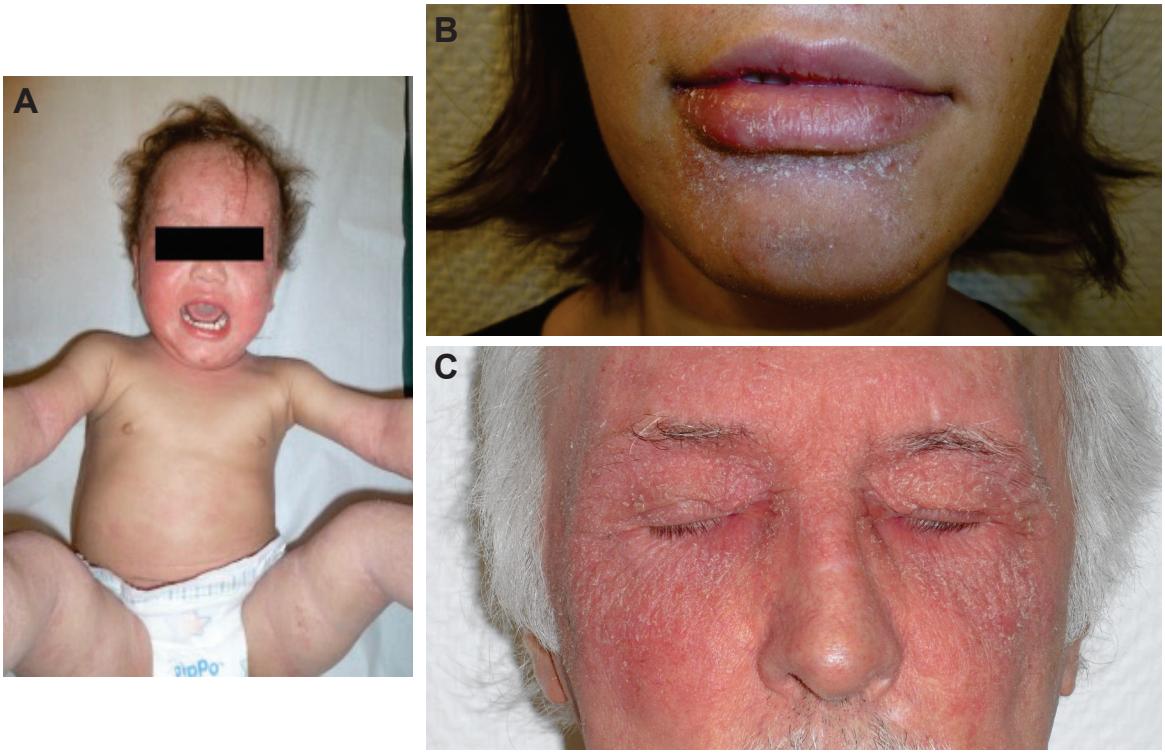


FIGURE 1: REPRESENTATIVE PHOTOGRAPHS OF AD.
During infancy, affected areas can be observed in the face as well as the extensor surfaces of the extremities (A). Head dermatitis in adults affecting the lips (B) and eyelids of the patients (C). Clinical images courtesy of Audrey Nosbaum, M.D., Ph.D.

2 Prevalence and socio-economical aspects of ACD and AD

ACD an AD are complex diseases and even though they are not life threatening they have an immense impact on the patients' quality of life and subsequently put a high burden on the public health system [36], [37]. The diseases can affect life on many personal levels, ranging from sleep, study or work, interpersonal relationships and also intimate behaviors but also the patients' family might be influenced [38], [39], [40], [41]. While AD often develops in early childhood, the onset of ACD can occur at any point of life. Also, ACD belongs to the most common occupational health diseases due to allergen exposure at work places. Estimating the prevalence of ACD and AD which can both be present in patients for very long time, is also important from an economical point of view, since they are both associated with considerable direct costs [42], [43].

Recent studies investigating the prevalence of ACD showed that approx. 20-27% of the overall European population suffers from allergy against at least one contact allergen [44], [3]. If this would be accounted for all people living in Europe in 2010 (508 million, https://europa.eu/european-union/about-eu/figures/living_en) up to 137 million people would be affected by ACD with a higher prevalence in women than in men. The highest prevalence was found for nickel (14.5%), followed by thiomersal (5.0%) a preservative used in vaccines, cobalt chloride (2.2%), different fragrance mixes (1.8-1.9%), and para-phenylenediamine (PPD) free base (1.0%) a constituent of hair dyes [44], [45].

Most studies only investigate the prevalence of ACD in adults (>18 years). However, a review of the literature regarding ACD in children (mainly in the United States of America (USA)) over one decade starting in 2008 revealed that an increasing number of children has confirmed cases of ACD [46]. Similar to the results from adults, the highest prevalence was found for nickel (17.5-29.7%), neomycin (6.0-16.3%), cobalt chloride (8.8-17.8%), fragrance mixes (5.1-12.9%), and thiomersal (4.0-15.8%).

The fact that allergies against nickel are predominantly observed in most of the studies, prompted the European Union (EU) to release a nickel directive in 1994. The directive prohibits the marketing in the EU of metallic items with nickel release of $>0.5\mu g/cm^2$/week and $>0.2\mu g/cm^2$/week if they are intended for direct and prolonged skin contact or insertion into pierced holes in the skin, respectively. A study investigating the effect of the EU nickel directive on the prevalence of nickel allergies showed a significant decrease (19.8% to 11.4%). Still the prevalence is higher in young women compared to men. In the comparison between regions, a higher prevalence was found in southern compared to northern EU countries. Nevertheless, despite the overall decrease the prevalence of nicker allergies

remained relative high in the general European population (8-18%) [47].

In general, AD has an onset in infancy with a variable disease progression during adolescence and adulthood. The prevalence of AD ranges from 15-30% and 2-10% in children and adults, respectively. Epidemiological studies revealed that the incidence of AD increased 2-3-fold during the past 30-35 years in industrialized countries and tends now to have reached a plateau [2], [48]. While the prevalence of AD in children is relatively high, it has been observed that half of them develop symptoms within the first year of life and 85-95% experience a disease onset before 5 years [49]. However, approx. 70% of patients with onset in early childhood outgrow the disease in late childhood before adolescence. The remaining 30% have continuous AD or spontaneous relapses after some symptom-free years [50]. Nevertheless, around 30% of all children with AD also develop food allergies. Also, the risk to develop other atopic diseases such as asthma (50%) and allergic rhinitis (75%) increases in children with AD [31], [32].

The risk for children to develop AD increases with an AD-positive parental history since the incidence rate is double or tripled if in one or both parents AD is present, respectively [2]. Overall the AD prevalence is lower in rural and in non-affluent or developing countries which emphasizes the importance of environmental factor for the disease development. In light of these findings, the hygiene hypothesis is often challenged which postulates an inverse relationship between AD and an environment with increased pathogen exposure [51], [52].

3 Causative factors of ACD and AD

3.1 Haptens

The relevant allergens in ACD are contact allergens also referred to as haptens, a diverse group of chemicals which are not immunogenic by themselves but must form a link to epidermal protein to form new antigenic determinants [28]. The newly formed hapten-protein complexes are engulfed by epidermal dendritic cells (DCs) and presented to naïve T lymphocytes via the major histocompatibility complex (MHC). In order to penetrate the intact skin barrier and to form such hapten-protein complexes, haptens must be smaller than 500 Dalton (Da) which is actually true for all common contact allergens [53]. Based on their potential to enter the skin and to sensitize an individual, haptens are categorized into extreme, strong, moderate, and weak sensitizers (see Table 1) [6]. While there are few extreme sensitizers usually used in ACD-like pre-clinical mouse models, thousands of weak sensitizers exist that are responsible for human ACD [28], [54].

Most haptens contain hydrophobic residues, which enable them to cross the corneal barrier of the skin. Also they bear electrophilic residues while skin proteins have electron-rich nucleophilic functional groups, which are present in the side chains of the amino acids. Amino acids with nucleophilic properties are cysteine, lysine, histidine, methionine, and tyrosine. Hapten-protein complexes are formed by covalent bindings between the electrophilic residues and specific amino acids of the protein [55], [56], [57]. In addition, covalent bindings can also be formed directly by radical coupling in the case of hydroperoxides [58]. Another group of contact allergens are metal ions such as nickel, cobalt, and chrome, which form stable protein-metal complexes. Most likely these protein-metal complexes form directly at the MHC molecule where the epidermal peptide is already residing [59], [60].

Chemical	Sources	Sensitizing capacity
Oxazolone	Chemistry	Extreme
2,4-Dinitroflurobenzene	Chemistry	Extreme
2,4-Dinitrochlorobenzene	Chemistry	Extreme
Gluteratadehyde	Conservator, antiseptic	Strong
Formaldehyde	Cosmetics, textile, resin	Strong
Cinnamaldehyde	Perfume, fragrance	Moderate
Hexyl cinnamaldehyde	Cosmetics (perfume)	Moderate/weak
Eugenol	Cosmetics, antiseptic	Weak
Hydroxycitronellal	Cosmetics (perfume)	Weak
Linalool	Cosmetics (perfume)	Weak
Citral	Perfume, fragrance	Weak
Vanillin	Perfume, fragrance	Weak
Propyl paraben	Cosmetics	Weak
Amoxicilline, cynide, cetrimide	Medicine	Weak

TABLE 1: EXAMPLES OF CHEMICAL SKIN SENSITIZERS AND DESCRIPTION OF THEIR SOURCES AND SENSITIZING POTENTIAL.

3.2 Pre-haptens and Pro-haptens

While haptens are directly able to form immunogenic complexes with epidermal proteins, some non- or only low-sensitizing substances can undergo transformation into haptens either by oxidation prior to entering the skin or by enzymatic activation once they are inside the skin. These substances are called pre-haptens or pro-haptens (see FIGURE 3), respectively [61], [62].

Pre-haptens are found among terpene fragrances such as linalool, limonene, linalyl acetate etc. [61], [63]. Also, substances that are transformed by ultraviolet radiation belong to the group of pre-haptens and are known as photosensitizers [64].

Pro-haptens are activated by the enzymatic skin system, which participates in the detoxification of the skin by metabolizing foreign chemical compounds e.g. drugs. The skin enzymes induce structural conformation of harmless compounds into reactive species that can form complexes with the epidermal proteins. Pro-haptens can also be found among natural products and fragrances (terpene, eugenol, cinnamaldehyde), dyes (disperse blue), drugs (hydrocortisone, sulphamethazole), and chemicals (ethylendiamine, vinyl pyridine) [61], [63].

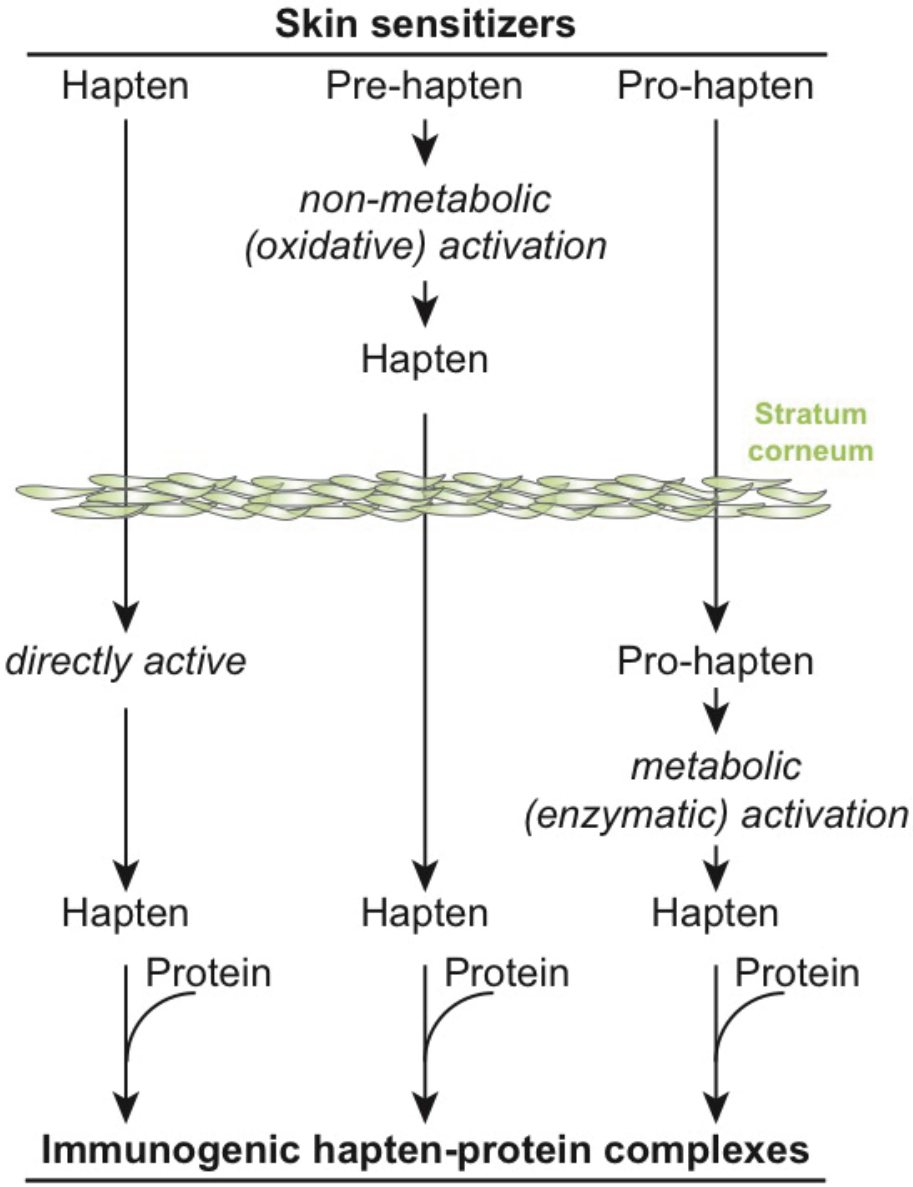


FIGURE 3: HAPTENS, PRE-HAPTENS, AND PRO-HAPTENS.
Haptens can directly enter the skin and form immunogenic hapten-protein complexes without further modification. Pre-haptens and Pro-haptens must undergo oxidative or metabolic activation before or after skin penetration, respectively, before they can engage in the formation of immunogenic hapten-protein complexes.
Adapted from Karlberg et al., 2013, and Urbisch et al., 2016.

3.3 Protein-allergens

The causative agents in ACD are low molecular weight haptens that are <500 Da so that they can even penetrate an intact skin barrier. Contrary to ACD, AD is associated with airborne protein allergens, derive from different sources such as house dust mites (HDMs), insects, and pollen. They are actually too big to enter the skin but they might do so anyway due to the skin barrier effects [65]. In addition, all of these sources equip their allergens with proteases which have the ability to further disrupt connection between the keratinocytes promoting their skin penetration [66]. Also, allergens deriving from the skin flora e.g. from *Staphyolococcus. aureus* (*S. aureus*) or from yeast *Malassezia* are thought to be causative of AD [67], [68], [69]. The structures of the protein-allergens are as diverse as their sources. For instance, more than 20 different groups of allergens are known to derive from the HDMs species *Dermatophagoides farina* (*Der f*) and *Dermatophagoides pteronyssinus* (*Der p*). Allergens from group 1 and 2 are known to cause the strongest immune response but all other groups can elicit reactions as well. They are small proteins or glycoproteins with different biological functions e.g. enzymes or lipid transport [70], [71]. Allergens derived from *Malassezia* are uniquely structured proteins localized in the cell wall of yeast cells. They can bind to phospholipids and cause the release of histamines [72], [73]

The most common protein allergen in AD is derived from HDMs. In order to minimize allergen contact, patients with a sensitivity to HDMs are recommended to take certain precaution such as enveloping their pillow and matrasses as well as to wash and vacuum them frequently. Also, minimal interior decoration such as carpeting, curtains, and drapes can help to reduce indoor pollution by allergens and to manage AD [74], [75].

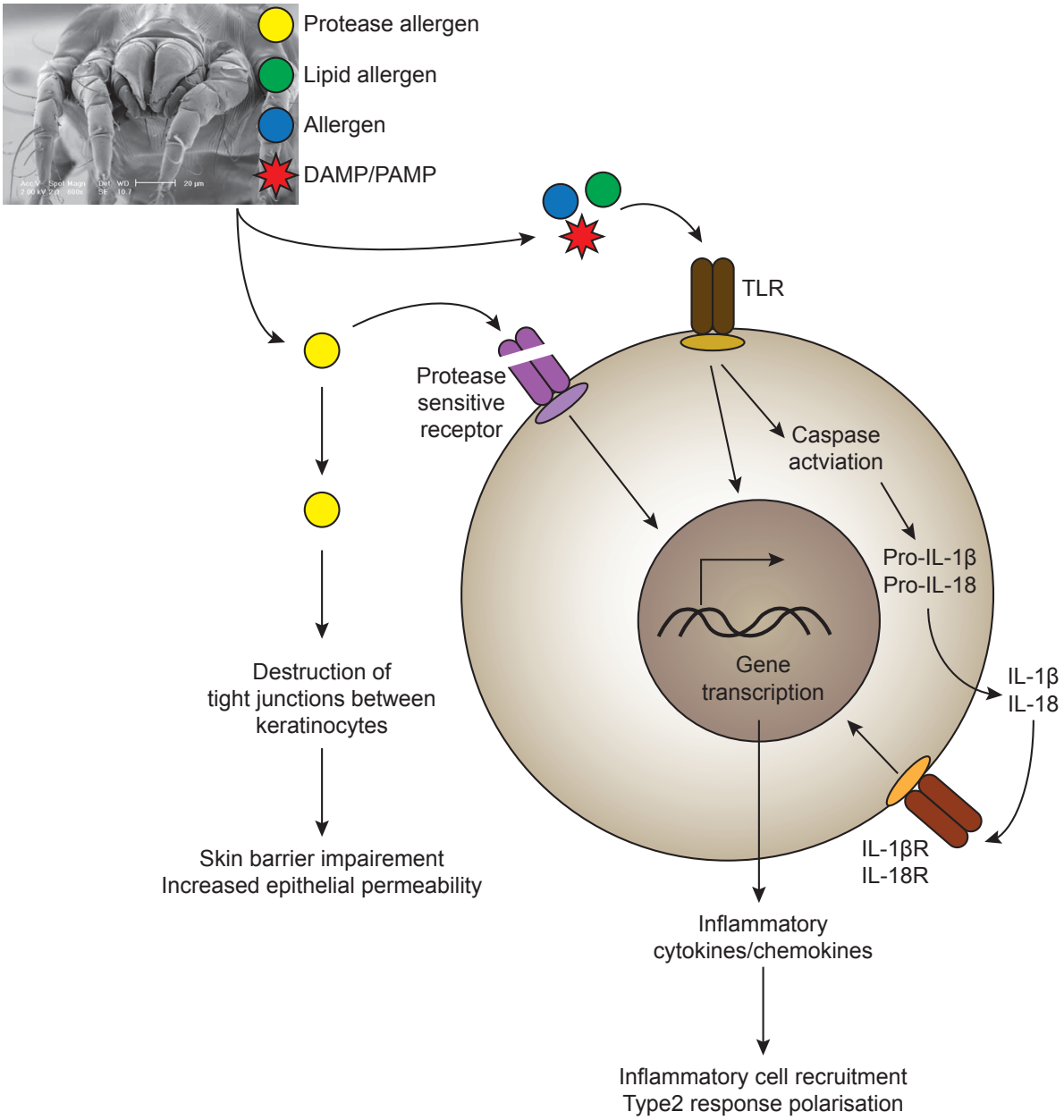


FIGURE 4: ACTIVATION OF INNATE IMMUNE CELLS BY DIFFERENT ALLERGENS RELEASED FROM HOUSE DUST MITES.

Innate immune cells can be activated to initiate immune responses against house dust mites through several pathways. Activation requires the binding of lipid-binding and contaminating allergens or the recognition of pathogen-associated and danger-associated molecular patterns (PAMPs and DAMPs) by Toll-like receptors (TLRs). Protease allergens bind to protease sensitive receptors or engage directly in the destruction of connections between keratinocytes resulting in increased epithelial permeability. Receptor activation results in the up-regulation of inflammatory cytokines and chemokines promoting type2 differentiation.
Adapted from Jaquet, 2011. Electron microscopically photograph of house dust mite courtesy of Professor Tadj Oreszczyn and Professor David Crowther, UK.

4 Etiology of ACD

ACD is caused by the repeated contact of an individual with a molecule comprising sensitizing potential. During the sensitization step an adaptive immune response is generated, in which a delayed T lymphocyte-mediated allergic response to the molecule in question occurs [76].

Thousands of potential sensitizers are known today. They are present in our daily life products such as cloths, cleaning products, jewelry, cement, pharmaceutical drugs, cosmetics, hair-dyes etc. and with the advancing development of new compounds (mainly encouraged by the demands of the market) the risk of exposure to new potential sensitizers increases [77], [78].

In general, a substance is classified as sensitizer if there is evidence in humans or a positive result from an appropriate animal test that it can lead to sensitization by skin contact. However, not all of these compounds have the same sensitizer potential. Therefore, they can be categorized according to the frequency of sensitization and the severity of the skin reactions into strong, moderate or weak sensitizers [79].

While many individual factors such as genetic disposition, age, sex, stress, and environmental factors can influence the sensitization of an individual to a certain substance [5], [80] one main factor appears to remain long-during and repeated exposure to the respective substance. However, some circumstances can promote skin sensitization even to weak sensitizers e.g. prolonged periods of wet skin during the contact with the allergen. Therefore, ACD often concerns certain professional groups such as medical personal, bar tenders, hair dressers, and construction workers which are in contact with potential sensitizers under sensitization promoting conditions due to their work habits [81], [82], [30].

5 Etiology of AD

5.1 Inside-out or Outside-in?

While in ACD mainly the strength, the contact frequency, and duration with a sensitizer is crucial, AD is a rather multifactorial disease. In AD, breakdown of the skin barrier results in increased transepidermal water loss (TEWL), reduced skin hydration, and increased Ag-presentation by Langerhans cells (LCs), which eventually initiate the inflammation [83]. Even though the physiopathology is relatively well described (see FIGURE 6) it remains controversial whether AD is caused by inflammation triggering skin barrier breakdown (inside-out) or by skin barrier abnormalities triggering immunological alterations (outside-in) [84].

The inside-out hypothesis states that cutaneous inflammation precedes the impairments in skin barrier function. Inline with this, it has been demonstrated that inflammatory immune processes affect the skin barrier by down-regulating the filaggrin (FLG) production [85]. Such barrier disruption is accompanied by increased skin penetration of allergens as well as commensal microbiota [65], [86]. The link of mutations in genes associated with inflammation in AD patients emphasize the role of cutaneous inflammatory processes in the induction of AD [84].

The outside-in hypothesis on the other hand suggests that the skin barrier function must be already impaired for immune dysregulation to occur in response to allergens. This hypothesis was fueled by a study that showed mutations of the FLG gene in up to 20% of AD cases in northern Europe [87], [88]. However, the FLG mutation does not seem to account for AD development in all cases, since many individuals without such underlying mutation develop AD and carrier of the mutation can outgrow the disease [84].

In the end each hypothesis might be true for subsets of AD patients and further research regarding the underlying mechanisms of AD is needed. Based on the two different hypotheses, investigators have come up with a third hypothesis called outside-inside-outside. Here, the presence of minor barrier dysfunctions (outside) allow deregulated stimulation of immune responses (inside) which in turn further compromise the skin barrier function (outside) establishing a vicious cycle of the disease [89].

5.2 Genetic factors and impaired skin barrier

Lesional AD skin is characterized by disrupted terminal differentiation of keratinocytes, which form the stratum corneum, the outermost layer of the skin. Therefore, correct keratinocyte differentiation is necessary to develop a protective barrier against environmental challenges. One key protein in the differentiation process of keratinocytes is FLG. It promotes the characteristic shaping of keratinocytes and its metabolites act as osmolytes, which draw water into the stratum corneum, thus facilitating its hydration [90], [91]. There are many other factors which control the terminal differentiation of keratinocytes such as loricrin, involucrin, and periplakin [83]. However, after the description of the loss-of-function mutations in the gene encoding FLG, many other FLG mutations have been described and they are the strongest and most widely replicated genetic risks for AD identified to date [92]. It has been shown that patients with FLG mutations suffer from dry skin and they have a higher risk for early-onset of AD which is often associated with asthma and food allergies. Those observations strengthen the outside-in theory discussed above [31], [93]. The effects of FLG mutations are

numerous and include enhanced expression of inflammatory cytokines such as Interleukin (IL)-1 and Interferon (IFN)-γ [83]. Noteworthy, the frequency of FLG mutations in AD patients strongly depends on the investigated population and they can vary between 10 – 50%. Thus, there must be other mechanisms leading to decreased FLG expression than mutations in the FLG gene.

Although, FLG mutations are the most replicated ones associated with AD, other gene mutations, which are affecting the skin barrier and are connected to AD development have been found. For instance, mutations of the gene encoding laminin 5, a skin protein involved in connecting epidermis and dermis, have been suggested for increased susceptibility to AD [94]. Also, single nucleotide polymorphisms in the Late Cornified Envelope-like Proline-1 rich (LELP1) gene have been identified as risk factors for AD [95].

5.3 Skin colonization by *Staphylococcus aureus*

S. aureus and other *Staphylococcus* species are known to colonize skin and mucosal membranes where they have the ability to form biofilms [96], [97]. While *S. aureus* can be found on the skin of 10-20% od healthy individuals, the skin lesions of approx. 90% of all AD-patients are colonized by it [67]. In AD patients the malformation of the skin barrier, increased pH, and subsequently increased TEWL promote the skin colonization with *S. aureus,* that preferentially destroys FLG-deficient keratinocytes [98], [99]. The released enterotoxin B from *S. aureus* has been demonstrated to have properties to act as superantigens, which lead to an Ag-independent T cell activation, and it promotes the secretion of inflammatory mediators such as IL-4, IL-5, and immunoglobulin (Ig) E [100]. Thereby it generates a milieu for the generation of T helper (Th) 2 cell responses promoting the progression of AD [98]. In addition, the released superantigens promote sustained inflammation, pruritus, and skin lesions contributing to the self-maintained vicious circle of the disease. Interestingly, different species of Staphylococcus appear to have different effects on the severity of AD. A recent study revealed that in pediatric AD patients a pre-dominance of *S. aureus* on AD-lesions was associated with more severe case while a pre-dominance of *Staphylococcus epidermidis (S. epidermidis)* in the lesion colonization was seen in milder cases of AD [101].

5.4 Immune pathways associated with AD progression

Several arguments support that AD is not primarily driven by FLG gene mutations but that deregulated immune pathways are responsible for impaired keratinocyte differentiation and

thereby create a skin barrier effect, which explains the Inside-out theory. The most important arguments are that FLG mutations are absent in most of the AD patients [5], [102] and that most children who have AD outgrown the disease even if they carry a FLG mutation [103]. Also, AD lesions are associated with underlying immune activation and AD can be efficiently treated with immunosuppressive therapies indicating that these mechanisms might lead to impaired skin barrier functions [104].

Acute AD lesions are associated with Th2, Th22, and Th17 cytokines productions and in chronic AD a shift toward Th1 cells can be observed [105], [8]. The increase of IFN-γ released by Th1 cells has been demonstrated to enhance inflammation and to cause keratinocyte apoptosis. Also, Th2 and Th22 cell cytokines have modulating effects on the skin barrier, which include suppression of keratinocytes differentiation, epidermal hyperplasia, and keratinocytes apoptosis [104].

Noteworthy, although immune activation is significantly higher in lesional than non-lesional AD skin, non-lesional skin also shows characteristic changes that are usually associated with lesional skin, and which involve a reduction of several genes encoding for epidermal differentiation genes. Therefore, non-lesional AD skin has an AD- "background" phenotype which is clearly distinct from healthy non-AD skin [106].

6 ACD in AD: Is there a link?

Even though several studies have been performed in order to determine a potential relationship between AD and ACD, the results remain controversial [107], [108]. However, more recently the question of an association has been re-addressed [98]. Hence, risk factors such as impaired skin barrier functions caused by FLG gene mutations might promote both the pathogenesis and elicitation of AD and ACD by allowing increased epidermal passage of haptens. Moreover, decreased FLG production leads to increased pH of the skin which favors both increased formation of hapten-protein complexes and bacterial colonization e.g. by *S. aureus* [109], [110].

In addition to skin penetration of allergens associated with ACD, other immune mechanism might mutually promote the disease development. While skin penetration by haptens in ACD is usually associated with type1 responses, sustained hapten penetration through the impaired skin barrier might promote a shift from type1 to type2 cell responses which are associated with exacerbation of AD [85]. Conversely, in chronic AD a shift from type2 to type1 cell responses can be observed which might favor the development of ACD reactions [105].

Finally, sensitization against chemical allergens in AD patients may also occur due to the

increased usage of moisturizers and topically applied corticosteroids. Moreover, many of such contact allergens contained in commercial products are associated with ACD [107].

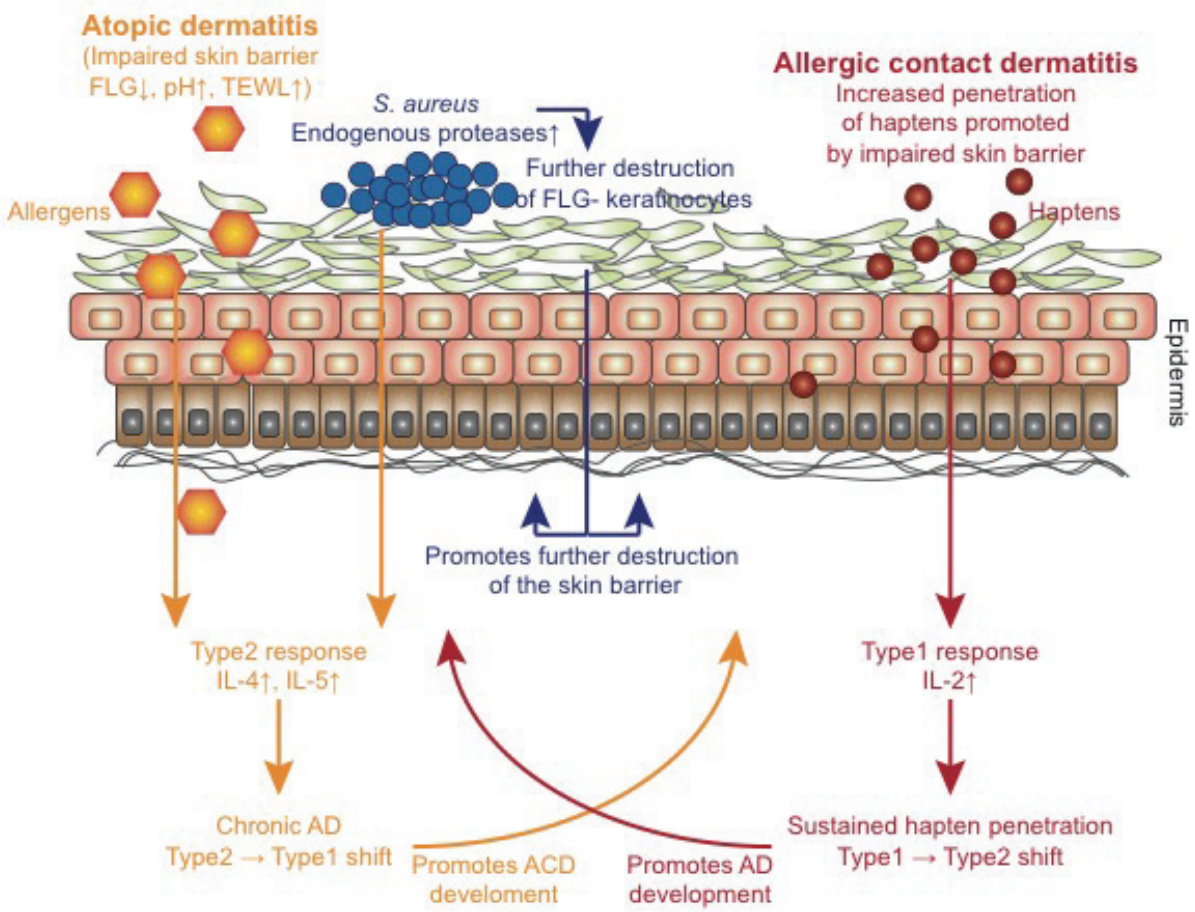


FIGURE 5: SCHEMATIC LINK BETWEEN AD AND ACD.
Impaired skin barrier leads to skin penetration of allergens (AD). Release of endogenous proteases by skin colonizing S. aureus leads to further impairment of the skin barrier. Increased penetration of haptens (ACD) is promoted by the impaired skin barrier. Chronic AD and sustained hapten penetration lead to a shift of type2 to type1 or type1 to type2 responses, respectively, which in turn promotes the development of the other skin condition. Adapted from Rundle et al., 2017.

7 Physiopathology of ACD and AD

In the following part, I will discuss the physiopathology of ACD and AD which are both considered to be delayed-type hypersensitivities (DTHs) that are characterized by a non-immediate but up to 72h delayed reaction against Ag [111]. The development of ACD and AD comprises three phases: 1.) The sensitization phase, which results in the generation of Ag-specific T lymphocytes, 2.) the challenge or elicitation phase during which Ag-specific T lymphocytes are activated, and 3.) the resolution phase [112], [113], [114].

7.1 Sensitization phase

The sensitization phase occurs at the first encounter on the skin with an Ag. Once the Ag has penetrated the skin, the innate immune system of the skin is activated and releases pro-inflammatory mediators to recruit skin-resident DCs [115]. The Ag is engulfed by skin resident DCs which process the Ag and migrate to the skin draining lymph node [116]. Inside the draining lymph node, the processed Ag is presented via MHC class I and II molecules to naïve CD8+ and CD4+ lymphocytes, respectively [117], [118]. In such way activated naïve T lymphocytes undergo proliferation and maturation processes and they become effector T (Teff) cells, which migrate back to the skin or stay in the circulation depending on their received homing signal. While in ACD the sensitization can occur anytime in life upon allergen contact, in AD these events mostly take place during early infancy.

In both ACD and AD the innate immune system plays a key role in the sensitization step. For instance, several innate immune mechanism are known, which promote the activation of DCs and the uptake and processing of Ag so that it can be presented to naïve T lymphocytes.

In ACD haptens mediate the activation of the innate immune response by initiating the production of reactive oxygen species (ROS) and the release of adenosine triphosphate (ATP) and other danger associated molecular patterns (DAMPs) from the affected skin cells. These molecules are sensed by neighboring cells via Toll-like receptors (TLRs) and other receptors, which results in the expression of pro-inflammatory mediators such as IL-1β, IL-18, IFN-α etc. and thereby the activation of skin DCs [115]. Moreover, haptens induce anti-oxidant responses, e.g. via the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, which initiate the cellular detoxification and the production of anti-oxidant enzyme [119], [120].

In AD, penetration of the allergen causes the keratinocytes to release numerous cytokines including tumor necrosis factor (TNF)-α, IL-1, granulocytes-macrophage colony-stimulating factor (GM-CSF), IL-25, and IL-33 [121], [122]. Also, thymic stromal lymphopoietin (TSLP),

which is produced at high levels by the keratinocytes of AD patients plays a key role in the sensitization phase [123]. Due to the impaired barrier function of AD skin, allergens but also microbial products can easily penetrate the skin and be recognized by pattern recognition receptors (PRRs) on the keratinocytes, which in turn release TSLP, a strong activator of skin DCs.

In general, the events described above promote in ACD the differentiation of Ag-specific CD4+ and CD8+ T lymphocytes that are associated with responses mediated by type1 (IFN-γ, TNF-α) or type 17 (IL-17) cytokines [124]. In contrast, in AD Ag-sensitization is considered to drive T lymphocyte responses with type2 (IL-4, IL-13, IL-31) and type22 cytokines (IL-22) [125], [126].

7.2 Challenge/elicitation phase

During the challenge or elicitation phase the individual that has been immunized against a certain Ag during the sensitization phase is re-exposed to the same Ag [113]. After skin barrier penetration, the Ag is again taken up by APCs of the skin and presented to Ag-specific CD8+ and CD4+ memory T (Tm) cells which were generated after the first Ag-contact and which are located mainly in the skin (CD8+ Tm cells in ACD and AD/ CD4+ Tm cells in AD) [127]. Skin inflammation is induced rapidly since Tm cells can respond more rapidly and efficiently to the Ag-challenge. In both ACD and AD CD8+ Tm cells are key for the triggering of inflammatory processes in the skin, which leads to the recruitment of CD8+ cytotoxic T lymphocytes (CTLs) which are indispensable Teff cells of the response [112], [128]. Their presence in the skin leads to further recruitment of neutrophils and eosinophils, which participate in the development of the eczematous lesion. Also, CD4+ Tm cells, including cells with both effector and regulatory functions, are recruited to the skin but their arrival is less important for the initiation of skin inflammation [128], [129], [112].

7.3 Resolution phase

The resolution of skin inflammation is mediated by several mechanisms. Initially, it was thought that in ACD Ag-clearance mainly contributes to the decrease of skin inflammation. However, this might only be partially true since some haptens have been described to persist for at least 2 weeks [113]. Also, our own observations regarding the elimination of a hapten allergen show a long-term persistence for up to 4 weeks. Nevertheless, the amount of hapten that can be detected decreases dramatically after few days and might be below a certain threshold needed for continuous skin inflammation. To my knowledge no data exist regarding the persistence of protein allergens in AD skin. However, due to the environmental nature of

the allergen, at least in AD patients, it might be difficult for it to be immediately identified and removed so that prolonged allergen contact could occur leading to prolonged skin inflammation.

Other regulatory mechanisms involve up-regulation of inhibitory ligands on skin-resident immune cells such as programmed death ligand-1 (PD-L1) on DCs [130] and T lymphocytes (our own observation), the release of anti-inflammatory cytokines by skin cells, and the recruitment and activation of CD4+Foxp3+ regulatory T (Treg) cells which exert suppressive functions on activated Teff cells [131]. Other cells are involved in the regulation of allergic responses against haptens. For instance, regulatory B cells, which produce anti-inflammatory IL-10 have been described [132]. Also, natural killer T (NKT) cells have been described to participate in the regulation of allergic responses at least in certain pre-clinical mouse models of ACD [133]. However, an interplay between NKT cells and regulatory B cells has been described suggesting that the regulative function of NKT cells occurs at least partially through the regulation of IL-10 production by B cells [134]. In addition, a recent controversial study described a regulatory effect of mast cells on the immune responses in a mouse model of ACD [135], although those cells have also been demonstrated to exacerbate skin reactions in ACD [136], [137].

7.4 Flare-up reactions

Even though macroscopically healed lesions appear similar to naïve skin, alterations in the cellular and molecular level can be observed, which promote the development of so called flare-up reactions in case the sensitized individual encounters the same Ag again [138]. Usually, flare-up reactions occur preferentially at the site of previously healed skin and they are associated with increased severity. Often, a vicious cycle of active flare-up and resolution of inflammation at the same skin site can be observed. This characteristic clinical feature, which occurs in ACD and AD but also in other skin inflammatory diseases such as psoriasis and fixed drug eruption (FDE), could be explained by the persistence of Ag-specific skin Trm cells [24], [25], [26], [139].

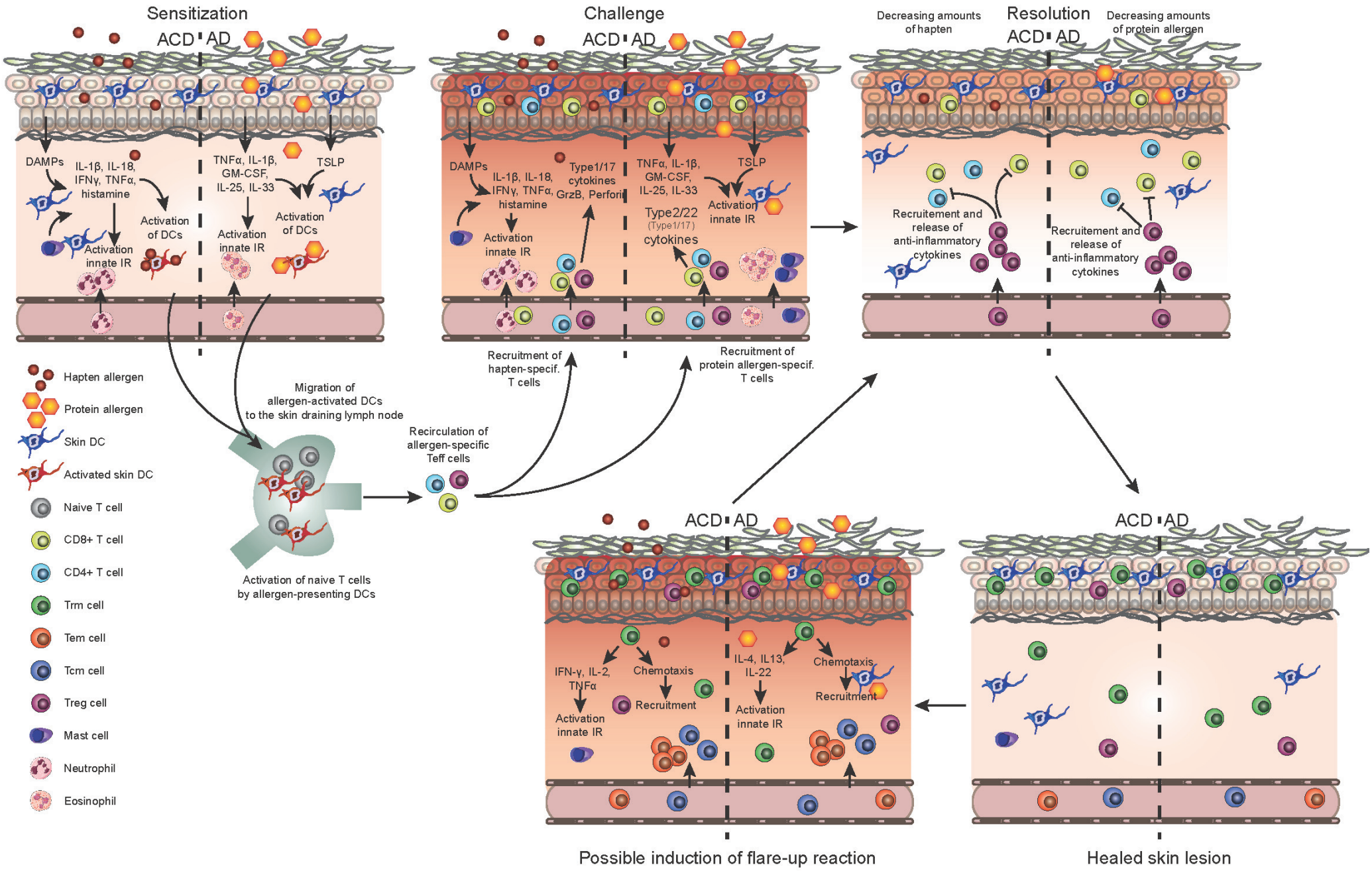

Sensitization
ACD
AD
DAMPs
IL-1β, IL-18, IFNγ, TNFα, histamine
Activation innate IR
Activation of DCs
TNFα, IL-1β, GM-CSF, IL-25, IL-33
TSLP
Activation innate IR
Activation of DCs
Challenge
ACD
AD
DAMPs
IL-1β, IL-18, IFNγ, TNFα, histamine
Activation innate IR
Type1/17 cytokines GrzB, Perfori
TNFα, IL-1β, GM-CSF, IL-25, IL-33
TSLP
Activation innate IR
Type2/22 (Type1/17) cytokines
Resolution
ACD
AD
Decreasing amounts of hapten
Decreasing amounts of protein allergen
Recruitement and release of anti-inflammatory cytokines
Recruitement and release of anti-inflammatory cytokines
Hapten allergen
Protein allergen
Skin DC
Activated skin DC
Naive T cell
CD8+ T cell
CD4+ T cell
Trm cell
Tem cell
Tcm cell
Treg cell
Mast cell
Neutrophil
Eosinophil
Migration of allergen-activated DCs to the skin draining lymph node
Activation of naive T cells by allergen-presenting DCs
Recirculation of allergen-specific Teff cells
Recruitment of hapten-specif. T cells
Recruitment of protein allergen-specif. T cells
ACD
AD
IFN-γ, IL-2, TNFα
Chemotaxis
Recruitment
Activation innate IR
IL-4, IL13, IL-22
Activation innate IR
Chemotaxis
Recruitment
ACD
AD
Possible induction of flare-up reaction
Healed skin lesion

FIGURE 6: PATHOPHYSIOLOGY OF ACD AND AD.

Sensitization: Hapten (ACD) or protein allergens (AD) pass the skin barrier, which leads to the activation of the innate immune response (IR). Activate skin DCs engulf the Ag and transport it to the skin draining lymph node. Here, the processed Ag is presented to naïve T lymphocytes and hapten- or protein allergen-specific T lymphocytes are released into the circulation.

Challenge: Upon Ag re-encounter, the innate IR is activated again, leading to the infiltration of neutrophils (ACD) and eosinophils and mast cells (AD) but also Ag-specific T lymphocytes are recruited rapidly from the circulation, initiating a type1/17 or type 2/22 response in ACD and AD, respectively. Noteworthy, type1/17 responses can be observed in chronic AD.

Resolution: Upon progressive elimination of the Ag, Treg cells infiltrate the affected areas but cell intrinsic regulatory mechanism, such as the up-regulation of inhibitory immune molecules, also contribute to the resolution of the inflammation.

Healed skin lesion: After the resolution of the skin inflammation, tissue-resident Ag-specific T memory cells (Trm) persist within the epidermis and dermis of healed lesions. In the circulation Ag-specific central (Tcm) and effector memory T lymphocyte (Tem) can be found.

Flare-up reaction: Upon re-encounter of the hapten or protein allergen, flare-up reactions occur. Possible mechanisms of flare-up induction involve the activation of persisting Trm cells by cognate Ag recognition resulting in a tissue-wide state of alert activating both the innate and adaptive IR. Flare-up reactions are followed by a resolution phase but the sites of healed lesions are not spared from recurring flare-up reactions.

8 Diagnosis of ACD

Diagnosis of ACD is made by clinical observations regarding the location and morphology of the skin inflammation but the most important criterion is patch testing. Patch testing should be performed in order to determine the causative allergens of the dermatitis [30], [46]. Indications for patch testing are given if the distribution, e.g. hands, feet, face or eyelid, of the dermatitis and the clinical history of the patient is suggestive for ACD. Also, patients working in a high-risk field for ACD such as hairdresser, health care worker, florists etc. should be tested by patch test. Finally, if the dermatitis is of unknown etiology or unresponsive to treatment, patch testing should also be considered [114].

During the patch test, eczematous reactions to a potentially causative allergen are reproduced on intact skin [29]. Therefore, the allergens are placed in patch test chambers, which provide inclusion of individual allergens while adhered on the skin. Reading of the patch test results occurs usually after 48h and is followed by a delayed reading after 72 to 168 hours in order to distinguish between irritant and real allergic reactions (see FIGURE 7) [114].

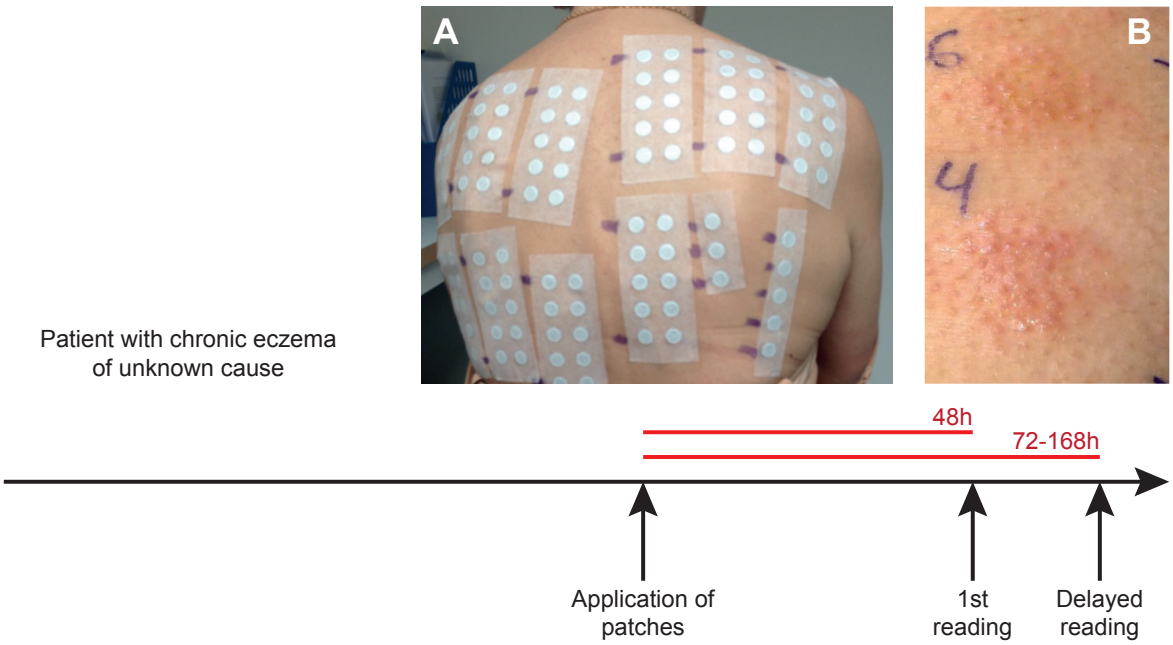


FIGURE 7: DIAGNOSIS OF ACD WITH THE HELP OF PATCH TEST APPLICATION.
Potential chemical allergens are applied on the skin for (A). After 48h a first read out of the patch test is performed, followed by a delayed reading at 72-168h. Clinical images courtesy of Audrey Nosbaum, M.D., Ph.D.

Grade	Observation	Description
0	Unchanged skin	No reaction
+?	Faint erythema only	Doubtful reaction, may be irritant
+	Erythema, infiltration, possibly papules	Weak allergic reaction
++	Erythema, infiltration, papules, and vesicles	Moderate/Strong allergic reaction
+++	Intense erythema, infiltration, and coalescing vesicles	Extreme allergic reaction
IR	Follicular, glazed erythema, and ulceration	Irritant reaction
NT		No tested

TABLE 2: CLASSIFICATION OF ACD PATCH TEST SCORE READINGS. ADAPTED FROM BASKETTER ET AL., 2012, AND MOWAD ET AL., 2016.

Other test to determine causative allergens for ACD are the Repeat Open Application Test (ROAT) and the Lymphocytes Transformation Test (LTT). ROAT is recommended in case the amount of chemicals is too small to cause an allergic reaction (e.g. patients that are allergic against chemicals present in low-doses in the commercial products they are using). During the ROAT, the product is repeatedly applied by the patients over up to 2 weeks, to determine whether the accumulation of the chemicals is causing the ACD reactions [114], [54].

The LTT is performed if e.g. the patient does not present enough unaffected skin and the patch test cannot be properly performed. During the LTT peripheral blood T lymphocytes isolated from the patients are incubated in the presence of the allergens in question. Proliferation of allergen-specific T lymphocytes indicate sensitization of the patient (see FIGURE 8) [140].

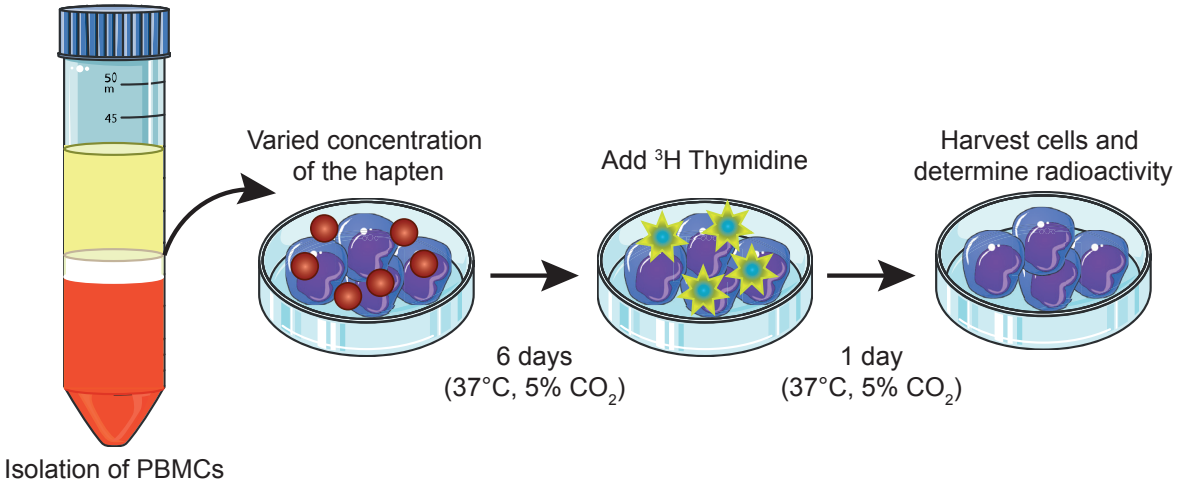


FIGURE 8: SCHEMATIC OF THE LYMPHOCYTE TRANSFORMATION TEST (LTT).
Peripheral blood mononuclear cells (including lymphocytes) are isolated from patients' blood by density gradient. They are incubated for 6 days in the presence of different concentrations of the allergen to be tested. After the incubation in the presence of radioactive ^{3}H thymidine, the incorporation of 3H thymidine into the DNA of proliferating cells is determined. Only lymphocytes specific for the allergen will proliferate and take up the ^{3}H thymidine.

9 Diagnosis of AD

The diagnosis of AD is made clinically and it is based on several features including the patients' history, the morphology and distribution of skin lesions as well as associated clinical results. Even though many cases are associated with increased IgE serum levels, to date no reliable set of biomarkers for the diagnosis of AD has been validated [27], [141] and clinicians depend on different set of features associated with AD. One of the earliest among many other sets of diagnostic criteria has been formulated by Hanifin and Rajka in 1980 [142]. It describes 4 major and 27 minor criteria that need to be analyzed and from both groups at least 3 are required to diagnose AD [143], [35]. However, some of the criteria described were not specific enough or not easily applicable in the practice and therefore have been re-evaluated and a distilled set of criteria has been proposed which consists of 1 mandatory, 5 major, and several associated features as well as exclusionary conditions (see Table 3) [144].

Several other tests can be performed to narrow down the manifestation of AD. The severity of the skin disease can be determined by different scoring systems. The most acknowledged tests are the Score in Atopic Dermatitis (SCORAD) and Eczema Area and Severity Index (EASI). More recently, patient-related scoring such as the Patient-oriented SCORAD (PO-SCORAD) have been described and help to evaluate the course of skin inflammation (see FIGURE 9). All these scoring systems combine the extent of the eczema, the severity, and the subjective symptoms and have be validated in several studies [145], [146], [147], [148]. In order to determine the causative allergen and to strengthen the diagnosis of AD, a specific Atopy Patch Test (APT) testing for protein allergens, prick test as well as skin biopsies can be performed [27]

Essential Features (must be present)
Pruritus Eczema (acute, subacute, chronic) • Typical morphology and age-specific patterns • Chronic or relapsing history
Important Features (seen in most cases)
Early age of onset Atopy • Personal and/or family history • IgG E reactivity Xerosis
Associated Features
Atypical vascular responses Keratosis pilaris/pityriasis alba/hyperlinear palms/ichthyosis Ocular/periorbital changes/periauricular lesions Other regional findings (e.g. perioral changes) Perifollicular accentuation/lichenification/prurigo lesions
Exclusionary conditions
Scabies Seborrheic dermatitis ACD Ichthyoses Cutaneous T-cell lymphoma Psoriasis Photosenstitivity dermatoses Immune deficiency diseases Erythrodema of other causes

TABLE 3: FEATURES TO BE CONSIDERED IN THE DIAGNOSIS OF PATIENTS WITH AD. ADAPTED FROM EICHENFIELD ET AL., 2014.

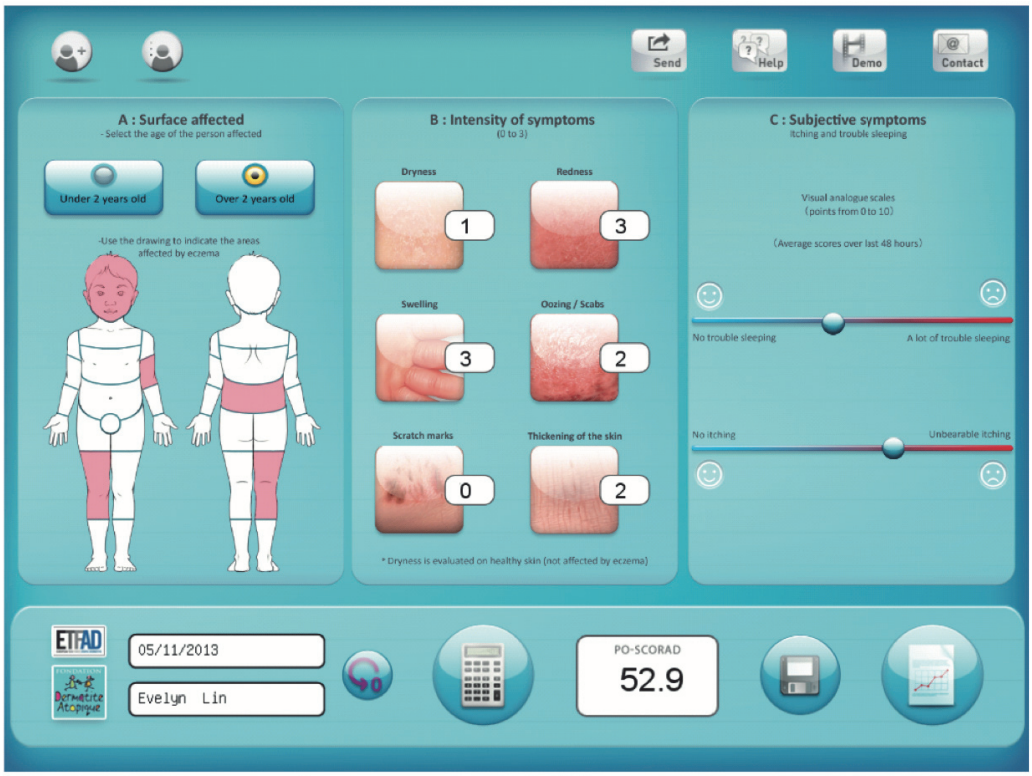


FIGURE 9: PO-SCORAD.
User interface of the web-base PO-SCORAD tool, which facilitates easy communication between AD patients and physicians. Patients enter several parameters evaluating their eczema, they can add pictures of affected skin areas, and directly determine their own PO-SCORAD. Furthermore, the entered data can be directly transmitted to the clinician.

10 Treatments of ACD and AD

The most efficient treatment for ACD is identification and subsequent avoidance of the causative allergen, which is possible due to the chemical nature of the allergens [54]. In AD, reduction of the allergens helps the patients but, since they are present in our every day environment, complete avoidance cannot be achieved for them. Medications for both ACD and AD exist which mainly treat the skin inflammation or that are prophylactic and target the skin barrier effect. However, in most of the cases the symptoms recur after discontinuation of the treatment or in case of ACD, after re-exposure to the allergen. Several topical treatments for ACD and AD are overlapping and discuss below.

10.1 Topical treatments for ACD and AD

10.1.1 Corticosteroids

Topical corticosteroids have been the main treatment of ACD and AD during the last decades. They are recommended for active skin lesions, chronic cutaneous manifestations, and management of pruritus [149]. They are highly anti-inflammatory and their predominant effect is to switch off inflammatory genes encoding cytokines, chemokines, adhesion molecules, inflammatory enzymes, receptors, and proteins that have been activated during chronic inflammation [150]. Their efficacy in decreasing signs of AD and ACD has been demonstrated in many trials [151], [152] and according to their potency they can be organized into 7 groups, ranging from low to very high potency [153]. The treatment plans are variable and in the case of ACD mainly short-termed. In AD they can include the short-term use of high potency agents in the beginning, with subsequent decrease in the potency for long-term use. Also, long-term use of low potency product with gradual increase depending on the patients' tolerance might be applicable [149]. However, the choice of which corticosteroid is used should include several points such as the severity of ACD or AD skin lesions, the age and body weight of the patient, and the application frequency [153], [154]. Side effects caused by topical corticosteroids are relatively low but of greatest concern is the occurrence of reversible skin atrophy which is increased in older patients or by the use of high potency agents [155]. Noteworthy, topical application of corticosteroids on lesional AD skin reduces colonization with *S. aureus* potentially due to decreased release of inflammatory cytokines, which inhibit the production of antimicrobial peptides [156].

10.1.2 Calcineurin inhibitors

Topically applied calcineurin inhibitors are a second class of inflammatory therapy for AD patients. They are not validated for the treatment against ACD. They inhibit the protein phosphatase calcineurin, which subsequently prevents the dephosphorylation of the nuclear factor of activated T cells (NF-AT), a transcription factor (TF) necessary for T cell activation. Subsequently, signal transduction in T cells is blocked which prevents the production of pro-inflammatory cytokines such as IL-3, IFN-γ, TNF-α, and GM-CSF [157], [153]. Currently, two calcineurin inhibitors are commercially available: tacrolimus for moderate to severe AD and pimecrolimus for mild to moderate cases [158]. Even though they are not validated for ACD yet, both tacrolimus and pimecrolimus have also been shown to be effective in ACD [159], [160]. Topical use of calcineurin inhibitors is useful to treat sensitive skin sites such as the face, since, unlike corticosteroids, it does not cause skin thinning. However, they have often been associated with burning of stinging sensations [149]. Also, rare cases of malignancies such as skin cancer and melanoma have been reported in patients treated with calcineurin inhibitors but until now no causal relationship could be established between them [153].

The use topical use of corticosteroids and calcineurin inhibitors might be combined to obtain optimal therapeutically results. Since corticosteroids have a higher potency, they can be used in order to reduce acute symptoms. Subsequently, calcineurin inhibitors could be used to prevent relapses and to spare the use of corticosteroids especially in AD [153].

10.2 Phototherapy for ACD and AD

Several decades ago it was observed that AD symptoms improved after patients spend time in sunny climates. This led to the treatment of AD patients with ultraviolet (UV) light which has intrinsic immunosuppressive properties [161]. Since then, several forms of light therapy have been demonstrated to be beneficial for both ACD- and AD-patients including natural sunlight, narrowband UV-B, broadband UV-B, UV-A, topical and systemic psoralen in combination with UV-A, and combinations of UV-A and UV-B light [162], [163]. Phototherapy treatments are numerous and depend on the patients' history and clinical presentation of ACD or AD [164]. Also, they can be used as monotherapy or combined with topical corticosteroids. Moreover, phototherapy is considered to have a relatively low rate of side effects, which might include local erythema, pruritus, burning, and stinging [163].

10.3 Non-pharmacological treatments for AD

Other treatments in order to reduce the symptoms of AD include the use of moisturizers, the wet-wrap therapy or special bathing therapy. They all intend to reduce xerosis, which is a hallmark of skin form in AD-patients that is caused by TEWL due to the impaired epidermal barrier [165].

In general, moisturizers contain emollients that lubricate and soften the skin, occlusive agents that prevent evaporation of water, and humectants that attract and retain water [153]. Since moisturizers have been demonstrated to increase skin hydration and to reduce signs of AD (e.g. pruritus) they also reduce inflammation and AD severity [166], [167]. Therefore, moisturizers are the primary treatment of all case of AD [168]. Moreover, a study investigating the effect of emollients in preventing AD in high-risk neonates having either at least one parent or one full sibling diagnosed with AD, asthma, or allergic rhinitis showed that the treatment from birth on is an effective approach to reduce the onset of AD [169].

In the wet-wrap therapy, a topical agent such as a moisturizer is covered by a first wetted layer of bandages, gauze or a cotton suite, followed by a second dry layer. The duration of the wrap depends on the patients' tolerance and can last from several hours to several days [149], [153]. It has been demonstrated that the wet-wrap therapy was more efficient when a topical corticosteroid is applied in addition to the moisturizer. However, such combinations have to be carefully chooses, since due to wet-wrap increased absorbance of the agents might occur [170], [171].

In AD short term showering for less than 5 minutes with water can be helpful for the patient since it can hydrate the skin and remove crust, scale, irritant, and allergens. However, in order to prevent further water loss after the bath due to evaporation from the skin, moisturizers should be applied immediately to maintain the obtained hydration status [172]. Also, soaps with an alkaline pH should be avoided since the natural skin pH is 4 to 5,5. However, non-soap-based surfactants and detergents may be added to the bathing water [173].

10.4 Immunotherapy for ACD and AD

Immunotherapy of ACD and AD aims to induce unresponsiveness to allergens through induction of immune tolerance. The aim is either to prevent sensitization or to induce hypo-sensitization in order to reduce existing hypersensitivity to an allergen [174]. This means immunotherapy can occur either before the sensitization phase or after to prevent elicitation phases.

In pre-clinical ACD mouse models, tolerance toward nickel could be induced in a dose-dependent manner after oral nickel administration [175], [176]. Moreover in patients, induction of tolerance could also be observed after oral intake of nickel [177], [178]. Similarly, in animal models it has been shown that cutaneous application of hapten in low doses can induce tolerance toward it [179], [180]. Investigators from the same group were able to demonstrate that Treg cells are key in this model to instruct DCs to become tolerogenic for the allergen [181].

In two studies investigating canine AD, it has been demonstrated that the continuous s.c. administration of AD-associated allergens from *Der f* and *Malassezia* is effective in reducing allergic reactions. Moreover, an AD-mouse model using s.c. Ag applications could demonstrate that immunotherapy led to decreased serum IgE levels which is associated with an amelioration of the disease [182]. Also in humans immunotherapy has been shown to have beneficial effects in AD but due to the complexity of the disease the investigators recommended to further investigate the combination of immunotherapy with other treatments such as mAb therapy [183].

Since in most cases the event of sensitization can hardly be foreseen, the necessity of tolerance induction might be difficult to anticipate. Therefore, ongoing strategies to re-induce tolerance aim at improving the existing methods by oral or cutaneous hapten application as well as to develop new drug in order to activate regulatory mechanisms e.g. by Treg cell activation [184]. Also our own observations show that low dose application of haptens in allergic mice can reduce the responses to high dose challenges.

10.5 Systemic treatments for ACD and AD

Systemic treatments (e.g. with methotrexate or cyclosporine) of ACD and AD should be considered for severe cases and AD-patients for whom an optimized topical treatment with moisturizer and/or phototherapy did not lead to adequate management of the disease [163].

In the case of AD, systemic administration of corticosteroids only leads to short-term suppression of the disease. However, it should be avoided since both short- and long-term side effects and general immunosuppression have been observed [185], [163]. Similarly, the systemic administration of broad immunosuppressant drugs bears the risk of side effects.

10.6 New Treatments for ACD and AD

The relative high rate of unwanted side effects of systemic administration of corticosteroids and other immunosuppressant drugs indicates the need for new medications to treat both ACD

and AD. Current approaches include new topical products but also systemic treatments by monoclonal Abs directed against inflammatory mediators [158], [154].

Phosphodiesterase (PDE)-4 inhibitors, that can be applied topically, reduce the production of inflammatory cytokines by targeting the regulation of intracellular cyclic adenosine monophosphate (AMP), an intracellular signaling molecule. Noteworthy, increased PDE-4 levels that promote the production of inflammatory cytokines have been reported in AD-patients [186]. However, PDE-4 inhibitors are able to reduce the inflammatory responses by suppressing the production of cytokines such as IFN-γ, TNF-α, IL-4, IL-5, and IL-13 in ACD and AD [187], [188], [189].

Another pathway that can be targeted topically is the Janus kinase (JAK) signal transducer and activator of transcription (STAT) by JAK inhibitors. Many inflammatory cytokines such as IL-4, IL-5, IL-13, and IL-31 are induced by the JAK-STAT pathway [190]. Clinical trials demonstrated that the application of JAK inhibitors has been associated with a decrease in the production of these cytokines and improved skin barrier function in AD skin[191]. Also, pruritus could be reduced by JAK inhibitors, most likely due to the suppression of IL-31 which is a cytokine associated with the occurrence pruritus [192].

Also the aryl hydrocarbon receptor (AhR) has become a target for new topical therapeutics. AhR is a ligand-activated TF that is expressed in the skin and that acts as a receptor for xenobiotics. In the skin of AD-patients AhR is increased and its activation seems to not only interfere with Th2 cell signaling but also improves epidermal functions by restoring FLG expression [193], [194]. Treatment with AhR agonists showed promising results in early clinical trials [195]. New systemic treatments of AD are mainly based on the development of monoclonal antibodies (mAbs), targeting specific cytokines or molecules associated with the development of inflammation in AD. Currently, mAbs abrogating signaling of IL-4, IL-5, IL-13, IL-12/IL-23, IL-17A, IL-22, and IL-31 are investigated [158]. So far, only one mAb targeting IL-4/IL-13R (dupilumab) has been approved for severe cases of AD in the USA but not yet in the EU [196]. Other targets of mAB therapy are TSLP and IgE which both play an important role in the polarization of immune responses and the development of skin inflammation [197]. However, anti-TSLP might only affect sensitization pathway by influencing the polarization towards Th2 cell responses. However, it could be combined with other mAb treatment for more efficacy [198]. Also, anti-IgE treatments do not show the desired efficiency in AD treatment [199].

11 Pre-clinical mouse models to investigate ACD and AD

11.1 ACD-like contact hypersensitivity mouse model

To study the underlying mechanisms of ACD, haptens are use to induce contact hypersensitivity (CHS) in mice. Similar to ACD, CHS consists of two different phases: the sensitization and elicitation phase during which the first skin contact and re-exposure with a hapten occur, respectively [200]. In general, CHS in mice is induced by haptens with strong sensitizing potential such as Oxazolone (Oxa), 2,4-Dinitrofluorobenzene (DNFB), 2,4 Dinitrochlorobenzene (DNCB), 2,4,6-Trinitrochlorobenzene (TNCB), and Fluorescein isothiocyanate (FITC) [201], [113]. However, CHS can also be induced by weak haptens e.g. α-Hexylcinnamaldehyde (HCA), eugenol (EUG), and hydroxzcitronellal (HDCL) which are involved in fragrance allergy [112]. The application protocols in order to sensitize mice and to elicit a CHS response are manifold and depend on the scientific problem that is addressed by the experiments. Moreover, depending on the mouse strain used, reactions to the haptens might differ. For instance, some transgenic mice such as Nrf2-/- lack a functional cellular detoxification machinery. They are more sensitive to haptens and show strong CHS responses even in response to weak haptens [202], [203]. Regarding the choice of a hapten to use in a CHS model, it is also noteworthy that the roles of cytokines and chemokines and their corresponding receptors might differ between haptens and mouse strains [201], [204]. For instance, it has been described that Oxa induces normal CHS responses in B6x129Sv IL-1β-/- mice while the reactions against TNCB are impaired. Similarly, in Balb/c IL-4-/- mice reactions against Oxa are normal but decreased against DNFB and DNCB [205].

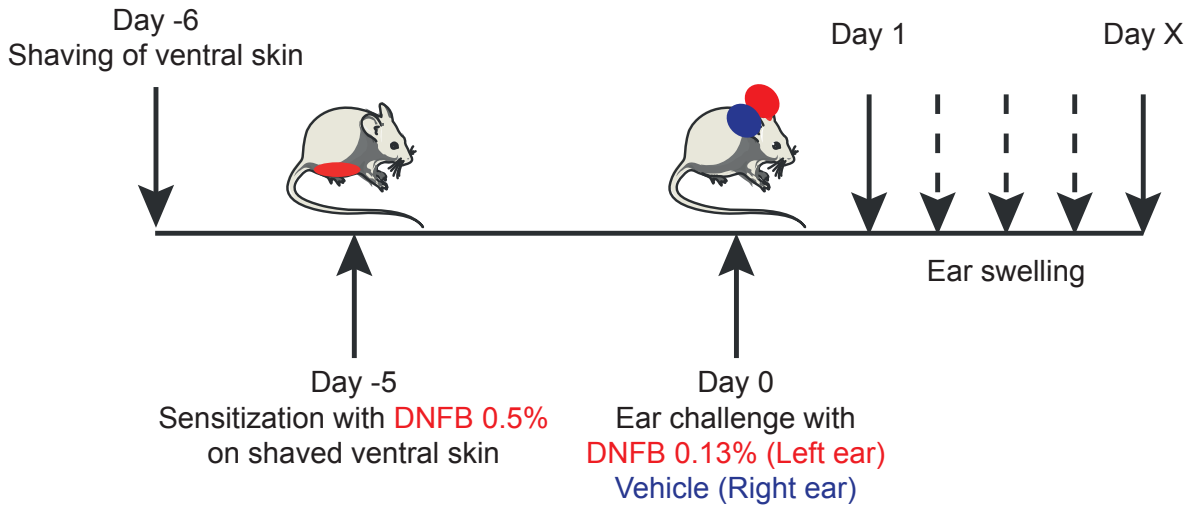


FIGURE 10: EXAMPLE OF ACD-LIKE CHS MODEL IN MICE.
DNFB diluted in Aceton/Olive oil (Vehicle) is applied on the shaved belly. 5 days later DNFB is applied on the left ear and vehicle on the right ear. In the following days, ear swelling is determined by standard mouse ear swelling test (MEST). The duration and adaption of the protocol depends on the experimental outline.

11.2 AD-like mouse models

Pre-clinical mouse models to investigate the underlying mechanisms of AD can be categorized in three goups: Induced, mutant, and spontaneous AD-like models.

11.2.1 Induced AD-like mouse models

Among the induced models, two subtypes of sensitization can be distinguished. Passive sensitization is based on intravenously i.v. injection of murine monoclonal IgE α-allergen antibodies prior to challenge with the respective allergen. Since inflammatory reactions appear within minutes after challenge, passive immunization represents immediate hypersensitivity [206]. In contrast, epicutaneous sensitization is induced by the repeated application of the allergen to the skin. It requires a break-down of the skin barrier which can either be achieved by tape-stripping of the skin or, if tape-stripping might not be possible, by dissolving the allergens in vehicles such as dimethyl sulfoxide DMSO that lead to impaired skin stability. The allergen can be applied directly in a dissolvent or on patches that will stick to the skin. Allergens that are frequently used in AD-like mouse models are extracts from HDMs such as *Der f* and *Der p* but purified ovalbumin (OVA) protein [207] or enterotoxins from *S. aureus* strains can also be used to initiate AD-like skin reactions [101], [208].

11.2.2 Mutant AD-like mouse models

Several genetically engineered mutant mouse models that lack or over-express molecules have been observed to develop AD-like skin conditions indicating that these molecules are

involved in the pathogenesis of AD. For instance, increased expression of TSLP in mice either by tetracycline-induced expression or by ablation of retinoid X receptors leads to the development and progression of chronic AD-like skin lesions. In this model, mice exhibit a Th2 cell immune response with up-regulation of IL-4, IL-5, IL-13 and IL-10 [209], [210]. Similarly, mice over-expressing IL-4 [211], IL-31 [212] or Caspase 1 [213] develop AD-like lesions. Interestingly, TSLP expression can also be induced by cutaneous application of calciprotiol, a low-calcemic analog of vitamin D3, which results in the development of AD-like skin inflammation [214], [215].

Flaky tail mice carry a loss-of-function mutation of the FLG gene, which is as described above an important component to maintain skin integrity. Flaky tail mice develop AD-like skin lesions upon day 28 of life [216], [217]. Cathepsin E knockout mice lack the aspartic proteinase cathepsin E, which is involved in the degradation of colonizing skin microbiota. Therefore, these mice develop AD-lesions that are colonized with *S. aureus* [218].

11.2.3 Spontaneous AD-like mouse models

Spontaneous AD development can be observed in Nc/Nga mice, an inbred mouse strain. Under specific pathogen free (SPF) and air-controlled housing conditions, this mouse strain is not affected by AD development. However, when the mice are kept under conventional conditions and are infected with mites, they develop AD-lesion lesions within week [219].

Even though, histological analysis revealed close proximity of the AD-like lesions from the mentioned as well as other mouse models to AD-lesions from patients, analysis of the underlying immune responses revealed differences regarding Th cell polarization, cytokines release, accumulation of serum Igs etc. Therefore, similar to the choice of haptens in pre-clinical ACD mouse models, the choice of AD models depends on the experimentally addressed question.

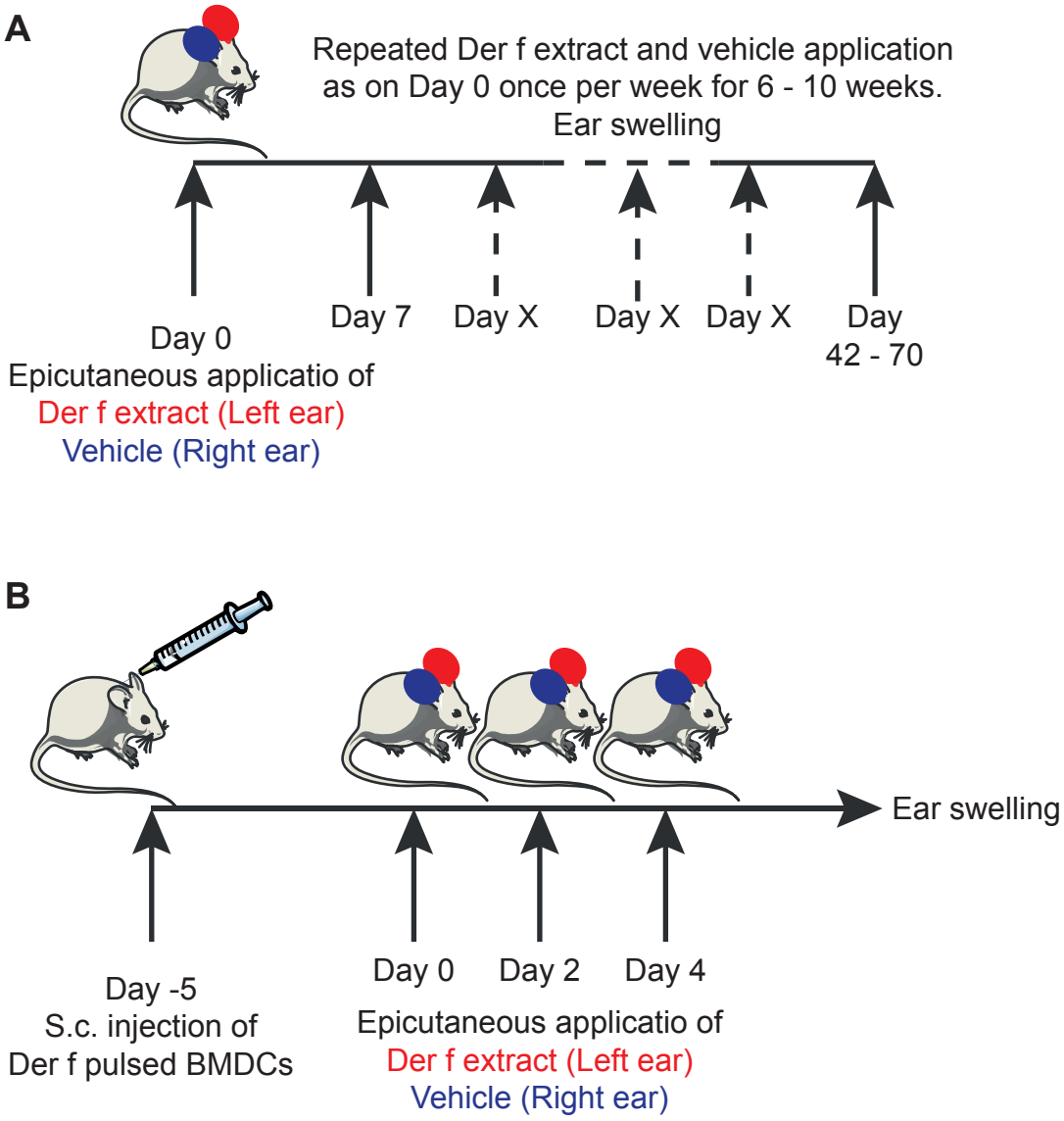


FIGURE 11: EXAMPLES OF INDUCED AD-LIKE MODEL IN MICE.
(A) Der f extract diluted in DMSO/Water (Vehicle) is applied on the ear once a week for 6-10 consecutive weeks until skin inflammation is observed. (B) Mice are injected s.c. with Der f extract-pulsed BMDCs. Five days later, Der f extract diluted in DMSO/Water (Vehicle) is applied on the ear every other day for 3-4 times until skin inflammation is observed. In both models, skin inflammation is determined by standard mouse ear swelling test (MEST). The duration and adaption of the protocol depends on the experimental outline.

Part 2: Tissue-resident memory T cells – Protective functions and detrimental potential

12 T cell-mediated immunity: From naïve to memory

Ag-specific T lymphocytes are central players in the generation of protective immune responses against numerous microbial infections. While cells of the so-called innate immune system recognize pathogen associated molecular patterns (PAMPs) that are expressed by large groups of Ag, cells of the adaptive immune system recognize specific Ag via their T cell receptor (TCR) [220], [221]. Both the innate and the adaptive immune system are functioning in a synergistic way to generate an appropriate immune response. Following exposure, the Ag is engulfed and processed by DCs, which are professional Ag-presenting cells (APCs) and belong to the innate immune system. In such way activated DCs traffic to the draining lymph nodes where they present the processed Ag to naïve T lymphocytes [222].

Naïve T lymphocytes that are highly diverse in their TCRs specificities constantly recirculate between the blood stream and lymph nodes, probing them for their target Ag. Activated T lymphocytes undergo clonal expansion giving rise to cast numbers of Teff cells expressing the same TCR [14], [15]. Of note, the Ag is also presented to naïve B lymphocytes which comprise the humoral arm of the adaptive immunity. Activated B lymphocytes will develop into cells secreting Ag-specific antibodies which can bind to the Ag in order to neutralize it and to prevent infections [223]. However, B lymphocytes will not be further discussed here. The T cell-mediated site of the adaptive immune response consists Teff cells and Tm cells.

Teff cells can be further distinguished into CD8+ cytolytic T lymphocytes (CTLs) and CD4+ Th cells, which differentiate depending on their initial signal into different subsets. Th cell subsets release different cytokines which mediate CTL responses against pathogens but can also promote immunopathologies [224], [225], [226].

Th1 cells produce high levels of IFN-γ but also IL-2 and TNF-α. They have been shown to be responsible for phagocyte activation and thereby protection against intracellular pathogens but they also contribute to the development of autoimmune diseases and chronic inflammatory disorders [227], [226].

Th2 cells produce IL-4, Il-5, IL-6, IL-10, and IL-13. Their differentiation is induced in response to helminth infection against which they also protect and in response to IL-2 and IL-4 signaling. Th2 cells are also known to mediate allergic inflammation e.g. asthma [228], [229].

Th17 cells produce IL-17, IL-21, and IL-22 and their main role is the clearance of

extracellular bacteria and fungi, which are mainly mediated by the recruitment and the activation of neutrophils. However, Th17 cells also play a role in the pathogenesis of autoimmune diseases such as psoriasis [230], [231], [232].

So far Th1, Th2, and Th17 cells are the best described subsets of Th cells but others subsets, such as Th22, Th9, and T follicular helper (Tfh) cells, also exist. They are known to be involved e.g. in maintaining mucosal barrier functions, autoimmune inflammation, and the formation of germinal centers in secondary lymphoid organs (SLOs) [233], [227].

In addition, CD4+ T lymphocytes can also differentiate into Treg cells in response to transforming growth factor (TGF)-β and IL-2 signaling [234], [235], [236]. Treg cells are important to maintain immune tolerance and lymphocyte homeostasis. They have suppressive functions which regulate the effector functions of Th cells and CTLs during immune responses in order to prevent excessive tissue damage or immunopathologies [235], [234], [236].

The cytokines released by Th cells are key to orchestrate strong responses of CTLs. CTLs use mainly two mechanisms to eliminate microbially infected cells: the perforin granule exocytosis pathway and FasL/Fas mediated apoptotic cell death [237]. In addition to their cytolytic capacity, CD8+ T lymphocytes also release anti-microbial and inflammatory cytokines such as IFN-γ and TNF-α [238].

After fulfilling their effector functions, most Teff cells (both CD4+ Th cells and CD8+ CTLs) undergo cell death leaving behind a small but long-living population of Tm cells. Compared with naïve T lymphocytes, Tm cells have the advantage to survive and to respond more rapidly and efficiently to any further Ag exposure [14], [15].

12.1 The different phases oft the adaptive immune response

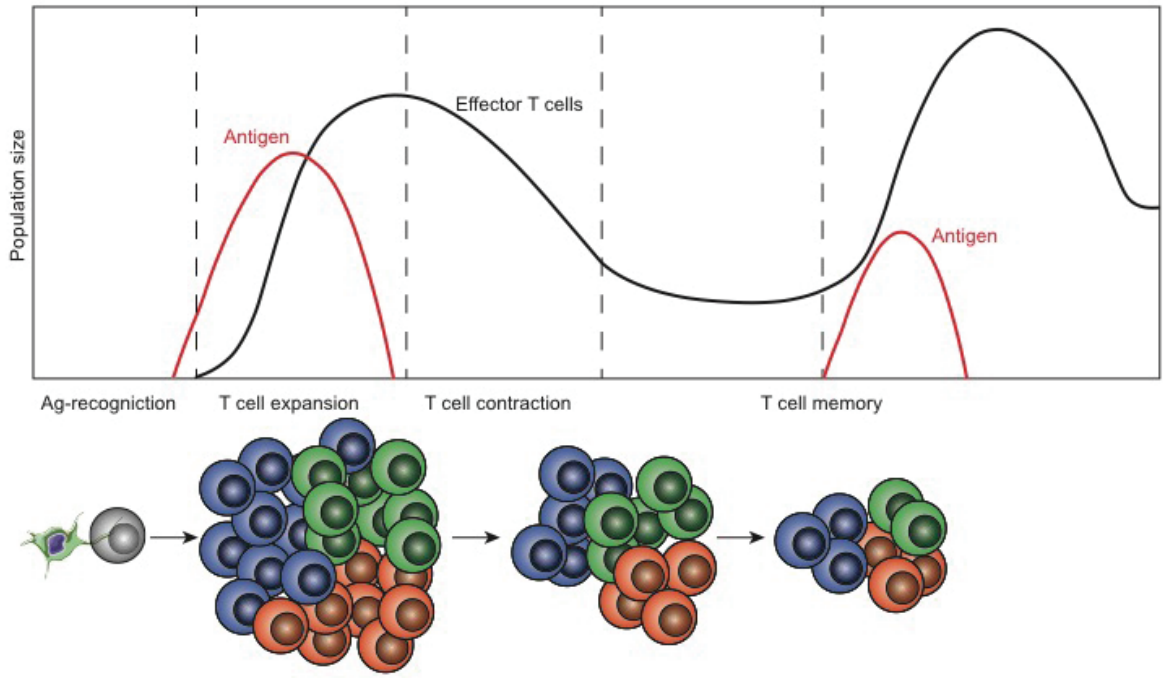


FIGURE 12: THE DIFFERENT PHASES OF THE ADAPTIVE IMMUNE RESPONSE.
Ag-recognition - In the periphery, DCs are the first cell population to encounter the Ag. After Ag- engulfment they transport processed fragments of the Ag to the tissue-draining lymph node where they present it to naïve T lymphocytes which circulate between the blood stream and lymph nodes where they probe the microenvironment for their target Ag.

Clonal expansion: Ag recognition by naïve T lymphocytes leads to their activation. Activated T lymphocytes undergo clonal expansion giving rise to numerous Teff cells comprising Th cells and CTLs with the same TCR that mediate Ag elimination.

T cell contraction: Following the Ag elimination during the contraction phase most of the Teff cells die after fulfillment their effector functions. Only a small fraction of the Teff cells survive and develop further into mature Tm cells.

T cell memory: Long-living Tm cells that developed after the first encounter with an Ag have the ability to rapidly generate effector functions and to produce a new wave of CTLs in order to efficiently respond to re-exposure of the same Ag.

Adapted from Kaech&Cui, 2012.

13 Memory T cells: A heterogeneous population

Despite their uniform Ag-specificity it became evident that Tm cells comprise a very heterogeneous group of cell populations that can be distinguished according to their phenotype, function, and location.

Until recently, Tm cells were categorized into two different groups depending on their function and homing properties. The two subsets were designated central (Tcm) and effector (Tem) memory T cells [239], [240]. Tcm cells are predominantly found within lymphoid organs and they can circulate into the blood stream. Tem cells on the other hand are mainly present within peripheral tissues but they are found in the blood as well. Tcm cells express the vascular addressin CD62L as well as the chemokine receptor CCR7 which allows them to enter lymphoid tissues from the blood stream [241], [16]. Tem cells do not express CD62L and they are variable in their expression of CCR7. However, Tem cells have been demonstrated to express receptors permitting them to enter peripheral tissue (e.g. Cutaneous Lymphocyte Antigen (CLA) to enter the skin). Therefore and for their capacity to produce rapidly IFN-γ, Tem cells have been thought to be rapidly recruited to peripheral tissues upon Ag-challenge in order to provide protection [242] (see Figure). While Tem cells only show small proliferative potential, Tcm cells can strongly proliferate and release IL-2 upon Ag-encounter and activate further Th cell responses.

However, due to the diverse migration phenotype of Tem cell subpopulations indicated (e.g. by different level of CCR7 expression), it became clear that another important subset of Tm cells exists which has been described as tissue-resident memory T cells (Trm). Trm cells neither express CD62L nor CCR7 but they are positive for CD69 and CD103 two important Trm cell markers that are not expressed by Tcm or Tem cells. The role of CD69 and CD103 in the development and maintenance of Trm cells will be discussed later in more detail. Under physiological conditions, Trm cells survive for several months inside the epithelia of various organs of the organism without recirculating throughout the body. Trm cells are considered to be precious sentinels which are positioned at the interphase with environment and entry points of pathogens, providing efficient protection against infectious agent, e.g. viruses, that penetrate locally through the epithelia [243], [244], [13], [16] (see FIGURE 13 and Table 4).

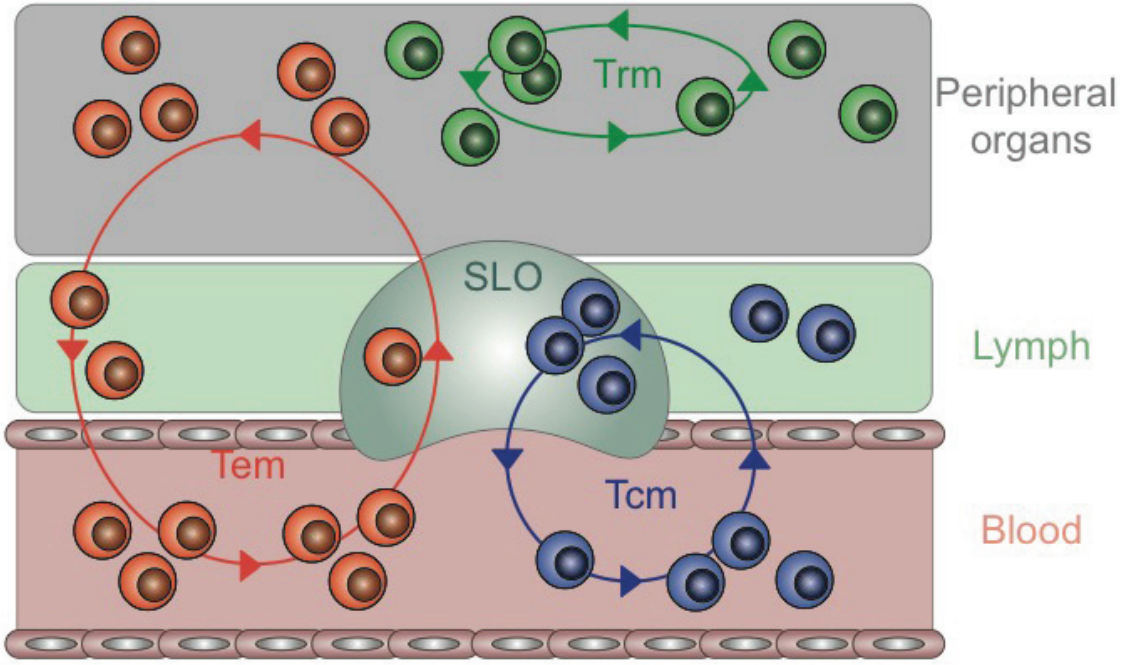


FIGURE 13: MIGRATION PATTERN OF TM CELL SUBSETS.
Tcm cells (blue) migrate between the blood, the lymph, and secondary lymphoid organs. Tem cells are present in the blood but can also enter inflamed peripheral organs. Few are found within secondary lymphoid organs. Trm cell do not circulate and remain resident in peripheral organs. Adapted from: Carbone et al., 2013 and Schenkel&Masopust, 2014.

Subset	Phenotype	Location
Tcm cells	$CD44^{high}$, CD62L+, CCR7+, CD127+, CD69, CD103-, GzmB-, Ecad-, KLRG-1-	LNs, spleen (white pulp > red pulp, blood, bone marrow
Tem cells	$CD44^{high}$, CD62L-, CCR7+/-, CD127+, CD69-, CD103-, GzmB+, Ecad-, KLRG-1+	Spleen (red pulp > white pulp) few in in LNs and lymph, Blood, lung, liver, intestines, FRT, kidney, adipose tissue, heart
Trm cells	$CD44^{high}$, CD62L-, CCR7-, $CD11a^{high}$, CD69+, CD103+, CD127+, Ecad+, GzmB+, KLRG-1- *Note: CD69 and CD103 have been considered to be canonical Trm cell markers but recently also CD69- and CD103- cells been described* [245], [246]	Spleen, lung, intestine, skin FRT, brain, kidney, liver, salivary gland, LNs, tonsils, thymus

TABLE 4: PHENOTYPE AND LOCATION OF TM CELL SUBSETS.
ADAPTED FROM: GEBHARDT&MACKAY, 2012; MUELLER ET AL., 2013; AND GEBHARDT ET AL., 2013

14 The diverse niches of tissue-resident memory T cells

The concept of tissue specific immune systems in which lymphocyte subpopulations are long-term resident within certain areas of an organ has been described several decades ago [247]. Initially described in the skin [24] and gut [248] Trm cells have been found in a variety of tissues and organs in humans and mice. They can be clearly distinguished from their circulating counterparts (Tem and Tcm cells) by their lack of migration throughout the organism but also by their phenotype, function, and transcriptional signature. Based on the expression of CD69, a key marker of Trm cells that is involved in their tissues residency, a large study analyzing multiple organs from healthy human donors detected Trm cells, in all analyzed tissues, which included spleen, lung, three parts of the intestine (jejunum, ileum, and colon), as well as inguinal, mesenteric, and bronchial lymph nodes [249]. Those results are complemented by several studies in mouse and humans demonstrating the persistence of Trm cells in non-lymphoid tissues such as the skin [246], [250], the female reproductive tract (FRT) [251], [252], the brain [253], [254], the kidney [255], and the liver [256]. Even though Trm cells are generally associated with residency inside non-lymphoid tissues, recent studies provide evidence for Trm cell niches inside both primary and secondary lymphoid organs [257], [258], [259], [260]. It has been demonstrated that Ag-specific CD4+ and CD8+ Tm cells in primary and secondary lymphoid organs such as the thymus and lymph nodes express markers usually associated with Trm cells in non-lymphoid tissues and they localize preferentially at the entry point for peripheral Ag where they are thought to guard the lymphoid organs [258], [259], [260].

Although one major hallmark in distinguishing Trm cells from their circulating counterparts is their localization and their lack of migration throughout the organism, within certain tissues such as the skin, Trm cells display migration within their niche in order to identify rare APCs [261]. By doing so they increase the chances to provide rapid on-site immune protection against re-encountered pathogens.

15 Tissue-resident T lymphocytes: More than just Trm cells

Even though they are today the most described tissue-resident T lymphocytes, CD8+ and CD4+ Trm cells represent only two subsets of them. Further tissue-resident T lymphocyte populations include Foxp3+ Treg cells, Non-conventional T cells including subsets of TCRαβ or γδ T cells, and innate lymphoid cells (ILCs) [262]. Tissue-resident T lymphocytes share certain hallmarks including long-term maintenance and potential self-renewal, abundance in barrier tissues, sensing of microbial products, cytokines, alarmins, and stress ligands, as well

as the rapid provision of anti-microbial and tissue-protective factors making them key players of the immune system and linking its innate and adaptive branch [263].

15.1 CD4+ and CD8+ Trm cells

Even though the concept of Trm cells versus their circulating counterparts has been mainly established on research regarding CD8+ Trm cells, the existence of CD4+ Trm cells has been demonstrated as well. Experimental series in both pre-clinical mouse models as well as in humans demonstrated that Trm cells cannot be eliminated from their tissue niche by systemic antibody depletion most likely due to their restrained access to the bloodstream [246], [17], [264]. Our own observation showed that even direct intraepidermal application of depletion antibodies does not facilitates Trm cell depletion from the epidermis since it is directly removed via the draining lymph nodes resulting in depletion of circulating Tm cells. In addition, tissue persistence and longivity of Trm cells has been also demonstrated in mouse parabiosis experiments. Here, two sex- and age-matched mice, one mouse having Trm cells after e.g. herpes simplex virus (HSV) infection and one naïve mouse, are surgically joined on their flanks to establish a shared blood circulation. After equilibration of cells with circulation potential, the two mice are separated again and the Ag-specific cells are analyzed. Only circulating Tem and Tcm cells will reach an equilibrium between the two mice, while Trm cells will only be found inside the mouse immunized previously to parabiosis [24], [20].

While today tissue residency of CD8+ Trm cells is well demonstrated, less is known about CD4+ Trm cells. However, similar to CD8+ Trm cells they have been described within multiple non-lymphoid organs such as the gut [265], the lung [266], the genital mucosa [267], the salivary gland [268], and the skin [246]. However, since CD4+ T cells appear to maintain their capacity to reenter the circulation, it remains difficult to determine whether for some CD4+ Trm cell subpopulations tissue residency is a permanent state or whether they all remain in contact with the circulation. For instance, in humans it has been demonstrated that the chemokine receptor CCR7 which is key for tissue egress remains up-regulate on CD4+ skin Trm, cells indicating their potential to re-enter circulation at any given time point [269]. Noteworthy, the remaining ability or the complete loss of migration of Trm cells might influence the final localization inside their tissue niche. For example, in skin it has been shown that CD8+ Trm cells localize in the epidermis at the basal membrane while CD4+ Trm cells can be found in the dermis which is more accessible that the epidermal compartment [270]. Preferential regionalization of two Trm cell subsets has also been found in the intestine, lung and FRT [19].

15.2 Tissue-resident regulatory T cells

As mentioned above Foxp3-expressing Treg cells are key mediators in establishing and maintaining immune homeostasis. In the blood they have been demonstrated to be a heterologous population of resting Treg cells displaying a "naïve" phenotype, activated Treg cells displaying a phenotype associated with Tm cells, and Foxp3+ T cells which lack suppressive functions but instead secrete effector cytokines [271]. Within peripheral tissues, Treg cells appear to represent a significant fraction of CD4+ Trm cells since for example, in human skin it has been shown that approx. 20% of CD4+ T cells in adults express Foxp3. Moreover, almost all of them have previously seen Ag, as indicated by their expression of CD45RO and their lack of expression of the chemokine receptor CCR7, which prevents them from reentering the circulation [269]. By using a mouse model of inducible cutaneous self-Ag it has been demonstrated that Treg cells with an effector memory-like phenotype are, similarly to Trm cells, accumulating progressively and persist for long periods in the skin where they most likely fulfill effective suppression of Trm cells [272].

15.3 Tissue-resident non-conventional T cells

Several non-conventional or innate like T cells such as the TCRαβ expressing NKT cells or TCRγδ T cells have been found to be tissue resident. NKT cells play a role in tumor surveillance and controlling microbial infections and they have been described in several tissues such as liver and lung [12], [273]. NKT cells express integrins necessary for tissue retention and they express CD69, which is associated with the Trm cell phenotype. TCRγδ T cells are prominent within barrier tissues. They are especially enriched among the intraepithelial lymphocytes (IELs) that reside in the intestine. Also, TCRγδ T cell can be found in the epidermis as dendritic epidermal T cells (DETCs) which exhibit limited TCR diversity and arise from fetal thymic precursors that seed the intraepithelial tissue early during development [274], [275]. Both, IELs and DETCs express CD69 and CD103, they are maintained in the tissue without further help from circulating cells and have been show to be involved in both tissue homeostasis and immune responses [276], [277].

15.4 Tissue-resident innate lymphoid cells

ILCs are a diverse family of lymphocytes, which are involved in various functions including immune cell recruitment, lymphoid tissue organization, tissue homeostasis, tumor surveillance and anti-microbial immunity. ILCs can be subdivided based on their differential

expression of specific TFs and production of different effector cytokines into types 1 including tissue-resident natural killer (NK) cells, 2, and 3 including lymphoid tissue inducer cells (LTi) (ILC1, ILC2, and ILC3) [278]. Long-term parabiosis experiments have demonstrated their tissue-residency inside non-lymphoid tissues and SLOs, without help from circulating cells [279].

The summarized findings above depict only the tip of the iceberg from what is already known regarding tissue-resident T lymphocytes.
Since my research concentrates on CD8+ and CD4+ Trm cells in the skin, I will focus on these two populations only and discuss in the following part their development and tissue retention, their activation and function, their transcriptional signature, and their regulation.

16 Differentiation and maintenance of Trm cells

The molecular mechanisms that are required to control Trm cell development are just beginning to be determined. However, Trm cell development involves several checkpoints, namely entry into non-lymphoid tissues, local retention by prevention of tissue egress, and subsequent responsiveness to locally-driven signals that support Trm cell differentiation and maintenance.

16.1 Tissue entry of activated effector T cells

As described above, naïve T cells are activated to differentiate into Teff cells inside the draining lymph nodes. Furthermore, they are instructed to express tissue-homing molecules and that depend on the location and tissue-association of the draining lymph, these homing molecules are quite different [280], [281]. However, not only T cell activation inside the draining lymph node but also signals derived from the target tissue can induce the expression of homing molecules on activated T cells [282], [283]. Tissue-homing is mainly facilitated by several mechanisms: integrin-ligand interactions, chemokine attraction, and response to released cytokines. These signaling events are crucial for certain cell activities such as cell-cell interactions, cell migration, proliferation, survival, and gene expression [284], [285]. Together, the induced signaling pathways orchestrate optimal tissue-homing of activated T cells.

Specialized homing mechanisms are thought to exist for each individual tissue but until now the processes directing activated cells into the gut tissue and the skin are the best described.
T cell priming inside the gut the draining lymph node especially promotes the expression of

α4β7 integrin on Teff cells [286]. α4β7 integrin is the receptor of mucosal vascular addressin cell adhesion molecule (MAdCAM)-1 that is expressed on post-capillary venules in the lamina propria [287]. Once the activated T cells are recruited to the gut tissue, the production of the cytokine IL-21 by CD4+ Th cells and NK aids the distribution of activated CD8+ T cells since it is associated with the increased levels of α4β7 integrin on Teff cells [288]. Nonetheless, α4β7 integrin expression also strongly depends on the tissue environment which influences its up- or down-regulation in response to the same cytokine. While in the gut TGF-β signaling induces enhanced expression and thereby stronger tissue retention, inside secondary lymphoid organs it dampens α4β7 integrin expression and thereby prevents migration of CD8+ Teff cells to the intestine [289]. However, after settling inside intestinal mucosa, Teff cells down-regulate α4β7 integrin and differentiate in response to further tissue derived signals into long-lived CD8+ Trm cells that remain resident without recirculation throughout the organism [248]. Another important molecule for gut tissue entry and retention is chemokine receptor CCR9. Similar to α4β7 integrin it is expressed after the activation of naïve T cells in gut-draining lymph nodes. CCR9 binds to the ligand CCL25 that is produced by the intestinal epithelium and thereby facilitates Teff cell settling inside the gut tissue [290].

In order to permit cutaneous accumulation of Teff cells, the skin-draining lymph node induces the expression of the P-selectin ligand CLA on activated CD8+ T lymphocytes [291]. The P- and E-selectines are highly up-regulated in skin after virus infection and the expression of both P-selectin and E-selectin ligands is required for CD8+ T lymphocyte recruitment into the skin [20]. Selectin-ligand interactions coordinate the extravasation of lymphocytes from the blood stream into the tissue [292]. In experimental mouse model of both epidermal and mucosal recruitment of CD8+ Teff cells it has been demonstrated that the action of the CXCR3 ligands CXCL9 and CXCL10 is involved to efficiently guide the cells into the tissues since less Trm cells formed from transferred CXCR3-deficient precursor cells [293], [252].
In addition to signals derived from the draining lymph node, after skin infection Teff cells are also instructed to express skin-homing molecules in response to skin-derived signals. In 2015 It has been shown in both mouse and humans that soluble keratinocyte-derived vitamin D metabolites induce the expression of CCR8 and CCR10 [282], [294]. Interactions of CCR8 with the respective ligand CCL1 expressed in the epidermis control efficient Teff cell seeding of the skin [282]. Furthermore, during inflammatory conditions, the interactions between the chemokine receptor CCR10 of activated T lymphocytes with the ligand CCL27 which is also

expressed in the epidermis regulates cutaneous T cell migration [295], [296]. In line with these findings a recent study demonstrated that CCR10 indeed is necessary for Trm cell formation but the lack of CCR10 does not influence the migration [297].

In the lung it was shown that the collagen binding α1β1 integrin (Very Late Ag (VLA)) which is expressed by Ag-specific CD8+ T lymphocytes after influenza infection mediates the retention of protective Teff cells in the lung [298]. However, a recent study employing a tuberculosis infection model in mice demonstrated that VLA is not involved in the trafficking of Teff cells to the lung but it plays an important role regarding the regulation of T cell contraction during the transition into the memory phase of the immune response [299].

In non-barrier tissues such as the kidney, TGF-β signaling is required for trans-endothelial migration of CD8+ Teff cells. Similarly to mechanisms in cutaneous cell recruitment, TGF-β induces the expression of E- and P-selectin ligands on Teff cells and thereby facilitates the accumulation of T lymphocytes in the tissue. In addition TGF-β enhances the expression of the inflammatory chemokine receptor CXCR3 that aids the seeding of the tissue by Teff cells via binding to the ligands CXCL9 and CXCL10 [255].

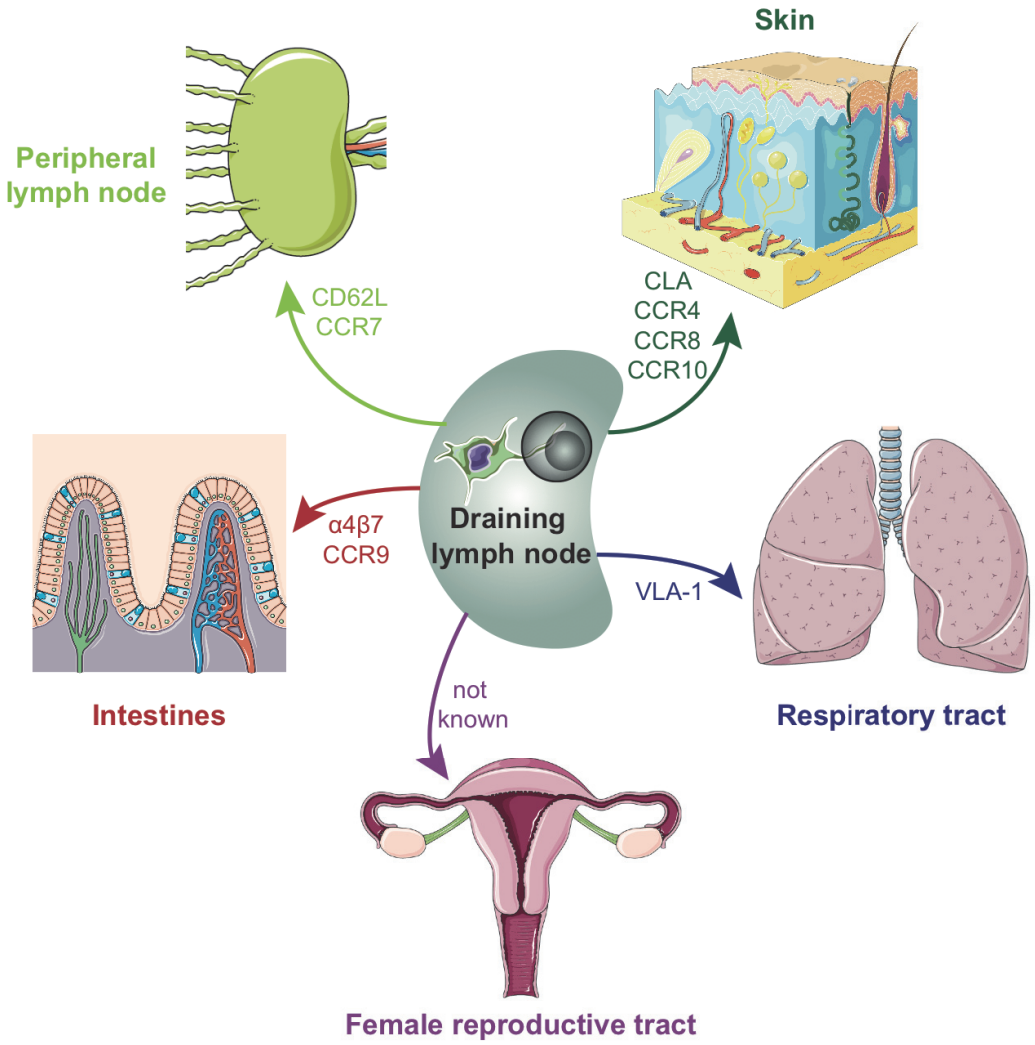


FIGURE 14: TISSUE HOMING OF ACTIVATED T CELLS.
After the activation of naïve T cells in the draining lymph node by APCs, they are equipped with different homing molecules, which permit them to enter their respective tissues. Adapted from Mueller et al., 2016, and Park et al., 2015.

16.2 Prevention of tissue egress

Once homed inside the tissue, Teff cells fulfill their function by eliminating the Ag. Afterwards, most of them undergo cell death during the contraction phase [14]. However, it is now clear whether a small fraction of the Teff cells remains inside the tissue and differentiates into Trm cells after the Ag is eliminated [300] or only small amounts of Ag persist (our own observation). In order to be retained inside the tissue, egress has to be prevented by the up- and down-regulation of certain molecules.

CD69, a membrane-bound type II C-lectin receptor, and CD103, the α-chain of the E-cadherin binding $\alpha_E\beta_7$ integrin, are the best described markers of Trm cells. Both CD69 and CD103 are associated with tissue retention of T lymphocytes after Ag elimination but while CD69 appears to play an important role in all tissues, the role of CD103 is rather tissue depended and less clear.

CD69 is classically associated with recent T lymphocyte activation due to rapid surface expression after their stimulation [301]. However, most of the cells located inside peripheral tissues have not been activated recently but still express CD69. Therefore, it has been suggested that CD69 is involved in tissue retention of the cells. Several studies demonstrated that CD69 expression is regulated at several levels after the activation of naïve T lymphocytes and the subsequent Trm cell development [302]. Also, CD69 expression is associated with both CD4+ and CD8+ Trm cells.

CD69 expression is regulated via Kruppel-like factor (KLF)-2, a TF that is down-regulated upon primary T lymphocyte activation or following non-lymphoid tissue entry in a TGF-β, IL-33, and IFN-α-dependent manner [303], [304]. The down-regulation of KLF-2 results in the up-regulation of CD69 which in turn induces the down-regulation of the G protein-coupled sphingosine 1 phosphate receptor 1 (S1PR1), which normally mediates tissue egress from both lymphoid and non-lymphoid tissues [305]. By down-regulating S1PR1, CD69 mediates retention of recently activated T lymphocytes in the lymph node in order to facilitate proper differentiation. Following that step, KLF-2 is up-regulated again, which induces CD69 down-regulation and subsequent S1PR1 up-regulation and allows cells to migrate from the lymph node into peripheral tissues according to the homing molecules. Once inside the tissue, CD69 and S1PR1 are up- and down-regulated again, respectively, and tissue egress is prevented [304], [306].

While CD69 plays a key role in the prevention of Trm cell tissue egress in all described tissues, CD103, the other well described marker of Trm cells, seems to play a less important role in Trm retention. CD103 is predominantly expressed on CD8+ Trm cells compared to CD4+ Trm cells and it is a ligand of E-cadherin an adhesion molecule expressed by epithelial cells [307]. Experiments using CD103-deficient mouse models demonstrated that CD103 expression is not required for Teff cell tissue entry. However, in some peripheral tissues such as the skin, CD103 expression is required for Trm cell maintenance since mice transferred with CD103-deficient CD8+ T lymphocytes before skin infection showed a reduced Trm compartment [293]. Nonetheless, in human skin it has been demonstrated that both CD103+ and CD103- Trm cells exist. While CD103+ expression on both CD4+ and CD8+ Trm cells showed a stronger association with epidermal localization, CD103- Trm cells were mainly found in the dermis [308]. A study investigating the mobility of CD8+ skin Trm cells demonstrated that CD103- Trm cells have a higher mobility than CD103+, indicating a contribution of CD103 to their adhesion [297].

Another tissue that shows different CD103 expression depending on the compartment in which the Trm cells are localized is the gut tissue. Similar to the skin, CD103 expression is needed for Trm cell maintenance inside the IEL population but it is dispensable for Trm cell in the lamina propria [309]. In addition, in some tissues such as the salivary gland CD103 expression is completely dispensable for the maintenance of Trm cells [310].

Similarly to certain peripheral tissues such as the gut and the skin, a population of CD69+CD103- Trm cells can also be found in the thymus, in addition to the CD69+CD103+ Trm cells. However, unlike in non-lymphoid organs these CD69+CD103- Trm cells displayed a profile that was rather associated with circulating Tcm cells and they were also eliminated from the thymus after mAb-mediated depletion indicating their probable localization within the blood stream [258].

The theory regarding the functional role of CD103 to potentially bind to its ligand E-cadherin is flawed by the fact that certain Trm cell populations express CD103 in tissues, which do not express E-cadherin. For instance, CD8+ brain Trm cells express CD103 [253] while the central nervous system (CNS) expresses E-cadherin only transiently during development [311]. Either CD103 has another unidentified ligand or instead of equipping Trm cells with functional requirements for tissue residence, CD103 might rather be a marker of terminal differentiation. In line with this argument are data demonstrating that CD103+ Trm cells have higher capacity to produce effector cytokines but show less proliferative potential than their CD103- counterparts in both mouse and humans [253], [312], [313].

Another important regulator of tissue egress is the chemokine receptor CCR7 which directs T lymphocytes and DCs toward lymph nodes [314]. It is expressed on Tcm cells and a special population of CD4+ Tm cells that has been demonstrated to reenter circulation from the skin and migrate towards the draining lymph node, the so called recirculating memory T cells (Trcm) [315]. Employing a model of human skin grafts on mice it has been shown that only CD69+CD103+ or CD69+CD103- Trm cells that also lacked CCR7 expression remained in the skin and did not belong to the circulating pool of Trcm cells [246].

16.3 Response to tissue-derived signals

The regulation of Trm cell markers such as CD69 and CD103 depends on the response of tissue-homed activated T lymphocytes to tissue-derived signals and cytokines. One of the key signal in Trm cell development is TGF-β which is expresses in its latent form by many tissues. In order to be activated, latent TGF-β has to undergo proteolytic cleavage, interactions with integrins or pH changes in the local environment and this will induce the conformational changes needed for receptor binding [316], [317]. Several studies demonstrated that TGF-β is required for the formation of Trm cells since it regulates the expression of CD69 and CD103 [309], [293], [304]. During infection, Ag-specific CD8+ T lymphocytes from the gut have been shown to enhance their TGF-βRII expression. Therefore they can receive stronger signals after TGF-β activation and binding, which leads to the up-regulation of CD69 and CD103. Conversely, defects in the TGF-βRII expression inhibited optimal CD69 and CD103 expression and lead to decreased retention of Trm cells in the gut [289]. Another study shows that in the gut a CD69+CD103- Trm cell population is generated independently of TGF-β signaling, indicating that redundant mechanism might exist which regulates the expression of CD69 [318]. In line with these findings, it has been reported that the effect of TGF-β on the up-regulation of CD103 on gut Trm cells is inhibited by IFN-γ and IL-12 resulting in the formation of CD69+CD103- Trm cells within the lamina propria. Also, in the presence of these cytokines in addition to TGF-β CD69 expression was increased [319]. In addition to responses to the cytokine milieu, CD69+CD103- Trm cells have been demonstrated to develop in clusters, in which CD4+ T lymphocytes but also CX3CR1+ macrophages and DCs are found, which might be Ag-specific as well or present Ag to the T lymphocytes, respectively. Moreover, CXCR3-driven T lymphocyte recruitment was critical for CD69+CD103- Trm cell development and subsequent pathogen clearance [318].

In the brain, TGF-β derived from Treg cells results in increased expression of CD103 in CD8+ T cells, which allows Trm cells to be maintained in large quantity after viral infection. However, in the brain it is not known if Ag-specific Teff cells also up-regulate TGF-βRII in order to be more receptive to TGF-β signaling [254].

In the skin TGF-β is activated by keratinocytes via the expression of $\alpha_v\beta_6$ and $\alpha_v\beta_8$ integrins. By employing TGF-βRII-deficient mice or an UV treatment mouse model which leads to reduced $\alpha_v\beta_6$ and $\alpha_v\beta_8$ integrin expression and subsequently reduced activation of TGF-β, it has been shown that TGF-β is required for maintenance of CD8+ Trm cells as well as other skin resident cells such as LCs [293], [320].

Moreover, TGF-β in combination with TNF-α and IL-33 leads to decreased KLF-2 expression in *ex vivo* cultures of activated CD8+ T lymphocytes. Similarly, the combination of IL-12 an IL-18 decreased the KLF-2 expression which is important for increased CD69 expression [304].

While the role of TGF-β signaling is well describe in the expression and thereby in the maintenance of the CD69+CD103+ Trm cell phenotype, the role of other cytokines remains less clear. For example, the role of IL-15 appears to depend on the tissue in which the Trm cells are establishing. In the skin IL-15 expression in combination with IL-7 is needed for CD103 expression and thereby for the maintenance of Trm cells [293], [321]. Conversely, IL-15 is dispensable in the mucosa of the FRT and the gut as there was no reduction of CD103 expression observed in the absence of IL-15. [322].

Moreover, IL-15 deficiency lead to increased number of Trm-like cells inside the lymphoid organs. It seems to be attractive to speculate here that the overrepresentation of Trm cells in lymphoid organs of IL-15 deficient mice might be due to the fact that IL-15 is needed to establish Trm cells in certain non-lymphoid tissues since it is needed for the up-regulation of CD103 in the skin as mentioned above [321]. In the absence of IL-15, Teff cells that do not settle inside the peripheral organs but have received signals for Tm cell differentiation could migrate into the lymph nodes. Conversely, another study revealed that IL-15 was expressed within tonsils on regions where CD8+ Trm cells localized, indicating a certain heterogeneity regarding the required signals for tissue-residency between different secondary lymphoid organs [323].

In both the gut and the mucosa of the FRT the development of CD8+ Trm cells depends on the mammalian target of rapamycin (mTOR) which is a regulator of cell proliferation, differentiation, and cell survival. mTOR activity can be selectively inhibited by the drug of rapamycin which prevents Trm cell development by disrupting the formation of functional CD8+ Teff cells that seed the tissues prior to Trm cell establishment [324]. In contrast to the findings that IL-15 signaling is dispensable for CD103 expression in the gut, a recent study indicates that IL-15 promotes CD8+ Trm cell accumulation by promoting mTOR activation [325].

Often the expression of CD103 on CD8+ Trm cells depends on proper CD4+ T lymphocyte help. In the lung CD4+ Th cells produce IFN-γ which is needed for efficient development of CD8+ Trm cells. In the absence of IFN-γ, CD103 expression was decreased on such "unhelped" lung Trm cells and they were mislocalized within the tissue compared to Trm cells receiving the complete set of signals [326].

After establishment in the tissue, maintenance mechanisms also include the metabolism of Trm cells. In skin and lung CD8+ Trm cells have been shown to depend on the uptake and metabolism of exogenous lipid. Therefore, they express high levels of molecules facilitating the uptake of free fatty acids (FFAs). These molecules are not needed for the survival of circulating Tcm cells. Moreover, deficiency in the uptake of FFAs leads to decreased protective functions of Trm cells [327].

As mentioned above, Trm cells are not the only tissue-resident T lymphocyte population and some factors needed for their maintenance are shared between them in order to guarantee an equilibrium in the restricted tissue niches. For instance in the skin, Trm cells compete with DETCs for local survival signal since the expression of AhR, a TF known to be important for the maintenance of both cell types [328], [250].

16.4 The role of Ag in the development and maintenance of Trm cells

The development and mainly the maintenance of Trm cells are also strongly influenced by the presence and persistence of Ag. It is interesting to speculate that need for the Ag might depend on the used model as well as on the accessibility of a certain tissue. While in some tissues such as the brain [329] the local Ag is obligatory for the development, maintenance and later effector function of Trm cells, in other tissue such as the skin local Ag is dispensable for

Trm cell lodgment [330]. Also it has been described that local Ag is dispensable and indispensable within the same tissue since e.g. in the salivary gland generation of CD4+ but not CD8+ Trm cells depends on it [268].

In the brain, Trm cell development and effector function not only depend on the presence but also on stimulatory potential of the Ag. By using a brain infection in mice employing different variant with different stimulatory potential of the same virus, it has been shown that even weak responses were sufficient to induce Teff cell seeding of the brain. Moreover, the lower the stimulation was the greater was the functionality of Trm cells [329]. However, upon re-infection CD8+ Trm cell function depends on the Ag-presentation on MCH class I molecules since bystander activation or expression of antiviral genes in surrounding tissues is not sufficient to mediate viral clearance during an infection with a genetically engineered lymphocytic choriomeningitis virus LCMV variant in which MHC class I recognition is prevented [331]. Interestingly, by using an adhesion frequency assay it has been shown that Trm cells have TCRs with higher affinity for MHC class I molecules than circulating Tm cells. Additionally, over time CD8+ Trm cells in the brain show progressively increased MHC class I molecule binding and CD8 co-receptor expression indicated by increased tetramer binding. The high affinity for MHC class I molecules equips CD8+ Trm cells ideally to detect invading pathogens [332].

In the lung Trm cell activation is initiated in both the presence and absence of local Ag. However, if cognate Ag cannot be detected other requirements must be met. For instance, IFN-γ signaling is needed to induce potential cytolytic activity indicated by expression of granzyme (Gzm) B [333]. Moreover, if cognate Ag is present it enables the conversion of recruited Tem cells during re-call responses into Trm cells by creating tissue damage associated new niches inside the lung [245]. Interestingly, the number of virus-specific CD8+ Trm cells in the lung can be increased not only by re-challenge with their cognate Ag but also by heterologous virus infections an thereby leading to increased protection [334].

In the skin as well as in the mucosa of the FRT, local Ag-recognition is dispensable since Ag-specific Trm cell develop as in a skin inflammation-dependent manner and show long-term persistence [335], [252]. However, local presence of the Ag resulted in higher density of skin Trm cells [335], [330]. Moreover, repeated skin infection or induced inflammation leads to progressive accumulation of protective Trm cells in the skin even in non-involved areas. Nonetheless, active recruitment of Ag-specific Teff cells by local Ag results in superior protection against

infection since the developing Trm cells are more potent stimulators of inflammatory responses [20], [336]. In addition to the presence of Ag in the skin, it has been shown in a recombinant virus vector model that co-expression of Ag by infected cells leads to Ag-dependent competition between CD8+ T cells of different specificity, which in the end shapes the local pool of Trm cells. Moreover, Trm cell development occurred undisturbed when instead of being co-expressed, the different Ags where expressed on distinct cells [337].

Aside from the local presence, the duration of Ag persistence also influences the characteristics of the Trm cell population. Similarly to the skin, Teff cell lodgment also occurs in the gut, even in the absence of cognate Ag. However, while acute virus infection promotes the canonical phenotype of intestinal lymphocytes, which includes the up-regulation of CD103, prolonged Ag persistence during a chronic virus infection prevents the up-regulation of CD103 indicating a phenotype rather associated with Tcm than Trm cells [309]. In several mucosal sites it has been observed that chronic inflammation leads to a shift within the immunodominance of the mucosal Trm cell population as they are dynamically maintained by circulating Tm cells that develop into Trm cells throughout the persistent infection [338].

Taken together, these data suggest that in some but not all tissues the presence of Ag is needed neither for the lodgment nor for the maintenance of Trm cells even though they bear markers that are associated with recent activation by Ag-recognition [301]. In the tissues where the Ag is dispensable, the cytokine milieu is thought to induce the canonical phenotype of Trm cells. This diversity might reflect some needs of the different tissues discussed above. While some tissues are more likely to encounter and re-encounter Ag such as the skin, it is important to establish an effective defense network even in the absence of local Ag. Other non-barrier tissues such as the brain might be more protected due to their location. Therefore, in these site-protective tissues the developmental pathway and activation for Trm cells is only engaged when the necessity is indicated e.g. by the local recognition of Ag. The need of local Ag-presence either for the development or for the activation of Trm cells might also be considered for vaccine approaches which aim to increase the Ag-specific Trm cells in certain areas of the body such as the lung or the mucosa of the FRT.

17 The pilikia of becoming a Trm cell: Factors influencing Trm cell development

Aside from their capacity to enter peripheral tissues and respond to tissue-derived signals and local Ag in order to develop into Trm cells, other factors influencing T cell homing and subsequent Trm cell development have been reported.

For instance, not only the location of the draining lymph node but also the time of arrival of the naïve T cells inside the lymph node and thereby their time of contact with the presented Ag by DCs plays a role on the developmental fate of Tm cells. It has been shown that naïve CD8+ T lymphocytes that arrived late in the lymph nodes compared to T lymphocytes that were already present or arriving immediately after the arrival of APCs were more likely to express CD62L and to be recruited to other secondary lymphoid organs from where they enter the Tcm cell pool [339]. Similar results were found for CD4+ T lymphocytes entering the skin draining lymph node late after Ag contact [340].

In addition, two studies suggest that the TCR signal strength during Ag-presentation influences the effector-to-memory transition and cross-presentation of the Ag influences the Trm cell development [337], [309]. In line with these findings, [341] show that optimal differentiation of Trm cells depends on their activation by DNGR-1+ DCs inside the draining lymph node. DNGR-1+ is a C-type lectin receptor expressed by DCs that favors cross-presentation of Ag. In a mouse model of viral skin infection they demonstrated that defective cross-presentation due DNGR-1-deficiency resulted in impaired Trm cell responses.

Another key point in Trm cell development is phenotype of the Trm-precursor cell. For example, it has been shown that activated T lymphocytes with low expression of the killer cell lectin-like receptor subfamily G member 1 (KLRG1) give rise to either Tcm or Trm cells but not Tem cells [293]. The transcriptional signature of Trm cells will be discussed in more detail below. However, in line with this finding Trm and Tcm cells have been demonstrated to be of the same clonal origin. Moreover, Ag-specific Trm cell populations inside peripheral tissues are mirrored by a circulating pool with an identical TCR repertoire [24]. However, the decisive factors about the final fate of these common pre-cursor cells remain to be determined.

18 The transcriptional signature of Trm cells

Trm cells are distinct from their circulating counterparts on many levels such as migration pattern, phenotype and effector functions. Also, they have to meet certain requirements in order to enter peripheral tissues and to respond to tissue derived signals for their optimal development and maintenance. Moreover, as discussed above, these requirements can be very distinct in different tissues. Recent transcriptomic analysis of Trm cells from various tissues showed that their molecular signature is different from their circulating counterparts. Mainly these differences affect a variety of expressed genes that are associated with tissue retention after entry as well as differentiation into Trm cells, Trm cell maintenance and metabolism. However, differences in the transcriptome are not only found between Trm cells and circulating Tm cells, they can also be detected between Trm cells isolated from different tissues reflecting their ability to respond to local tissue environments [262].

18.1 Regulation of Trm cell differentiation

18.1.1 KLRG-1, Blimp-1, and Hobit

Already at the stage of activated Teff cells, differences in gene expression determine the fate of the cells. In the skin, CD69+CD103+ Trm cells exclusively develop from precursor cells that express low levels of KLRG-1 at very early time points after viral infections [293]. Two other central regulators of Trm cell development are B lymphocyte-induced maturation protein (Blimp) -1 and the homolog of Blimp-1 in T cells (Hobit). They are up-regulated in CD8+ Trm cells of the skin, IEL population, lamina propria, kidney, and liver [342], [343]. Moreover, they are also important for the development of tissue-resident and non-resident NKT cells of the liver [344]. While Hobit is indispensable for Trm cell development, which was demonstrated by reduced Trm cells in the skin after intradermal transfer of Hobit-deficient CD8+ cells, Blimp-1 was not essential for skin Trm cell formation by itself but its presence is beneficial as more Trm cell developed. Combined loss of both Hobit and Blimp1 leads to the expression of several genes, which are usually down-regulated during Trm cell development and that are related to the ability to exit peripheral tissues and to home back to lymph nodes (LNs) such as Klf2 and its target gene S1PR1 or CCR7 [342]. Hobit and Blimp-1 are expressed at different stages of the Trm cell development. While Hobit is expressed early after T cell activation in a T-box protein expressed in T cells (T-bet) independent manner and in a IL-2 and IL-12 prone cytokine milieu, T-bet expression is driven after tissue entry in a T-bet dependent manner by IL-15 [342].

18.1.2 T-bet and Eomes

Two other TFs which are key for the development of CD8+ Trm cells are T-bet and Eomesodermin (Eomes), two molecules of the T-box factor family [345], [346]. Their combined down-regulation controls the CD8+CD103+ Trm cell development and forced up-regulation results in decreased Trm cell formation. Down-regulation of T-bet and Eomes occurs in a TGF-β-dependent manner and the complete down-regulation of Eomes and partial down-regulation of T-bet enforces enhanced TGF-β responsiveness of the cells which creates a feedback loop allowing Trm cell development [293], [253]. Residual expression of T-bet also maintained IL-15R expression on Trm cells and thereby their IL-15 responsiveness. Both IL-15 and TGF-β signaling are needed for proper Trm cell development and their long-term survival e.g. by the expression of CD103 [347], [326]. Noteworthy, T-bet and Eomes are not expressed on CD103+ lung Trm cells. Their regulation of responses to tissue-derived signals might be compensated by other TFs such as Basic Leucine Zipper ATF-like TF (BATF) expressed on lung Trm cells [348].

18.2 Regulation of Trm cell maintenance

18.2.1 Notch

A key regulator of Trm cells maintenance is the TF Notch. It has been demonstrated that Notch controlled the maximal expression of CD103 in CD8+ lung Trm cells, which might help the Trm cells to anchor in the epithelia [348]. However, most of the other expression programs on Trm cells associated with adhesion molecules were shown to be independent of Notch.

18.2.2 Ahr

As mentioned above, Trm cells express AhR, a TF important for their survival but not their development. Employing a mouse CHS model using DNFB, it has been demonstrated that both AhR+ and AhR- CD8+ T lymphocytes enter the skin and differentiate into Trm cells but AhR-deficient cells are not maintained [250]. Also in the gut and in the liver Trm cells depend on AhR-ligand interactions for their maintenance [349], [350].

18.3 Regulation of Trm cell metabolism

As mentioned above, genes associated with cell metabolism can also show different expression between Tem, Tcm, and Trm cells. A recent study showed that skin Trm cells

express high levels of molecules facilitating the uptake of FFAs (FABP4 and FABP5). These molecules are needed for the long-term survival of Trm but not Tcm cells. The study also demonstrated that Trm cells but not circulating Tm cells showed increased mitochondrial activity in the presence of FFAs, which is most likely due to their increased expression of FABP4 and FABP5. Deficiency in these molecules leads to decreased Trm cells which are less effective at protecting mice from cutaneous viral re-infection [327].

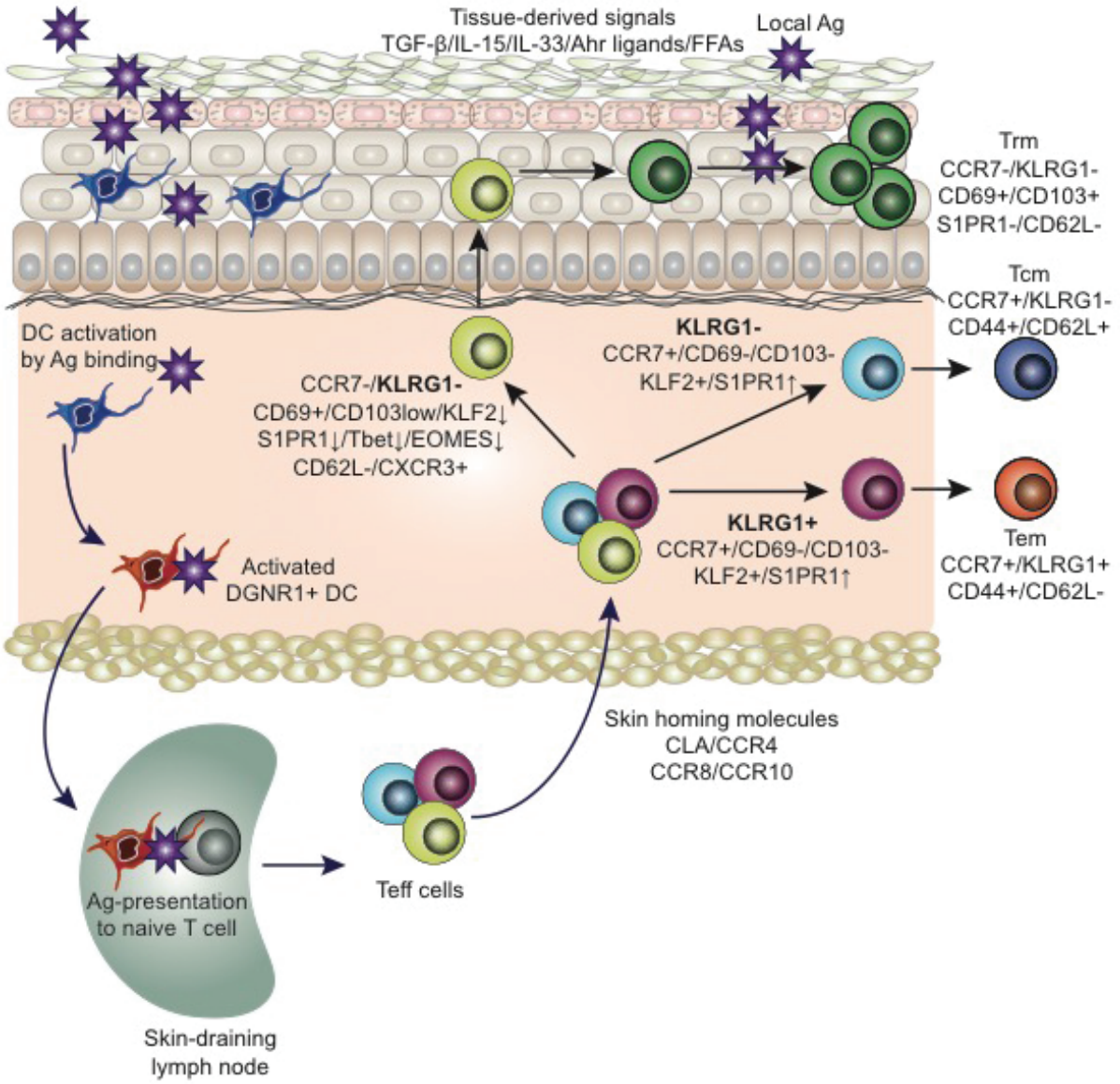


FIGURE 15: DEVELOPMENTAL PATHWAY OF SKIN TRM CELLS.

After skin penetration, Ag is engulfed by skin resident DNGR1+ DCs and transported to the skin draining lymph node where it is presented to naïve T cells. After T cell activation, Teff cells are recruited to the skin via their homing molecules. Following tissue entry further differentiation into Trm, Tcm, and Tem cells occurs depending on the phenotype of the precursor cells and their ability to respond to tissue derived signals e.g. TGF-β, IL-15, IL-33, and Ahr ligands. Local recognition is dispensable for the acquisition of Trm phenotype but its presence leads to increased Trm cell numbers in the skin.

Adapted from Mackay&Kallies, 2017, and Mueller&Mackay, 2016.

19 Re-activation and protective functions of Trm cells

As mentioned above, Trm cells can be distinguished from their circulating counterparts by their functional properties. While Tcm cells have a high proliferative potential they have weak effector function and low cytotoxicity. Conversely, Tem cells are strong producers of cytokines and have high cytotoxic but low proliferative potential [243]. Upon re-exposure to pathogens, circulating Tm cells are re-stimulated inside the secondary lymphoid organs. Here they proliferate (mainly Tcm cells) to generate secondary Teff cells that will migrate to the infected tissue. While this generation of secondary Teff cells is much faster than the primary generated Teff cells it can still take several days [15]. Tem cells can be recruited to infected tissue within hours. However, even the delay in Ag-detection and the arrival of Tem cells in the infected tissue can lead to spreading of the Ag which might be detrimental for the host [351]. Trm cells on the other side have been described to play crucial roles in the protection against infection and cancer since they are ideally positioned inside the barrier tissues to survey epithelial interfaces and to mount rapid immune responses upon re-infection. Thereby they provide the host with enhanced regional protection by having strong effector functions and high cytotoxicity [18], [19], [20]. Even though they respond more rapidly to re-encountered Ag than cells recruited from the circulation, their mode of action has not been clearly demonstrated yet and might even differ between certain tissues. More and more studies indicate that Trm cells fulfill actions of both the innate and adaptive immune response and thereby build a link between these to parts of the immune response [352].

Several studies demonstrated that Ag-recognition by Trm cells triggers a state of tissue-wide pathogen alert of the skin as well as the mucosa of the FRT [251], [352], [353]. They showed in two different models of viral infections that CD8+ Trm cell activation altered the gene expression profile on the skin and induced fast and robust cytokine secretion, which triggered both adaptive and innate responses including local humoral responses, maturation of local DCs, and activation of NK cells. Activated CD8+ Trm cells orchestrate the recruitment of Tm cells from outside the tissue in an IFN-γ-dependent manner.

Also in viral models, it has been shown that after skin inflammation both CD8+ Trm and Tcm cells are developing. However, Trm cells in the skin provide superior protection over Tcm cells by producing multiple cytokines such as IFN-γ, IL-2, TNF-α [246] even though Tcm cells can "catch up" and induce a delayed response in the absence of Trm cells [24]. Moreover, the protection is not just limited to the infection site since Trm cells are generated throughout the whole skin especially after multiple contacts with the Ag [20], [330], [336].

In addition, studies demonstrated that CD4+ Trm cells in the skin enhance the protection against Leishmania major, the parasite which causes cutaneous leishmaniasis, a lesional skin infection [354], [355]. Similar to their CD8+ Trm cells counterparts in the skin, Ag-specific CD4+ Trm cells produce IFN-γ and function to recruit circulating Tm cells to the skin in a CXCR3-dependent manner which leads to enhanced control of the parasites [355]. However, this protection is also mediated alone by CD4+ Trm cells without the help of Tm cells but it requires the recruitment and activation of inflammatory monocytes [354]. In the skin, CD4+ Trm cells generated in a *Candida albicans* (*C. albicans*) models are activated to proliferate and to produce IFN-γ and IL-17 by LCs in the presence of the Ag. In steady state, LCs only promote proliferation of skin resident Treg cells [356]. Also other professional APCs reside in the skin aside from LCs, which are orchestrating the response of Trm cells upon Ag re-encounter: dermal Langerin (Lang)+ DCs and dermal Lang- DCs. For the induction of CD8+ Trm cell mediated responses in virus infection Lang+ DCs but not LCs are indispensable. [357]. Interestingly, employing a virus infection model of skin it has been demonstrated that APCs accumulate similarly to Trm cells at the site of resolved inflammation and that they persist for prolonged periods of time. The presence of the persisting APCs can influence the effector functions of skin Trm cells, e.g. it promotes the IFN-γ production of Ag-specific CD4+ Trm cells upon viral re-challenge [358].

In the lung, Trm cells are also mainly associated with protective functions, since they are mediating enhanced viral clearance and survival to lethal influenza infections [266], [359]. Here, the compartmentalization of CD4+ Trm cells inside the lung is a major factor for immune protection, since circulating influenza-specific CD4+ Tm cells from the spleen failed to protect even though they can migrate to and expand in the lung [266]. Influenza-specific CD4+ Trm cells of the lung have been shown to produce IFN-γ and several other cytokines e.g. TNF-α and IL-2 and thus they are defined as multifunction Th1 cells [359]. Also, some of these CD4+ Trm cells are displaying a highly activated phenotype for several months after the initial infection. They express immediate effector function *in vitro* and produce IFN-γ within hours after re-stimulation [360]. CD8+Trm in the lung were poorly cytolytic upon viral re-challenge. Also, they do not proliferate inside the lung tissue. However, they rapidly produce IFN-γ after Ag encounter and thereby promote an early anti-viral state in the tissue which makes them an important key player in secondary immunity against respiratory viruses [361].

Not only in microbial re-infection Trm cells provide superior protection over circulating Tm cells but they also appear to play a key role in cancer immune surveillance. It has been demonstrated that tumor therapy, which boosts the anti-tumor responses of CTLs are promising and there are even more efficient when the CTLs showed an enrichment for transcripts associated with Trm cells [362]. Another study investigating the efficacy of an intranasal vaccine revealed that it was less efficient after the blockage of TGF-β signaling which as described above leads to decreased numbers of Trm cells [363].

To date, it is not clear whether or not Trm cells have the capacity to efficiently lyse infected cells even though they are able to produce GzmB in several tissues [309], [352], [288]. However, a recent study demonstrated cytolytic capacity of CD8+ Trm cells in the brain upon re-infection, which was perforin- and IFN-γ-mediated but independent of the recruitment of circulating Tm cells [331].

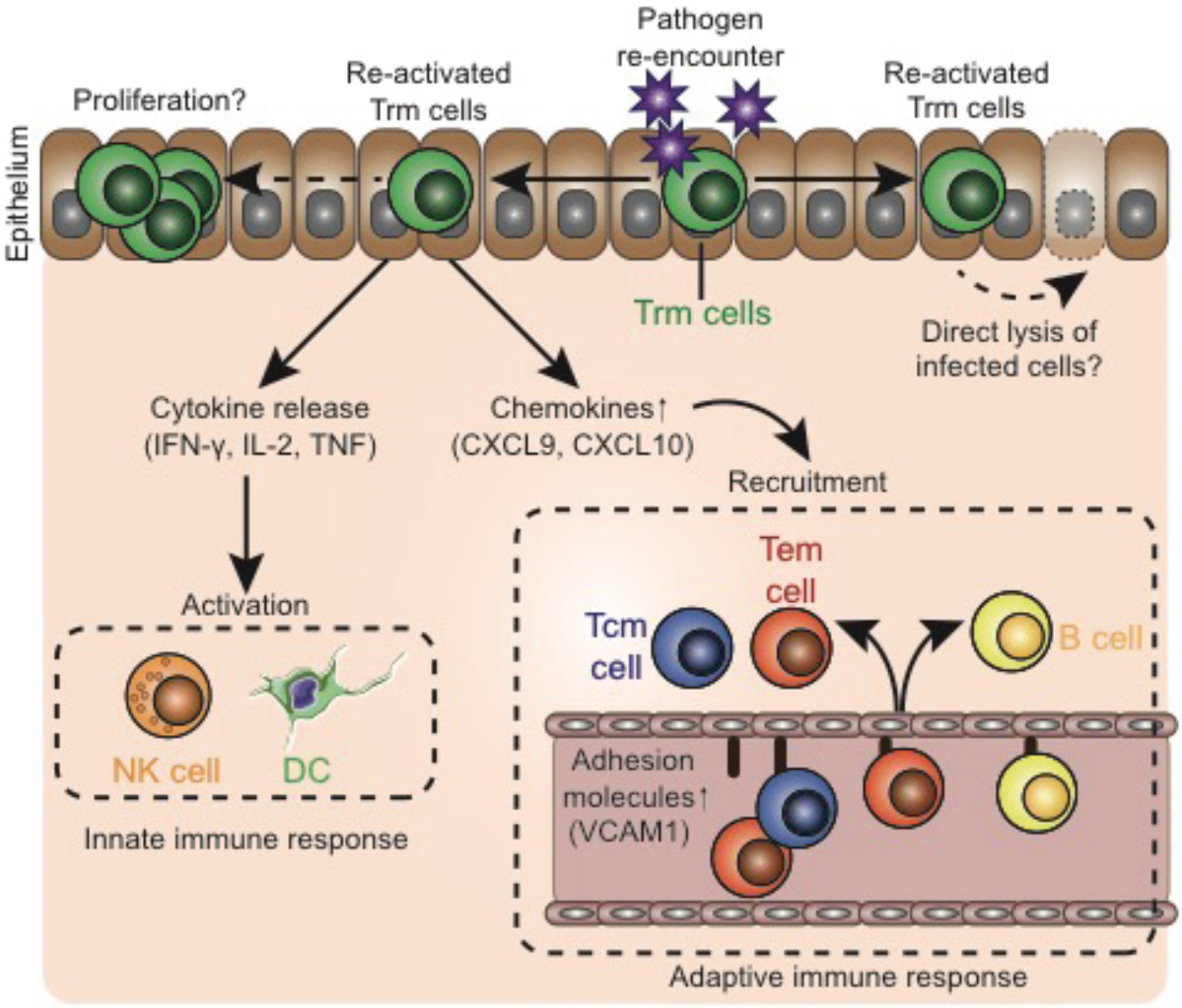


FIGURE 16: MODE OF ACTION OF TRM CELLS.

Trm cells are reactivated upon cognate pathogen recognition and trigger a cascade of innate and adaptive responses at the site of re-exposure. After re-activation chemokine release by Trm cells and adhesion molecules (VCAM1) on endothelial cells are up-regulated which results in the recruitment of Tem, Tcm, and B cells from the circulation and initiation of adaptive immune responses. Cytokine release activates natural killer (NK) cells and DCs. Although, granzyme B and perforin expression by Trm cells has been reported, their capacity to directly lyse infected epithelial cells has not been demonstrated. Similarly, proliferation of Trm cells upon pathogen re-exposure seems likely but formal demonstration is still missing. Adapted from Mueller&Mackay, 2016, and Rosato et al., 2017.

20 Trm cells and immunopathology

In infectious diseases the beneficial effects of Trm cells have been clearly demonstrated as discussed above. They have been shown to be invaluable for the occurrence of secondary immune responses against invading pathogens since they provide the organism with enhanced immune-surveillance and protection against re-infection [17], [18], [19], [20]. However, more recently dysregulated Trm cells have also been associated with detrimental effects in inflammatory and autoimmune diseases of barrier and non-barrier tissues. The clinical characteristics and immunopathology of those diseases reflect the biology and behavior of Trm cells [22], [21], [9].

Certain T cell-mediated chronic skin inflammatory diseases such as ACD, AD, FDE, psoriasis, and cutaneous T-cell lymphoma (CTCL) share the hallmark that skin lesions usually recur at the same anatomical site and for all of them it has been demonstrated that there is a link between their chronicity and the presence of Trm cells [364], [365], [17], [24], [25], [366], [367], [368].

Aside from the skin, also other diseases are associated with Trm development during the pathogenesis. Data show that HDM-specific CD4+ Trm cells in the lung which develop in an IL-2 dependent manner are sufficient to induce asthma without help from circulating Tm cells [369]. Moreover, also diseases of non-barrier tissues such as the multiple sclerosis, a degenerative disease of the central nervous system, or type 1 diabetes, developing after the destruction of pancreatic Beta islet cells, might potentially be induced by Trm cells [22].

Noteworthy, pathogenic Trm cells mediating inflammatory diseases are thought to be generated similarly to protective Trm cells since the Trm cell generation program can also be induced after exposure to allergens or auto-Ags [21].

In view of our own research, below I will discuss the role of Trm cells in immunopathology only in skin inflammatory diseases.

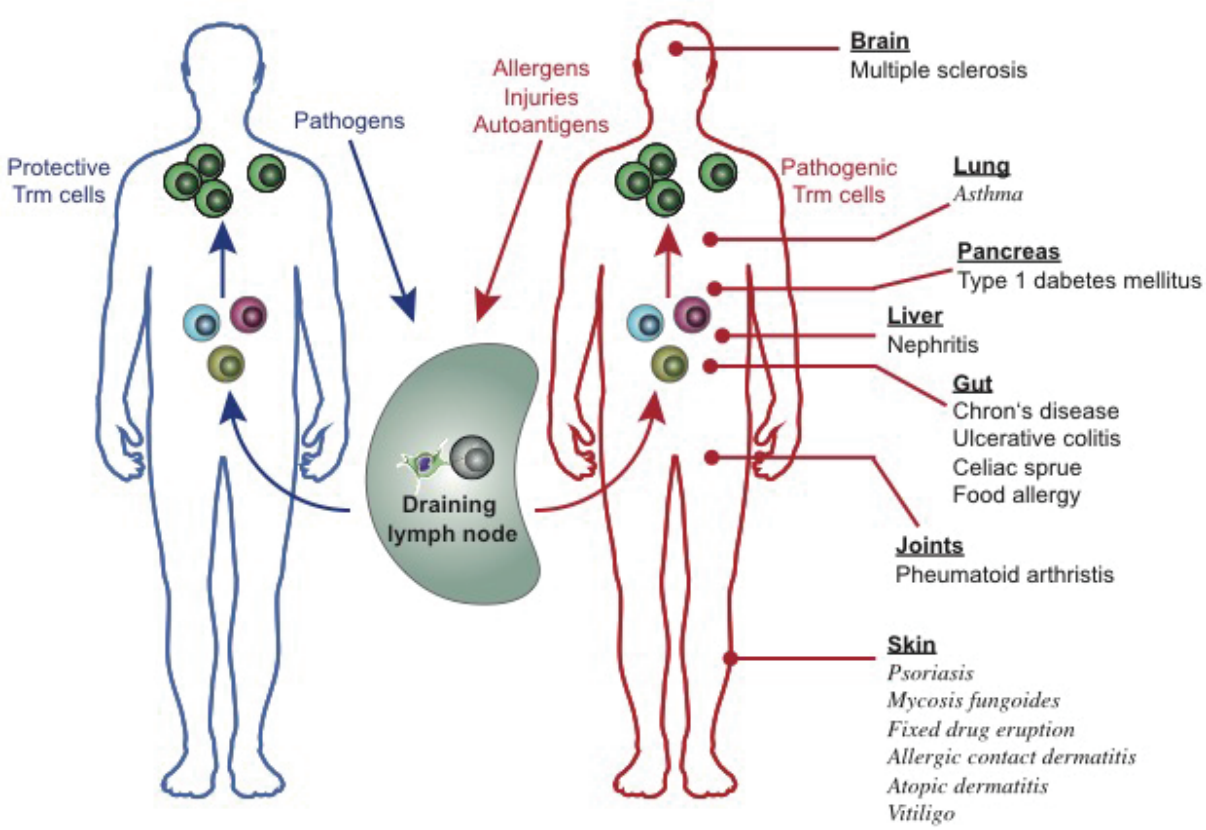


FIGURE 17: PROTECTIVE VERSUS PATHOGENIC TRM CELLS.
Protective and pathogenic Trm cells engage in similar developmental pathways after the exposure of an individual to a pathogen, allergen, or auto-Ag. While protective Trm cells provide the host with more rapid and more efficient adaptive immune responses upon pathogen re-exposure, pathogenic Trm cell are thought to actively contribute to the development of human inflammatory, autoimmune, and allergic disorders. For diseases indicated in italic fond on the figure their contribution has been demonstrated, however, the clinical characteristics of other human diseases suggest a major role for Trm cells in their pathophysiology as well.
Adapted from Clark, 2015, and Park&Kupper, 2015.

20.1 Trm cells in Psoriasis

Psoriasis is a complex inflammatory autoimmune skin disease with characteristic clinical features and histological phenotype. Psoriasis patients develop dry, well-demarcated, bright-red plaques that have thick non-adherent, white scales. Usually these plaques are not itchy. Also, the nails of psoriasis patients can be involved and present pits and spots. Histologically, confluent parakeratosis, hypogranolosis, and angiogenesis can be observed [370], [8].

More than a decade ago, it has been shown by using a mouse model of transferred human skin from psoriasis patients, that psoriatic lesions develop dependently on the proliferation of skin-resident T lymphocytes that pre-existed in the skin upon transfer and did not circulate into the recipient mouse. Moreover, the proliferation of these skin Trm cells depends on TNF-α signaling and can be inhibited by TNF-α neutralizing mAbs [371]. By comparing healthy skin

with psoriatic lesions, IL-17 and IL-22 producing CD8+ and CD4+ Trm cells, respectively, were found in both lesional and healed skin [364], [372]. Even when psoriasis lesions are resolved, the healed skin differs form healthy skin indicated by prolonged up-regulation of disease-related T cell (LCK and TRCB1) and inflammatory (IL-17, IL-22, IFNγ) genes up to at least three months after initiation of systemic TNF-α inhibition treatment [373]. In healed psoriatic skin up to 20% of the CD8+ T lymphocytes expressed CD103 and CD49a, a functional Trm cell marker. In active lesions massive infiltration of CD8+ T lymphocytes into the epidermis occurs and up to 50% of them express the Trm cell markers, indicating an increase of Trm cells during the active form of the disease. CD4+ T lymphocyte infiltration was dominant into the dermis. Gene expression analysis revealed that epidermal cells in active psoriasis were highly activated and produced inflammatory mediators such as IL-17, IFNγ, IL-22 but also regulatory molecules such as Foxp3 and IL-10 (CD4+), or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (CD4+ and CD8+). Their dermal counterparts were less activated [364]. Moreover, CD8+ Trm cells localize according to the expression of CD49a in the epidermis (CD49a+) or in both epidermis and dermis (CD49a-). CD8+CD49a- Trm cell from healed psoriasis skin predominantly produce IL-17 while in active psoriasis Trm cells produce both IL-17 and IFNγ. Of note, in vitiligo CD8+CD49a+ Trm cells that constitutively express perforin and granzyme B were enriched in the dermis and epidermis. The functional association of CD49a expression is maintained in pathogenic Trm cells and equips the two populations with different effector profiles and functions in skin disease [26].

Noteworthy, it has been shown that Trm cells express high levels of molecules that facilitate the uptake of FFAs and that Trm cells deficient in FFA uptake are less protective against viral re-infections. Interestingly, Trm cells from psoriatic skin lesions have be shown to have an increased FFA metabolism indicating their increased reactivity [327].

20.2 Trm cells in fixed drug eruptions

Another example for recurrent cutaneous reactions that are likely to be caused by skin Trm are FDE. FDE is characterized by a cutaneous hypersensitivity response against systemically administered drugs. FDE patients develop dusky, usually solitary, skin lesions, which resolve once the offending drug is removed. However, if the drug in question is taken again by the patient, even years or decades later, the recurrence of skin lesions often at the same anatomical site can be observed. Usually, FDE appears as one or few pruritic, erythematous macules that can develop into edematous plaques on the skin or mucous membrane. However, in some more severe cases the lesions can spread and become bullous, also systemic

manifestation is observed, mimicking Stevens-Johnson syndrome or toxic epidermal necrolysis [7].

While in healthy human skin more CD4+ Trm cells can be found in epidermis as well as in dermis [246], in resting FDE lesions a shift towards the CD8+ Trm cell population (approx. 90%) in the epidermis is observed. As typical skin Trm cells, they localize mainly at the epidermal/dermal junction. Moreover, upon *in situ* re-stimulation, only the intra-epidermally located CD8+ Trm cells produced IFN-γ which leads to the recruitment of secondary effector cells from the circulation [367].

CD8+ Trm cells in epidermis of healed FDE lesions produce IFN-γ and TNF-α. While only few CD4+ Trm cells are observed in the epidermis of resting lesions, upon activation by drug administration both CD8+ and CD4+ T cell infiltrated the epidermis. However, approx. 70% of these infiltrating CD4+ T cells are Treg cells and are capable to produce anti-inflammatory IL-10 which probably enhances the spontaneous resolution of the lesion upon drug withdrawal [368]. This stand in contrast to the accumulation of pathogenic Treg cells in other inflammatory skin diseases such us psoriasis [269].

In active FDE lesions at least some CD8+ Trm cells acquire a NK-like phenotype indicated by the expression NK-markers such as CD56 and GzmB which is reversed to the Trm cell phenotype in resting FDE lesions. Such CD8+ Trm cells can be found in the epidermis of FDE lesions for at least four years [374].

The knowledge of the pathophysiology of FDE is still limited due to the lack of appropriate experimental model. However, a model of T-cell mediated drug allergy in skin developed in our group demonstrated the preferential development CD8+ Teff cells in the skin upon sensitization [375].

20.3 Trm cells in mycosis fungoides

Cutaneous T-cell lymphomas comprise malignancies of skin homing T lymphocytes with divers presentation. Sézary syndrome is a leukemic variant (L-CTCL) in which malignant T cells accumulate in the blood and lymph nodes and give rise to diffuse erythema of the skin. Mycosis fungoides (MF) is variant in which malignant T lymphocytes reside primarily in infiltrated skin lesions [365], [376].

It has been demonstrated that in Sézary syndrome erythematous skin contains malignant CD4+ T lymphocytes expressing a Tcm cell associated phenotype. In addition to co-expression of the lymph node homing receptor CCR7 and CD62L, they also express the skin

homing molecules CLA and CCR4, indicating their ability to enter both secondary lymphoid organs and skin. In contrast, in MF skin malignant CD4+ T lymphocytes do not express the lymph node homing receptors CCR7 and CD62L but high levels of CLA and CCR4, indicating a Trm cell phenotype. The sessile non-motile nature of the majority of the detected Trm cells in MF skin explains the fixed anatomic location of MF skin lesions for many years [365]. In addition, mAb treatment with aCD52, a T lymphocyte depleting antibody, revealed that MF cannot be treated with it due to the inaccessibility of the skin resident Trm cells for the antibody. Sézary syndrome patients on the other hand respond well to the therapy during which malignant as well as benign Tcm cell that re-enter the circulating from the periphery have been depleted from the organism. Moreover, in those patients protection against infections could be observed due to persistent non-malignant skin Trm [17].

20.4 Trm cells in ACD

ACD is a T cell mediated skin disease, which is induced following the exposure to contact allergens. The concept that ACD could be caused by the induction of local memory responses specific for such contact allergens has been around for more than three decades [377] and it becomes more and more elucidated.

In the ACD-like CHS mouse model using DNFB it has been demonstrated that multiple applications lead to abundance of certain T cell clones in the skin and the draining LNs (dLNs) indicating a common clonal origin for them. However, the most abundant clones were 7-17x more numerous in the skin than in the LN, indicating that multiple exposures to the same Ag favors the expansion of Trm cells at the site of Ag encounter. Such an increase could also be observed in humans after multiple applications of contact allergens.

To determine the contribution of circulating and skin-resident Ag-specific T lymphocytes, investigators combined the DNFB mouse model with parabiosis. Thereby it was demonstrated that mice containing both Tcm and Trm cells mice mounted a robust skin inflammation within 24h after DNFB re-exposure. In mice deficient of Trm cells, the development of skin inflammation was up to five days delayed after DNFB re-exposure. However, *de novo* generation of Trm was confirmed by another DNFB re-exposure in those mice four weeks after the initial challenge following the separation, which resulted in immediate skin inflammation comparable to the mice containing both Tcm and Trm cells [24].

In line with these findings, another study demonstrated that local immune responses against DNFB induce stronger inflammation than global responses. The local response was maintained longer than the global response and occurred in an Ag-specific and IL-1β

dependent manner, which was produced by keratinocytes and mainly localized in the epidermis of the mice. However, Trm cells extracted from healed skin lesions also responded stronger to un-specific stimuli with the production of IL-17 and IFN-γ than cell from vehicle treated skin sites. Those results were confirmed in patients known to be allergic against nickel. Here, IL-1β also accumulated in the epidermis from healed lesions and the presence of IL-17 correlated with the clinical score of the patients [25].

These two studies suggest that after skin immunization, the immune system generates and distributes identical, Ag-reactive T cell clones to both the LNs (Tcm cells) and the peripheral tissues (Trm), thereby generating two location sites of Tm cells with identical Ag-specificity but different effector functions. Such a back-up system seems to be important for the development of effective immune responses against pathogens but it also helps to explain the recalcitrant, recurrent, and site-specific nature of ACD and other allergic skin diseases.

20.5 Trm cells in AD

Also the clinical characteristics of AD suggest a contribution of Trm cells to the pathogenesis. Recently, a study demonstrated that CD4+ Trm cell in the lung that are specific for HDMs are sufficient to induce asthma, a disease that like AD belongs to atopic diseases which are related to each other [369].

Similarly to findings in psoriasis patients, not only lesional but also non-lesional AD skin differs from healthy non-AD affected skin. It shows increased thickness, an increase of infiltrating T lymphocytes and DCs. Moreover, gene expression analysis revealed that non-lesional AD skin displays the phenotype of lesional AD skin and has only small similarity with healthy skin. In lesional and non-lesional AD skin down-regulation of genes associated with terminal differentiation of keratinocytes could be detected. Proper differentiation of keratinocyte is key to establish a functional skin barrier [106]. However, the expression profile of non-lesional skin resembled normal skin in a set of genes containing inflammatory gene products, such as cytokines, chemokines, and cytokine-induced gene products in keratinocytes which were all up-regulated in lesional AD skin. Interestingly, the extend of the disease on lesional skin influenced the phenotype of non-lesional skin in a way that higher disease scores cluster it closer to lesional skin and lower scores closer to normal skin [138].

Similar to findings in ACD [24], T lymphocyte counts were higher in lesional than non-lesional skin of AD-patients. Even though less T cell clones could be detected in non-lesional AD skin, there is no significant difference in the diversity of the TCR repertoire between lesion and non-lesional AD skin. Also, there was no subset of T cell clones that was enriched in

lesional or non-lesional AD skin. Importantly, in almost all of the analyzed individuals the three tops T cells clones found in lesional AD skin were also found in corresponding non-lesional biopsies, indicating that both sides are equipped with nearly the same top expanded T cell clones. Even after topical glucocorticosteroid treatment, the top clones were still present in both sites of the skin [378].

Finally, a study identified a population of skin resident group 2 ILCs in healthy human skin that was enriched in human AD lesions. In a comparative AD-mouse model they demonstrated that this group 2 ILC population was critical for the induction of skin inflammation and that they were dependent on TSLP.

Even though in AD a clear demonstration of the contribution of Trm cells to the pathogenesis is missing the summarized finding above suggest that in this inflammatory disease they most likely play a key role as well.

21 The regulation of Trm cells

Trm cells are key players in the adaptive immunity by providing on-site protection against invading pathogens. However, aside from their beneficial functions more and more evidence point to a rather detrimental role for Trm cells in inflammatory diseases [22], [21], [9]. Nevertheless, both protective and pathological Trm cells are not constantly active, as indicated by the absence of effector functions after pathogen clearance or during the latent phases of chronic inflammatory diseases. This indicates that Trm cells are subjected to regulation inside the tissue. Such regulation could be either tissue-derived e.g. anti-inflammatory cytokines, cell-mediated e.g. by Treg cells [379], and endogenous e.g. by the expression of inhibitory checkpoint receptors (ICRs) on the Trm cells. Since my research addresses the potential regulation of Trm cells in the skin by the expression of ICRs, in the following I will discuss their role in immune regulation.

The expression of ICRs on T lymphocytes is associated with a phenomenon referred to as T cell exhaustion, which is a state of T lymphocyte dysfunction usually arising during chronic infections and cancer [380]. T cell exhaustion is defined by the progressive loss of effector functions and the sustained expression of ICRs such as programmed cell death protein 1 (PD-1), lymphocytes activation gene 3 (LAG-3), T-cell Ig and mucin-domain containing protein 3 (TIM-3), 2B4 (CD244) etc. [381]. Ligands for the ICRs are either expressed on APCs or on target cells of the Teff cells, which can be virus infected or tumor cells. The molecular mechanisms initiated by ICRs binding to their respective ligands can be classified as ectodomain competition, modulation of intracellular mediators, and induction of inhibitory

molecules.

During an acute infection the Ag is cleared and Teff cells develop into the heterogeneous and highly polyfunctional Tm cell population consisting of circulating Tem and Tcm and tissue-resident Trm cells. As discussed above, depending on their affiliation they comprise certain proliferative, migratory, and effector qualities [382], [16]. However, during chronic infection or tumor growth the Ag-load persists and increases. Its constant presentation by APCs forces T cells into stages of progressive dysfunction and loss of effector functions during which a progressive increase in the expression and diversity of ICRs can be observed. Finally, if the severity of an infection or the presence of tumor-Ag is to high, it leads to the elimination of Ag-specific T cells and the loss of Ag-specific T cell responses [383].

The expression of ICRs is not only restricted to Teff cells but also affects Trm cells after their proper lodgment within the tissue, even though it might not lead to cell exhaustion and subsequent cell death. While several studies confirmed the expression of ICRs on Trm cells [253], the effect of ICR expression on Trm cells is controversial. A recent study demonstrated that CD8+ Trm cells in the brain which express ICRs such as PD-1, TIM-3, and LAG-3 were incapable of controlling latent HSV infections due to impaired effector functions indicated by decreased IFN-γ production [384].

However, other studies suggest that the ICR expression on Trm cells in the brain, and also in other organs such as the lung, presumably impose an effective restraint on the Trm cells and thereby immunopathology and tissue damage from excessive immunoreaction is prevented [253], [348]. While this may partially be true for infectious diseases during which the organism has to maintain a certain balance between eliminating and tolerating a pathogen in order to either survive a lethal infection or to prevent detrimental organ damage, it is a very attractive hypothesis in inflammatory diseases. A study investigating CD8+ Trm cells in experimental autoimmune uveoretinitis, a model of T cell-mediated autoimmune disease, showed that they express ICRs which limits local inflammation [385]. A more recent study using a pre-clinical showed that the stimulation of PD-1 by its ligand PD-L1 leads to less local inflammation during CHS responses of the oral mucosa compared to mice deficient in PD-L1. This suggests the occurrence of on-site regulation during inflammation [386]. Also, our own observations demonstrate the up-regulation of ICRs such as PD-1 and TIM-3 on Ag-specific CD8+ Trm cells in an ACD-like CHS mouse model. In tumor therapy blockage of ICRs has been proven to be very efficient to re-induce Teff cell functions [387] but limitations have been seen since the

re-induction is only transiently [388], [389]. However, for us it seems prospective to target Trm cells in inflammatory diseases via their ICRs as well. However, instead of blocking them like in the tumor therapy, stimulation would be needed in order to tighten their regulatory restrain and to prevent activation upon Ag-recognition.

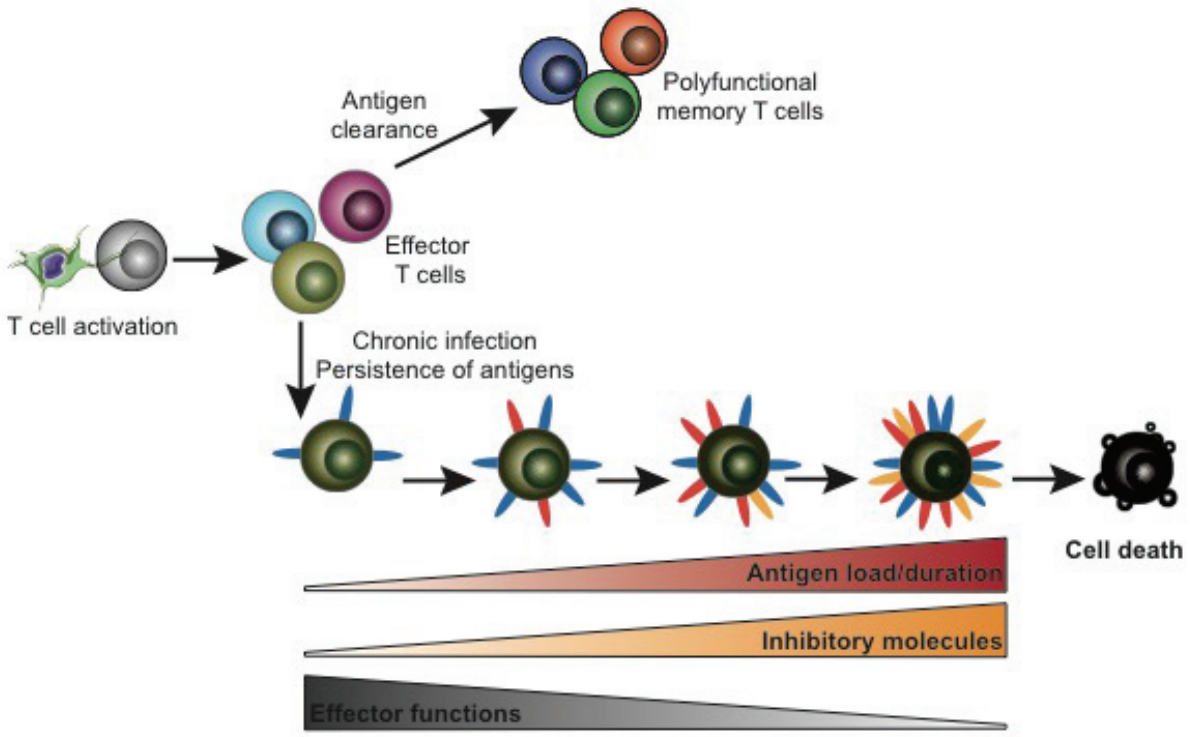


FIGURE 18: THE ACQUISITION OF INHIBITORY CHECKPOINT MOLECULES ON T CELLS.
After activation of naïve T cells they develop into Teff cells. Following sufficient Ag clearance, they further differentiate into polyfunctional Tm cells. However, in case of long-term persistence of Ag, e.g. during chronic infections, Teff cells progressively up-regulate inhibitory molecules. The up-regulation of those inhibitory molecules is inversely correlated with the effector function, putting the cells through divers' stages of dysfunction and eventually leads to cell death. Adapted from Wherry, 2011.

Part 3: Experimental results

22 Objectives of the project

The objective of this project was to gain deeper inside in the pathophysiology of ACD and AD, two chronic inflammatory skin disease mediated by Ag-specific T cells, in which skin lesions recur as flare-ups preferentially a previously affected sites. Even though in most cases, ACD and AD are not life threating diseases, they put a high burden on health-care resources and more importantly on the patients' quality of life. Here, we were especially interested in deciphering the contribution of local immune memory in the form of skin Trm cells to the chronicity and the increasing severity of such recurring skin lesions. The concept of local memory on healed skin lesions has been described more than three decades ago [377] and has been revisited more recently [390], [391], [392]. Moreover, several studies describe the presence of Trm cells in skin inflammatory diseases [24], [25], [26] and indirectly demonstrate their contribution. Therefore, in our project we intended to directly demonstrate the contribution to the development of flare-ups reaction upon re-exposure to the Ag. Furthermore, we investigated their Ag-specificity and aimed to determine certain protective regulatory mechanisms keeping skin Trm cells from constant activation despite their sentinel position at the interface with the environment and thereby preventing immunopathology.

In the following study we employed a pre-clinical model of ACD in CHS responses against the strong sensitizer DNFB were induced in mice. We confirmed a pre-dominant persistence of Ag-specific CD8+ Trm cells at former skin lesions for at least several weeks to months. Moreover, depletion of CD8+ Trm cells from the skin, by using an original inducible diphtheria toxin receptor (iDTR) approach, but not the elimination of circulating T cells was sufficient to prevent the re-induction of CHS responses in allergic mice which indicates their active participation in the development of flare-ups.

Interestingly, we observed that repeated low doses of the allergen was not sufficient to induce CHS responses in allergic mice. Moreover, we detected small but significant amounts of DNFB long after the resolution of skin inflammation which apparently was not sufficient to maintain the response. This led us to investigate whether Ag-specific skin Trm cells underlie tight regulatory mechanisms. In line with other findings [253], [385], [348], we observed that CD8+ Trm cells but not circulating T cells expressed several ICRs such as PD-1, TIM-3 and 2B4. We assume that these ICRs are up-regulated in response to persisting Ag and that they are

sufficient to prevent immunopathology caused by excessive inflammation by restraining the CD8+ Trm cells. This was confirmed since blocking of the ICRs with monoclonal antibodies induced increased reactivity of CD8+ Trm cells *ex vivo* and severe CHS reactions upon re-exposure to DNFB low doses.

In additional preliminary experiments carried out during this project, we could confirm the long-term persistence of Ag-specific CD8+ but but also CD4+ lymphocytes in healed lesion from mice subjected to a pre-clinical model of AD in which skin allergy is induced by epicutaneous application of *Der f* extract. Also, high frequencies of CD69+/CD103+ cells were detected among the CD8+ T cells confirming their Trm cell phenotype. Interestingly, the role of Trm cells in the induction of AD flare appears to be even more important than in the DNFB model, since a single application of *Der f* extract on healed skin leads to strong flare-ups but fails to induce any reaction at non-lesional skin sites. Similarly to the CHS model, the responses on healed lesions were dependent on the Ag dose, indicating a tight regulation of the Ag-specific skin Trm cells.

Adjacent to our studies in pre-clinical models of ACD and AD, we enrolled a clinical study with the objective to determine the phenotype of skin Trm cells from AD patients regarding their surface expression, transcriptional profile, Ag-specificity, and cytokine production. The study was initiated with our colleagues from the Hospital Lyon-Sud and is still ongoing. Even though the clinical work was already initiated in 2015, we were facing serious issues to recruit patients with accordant clinical criteria and had to adapt to that, the study is still ongoing. Therefore, only preliminary data of the so far three analyzed patients will be presented below.

The results, both presented in the following study and our complementary data, will meet our objectives by helping to further elucidate the pathophysiology of allergic skin diseases such as ACD and AD. In addition, our work will complement the knowledge about Trm cell biology which has been mainly gathered by employing virus models. We think that using pre-clinical models of skin allergies to determine the development and mode of action of Trm cells will be useful to develop new therapeutic and vaccine strategies of both allergic and infectious diseases, respectively.

23 Manuscript in preparation:

Inhibitory checkpoint receptors PD-1 and TIM-3 control CD8+ Trm reactivation to prevent skin allergy

Pia Gamradt[1-5*], Léo Laoubi[1-5*], Audrey Nosbaum[1-5], Virginie Mutez[1-5], Vanina Lenief[1-5], Sophie Grande[6], Daniel Redoulès[7], Anne-Marie Schmitt[8], Jean-François Nicolas [1-6] and Marc Vocanson[1-5]

Affiliations:

[1] CIRI, International Center for Infectiology Research, Université de Lyon, Lyon, France.

[2] INSERM, U1111, Lyon, France.

[3] Ecole Normale Supérieure de Lyon, Lyon, France.

[4] Université Lyon 1, Centre International de Recherche en Infectiologie, Lyon, France.

[5] CNRS, UMR 5308, Lyon, France.

[6]Allergology & Clinical Immunology, CH Lyon-Sud, Pierre-Benite, France.

[7] Pierre Fabre R&D Dermocosmétique, Toulouse, France

[8] Pierre Fabre R&D Pharmaceuticals, Toulouse, France

* Equal contributors

Correspondence:

Marc Vocanson, CIRI-INSERM U1111, 21 avenue Tony Garnier 69007 Lyon, France

Tel:+33 437 28 23 48; e-mail address: marc.vocanson@inserm.fr

Short title: CD8+ Trm in skin allergy.

Abstract

Tissue-resident memory T cells (Trm) are considered detrimental in numerous human inflammatory diseases, including eczemas, which are the most frequent skin allergies in children and adults.

To gain further insights into the contribution of skin Trm to the chronicity of eczema, we used an experimental model of allergen-induced contact hypersensitivity, which is mediated by CD8+ cytotoxic effector T cells.

A significant number of allergen-specific CD8+ Trm, originating from early effector T cells, accumulated in the epidermis during the development of the skin inflammation and stayed for several weeks after its resolution. Also, the allergen persisted in the epidermis for > 30 days. Trm participated actively to recurrent eczema exacerbations which did not occur when Trm were depleted from the skin. A substantial number of epidermal Trm expressed inhibitory checkpoint receptors (ICRs) such as PD-1 and Tim-3 which prevented Trm reactivation, since blocking of those ICRs using monoclonal antibodies was responsible for severe eczema exacerbations.

Therefore, CD8+Trm appear as key players in the chronicity and severity of eczema, although cell intrinsic mechanisms keep Trm in check to prevent damaging immunopathology. Developing therapeutic strategies targeting the reactivation of skin Trm *in situ* via their ICRs should open new avenues to restore tolerance in allergic individuals.

Keywords: Resident memory T cells (Trm), eczemas, atopic dermatitis, allergic contact dermatitis, inhibitory check point receptors, immunopathology.

Key Messages

• High density of allergen-specific CD8+ Trm accumulate at the sites of healed eczema lesion in response to the long-term persistence of allergen-moieties in the skin.

• These cells are key for the recurrence of the pathology.

• However, they express several inhibitory receptors, such as PD-1 or TIM-3, which keep them in check to preserve skin integrity and avoid the development of severe immunopathology.

Capsule summary

Recent works suggested the importance of local memory in the course of chronic inflammatory diseases. The present study confirms the crucial role of epidermal CD8+ Trm for the recurrences of eczema but also highlights cell intrinsic mechanisms, which prevent their uncontrolled (unnecessary) reactivation to preserve skin integrity and severe immunopathology. Developing Trm-targeting strategies should open new avenues to restore tolerance in allergic individuals.

Introduction

Initially described in the skin and the gut, resident memory T cells (Trm) constitute a recently described subset of memory T cells (Tm) that do not re-circulate the organism and persist long-term within peripheral tissues or organs, where they organize an optimal front-line defense against invading pathogens or neoplastic cells [1], [2], [3], [4], [5]. It is now clear that, both in mouse and human, Trm undergo a distinct differentiation program, involving notably the transcription factors (TFs) Hobit, Blimp-1 [6] Notch [7], KLF2 [8], Tbet, and Eomes [9], that discriminate them from central (Tcm) and effector memory T cells (Tem).

Phenotypically, CD8+ and CD4+ Trm are classically identified by their stable expression of CD69 and CD103, both implicated in lodging Trm inside peripheral tissues and enhanced functions [4], [10]. However, Trm lacking expression of CD69 or CD103 [11], [12] have also been reported, suggesting a substantial heterogeneity [13], [14].

The mechanisms that promote Trm differentiation are just beginning to be unraveled. Different studies conducted in murine models of virus infection have proposed that Trm and Tcm derive from common precursors [15] primed by DNGR-1+ dendritic cells (DCs) in the lymphoid organs [16], and are characterized by the low expression of Killer lectin-like receptor subfamily G member 1 (KLRG1) marker [17]. Further, Trm development involves several checkpoints, including tissue entry, local retention and subsequent responsiveness to locally-derived signals. Hence, (i) the ability of Trm precursors to enter non-lymphoid tissues, via chemokine receptors such as CXCR3, (ii) the modulation of their tissue egress by S1PR1-blocking mechanisms/programs (including CD69, KLF2) or (iii) their response to epithelia-derived cytokines such as TGFβ, IL-15, IL-7, IL-33 or to exogenous free fatty acid and aryl hydrocarbon receptor (AHR) ligands etc. were reported to be instrumental for subsequent Trm differentiation and long-term survival [17], [8], [18], [19], [20], [21], [22]. By contrast, the presentation of their cognate antigen to the site of residency does not appear to be an absolute necessity [23], [24].

Beyond their demonstrated active contribution to host protection, there is today an emerging understanding that Trm also participate in several autoimmune/inflammatory diseases, in which they can instigate or augment immunopathology [25], [26]. Hence, Trm were described in different skin diseases such as psoriasis, vitiligo [13], or leukemic cutaneous T cell lymphoma [27], [12], and suggested

to be important in a variety of intestinal, brain or joint diseases (Crohn's disease, ulcerative colitis, multiple sclerosis, human ankylosing spondylitis etc.) [25].

Similarly to autoimmune pathologies, Trm are thought to be major actors of allergen-mediated skin diseases, with fixed drug eruption being the first and best illustration of their detrimental activities [28]. However, very few is known about the nature and the contribution of Trm in eczemas which comprise allergic contact dermatitis (ACD) and atopic dermatitis (AD), two common chronic and relapsing T cell-mediated skin allergic diseases in children and adults [29]. Eczema flares (also referred to as exacerbations, relapses, recurrences) consist in the worsening of the skin inflammation upon allergen exposure. These exacerbations are followed by an improvement of the eczema leading to totally- or well-control disease activity during which the skin has a normal aspect. A major clinical feature of eczema is the fact that exacerbations develop usually on the same body sites, i.e. on previously affected but healed skin [30]. This observation has led to the concept of a “local memory to allergens” postulating that specific T lymphocytes (i) persist for several months in previous sites of inflammation and, (ii) are important in flare-up reactivity and in chronic inflammation [31]. Recent studies have described that similar T cell clones populate the sites of active and healed eczema lesions, but without demonstration of their phenotype, specificity and function [15], [32], [33].

In this study, we analyzed the contribution of skin Trm to the pathophysiology of eczema using the experimental model of contact hypersensitivity (CHS) to the chemical allergen 2,4-dinitrofluorobenzene (DNFB), which is relevant for both atopic dermatitis and allergic contact dermatitis [34], [35].

Results

Allergen-specific CD8+ Trm persist in the epidermis and dermis of healed eczema

To confirm that Trm persist at the sites of previously affected skin, the healed (30-60 days after the induction and resolution of an initial CHS response) and non-lesional skin of DNFB-allergic C57BL/6 mice were collected and processed for the presence of CD8+ and/or CD4+ T cell subsets by FACS and immunofluorescence microscopy (**Fig.1A)**. While the epidermis and the dermis of naive skin were nearly exclusively seeded with CD4+ T cells, significant numbers of CD8+ T cells were recorded in healed lesions (**Fig.1B and Fig.S1)**. Comparatively, the number of CD4+ T cells was only modestly augmented in these areas. Further analysis indicated that approx. 87% and 75% of epidermal and dermal CD8+ T cells, respectively, co-expressed the two canonical Trm markers CD103 and CD69 [36], emphasizing the residency properties of these cells (hereafter referred to as Trm) (**Fig.1C**). By contrast, we only detected approx. 3.5% of cells from the dLN co-expressing the two canonical Trm markers.

We next aimed to determine the proportion of allergen-specific Trm among all CD8+ Trm present in the healed lesion of DNFB-allergic mice. Thus, skin CD8+ and CD4+ T cell subsets were FACS sorted and re-activated *ex-vivo* with allergen-pulsed bone marrow-derived dendritic cells (BMDCs), or un-pulsed BMDCs as control. The following day, the number of spot forming cells (SFCs) producing IFN-γ, IL-17a or IL-4 was determined by enzyme-linked immunospot (ELISpot) assay. While approx. 1% ($1x10^4$ SFC cells/10^6 cells) and 0.2% ($2x10^3$ SFC cells/10^6 cells) of DNFB-specific CD8+ T cells from the epidermis and dermis of healed DNFB lesions produced IFN-γ (**Fig.1D**), IL-17a- or IL-4- were not detected (**data not shown)**. Comparatively, the frequency of DNFB-specific cells among total CD8+ T cells from dLNs, which comprise a mix of Tem, Tcm and dLN Trm was <0.1% ($5x10^2$ cells/10^6 cells). These data, thus, show that healed DNFB lesions are enriched for specific Trm.

To confirm our observation, we designed a new CHS model in C57BL/6 mice based on the s.c. injection of BMDCs pulsed with a MCH class I-restricted ovalbumin peptide bearing a dinitrophenyl (DNP)-moietie (DNP-peptide) [37]. In such way, immunized animals developed standard CHS responses when they were challenged on the ear with the respective DNP-peptide (**Fig.S2A**). The advantage of this model

is that it allows the tracking of allergen-specific CD8+T cells by using fluorescently labeled dextramers loaded with the cognate DNP-peptide. Using this approach, we detected approx. 30% of specific cells in the epidermal CD8+ Trm compartment of healed lesions, versus 0.4% in the dLNs (**Fig.S2B**). Moreover, we confirmed that the majority of epidermal CD8+ Trm were type 1 cells, since around 18% of them expressed IFN-γ or Granzyme-B after *ex-vivo* re-activation with DNP-peptide-pulsed BMDCs (**Fig.S2C)**.

Collectively, our data show that CD8+ type 1 cytokine-producing Trm persist in the epidermis and dermis of healed DNFB lesions for several weeks after the development and resolution of the skin allergic inflammation.

Epidermal CD8+ Trm accumulate from the CD8+ early effector T cells during the resolution of the skin inflammation

We next examined the formation and accumulation of epidermal CD8+ Trm during the course of the CHS response to DNFB. Hence, sensitized mice were challenged on the ear and total epidermal CD8+ T cells and CD8+Trm in the epidermis were determined at days 2, 4, 8, 15 and 30 post challenge. Surprisingly, we observed a gradual increase in the number of CD8+ T cells in the epidermis between day 2 and day 15, which paralleled the down-regulation of the acute CHS response from day 2 on (**Fig.2A**). Besides, the number of epidermal CD8+ Trm increased dramatically from day 2 on (**Fig.2A and Fig.S3A**), before it stabilized between day 15 and 30 alongside with the complete resolution of skin inflammation.

Next, we aimed to decipher whether Trm accumulating from day 2 on derived from *in situ* proliferation of early effector cells present at the peak of CHS response (at day 2), or from late arriving circulating CD8+ T cells infiltrating the skin during the resolution of inflammation. To this end, we examined the cell cycling and cytotoxic phenotype of epidermal CD8+ T cells during the days following DNFB challenge. While granzyme B+ cells were present only at day 1 and 2 post DNFB challenge, which corresponds with the augmentation of skin inflammation, a significant percentage of CD8+ T cells expressed the cell cycle marker Ki-67 from day 2 to 8, indicating ongoing proliferation despite the resolution of the skin inflammation (**Fig.2B**). Interestingly, part of the proliferating Ki-67+ cells co-expressed CD103 and CD69 at day 4 and 8 (17% and 31%, respectively), suggesting progressive acquisition of residency markers by epidermal CD8+ T cells at these time points

(**Fig.S3B**). To confirm this hypothesis, we capitalized on the fact that anti-CD8b mAb treatment via i.p. injection depletes circulating CD8+ T cells only, sparing the majority of their dermal and epidermal counterparts, to determine whether this treatment could prevent the progressive accumulation of epidermal CD8+ Trm. The depletion of circulating CD8+ T cells at day 2 (peak of skin inflammation) dramatically accelerated the resolution of inflammation **(Fig.S4**). However, it did not prevent the accumulation of epidermal CD8+ T cells and even promoted their transformation into Trm since we observed an increased percentage of epidermal CD8+ T cells co-expressing CD103 and CD69 at day 8 (**Fig.2C**). However, we did not observe any significant change in the numbers of epidermal Trm at day 15 post challenge, in anti-CD8b+ mAb treated-animals, when compared with un-depleted controls (**Fig.2D**).

Altogether, these results suggest that CD8+ early effector T cells present in the epidermis at day 2 of the CHS response to DNFB represent a major source of CD8+ Trm. Moreover, the fact that those cells continue to proliferate during the resolution of the skin inflammation, before they acquire Trm markers, suggests that they meet a nurturing environment promoting the acquisition of Trm marks [17].

Allergen persists for several weeks in the epidermis

Pivotal works conducted in viral models described that key cytokines, such as TGF-β or IL-15, produced by the epithelial cells prompt the generation of skin Trm [17], [8], [20]. Besides, it has been reported that, while Trm can differentiate inside the skin in absence of cognate antigen [23] [24], its presence strongly amplifies the number of specific Trm that will persist at the site of initial inflammation [38], [39], [40]

We therefore investigated the ability of keratinocytes to produce TGF-β or IL-15 during the course of the CHS response to DNFB. Interfollicular keratinocytes were FACS sorted at day 2, 4, 8 and 23 post DNFB challenge, and TGF-β or IL-15 gene expression was quantified by qRT-PCR. A significant increase of TGF-β and IL-15 transcripts was detected in keratinocytes collected from day 8 post DNFB challenge, comparatively to cells recovered at day 2, or from non-lesional skin (before DNFB application) (**Fig.S5**). While we cannot exclude that other skin cells also participate to produce seminal mediators promoting Trm development, these data indicate that keratinocytes, through the expression of TGF-β and IL-15, are certainly key players in the generation of epidermal Trm in our model.

To determine the role of allergen in skin Trm development, we also checked

how long DNFB, that is administrated epicutaneously, persists in this tissue. Using standard immunofluorescence staining of DNP-moieties generated at different time points after DNFB painting and confocal or light sheet fluorescence microscopy (LSFM), we observed that around 40% of the epidermal compartment were stained by anti-DNP mAbs at day 2 post challenge. Interestingly, approx. 10% are still stained at days 8 and 15 and about 5% remain labeled at day 30 post challenge, with a pronounced accumulation of CD8+ T cells (detected by using anti-CD8b mAb staining) around DNP-stained areas at each of the indicated time points (**Fig.S6**). Our results thus revealed an underestimated persistence of chemical allergen-moieties in the skin for at least one month, despite the complete resolution of skin inflammation.

Thereafter, we aimed to estimate whether the long-term persistence of the allergen has an impact on the amount of CD8+ Trm which populate the epidermis at day 30 post challenge by comparing the number of Trm generated via the application of cognate Ag or via a prime and pull strategy. The latter allows to recruit Ag-specific T cells in the skin using an inflammatory but structurally irrelevant molecule [23]; here, we used the strong contact sensitizer oxazolone (OXA). Capitalizing on our model of CHS response to DNP-peptide, we observed by FACS that similar numbers of specific CD8+ T cells are recruited into the epidermis 2 days post challenge with DNP-peptide or OXA. However, 40 days post challenge, only $^1/_4$ of the number of epidermal DNP-peptide-specific CD8+ Trm induced by cognate Ag challenge was detected after OXA challenge, confirming that the presence of allergen-moieties in the skin strongly amplifies the number of skin Trm which settle permanently in healed lesions (**Fig. S7**).

In short, our results demonstrate that CD8+ effector T cells that infiltrate the epidermis at day 2 post challenge encounter an adequate environment within the resolving skin inflammation to gradually acquire Trm features.

CD8+ Trm participate actively to recurrent flare-up reactions.

We next examined the contribution of CD8+ Trm to the allergen-induced eczema flares. When DNFB-allergic animals were re-challenged on the previously affected skin (30-60 days after the resolution of the skin inflammation), they developed potent flare-up reaction (maximal 24-48hrs post re-challenge), whose magnitude was more severe than the response induced on non-lesional skin and the

background response induced in non-allergic animals (**Fig.3A, left part**). Moreover, such flare-up reactions were antigen-specific, since the application of the irrelevant strong sensitizer OXA on the healed or naive skin of DNFB-allergic animals triggered background responses of similar magnitude than the one induced in non-allergic animals (**Fig.3A, right part**).

To define the contribution of DNFB-specific CD8+ Trm to the exaggerated reaction recorded in healed lesions upon allergen-retest, we next designed a set of two complementary experiments. First, we examined the flare-up reactions induced in animals injected with anti-CD8b and/or anti-CD4 mAbs one day before re-challenge. Interestingly, responses of relative similar magnitude were recorded in depleted mice comparatively to non-depleted control mice, despite the acute depletion of all circulating but not skin-resident CD8+ and/or CD4+ T cells (**FigS8**). This indicated that resident skin T cells, which are not depeleted by this treatment, are key to the flare-up reactions. Second, we developed an inducible diphtheria toxin receptor (iDTR)-based approach, using DNFB-allergic CD3ε-/- lymphopenic recipient mice, reconstituted prior to the induction of DNFB-allergy with iDTR-CD8+ and WT-CD4+ T cells collected by FACS sort from iDTR/CD4cre and C57BL/6 donor mice, respectively. Flare-up reactions upon retest were completely abrogated in recipient mice injected with diphtheria toxin (DT) (one day before re-challenge), while untreated or anti-CD8 mAb treated control mice developed full flare-up response (**Fig 3B**). Of note, DT injection depleted all the circulating and skin CD8+ T cells, but sparing non-CD8+ T cells in these recipients (**Fig.S9**).

Taken together our results show that antigen-specific CD8+ Trm are of prime importance for the development of recurrent flare-up reactions recorded on healed lesions of DNFB-allergic mice.

Epidermal CD8+ Trm but not circulating memory CD8+ T cells express several inhibitory check point receptors.

Even though our results demonstrated a crucial role for CD8+ skin Trm in the development of flare-up reactions induced by DNFB challenge at the optimal dose (0.13%), specific CD8+ Trm failed to induce skin inflammation upon repetitive exposures to low doses of DNFB. Indeed, the repeated application of low doses of DNFB (0,05%) required 3 consecutive administrations (on day 0, 2, and 6) to generate flares of similar magnitude than the one provoked by the optimal dose

(0.13%) (**Fig.3C**). Moreover, even 6 consecutive administrations (on day 0, 2, 6, 8, 13, and 15) of DNFB 0.01% failed to induce a flare-up reaction, suggesting that DNFB-specific Trm are actively restrained in the skin to prevent excessive immunoreactivity in response to DNFB application below a certain threshold (**Fig.3C**). In this respect, we examined the expression of different inhibitory checkpoint receptors (ICRs) by CD8+ skin Trm. We observed that these cells expressed high levels of PD-1, TIM-3 or 2B4 (**Fig.4A**), as well as CD5, but not TIGIT, LAG-3, CTLA-4 (**Fig.S10**), comparatively to CD8+ Tem or Tcm or naïve CD8+ T cells isolated from the dLNs of DNFB-allergic mice. CD160, VISTA or BTLA were also not detected on CD8+ skin Trm (data not shown). Further analysis showed that approx. 55% of CD8+ Trm expressed PD-1 and 40% were PD-1 negative (**Fig.4B, top**). In both the PD-1+ and PD-1- subpopulations, a majority of cells (approx. 37% and 53%, respectively) co-expressed TIM-3 and 2B4 [**Fig.4B, bottom;** similar results were observed in our DNP-peptide model **(Fig.S11)**].

Next we aimed to determine whether those ICRs are already expressed during cell infiltration or acquired during the resolution of skin inflammation. We noticed that the same percentage (approx. 50-60%) of epidermal CD8+ T cells express PD-1 throughout the CHS response to DNFB (from day 2 to 30 post challenge) whereas a slight increase in the percentage (from approx. 50% at day 2 to approx. 70% on day 30) of TIM-3+ cells was detected. By comparison, the percentage of 2B4+ cells increased dramatically from around 10% at day 4 to almost 80% at day 30 post challenge (**Fig.4C**). Time course analysis of the ICR expression by epidermal CD8+ Trm at indicated time points post DNFB challenge confirmed the previous findings. Here, the rise of PD-1+ and TIM-3+ Trm subsets paralleled the rise of total CD8+ Trm (**Fig.2A**), while 2B4+ Trm clearly appeared from day 4 on (**Fig.4D)**. Thus, only the acquisition of 2B4 but not PD-1 or TIM-3 follows the acquisition of canonical Trm markers. This result confirms a recent study, which reported that 2B4 expression is part of the specific Trm genetic program [17]. In contrast, the expression of the ICRs PD-1 and TIM-3 have previously been associated with recent or chronic stimulation of T cells by their cognate Ag [41].

We subsequently investigated whether the long-term persistence of the allergen in the skin could sustain the expression of these markers. Previous live imaging explorations reported that epidermal CD8+ Trm are not sessile cells and continuously patrol and scan the tissue to the search of their cognate Ag (Ariotti et

al., 2012). Using our model of CHS response to DNP-peptide, we compared the expression of PD-1, TIM-3 and 2B4 on specific CD8+ T cells recruited via peptide application or via prime and boost strategy. Significantly less TIM-3 but not PD-1 expression was detected on CD8+ T cells at day 2 post OXA challenge versus DNP-peptide setting indicated by a drop in the GMFI from approx. 3000 to 2200 (**Fig.S12**). At day 30 post challenge, the expression of both PD-1 and TIM-3 was decreased. Conversely, we did not observe any difference in the expression of 2B4 at the different time points (**Fig.S12**).

Therefore, persisting allergen in the skin not only amplifies the number of CD8+ Trm that populate the epidermis (**Fig.S7**), but also partially modulates their phenotype.

Blocking ICRs in vitro or in vivo leads to increased reactivity of skin CD8+ Trm and the development of severe immunopathology.

Finally, we tested for the functional relevance of ICRs expressed on skin Trm. Blocking ICR *in vitro* using anti-PD-1 and anti-TIM-3 mAbs increased the reactivity of purified epidermal CD8+ Trm and their capacity to produce IFN-γ upon *ex vivo* re-activation with allergen-pulsed BMDCs (**Fig.5A**). Of note, the impact of blocked TIM-3 signaling was more modest than for PD-1, with an increase of approx. 40% and 160% in the number of IFN-γ-producing SFCs, respectively. More importantly, we next demonstrated that *in vivo* blockage of these two ICRs triggered severe flare-up reactions, when DNFB-allergic animals (depleted of all circulating CD8+ T cells by anti-CD8 mAb treatment just before re-challenge) are re-exposed repeatedly to DNFB low doses (0.05%), which induced a very mild inflammation in untreated animals (**Fig.5B**). Of note, the acute depletion of skin CD8+ Trm by DT injection using our iDTR-based model abrogated the development of inflammation induced by anti-PD-1 and anti-TIM-3 blockage (**Fig.5C**).

In summary, PD-1 and TIM-3 expression controls the reactivation of epidermal CD8+ Trm and prevent/limit the development of severe immunopathology when individuals are re-exposed to low dose of allergens.

Discussion

Tissue-resident memory T cells are considered detrimental in numerous human inflammatory diseases, including skin allergies. In the present study, we capitalized on a reference mouse model of eczema to study the local parameters regulating Trm development and functions during pathological processes. We report that, in allergic animals, the sites of previously inflamed skin are seeded with a high frequency of specific CD8+ Trm, both in the epidermis and dermis. These cells are instrumental for the recurrence and severity of ACD, since they are sufficient to evoke intense flare-up reactions upon allergen challenge, without the help of their circulating counterparts. However, albeit they are highly pathogenic for the host, specific CD8+ Trm display certain levels of hypo-responsiveness. They express a large pattern of inhibitory receptors such as PD-1 and TIM-3 (but also high levels of CD5), which attenuate their reactivity and prevent excessive responses when individuals are re-exposed to the allergen. Finally, a major observation was the underestimated long-term persistence of the allergen inside the epidermis, which drives the expansion of early CD8+ T cell effectors and their differentiation into Trm, but also modulates their ICR expression to kept them in check. Our results therefore identify intrinsic factors that control pathogenic Trm to preserve skin integrity and to avoid the development of chronic and severe immunopathology.

1.CD8+Trm are major drivers of the chronicity and severity of eczema

In this study, allergic skin responses were triggered by the epicutaneous application of DNFB, a chemical allergen/hapten endowed with strong sensitizing properties. Cehmical allergen and virus immunity share several similitudes, notably the priming of specific effector/memory cytotoxic CD8+ T cells, which exert a central role in respective immune responses [42], [34]. Hence, it was not surprising to find that the majority of local memory cells which populated the epidermis and dermis of previously affected skin were CD8+ Trm. We failed to detect allergen-specific CD4+ T cells producing type-1, type-2 or type-17 cytokines.

We demonstrated that epidermal CD8+ Trm are sufficient to prompt severe eczema exacerbations, which in consequence categorize them as prominent drivers of the chronicity and severity of eczema. The concept of local skin memory, to explain the strong and accelerated allergen-induced inflammatory reaction at the site

of previously affected skin lesions, has been introduced more than two decades by Scheper et al. [31]. So far, major but only indirect demonstrations suggested that skin Trm, and notably CD8+Trm, are responsible for the development of these early and intense flares [15], [31], [32], [43]. The most solid evidence came from the study by Gaide et al., which reported that flare-up reactions are not prevented when allergic animals are injected with drug compounds that block the egress of memory T cells from the lymphoid organs [15]. In the present study, the sharp elimination of iDTR+ CD8+ Trm from the skin and the subsequent abrogation of allergen-induced flares firmly demonstrated the contribution of Trm to the allergen-induced exacerbations, excluding the potential involvement of other tissue resident cells such as dermal ILCs or liver NK memory cells (Peng H. JCI 2013, Rouzaire P. EJI 2012, Hartwig EJI 2015). Besides, the fact that the application of the irrelevant allergen Oxazolone gave similar inflammation on healed and naive skin in DNFB-allergic mice, enabled us to exclude the possibility that the intense flares recorded at the sites of healed lesions were due to bystander activation of non-allergen-specific Trm, solely as the results of the inflammatory environment and cytokine signaling on CD8+ Trm [44], [45], [46].

2. The long-term persistence of chemical allergen-moieties in the skin promotes the formation of CD8+ Trm

That the organism places troops endowed with potent killing arsenal at the skin interface to fight more efficiently against re-invading microbes is easy to understand, but why it does the same to struggle against non-infectious antigens such as chemicals allergens?

The mechanisms that drive the formation and fate of skin Trm are complex and so far only partially understood. Here, we observed that the accumulation of epidermal CD8+ Trm correlated with the long-term persistence of the allergen in the skin (at least > than 30 days), with a progressive decline from day 0 to day 30. Noteworthy, the allergen persistence at day 30 was mainly observed in keratinocytes from hair follicles (**data not shown**). Since epidermal renewal needs only 8-10 days in mice, this observation strongly suggests that the allergen is stocked in long-lived slow-cycling epidermal stem cells (SC), which represent a small fraction of interfollicular epidermis and hair follicles [47], [48], [49]. Hence, one could speculate that, mimicking viral immunity controlling virus integrated in our genomes [50], allergen-specific CD8+Trm are placed here to survey [51] long-lived skin cells that continuously

present allergen-epitopes. It will be crucial to determine exactly how long an allergen persist in the skin and whether its elimination correlates with progressive attrition of specific Trm pools from the epidermis. Here, confirming previous studies, we demonstrated that while a significant number of allergen-specific Trm can be recruited to the skin upon prime and pull strategy, this number is dramatically amplified when cells recognized their cognate Ag in the skin [38], [52].

3.Killers are placed at the skin interface, but they are kept in check

The most striking finding of our study was certainly the demonstration that, despite their key role in the recurrence of the pathology, allergen-specific CD8+ Trm are tightly regulated, and express ICRs, which prevent them from unnecessary strong reactivation upon allergen exposure. Several investigators have previously reported the expression of different inhibitory molecules by CD8+ Trm, not only in cancer [53], [54], but also in viral infections [55],[56], [57], [58], autoimmune uveoretinitis [59] or at the homeostasis [60], [7]. Besides, recent comparison of gene-expression pattern of lung Trm versus circulating Tem in humans demonstrated that Trm expressed restrained genetic program with high expression of CTLA4, BTLA, LAG3, SPRY1, A2AR, PD-1, TWIST1, BACH2 genes [7]. Here, we reported that allergen-induced Trm expressed at their surface different levels of a restricted set of ICRs (PD-1, TIM-3, 2B4, but also NKG2A or high level of CD5, but not TIGIT, BTLA, LAG-3, CD160, VISTA or CTLA-4), corroborating the hypothesis that the reactivation of Trm must be tightly controled to ensure sufficient but limited reactivity. Interestingly, a previous study demonstrated that the pattern of ICR coexpression and the number of receptors simultaneously expressed by the same CD8+ Trm affect the magnitude of Trm restrained. Thus, it will be crucial to determine the environmental signals that participate in modulating the level of ICR expression and the functions of skin Trm. It was recently suggested that the acquisition of a specific inhibitory program could be associated to the affinity/avidity of the initial TCR-pMHC interactions between Trm precursors and antigen presenting cells [57]. It will be interesting to determine whether TGF-β and IL-15, two of the major cytokines involved in the generation of Trm [17], [8], and produced here by keratinocytes during the resolution of inflammation, participate in modulating their functions and the acquisition of specific ICR markers. In this study, we showed that the persisting allergen in the skin not only amplified the number of CD8+ Trm populating the epidermis, but also maintained their expression of PD-1 and TIM-3 markers. By

contrast, we noted that the 2B4 marker is acquired progressively from day 4 on, correlating with the acquisition of the genetic Trm program (Mackay Nat immunol 2013). Our observations argues against a recent study in a model of persistent murine polyomavirus infection which proposed that the maintenance of PD-1 on brain CD8+ Trm is antigen-independent and more likely linked to the epigenetic demethylation of the PD-1 promoter [58]. Moreover, we cannot exclude that the observed decline of PD-1 expression on specific T cells in absence of cognate allergen was due to a progressive attrition of PD-1+ Trm over time or due to an excessive stimulation. Therefore, further investigations are required to better understand the parameters that control the reactivation and long-term survival of specific Trm in the skin.

4. Allergen-induced skin inflammation prompts the accumulation of numerous CD8+ Trm with unrelated Ag-specificity

Finally, another interesting finding of our study was the observation that high numbers of CD8+Trm with unrelated Ag-specificity accumulate to the site of healed eczema lesions. Indeed, if we recorded approximately 30% (50% in some experiences) of allergen-specific cells using dextramers, this suggests that a majority of Trm do not recognized allergen epitopes. Several investigators reported the recruitment and differentiation in the skin of Trm with irrelevant specificity, when using prime and pull strategies (chemokine gradient signaling or non-specific inflammatory triggers) [24], [23]. Muschaweckh et al. also reported that the presentation of multiple epitopes in the skin shape the final Trm repertoire that settle inside a specific niche [40]. Our result thus questions about the nature of the non allergen-specific Trm. One could hypothesize that these cells are specific for skin flora, due to an increased presentation of commensal epitopes during skin inflammation. Epidermal CD8+ Trm recognizing bacterial Ag have been recently identified in normal skin and shown to become activated during skin injury [14]. Moreover, while allergic eczema can develop in germ-free animals [61], the magnitude of the inflammatory reaction was significantly increased when animals were bred in standard SPF-environment, suggesting that recognition of flora-specific epitopes participates in the overall reaction. Therefore, it will be important to test whether flora-specific Trm can be entrapped inside the skin during the resolution of allergic inflammation, and whether they participate in the installation of a vicious circle by increasing flare-up

reactions upon allergen re-exposure. Of note, we cannot also exclude that numbers of non-allergen Trm populating healed eczema lesions are specific for self Ag [62] or cryptic Ag [63].

In conclusion, our results provide important information for considering the unique nature of allergen-induced CD8+ Trm and the mechanisms which preserve their unwanted reactivation and subsequent development of chronic or severe skin allergy. The development of therapeutic strategies targeting the reactivation of skin Trm *in situ* via their ICRs should also open new avenues to restore tolerance in allergic individuals.

Materials & Methods:

A brief description of the Materials & Methods is provided below. See additional details in the Supplemental Materials & Methods section.

Animals and reagents

Mice, allergens (2,4-dinitrofluorobenzene [DNFB], 2,4-dinitrobenzenesulfonic acid [DNBS], or oxazolone) or mAbs used in this study are reported in the supplementary material & method section.

Animal experimental procedures were conducted with the approval of and in accordance with the guidelines for animal experiments of a local ethics committee (CECCAPP Lyon, France) and the French Ministry of Research (APAFIS#8832-201607011455243 v5).

Depletion of CD8+ T cells by anti-CD8 mAbs and DT

Acute depletion (routinely >95%, as estimated by FACS) of circulating CD8+ T cells was obtained by injecting mice intraperitoneally (i.p.) with 20µg of anti-CD8b (H35, home maid) mAbs in 200µl PBS 1x, at indicated time points before the ear painting. For maintenance of the cell depletion, injections were repeated every other day with ¼ of the initial dose of mAbs. In order to deplete skin cells expressing the iDTR, mice were given a maximum of two i.p. injections of 1µg of Diphteria Toxin (DT; Calbiochem/Millipore, San Diego – USA) in 100µl PBS 1x, at indicated time points before the ear-challenge.

Generation of mice with iDTR CD8+ Trm

Lymphopenic CD3ε-/- animals were transferred intravenously, one month before induction of DNFB CHS, with CD45+CD8b+ ($0.5x10^6$) and CD45+CD4+ ($1x10^6$) T cells FACS-sorted from naïve iDTR and WT C57BL/6 animals, respectively.

Contact hypersensitivity to DNFB and flare-up reactions

For most experiments, skin allergy was induced as previously described [64]. Briefly, animals were sensitized epicutanously on the shaved abdomen with 25µl of 0.5% DNFB on day -5, and ear (left)-challenged on day 0 with 2x5µl 0.13% DNFB. AOO vehicle was applied on the contralateral (right) ear.

Allergic animals were next retested on days 30-60 with graded doses (0.01, 0.05 and/or 0.13%) of DNFB or with 1% OXA, both on healed (left ear) and/or non-lesional (right ear) skin, as indicated.

In some experiments, DNFB-allergic C57BL/6 or reconstituted CD3ε-/- mice were depleted of circulating or of skin CD8+ T cells by anti-CD8 mAbs or DT injections, and treated with anti-PD-1 (RMP1-14, 200µg/ml), anti-TIM-3 (RMT3-23, 200µg/ml) or respective IgG2a isotype (2A3, 400 µg/ml) (all from BioXCell, West Lebanon, USA) at indicated time points.

Skin cell isolation

For epidermal and dermal cell isolation, the anterior and posterior layers of the ear biopsies were separated and incubated 1h at 37° floating with the interior side down on dispase II (1,2 U/ml; Roche, Diagnostics, Meylan, France). Epidermal and dermal layers were separated, diced into small pieces, incubated for 45min at 37°C in collagenase type 1 (121.9 CDU/ml; Sigma-Aldrich, Saint-Quentin Fallavier, France) and DNase (2 KU/ml; Sigma-Aldrich, Saint-Quentin Fallavier, France), and passed through a 100µm cell strainer to obtain single cell suspensions (ThermoFisher Scientific,Dardilly, France). Cell viability was determined by trypan blue exclusion.

Flow cytometry analysis

Flow cytometry experiments were conducted on draining lymph node (dLN), spleen or skin cells, harvested from sensitized animals. Isolated single cell suspensions were stained using fluorescently labeled mAbs and fixable viability staining was performed to exclude dead cells from the analysis. Stained cells were analyzed on a LSRFortessa flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA), and data were analyzed using FlowJo software (Treestar, Ashland, Oregon, USA).

Total cell numbers were calculated based on the numbers of viable cells determined by trypan blue exclusion during the isolation process. In some experiments, the numbers of skin Trm subsets present in the epidermis or the dermis of allergic animals were estimated using AccuCheck Counting Beads (Invitrogen, Cergy Pontoise) according to the manufacturers protocol.

DNFB-specific T-cell responses

Epidermal, dermal and dLN cells of DNFB-allergic animals stained with anti-CD4, anti-CD8b, and anti-CD45 mAbs were sorted on a BD Biosicences FACSAria, and next tested for their capacity to produce IFN-γ, IL-4 or IL-17a by ELISPOT (IFN-γ, R46A2; BD Pharmingen; IL-4, SEL421; R&D Systems, Minneapolis, USA; IL-17A eBio17CK15A5; eBioscience), in response to an overnight *in vitro* re-stimulation with DNBS-pulsed BMDCs (Vanbervliet et al., 2011). $5x10^3$ or $10x10^3$ sorted T cells were plated with BMDCs. In some experiments, rat anti-PD-1 (RMP1-14, 50 μg/ml), anti-TIM-3 (RMT3-23, 50 μg/ml) or respective IgG2a isotype (2A3, 100 μg/ml) were added to the co-cultures.

Statistical analysis

Statistics were calculated with GraphPad Prism (GraphPad Software, La Jolla, CA-USA) using the two-tailed Mann-Whitney U test, or multiple groups were compared using one-way ANOVA.

n.s. = not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

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Author contribution statements

PG, LL, VM, VL and PR carried out the experiments and analysed the data.

AN, SG, DR, AMS participated to the interpretation of the data.

JFN and MV designed the experiments and supervised this study.

PG, JFN and MV wrote the manuscript.

All authors had final approval of the submitted and published version.

Conflict of Interest: The authors declare no competing financial interests. DR and AMS are employed by Pierre Fabre. All other authors are public employees from INSERM, University and Lyon-sud hospital.

Abbreviations

ACD: Allergic contact dermatitis

Ag: Antigen

Trm: Resident memory T cells

DNFB: 2,4-dinitrofluorobenzene

dLN: Draining lymph node

IFN-γ: Interferon gamma

CHS: Contact hypersensitivity

ICR: Inhibitory checkpoint receptor

Figure 1: DNFB-specific IFN-γ-producing CD8+ Trm persist in the epidermis and dermis of healed DNFB lesions.

A. Protocol: CHS response to DNFB. C57BL/6 mice were sensitized with 0.5% DNFB on the belly at day-5 and challenged at day 0 on the left ears. CHS response, which peaked at day 2 post challenge, was resolved in approx. 10 days. Epidermis and dermis from healed lesion or non-lesional skin of DNFB-allergic mice were processed (mechanic/enzymatic digestion) and analyzed for the persistence and functions of DNFB-specific CD8+ or CD4+ Trm by FACS or ELISPOT assay, 30-60 days after induction of skin inflammation.

B. Quantification of skin T cell subsets. Histograms depict mean cell numbers ± SEM of CD8+ (left) and CD4+ (right) T cell subsets present in the epidermis or dermis of healed (black bars) or non-lesional skin (white bars) skin determined by FACS analysis.

C. Canonical Trm marker expression. Epidermal and dermal CD8+ T cells were analyzed for the expression CD103 and CD69 markers by FACS analysis. As control, CD8+ T cells from dLN were analyzed. Figures on the FACS plots and histograms depict mean values (percentage) ± SEM of cells co-expressing the two markers.

D. DNFB-specific T cells response. In some experiments, CD8+ and CD4+ T cell subsets from epidermis and dermis were FACS sorted and re-activated overnight by co-culture with haptenized (DNBS; 0.4mM) or non-haptenized (NH) BMDCs. As control, the respective cell subsets from the dLN were analyzed. The following day, the number of specific T cells producing IFN-γ was determined by ELISPOT assay. Results are expressed as the mean number of SFC/10^6 CD8+ or CD4+ T cells ± SEM.

For FACS, 4-5 individual mice per group were analyzed. For ELISpot assay, 8-10 mice were pooled.

***P<0.001. Mann-Whitney U test.

Figure 2: Epidermal CD8+ Trm accumulate from the CD8+ early effector T cells during the resolution of skin inflammation.

A. Afflux of epidermal CD8+ Trm during the course of a CHS response to DNFB. The number of epidermal CD8+ T cells (black curve) or CD8+ Trm (co-expressing CD103 and CD69 markers) (grey curve) were determined by FACS during the course of a CHS response to DNFB (white circles) detected by mouse ear

swelling test (MEST) measuring the mean ear thickness (µm). Results respectively depict mean ear eodema (µm) ± SEM or mean cell numbers/ear ± SEM at indicated time points post challenge.

B. Proliferating and cytotoxic CD8+ T cells present in the epidermis. The expression of Ki-67 and granzyme B (GrzB) on epidermal viable CD8+ T cells was determined by FACS throughout the course of a CHS response to DNFB.

C&D. **CD8+ T cells infiltrating the skin during days 0-2 gradually populate the epidermis independently of further infiltrating cells from the circulation.** DNFB-sensitized animals were depleted of circulating CD8+ T cells by i.p. injection of anti-CD8 mAbs at day 2 post DNFB challenge. The mean percentage (C) ±SEM and mean number (D) ±SEM of epidermal CD8+ Trm was analyzed FACS respectively at day 8 and day 15 post challenge. As control, cells from un-depleted mice were also analyzed.

All results are representative of 2-3 independent experiments with 4-5 mice per groups.

n.s. = not significant, *P<0.05, **P<0,01. One-way ANOVA.

Figure 3: CD8+ skin Trm mediate site and Ag-specific flare-up reactions in DNFB-allergic mice.

A) CHS response of DNFB-allergic mice to DNFB or OXA. DNFB-allergic C57BL/6 mice were re-challenged with DNFB or oxazolone (OXA) on healed skin lesions or non-lesional skin 30-60 days after the induction of skin inflammation. As control, DNFB and OXA were applied on ears of non-allergic mice. The following day, CHS reaction was determined by measuring the mean ear thickness (µm) ±SEM.

B) Depletion of CD8+ skin Trm abrogates CHS response to DNFB. Allergy against DNFB was induced in CD3ε-/- mice reconstituted with iDTR-CD8+ and WT-CD4+ T cells. 30-60 days after the induction of skin inflammation and 1 day after DT or anti-CD8 mAb i.p. injections, mice were re-challenged with DNFB. The following day, CHS reaction was determined by measuring the mean ear thickness (µm) ±SEM.

C) CHS response of DNFB-allergic mice to repeated applications of DNFB low doses. DNFB-allergic C57BL/6 mice were re-challenged with DNFB low doses (0.05% at days 0, 2, and 6, white circles; or 0.01% at days 0, 2, 6, 8, 13, and 15, grey circles). As control, DNFB-allergic mice were re-challenged with a single standard

DNFB dose (0.13% at day 0, black circles). CHS reaction was determined by measuring the mean ear thickness (µm) ±SEM on the days following the DNFB applications.

All results are representative of two independent experiments with 4-5 mice per group.

n.s.=not significant, *P<0.05, ***P<0.001, ****P<0.0001. One-way ANOVA.

Figure 4: Epidermal CD8+Trm but not circulating memory CD8+T cells express several inhibitory checkpoint receptors (ICR).

A. ICR expression by epidermal or dLN CD8+ memory T cell subsets. The expression of PD-1 (left), TIM-3 (middle) or 2B4 (right) by CD8+ Trm (black solid line) isolated from the epidermis of DNFB-allergic mice was determined by FACS >30 days after the induction of skin inflammation. As control, CD8+ Tem (dark grey, filled), Tcm (medium grey, filled) or naïve cells (light grey, filled) collected from respective dLNs were analyzed. Representative histograms are shown, with isotype controls (black and grey dotted lines for epidermis and dLN, respectively) for each ICR.

B. ICR co-expression by CD8+ Trm. The co-expression of TIM-3 and 2B4 on epidermal PD-1+ and PD-1- CD8+ Trm subsets (black) was determined. Figures on representative FACS plots depict mean values (percentage) ±SD. As control, the expression of ICRs was evaluated on dendritic epidermal T cells (DETCs, blue). PD-1 isotype staining (red) is also shown (A2).

C. ICR expression by epidermal CD8+ T cells during the course of CHS response to DNFB. The percentage of epidermal CD8+ T cells expressing PD-1, TIM-3 or 2B4 markers throughout the course of a CHS response to DNFB (from day 2 to day 30, post challenge) was determined by FACS. Results are shown as mean values (percentage) ±SEM for indicated time points.

D. Quantification of CD8+ Trm expressing ICRs during the course of CHS response to DNFB. The mean numbers ±SEM of PD-1+, TIM-3+ or 2B4+ epidermal CD8+ Trm subsets are shown throughout the CHS response to DNFB (from day 0 to day 30, post challenge).

All results are representative of two independent experiments with 4-5 mice per group or time point.

Figure 5: Blocking of ICR signaling leads to increased reactivity of CD8+ T cells *in vitro* and exacerbated flare-up reactions.

A. DNFB-specific T cells response in the presence of ICR blocking mAbs. CD8+ T cells from epidermis and dermis were FACS sorted and co-cultured overnight with haptenized (DNBS; 0.4mM) or non-haptenized (NH) BMDCs in the presence of anti-PD-1 and/or anti-TIM-3 mAb. IgG2a was added to control co-cultures. The following day, the number of specific T cells producing IFN-γ was determined by ELISPOT assay. Results are expressed as the mean number of SFC/10^6 CD8+ T cells ± SEM.

B CHS response to DNFB low doses during ICR blockage. Allergy against DNFB was induced in C57BL/6 mice. Thirty days after the induction of skin inflammation DNFB-allergic mice were re-challenged with three consecutive DNFB low doses (0.05%, at days 30, 32, 34), while ICR blockage was maintained by i.p. injection of anti-PD-1 and anti-TIM-3 mAbs at days 26, 28, 30 and 32. As control, DNFB-allergic mice were injected with IgG2a mAbs. In order to avoid effect of ICR blocking Abs, circulating non-Trm were depleted by i.p. injection of anti-CD8 and anti-CD4 mAbs. The day following DNFB applications, CHS reaction was determined by measuring the mean ear thickness (μm) ± SEM.

C. CHS response to DNFB low doses during ICR blockage in the absence of CD8+ skin Trm. Allergy against DNFB was induced in CD3ε-/- mice reconstituted with iDTR-CD8+ and WT-CD4+ T cells. 30-60 days after the induction of skin inflammation ICR blockage was initiated, as in **B**, by anti-PD-1/anti-TIM-3 mAbs i.p. injection, and 1 day after DT and/or anti-CD8 mAbs i.p. injection, mice were re-challenged with DNFB. As control, non-allergic mice were re-challenged with DNFB. The following day, CHS reaction was determined by measuring the mean ear thickness (μm) ±SEM.

For ELISpot assay, epidermal cells from 10-15 mice were pooled. For CHS response 5 mice per group were used.

All results are representative of one-three independent experiments.

n.s.=not significant, *P<0.05, **P<0.01. Mann-Whitney U test.

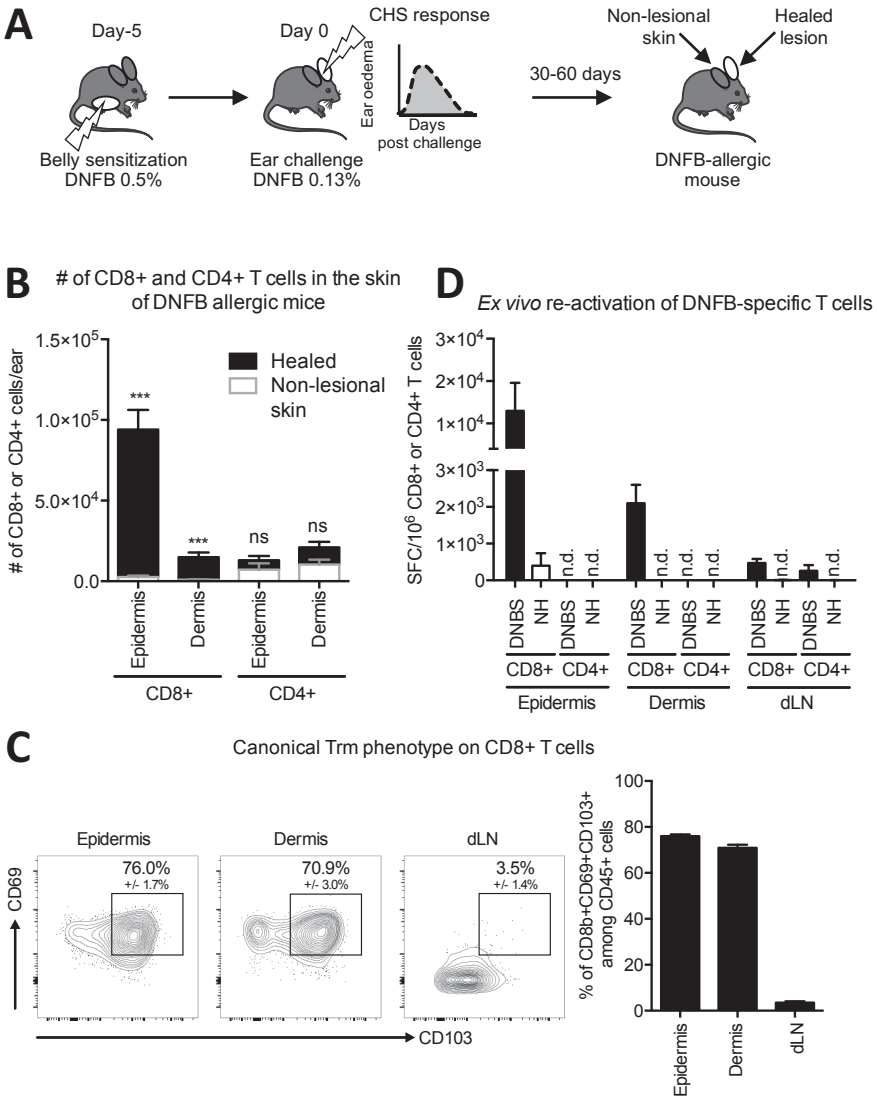

A
Day-5
Day 0
CHS response
Ear oedema
Days
post challenge
30-60 days
Non-lesional skin
Healed lesion
Belly sensitization
DNFB 0.5%
Ear challenge
DNFB 0.13%
DNFB-allergic mouse
B
# of CD8+ and CD4+ T cells in the skin of DNFB allergic mice
# of CD8+ or CD4+ cells/ear
Healed
Non-lesional skin
***
ns
Epidermis
Dermis
CD8+
CD4+
D
Ex vivo re-activation of DNFB-specific T cells
SFC/10^6 CD8+ or CD4+ T cells
n.d.
DNBS
NH
Epidermis
Dermis
dLN
C
Canonical Trm phenotype on CD8+ T cells
CD69
CD103
76.0%
+/- 1.7%
70.9%
+/- 3.0%
3.5%
+/- 1.4%
% of CD8b+CD69+CD103+ among CD45+ cells

Figure 1

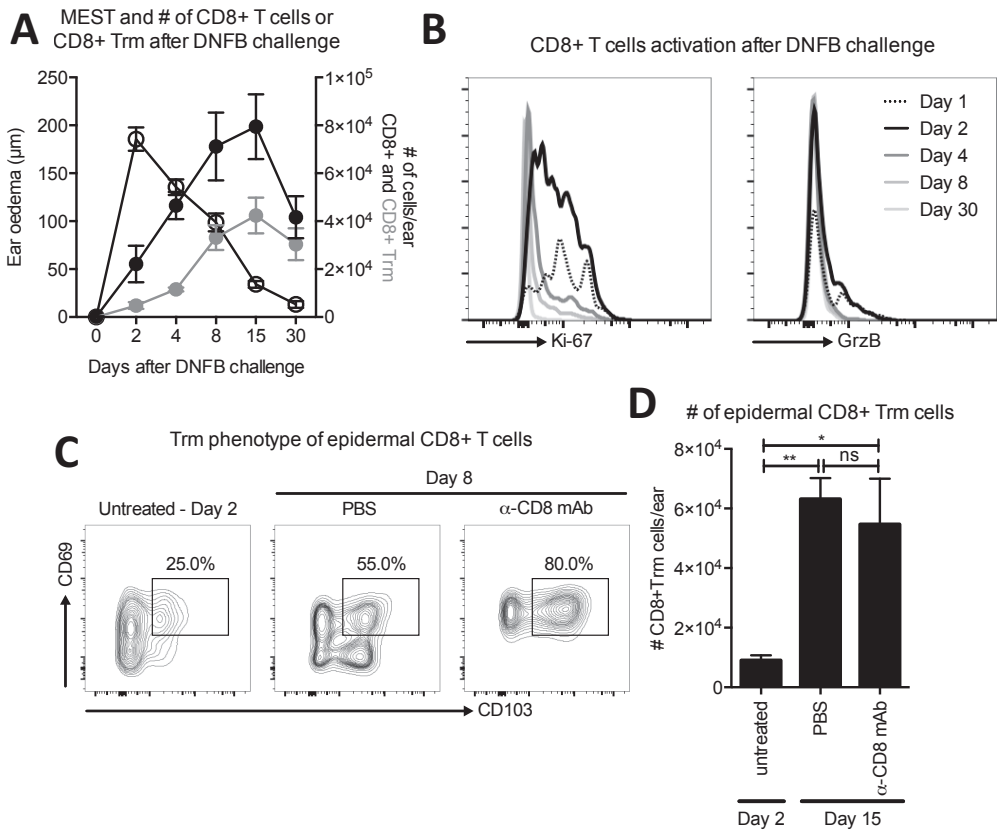

A
MEST and # of CD8+ T cells or CD8+ Trm after DNFB challenge
Ear oedema (µm)
# of cells/ear CD8+ and CD8+ Trm
Days after DNFB challenge
B
CD8+ T cells activation after DNFB challenge
Day 1
Day 2
Day 4
Day 8
Day 30
Ki-67
GrzB
C
Trm phenotype of epidermal CD8+ T cells
Untreated - Day 2
Day 8
PBS
α-CD8 mAb
25.0%
55.0%
80.0%
CD69
CD103
D
# of epidermal CD8+ Trm cells
CD8+Trm cells/ear
ns
untreated
PBS
α-CD8 mAb
Day 2
Day 15

Figure 2

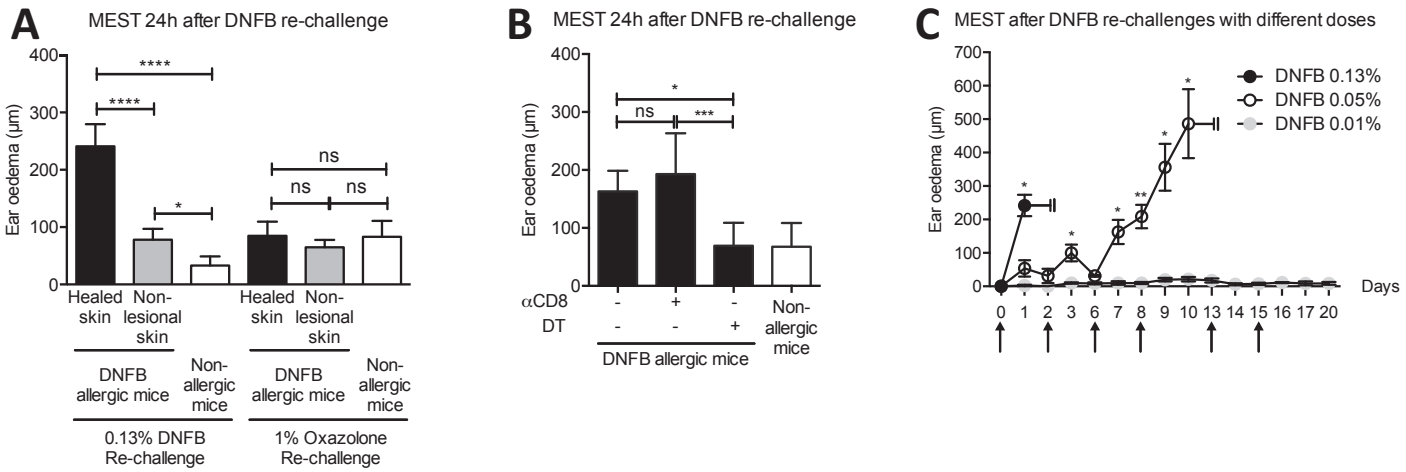

A
MEST 24h after DNFB re-challenge
Ear oedema (µm)
400
300
200
100
0
****
****
*
ns
ns
ns
Healed skin
Non-lesional skin
Healed skin
Non-lesional skin
DNFB allergic mice
Non-allergic mice
DNFB allergic mice
Non-allergic mice
0.13% DNFB Re-challenge
1% Oxazolone Re-challenge
B
MEST 24h after DNFB re-challenge
Ear oedema (µm)
400
300
200
100
0
*
ns
***
αCD8
DT
- + -
- - +
Non-allergic mice
DNFB allergic mice
C
MEST after DNFB re-challenges with different doses
Ear oedema (µm)
700
600
500
400
300
200
100
0
DNFB 0.13%
DNFB 0.05%
DNFB 0.01%
Days
0 1 2 3 6 7 8 9 10 13 14 15 16 17 20

Figure 3

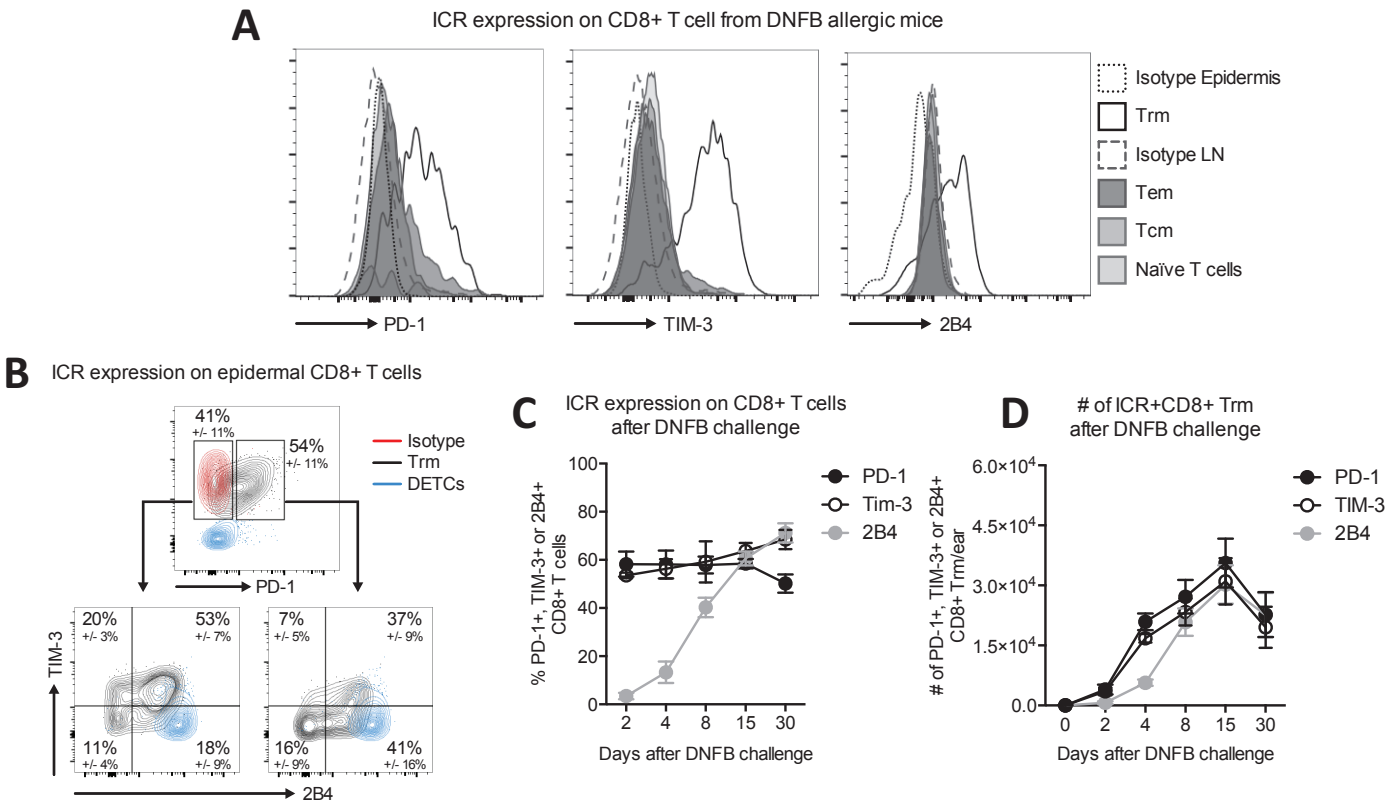

A
ICR expression on CD8+ T cell from DNFB allergic mice
Isotype Epidermis
Trm
Isotype LN
Tem
Tcm
Naïve T cells
PD-1
TIM-3
2B4
B
ICR expression on epidermal CD8+ T cells
Isotype
Trm
DETCs
41%
+/- 11%
54%
+/- 11%
PD-1
TIM-3
20%
+/- 3%
53%
+/- 7%
11%
+/- 4%
18%
+/- 9%
7%
+/- 5%
37%
+/- 9%
16%
+/- 9%
41%
+/- 16%
2B4
C
ICR expression on CD8+ T cells
after DNFB challenge
% PD-1+, TIM-3+ or 2B4+
CD8+ T cells
PD-1
Tim-3
2B4
Days after DNFB challenge
D
# of ICR+CD8+ Trm
after DNFB challenge
# of PD-1+, TIM-3+ or 2B4+
CD8+ Trm/ear
PD-1
TIM-3
2B4
Days after DNFB challenge

Figure 4

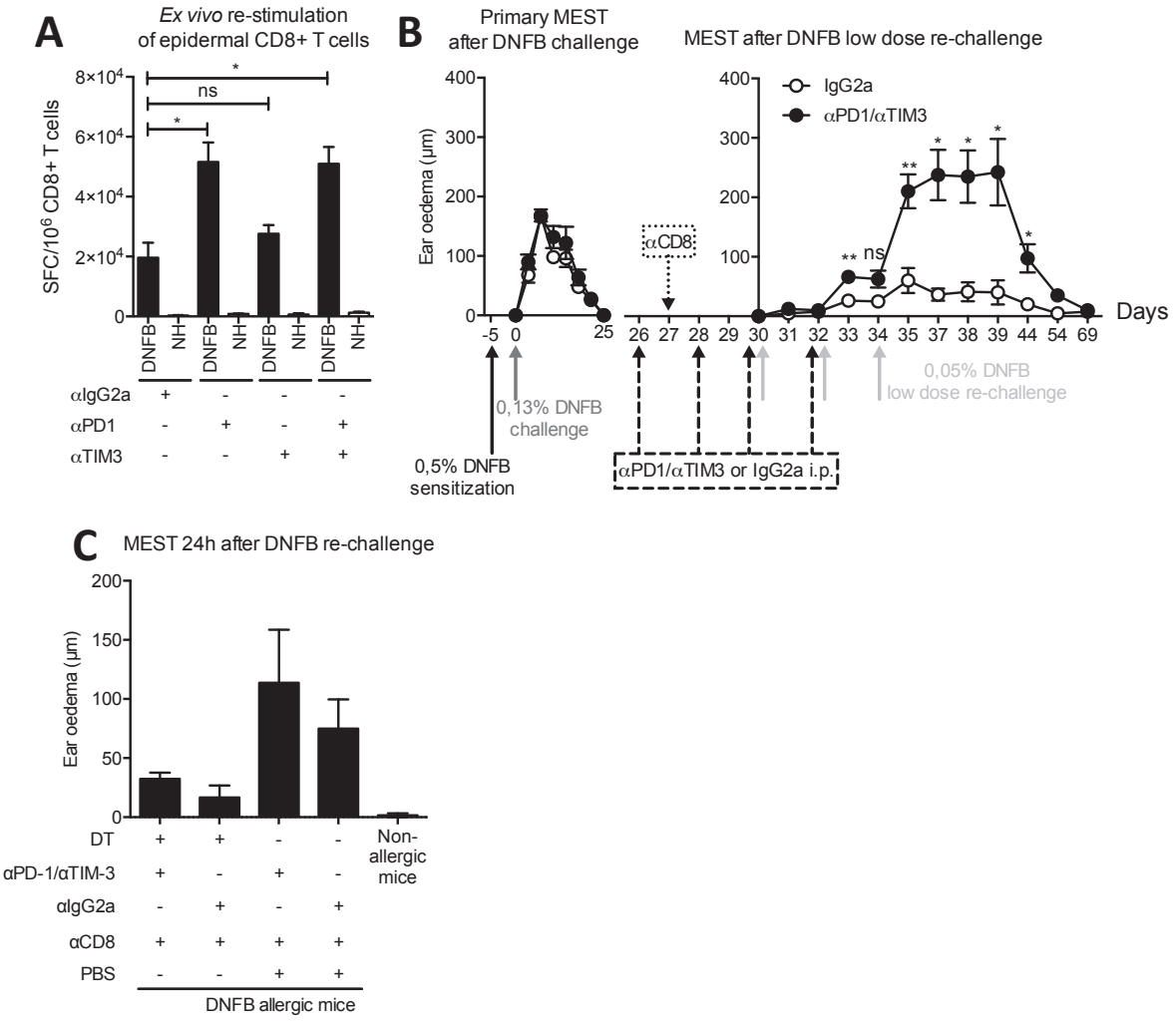

A
Ex vivo re-stimulation of epidermal CD8+ T cells
SFC/10^6 CD8+ T cells
8×10^4
6×10^4
4×10^4
2×10^4
0
ns
*
*
DNFB
NH
DNFB
NH
DNFB
NH
DNFB
NH
αIgG2a + - - -
αPD1 - + - +
αTIM3 - - + +
B
Primary MEST after DNFB challenge
MEST after DNFB low dose re-challenge
Ear oedema (µm)
400
300
200
100
0
IgG2a
αPD1/αTIM3
αCD8
** ns
**
*
*
*
*
-5 0 25 26 27 28 29 30 31 32 33 34 35 37 38 39 44 54 69
Days
0,13% DNFB challenge
0,05% DNFB low dose re-challenge
0,5% DNFB sensitization
αPD1/αTIM3 or IgG2a i.p.
C
MEST 24h after DNFB re-challenge
Ear oedema (µm)
200
150
100
50
0
DT + + - -
αPD-1/αTIM-3 + - + -
αIgG2a - + - +
αCD8 + + + +
PBS - - + +
Non-allergic mice
DNFB allergic mice

Figure 5

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Supplementary Material & Methods:

Allergens

2,4-Dinitrofluorobenzene (DNFB), dinitrobenzylsulfoxide (DNBS, the soluble equivalent of DNFB), and Oxazolone (OXA) allergens were from Sigma-Aldrich (Saint-Quentin Fallavier, France). DNFB and OXA were diluted in Acetone/Olive Oil (AOO) vehicle (4:1 vol/vol) for epicutaneous application.

H-2Kb-restricted DNP-conjugated ovalbumin octapeptides (SIIC^{D}FEKL or SIIK^{D}FEKL) were synthetized by standard Fmoc-chemistry, with DNP-Cysteine (C^{D}, 1717 CheMall Corporation, Mundelein, USA) or DNP-Lysine (K^{D}, 1717 CheMall Corporation) placed in position 4 [1]. DNP-peptides were solubilized in DMSO (Sigma) /water vehicle (3:1 vol/vol) for epicutaneous application.

mAbs used for IF staining

Mouse anti-CD3-AF700 (clone 17A2), anti-CD4-AF488 (clone RM4-5), anti-CD8b-AF647 (clone YST156.7.7), and anti-TCR$\gamma\delta$-PE (clone GLE) mAbs were purchased from Biolegend (Fell, Germany); anti-DNP-purified (polyclonal) and Chicken-anti-Rabbit secondary mAb-AF488 were purchased from Life Technologies (Saint-Aubin, France).

mAbs and dextramers used for FACS staining

Mouse anti-CD45 (clone 104), anti-TCR$\alpha\beta$ (clone H57-597), anti-TCR$\gamma\delta$ (clone eBioGL3), anti-CD4 (clone GK1.5 or RM4-5), anti-CD8b (clone H35-17.2), anti-CD69 (clone H1.2F3), anti-CD103 (clone M290), anti-granzyme-B (clone NGZB), anti-Ki67 (clone B56), anti-PD-1 (clone J43), anti-TIM3 (clone 215008), anti-2B4 (clones REA388 or m2B4(B6)458.1), anti-TIGIT (clone GIGD7), anti-LAG3 (clone C9B7W), anti-CD5 (clone 53-7.3), anti-MHC-class II (clone 2G9), anti-Sca-1 (clone D7), anti-CD34 (clone RAM34), anti-CTLA-4 (clone UC10-4F10-11) and anti-EpCAM (clone caa7-9G8) mAbs were purchased from BD Biosciences (Le Pont de Claix, France), Miltenyi Biotech (Bergish Gladbach, Germany), eBioscience (now ThermoFisher Scientific, Villebon-sur-Yvette, France), Biolegend (San Diego, California, USA) or (R&D Sytems, Lille, France).

Fluorescent H-2Kb-restricted SIIC^{D}FEKL or SIIK^{D}FEKL dextramers were synthetized

by Immudex (Copenhagen, Denmark).

Cell viability was analysed using a Fixable Viability Dye, purchased from eBioscience (San Diego, California, USA). Intracellular staining was performed using BD Cytofix/Cytoperm solutions (BD Biosciences, Franklin Lakes, New Jersey, USA).

Cell culture

Cells were cultured at 37°C, 5% CO_2, in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 µM β-mercaptoethanol (all from Life Technologies, Carlsbad, California, USA), and 10% fetal calf serum (FCS; Lonza, Basel, Switzerland).

Mouse strains and generation of iDTR mice

Female C57BL/6 mice (6-10 weeks of age) were purchased from Charles Rivers Laboratories (L'Arbresle, France). CD3ε-KO and Cre-inducible Diphteria Toxin Receptor (iDTR) mice were kindly provided by B.& M. Malissen (Centre d'Immunologie, Marseille-Luminy, France) and A. Waisman (Institute for Genetics, Cologne, Germany). DTR expression in iDTR animals is prohibited by a STOP cassette which is flanked by two loxP sites that are removable by Cre recombinase [2]. They were backcrossed with CD4-Cre animals [3], [4] to obtain CD4-Cre/iDTR animals in which all CD4+ and CD8+ T cells express the iDTR.

All experiments were conducted at the Plateau de Biologie Expérimentale de la Souris (PBES, SFR Biosciences Gerland-Lyon Sud UMS344/US8, France) under specific pathogen-free conditions with mice between 2 and 4 months of age.

BMDC generation and Ag-pulsation

Bone marrow-derived dendritic cells (BMDCs) were generated *in vitro* from C57BL/6 bone marrow progenitors with granulocyte macrophage colony-stimulating factor (GM-CSF) as previously described [5].

BMDCs were collected at day 7 of culture, and were pulsed with DNBS (1.4mM) for 15min, 37°C, pH=8, in the dark. In order to pulse BMDCs with $SIIC^{D}FEKL$ or $SIIK^{D}FEKL$ peptide, on day 6 of incubation, DNP-peptide was added to the BMDC culture at a final concentration of 5µM and incubated O/N at 37°C, CO_2 5%.

Depletion of CD4+ T cells by anti-CD4 mAbs

Acute depletion (routinely >97%, as estimated by FACS) of circulating CD4+ T cells was obtained by injecting (i.p.) mice with 100µg (and with 25µg for maintenance) of anti-CD4 mAbs (clone GK1.5; BioXCell, West Lebanon, NH, USA) in 200µl PBS 1x, at indicated time points.

Contact hypersensitivity to DNP-peptide

Mice were immunized by s.c. injection of $0.5x10^6$ DNP-peptide-pulsed BMDCs, and ear-challenge was performed with 2x10µl 10µM DNP-peptide. DMSO/water vehicle was applied on the contralateral ear.

Unspecific skin recruitment (prime-and-pull strategy) of DNP-peptide specific CD8+T cells was evaluated by challenging DNP-peptide-immunized animals with 2x5µl of 1% OXA.

DNP-peptide specific T-cell responses

Epidermal cells ($1x10^5$) from DNP-peptide allergic animals were cocultured overnight with DNP-peptide-pulsed or unpulsed BMDCs ($5x10^5$). The following day, stimulated cells were stained with fluorescent mAbs directed to CD45, CD8b, IFN-γ, Granzyme-B and PD-1 makers, and analysed by FACS.

Purification of interfollicular Keratinocytes

Single epidermal cell suspensions were stained with fluorescent mAbs directed to CD45, MHC-class II, Sca-1, CD34 and EpCAM makers, and interfollicular keratinocytes were FACS sorted as described in [6].

Quantitative RT-PCR

cDNA was prepared by using standard protocols from sorted interfollicular keratinocytes. RT-PCR was performed with platinum SYBR Green kit (Invitrogen, Cergy Pontoise, France) on an Applied Biosystems 7000 machine. A preliminary amplifcation step was conducted with kit Faststart Universal SYBR GreenMaster (Roche, Meylan, France) using a StepOne+ device and purified with ExoSap1 (ThermoFisher Scientific, Villebon-sur-Yvette, France). Relative gene expression was normalized to GAPDH and HPRT.

Primers used in this study are the followings:

GAPDH (Qiagen, Ref#PAMM-000Z

HPRT Qiagen, Ref#QT00166768
IL-15 Qiagen, Ref#QT00107653
TGF-b Qiagen, Ref#QT00145250

Preparation and IF staining of epidermal sheets

Mice were anesthetized to allow hair removal by application of depilatory cream (Veet-Reckitt Benckiser, Massy, France) for 1 minute before the skin was thoroughly washed with distilled water. Following, the mice were sacrificed by cervical dislocation and the ears were cut proximal. The ventral and dorsal leaves were separated along the cartilage and incubated for two hours at 37°C floating on 20mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, Saint-Quentin Fallavier, France) with the epidermal site up. Following incubation, the epidermal sheets were harvested, washed with PBS, and fixed in ice-cold acetone for 20 minutes followed by 2x5min washes in PBS. To block un-specific binding sites, the epidermal sheets were placed in blocking solution containing 2% bovine serum albumin (BSA) and incubated for 1h at 37°C. After incubation, the blocking solution was removed and the epidermal sheets were incubated with anti-DNP mAbs diluted in fresh blocking solution for 2 hours at 37°C followed by 3x5min washes in PBS. Next, the epidermal sheets were incubated for 2h at 37°C in fresh blocking solution containing secondary Ab followed by 3x5min washes in PBS. Finally, the epidermal sheets were incubated overnight at 4°C in fresh blocking solution containing directly labeled anti-CD8b and anti-TCRγδ, respectively. The next day, the epidermal sheets were washed 3x5min in PBS before counter staining with 4',6-Diamidino-2-phenylindole dichloride (DAPI, 1µg/ml; Sigma-Aldrich, Saint-Quentin Fallavier, France) diluted in Fluoromount medium (DAKO, Les Ulis, France). Epidermal sheets were stored at 4°C in the dark before images were acquired.

Acquisition of Light Sheet pictures

Image acquisition of the epidermal sheet stained with fluorescent labeled antibodies was perform on a Lightsheet Z.1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with 20×/1.0NA water-immersion detection optics and two-sided 10×/0.2 illumination optics, equipped with two PCO EDGE 4.2 cameras with scientific complementary metal-oxide-semiconducter (sCMOS) sensor (square pixels of 6.5× 6.5 μm side length, 1920×1920-pixel resolution, 4-channel images, 16-bit dynamic

range) (PCO AG, Kelheim, Germany). For all 3D datasets, a *z*-interval of 0.5 μ m with a zoom of 0.80 was applied. Volumetric images have a size of 553.2*553.2*1000 μ m (1920×1920×500 pixels). Directly before the acquisition, the epidermal sheets were immersed in 1% low-melting-point agarose 1% (Sigma-Aldrich, Saint-Quentin Fallavier, France) pre-warmed at 37°C and immediately loaded into a 1 mL syringe. Following solidification of the agarose, the syringe was introduced into the acquisition chamber containing 1X PBS and the images were acquired. Image acquisition was performed in triplicate. All acquired lightsheet fluorescence microscopy (LSFM) raw data images were processed using ZEN 2011 imaging software (Carl Zeiss, Oberkochen, Germany).

Images were deconvolved using classic maximum likelihood estimation (CMLE) with 20 iterations and theoretical point spread function (PSF) on Huygens software (Scientific Volume Imaging B.V., Hilversum, Netherlands). Threshold segmentation was performed using Imaris software (Bitplane, Zurich, Switzerland) for each fluorescence channel. The total volume of the imaged tissue was calculated on based on the DAPI channel. In all images we determined the mean fluorescence intensity (MFI) and the volume of DNP patterns.

Preparation and IF staining of frozen skin sections

Mouse ear biopsies were embedded in Optimal Cutting Temperature (OCT) medium (Sakukar, Leiden, Netherlands) and snap-frozen in liquid nitrogen. Frozen sections (5µm) were prepared, allowed to dry, and stored at -80°C until further use. Sections were fixed for 20min with paraformaldehyde (PFA; 2%; Sigma-Aldrich, Saint-Quentin Fallavier, France), rehydrated with PBS, and permeabilized with TritonX100 (0.1%; Sigma-Aldrich, Saint-Quentin Fallavier, France) before blockage with a solution of Bovine serum albumin (BSA, 1%; Sigma-Aldrich, Saint-Quentin Fallavier, France) and normal goat serum (10%; Sigma-Aldrich, Saint-Quentin Fallavier, France). Following, the sections were incubated for 2h at room in a wet chamber with a mixture of primary mAbs (see list above) diluted in blocking solution at a previously determined optimal concentration. After thoroughly washing with PBS, fluorescently labeled secondary Abs diluted in blocking solution at a previously determined optimal concentration were added on to the sections and incubated for 1.5h at room in a wet chamber. Sections were washed thoroughly washed with PBS and Fluoromount

medium (DAKO, Les Ulis, France) containing 4',6-Diamidino-2-phenylindole dichloride (DAPI, 1µg/ml; Sigma-Aldrich, Saint-Quentin Fallavier, France) was added before placing cover slips. Image acquisition of the frozen sections stained with fluorescently labeled antibodies was performed at a Confocal spectral Zeiss LSM710 microscope.

Acquisition and processing of IF pictures

Image acquisition of whole frozen sections was performed on a LSM710 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with an inverted stand with 20X/NA=0.5 air objective and numerical zoom 0.6, Acquired images have a size of 708.3x708.3µm. All acquired raw data images were processed using ZEN 2011 imaging software (Carl Zeiss Microscopy GmbH, Jena, Germany).

Image analysis

Acquired images were analyzed using ImageJ software (NIH Image, Bethesda, MD, USA). Briefly, epidermis and dermis surface (μm^2) were measured using the roi manager based on nuclear staining with DAPI, Thresholds were applied on splitted channels of utilized flurophores in the respective staining (DAPI, AF488, AF594/PE, AF647, and AF700) and converted into binary pictures. The number of DAPI+ and AF700+, AF594+/PE+ or AF647+ cells were determined by using the automated "Analyze particle" method for events bigger that 10μm^2.

Supplementary figure legends

Figure S1: Location and quantification of persisting CD8+ and CD4+ T cells in the skin of healed DNFB lesions.

Frozen sections obtained from the healed or naïve skin of DNFB-allergic mice 30-60 days after induction of skin inflammation were stained with fluorescent anti-CD3 (yellow), anti-CD8b (red) and anti-CD4 (green) mAbs prior to analysis by confocal microscopy. The epidermal-dermal junction is indicated by the white dotted line on each picture. Red arrows indicate the location of CD8+ T cells in merged pictures. As control, skin from active lesions 2 days after the induction of skin inflammation was analyzed.

Histograms depict mean cell numbers ±SEM of CD3+ (black), CD4+ (grey), and CD8+ (white) T cells present in the epidermis (left) and dermis (right) of skin sections. Three slide of each animal were analyzed and three animals per group and time point were used.

Figure S2: The persistence of allergen-specific Trm tracked in a model of DNP-peptide-induced skin inflammation.

A. CHS response to DNP-peptide. Naïve C57BL/6 mice were immunized i.p. with DNP-peptide pulsed-BMDCs and challenged 5 days later on the left ear with the DNP-peptide in DMSO. As control, DMSO vehicle was applied on the contralateral right ear. CHS reaction was determined by mouse ear swelling test (MEST) measuring the mean ear thickness (µm) ± SD at indicated time points post challenge.

B. Detection of specific CD8+ Trm using DNP-peptide dextramers. The presence of DNP-peptide specific CD8+ Trm in the epidermis was examined by FACS 30-60 days after induction of skin inflammation, using fluorescently labeled dextramers loaded with the respective DNP-peptide or control peptide. As control, CD8+ T cells from dLN were analyzed. Numbers on the FACS plots and histograms depict mean values (percentage) ± SD of CD8+/dextramer+ T cells.

C. *Ex-vivo* reactivation of specific Trm. Epidermal cells isolated from healed lesions were re-activated overnight with pulsed (DNP-peptide; 5 mM, red lines) or un-pulsed BMDCs (black lines). The following day, the expression of IFN-γ, granzyme B (GrzB), and PD-1 on viable CD8+ T cells was analyzed by FACS. Numbers on the FACS plots depict mean values (percentage) ± SD of cells co-expressing PD-1 and IFN-γ or granzyme B.

All results are representative of two independent experiments with 4-5 mice per group.

Figure S3: Acquisition of Trm phenotype in epidermal CD8+ T cells during the course of a CHS response to DNFB.

A. The acquisition of Trm phenotype by epidermal CD8+ T cells was analyzed by FACS during the course of a CHS response to DNFB. Figures on representative FACS plots, and the line graph (black line) depict mean values (percentages) of viable epidermal CD8+ T cells co-expressing CD103 and CD69.

B. Part of the proliferating Ki67+ CD8+T cells express CD69 and CD103 at day 4 and day 8 post DNFB sensitization. The acquisition of Trm phenotype by Ki67+ (black) and Ki67- (blue) CD8+ T cells was analyzed by FACS during the course of a CHS response to DNFB. Numbers and the line graph (black line) on representative FACS plots depict mean values (percentages) of Ki67+CD8+ T cells co-expressing CD103 and CD69.

All results are representative of two independent experiments.

*P<0.05, **P<0.01, Mann-Whitney U test.

Figure S4: Depletion of circulating CD8+ T cells accelerates the resolution of skin inflammation. DNFB-sensitized mice were injected with anti-CD8+ mAbs or PBS at day 2 post DNFB challenge. Results depict the mean ear thickness (μm) ± SD at indicated time points post challenge.

Results are representative of two independent experiments with 4-5 mice per group or time point.

Figure S5: TGF-b and IL-15 gene expression by interfollicular keratinocytes during the course of DNFB CHS.

Interfollicular keratinocytes were extracted and FACS sorted from the epidermis at day 2, 4, 8 and 23 post DNFB challenge. The expression of TGF-β or IL-15 mRNA transcripts were quantified by qRT-PCR. Results are representative of one preliminary experiment in which 10 mice were pooled.

Figure S6: Quantitative estimation of DNP moieties present in the skin during

the course of a CHS response to DNFB.

Illustrative and quantitative analysis of DNP moieties present in the skin during the course of a CHS response to DNFB.

A. Threshold segmentation images. Epidermal sheets collected before (a) and 2 (b), 8 (c), 15 (d) or 30 (e) days after DNFB challenge of DNFB-sensitized animals were explored for the presence of DNP moieties (green), CD8b+ T cells (red), and dendritic epidermal T cells (DETCs, yellow) by standard immunofluorescence staining. Representative photographs acquired on a Lightsheet Z.1 microscope after threshold segmentation using Bitplane Imaris are shown. As control, an anti-DNP isotype staining was performed on epidermal sheets collected at 8 days post DNFB challenge (f).

B. Quantification. The percentage of epidermal area stained by anti-DNP mAbs was calculated throughout the CHS response to DNFB by determining the total epidermal area (μm^3) analyzed based on nuclear staining with DAPI and the DNP-stained area (μm^3). For each individual mouse 3-4 pictures were analyzed.

All results are representative of two independent experiments with 3 mice per time point.

Figure S7: Epidermal DNP-specific CD8+ Trm recruited through prime and pull strategy do not accumulate in the healed lesion.

Quantification of DNP-specific CD8+ Trm. Naïve C57BL/6 mice were immunized i.p. with DNP-peptide pulsed-BMDCs and challenged 5 days later on the left ear with the DNP-peptide in DMSO or with an irrelevant hapten, oxazolone (OXA).

The number of DNP-peptide specific CD8+ T cells recruited in the epidermis via the Ag-specific or the non Ag-specific approach (the latter being referred to as “prime and pull strategy”) was measured by FACS using fluorescently labeled dextramers loaded with the respective DNP-peptide. Results are depicted as mean values (percentage) ±SEM.

Results are representative of two independent experiments with 4-5 mice per group.

Figure S8: Sustained CHS responses to DNFB after the depletion of circulating CD8+ and/or CD4+ T cells.

A. CHS responses to DNFB after depletion of circulating T cells. Allergy against DNFB was induced in C57BL/6 mice. 30-60 days after the induction of skin

inflammation and 1 day after anti-CD8 and/or anti-CD4 mAb i.p. injections, DNFB-allergic mice were re-challenged with DNFB. As controls, non allergic animals were challenged with DNFB. The following day, CHS reaction was determined by measuring the mean ear oedema (µm) ±SEM.

B. Detection of CD8+ and CD8- T cells after anti-CD8 and/or anti-CD4 mAb administration. DNFB-allergic mice were injected i.p. with anti-CD8 and/or anti-CD4 mAb and skin lesions were analyzed by FACS for the presence of CD8+ and non-CD8+ T cells in the epidermis and dermis. As control, circulating cells from the dLNs of the same mice were analyzed. Figures on representative FACS dot plots depict mean values (percentage) ±SEM of CD8+ and CD8- T cells.

All results are representative of two independent experiments with 4-5 mice per group.

Figure S9: Depletion of CD8+ skin Trm using an original iDTR-based approach. Lymphopenic CD3ε-/- mice were reconstituted with iDTR-CD8+ and WT-CD4+ T cells isolated by FACS sort from iDTR/CD4cre and C57BL/6 mice, respectively, prior to sensitization and challenge with DNFB. 30-60 after the induction of skin inflammation, in such way reconstituted DNFB-allergic mice were injected i.p. with DT or anti-CD8 mAb and, healed skin lesions were analyzed by FACS for the presence of CD8+ and non-CD8+ T cells in the epidermis and dermis. As control, circulating cells from the spleen of the same mice were analyzed. Figures on representative FACS dot plots (A) and histograms (B) depict mean values (percentage) ±SEM of CD8+ and CD8- T cells.

All results are representative of two independent experiments with 4-5 mice per group.

Figure S10: Phenotyping epidermal and dLN CD8+ memory T cell subsets. The expression of LAG3, CD5, TIGIT and CTLA4 by CD8+ Trm (solid black line) isolated from the epidermis of DNFB-allergic mice was determined by FACS 30-60 days after the induction of skin inflammation. As control, CD8+ Tem (dark grey, filled), Tcm (medium grey, filled) or naïve cells (light grey, filled) collected from respective dLNs were analyzed. Representative histograms are shown, with isotype controls (dotted black and grey lines, respectively for epidermis and dLNs) for each ICR.

Figure S11: ICR expression by epidermal CD8+ Trm tracked in a model of DNP-peptide-induced skin inflammation.

The co-expression of TIM-3 and CD244 on epidermal PD1+ and PD1- DNP-specific CD8+ Trm subsets (black) was determined by FACS. DNP-specific CD8+ Trm were tracked using fluorescently labeled dextramers loaded with the respective DNP-peptide. Figures on representative FACS plots depict mean values (percentage) ±SD. As control, the expression of ICRs was evaluated on dendritic epidermal T cells (DETCs, blue). PD-1 isotype staining (red) is also shown.

Results are representative of two independent experiments with 4-5 mice.

Figure S12: Epidermal DNP-specific CD8+ Trm recruited through prime and pull strategy do not accumulate in the healed lesion and express low level of TIM-3 and PD-1 markrs at day 40 post DNFB challenge

As in Figure S7, naïve C57BL/6 mice were immunized i.p. with DNP-peptide pulsed-BMDCs and challenged 5 days later on the left ear with the DNP-peptide in DMSO or with an irrelevant hapten, oxazolone (OXA).

A&B) ICR expression by DNP-specific CD8+ Trm. The expression level of PD-1, TIM-3, and 2B4 by DNP-specific CD8+ Trm after Ag-specific (DNP-peptide, light grey filled) or non Ag-specific (Oxazolone, dark grey filled) recruitment was determined FACS. Representative histograms at 40 days with isotype control (black line) for each ICRs (A) and mean values of respective geometric mean fluorescence intensity (GMFI) ±SEM (B) are shown.

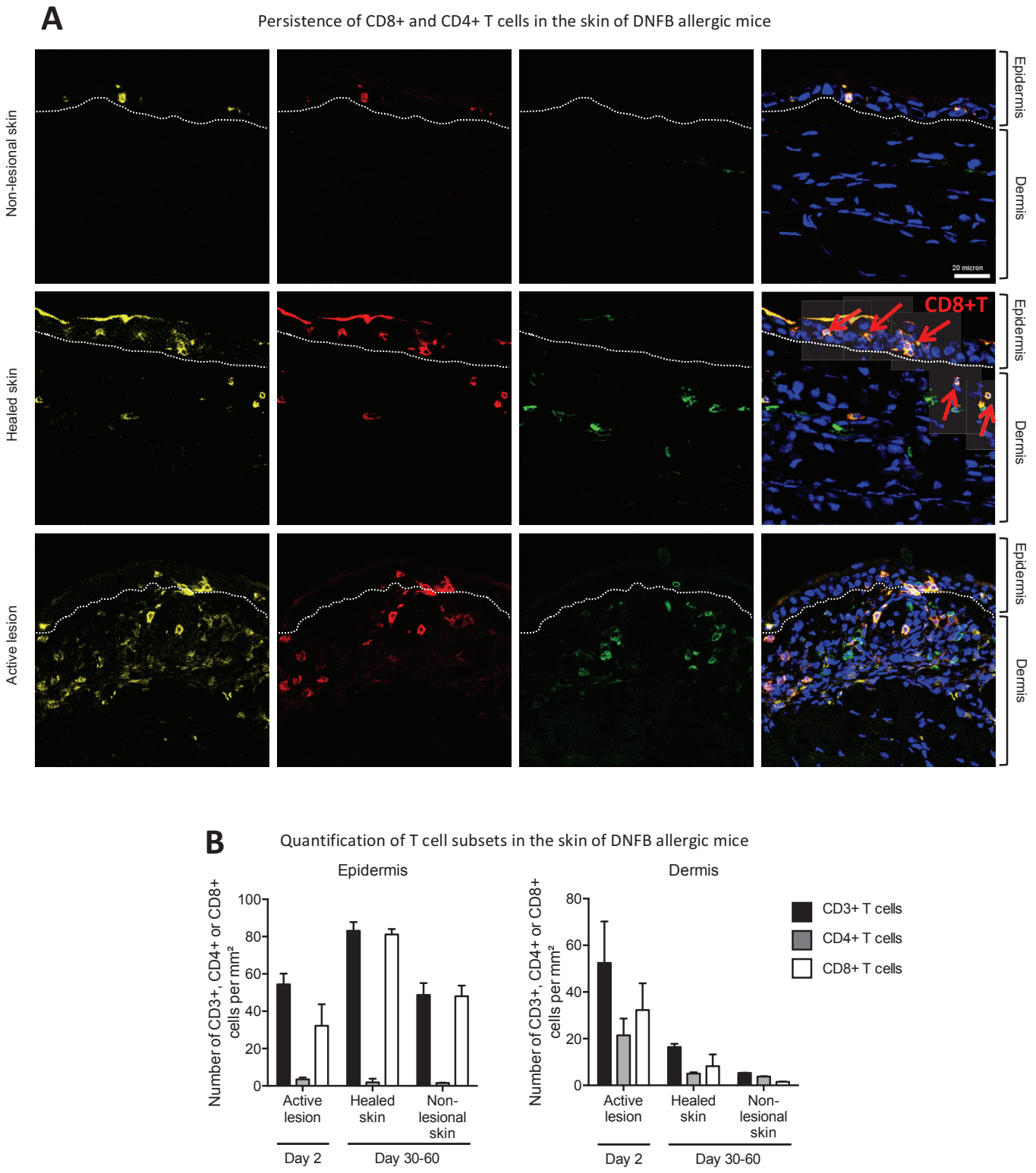

A
Persistence of CD8+ and CD4+ T cells in the skin of DNFB allergic mice
Non-lesional skin
Healed skin
Active lesion
Epidermis
Dermis
20 micron
CD8+T
B
Quantification of T cell subsets in the skin of DNFB allergic mice
Epidermis
Dermis
Number of CD3+, CD4+ or CD8+ cells per mm²
CD3+ T cells
CD4+ T cells
CD8+ T cells
Active lesion
Healed skin
Non-lesional skin
Day 2
Day 30-60

Figure S1

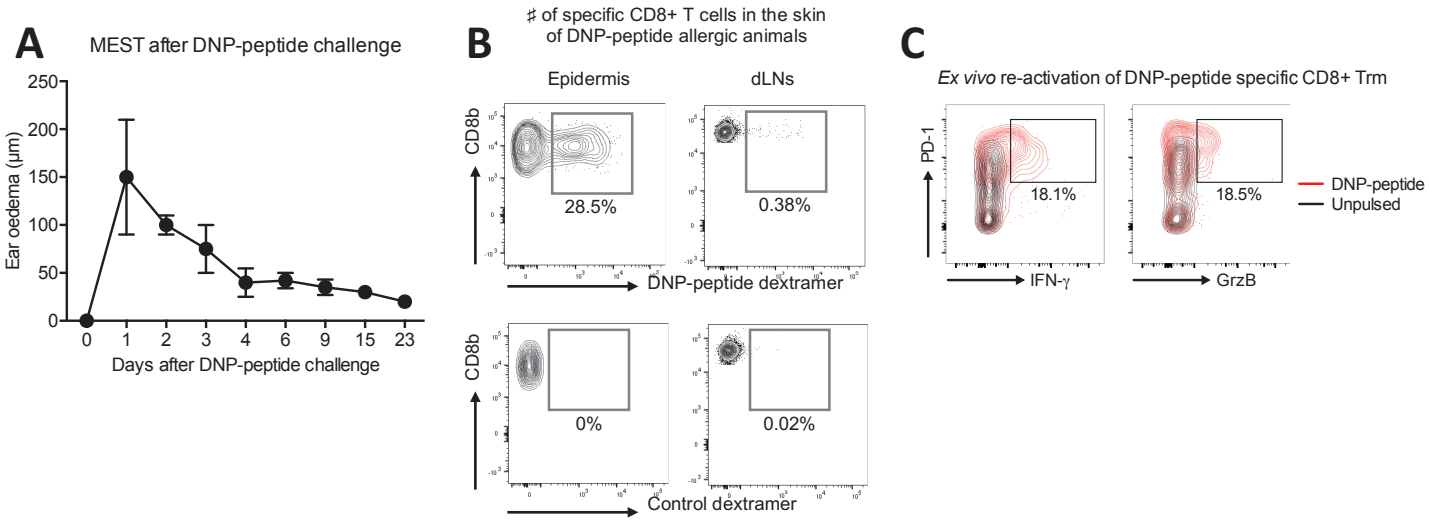


Figure S2

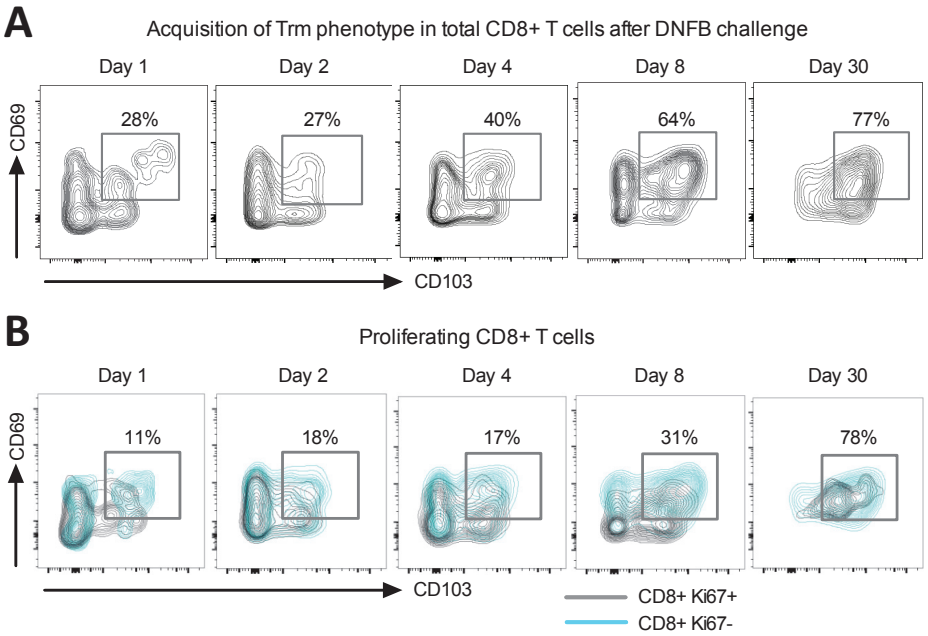


Figure S3

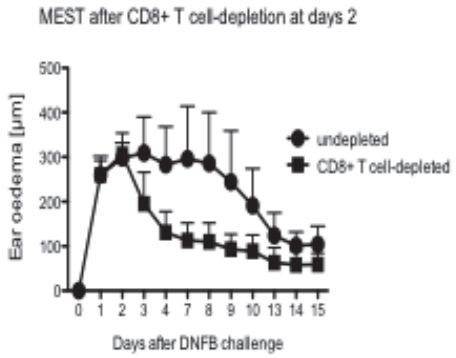


Figure S4

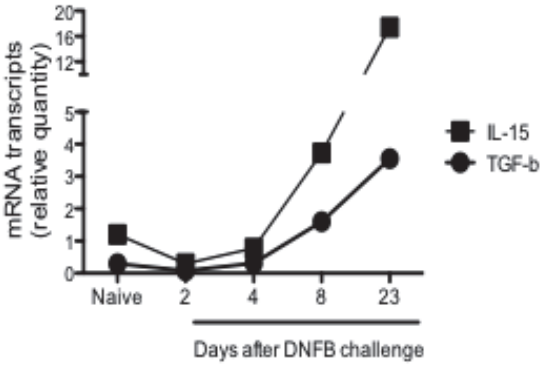
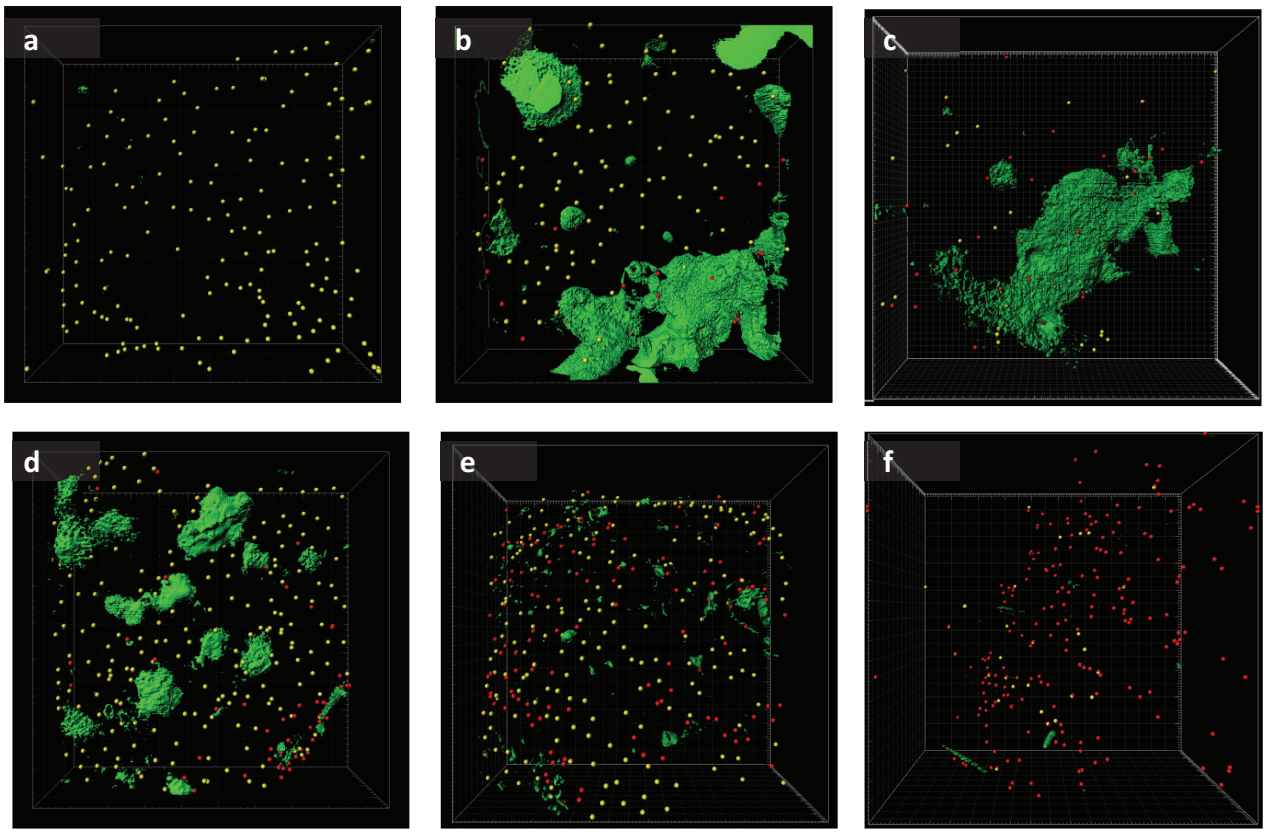


Figure S5

A

Longterm persistence of DNP moities in the skin



B

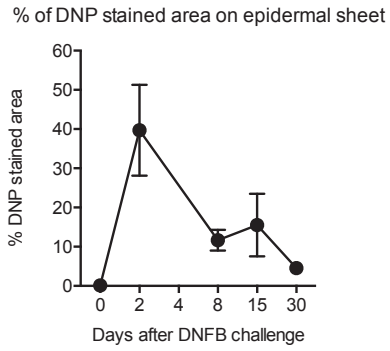


Figure S6

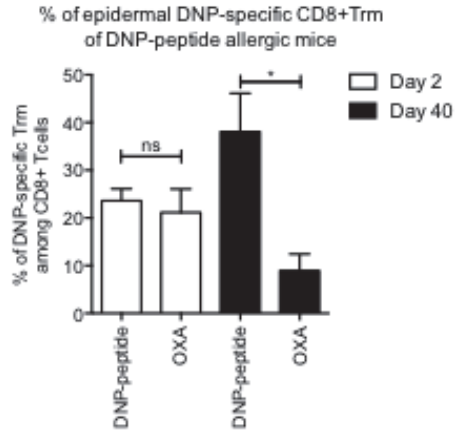
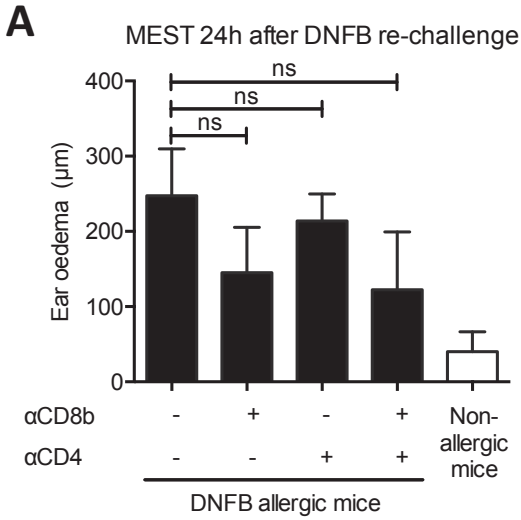


Figure S7



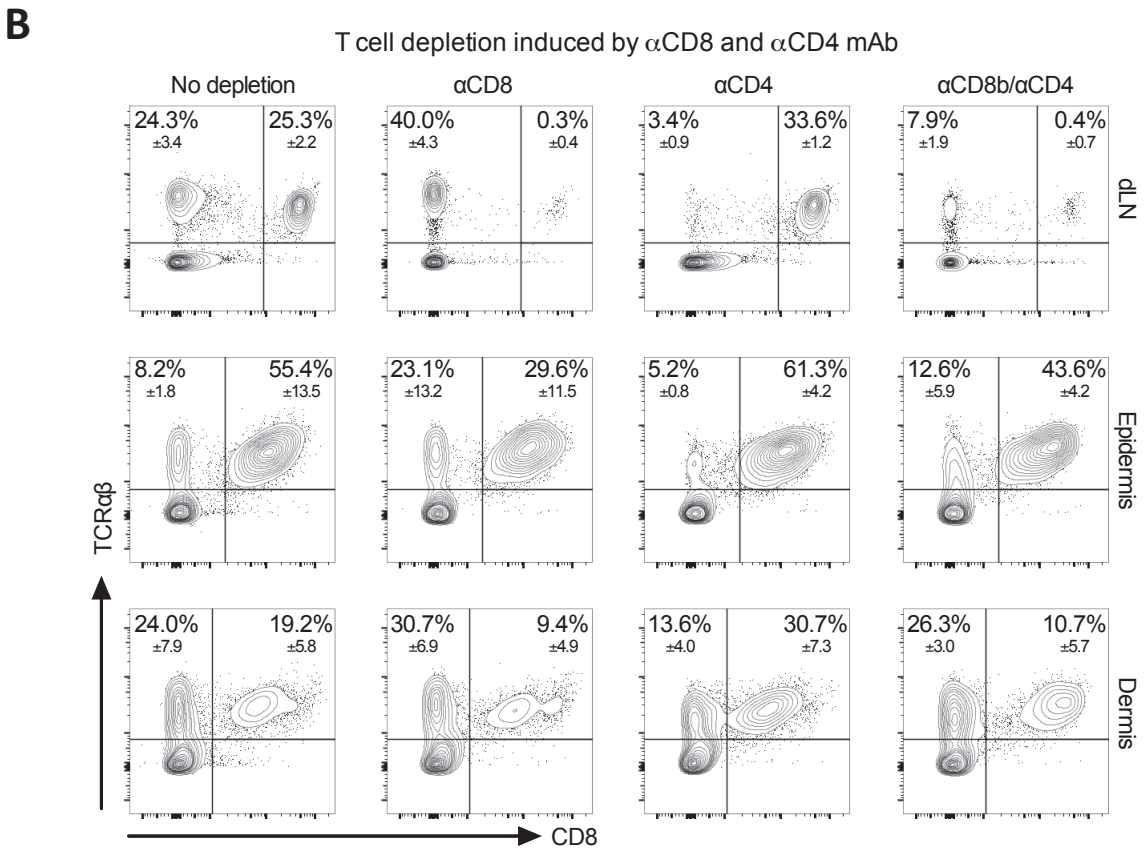


Figure S8

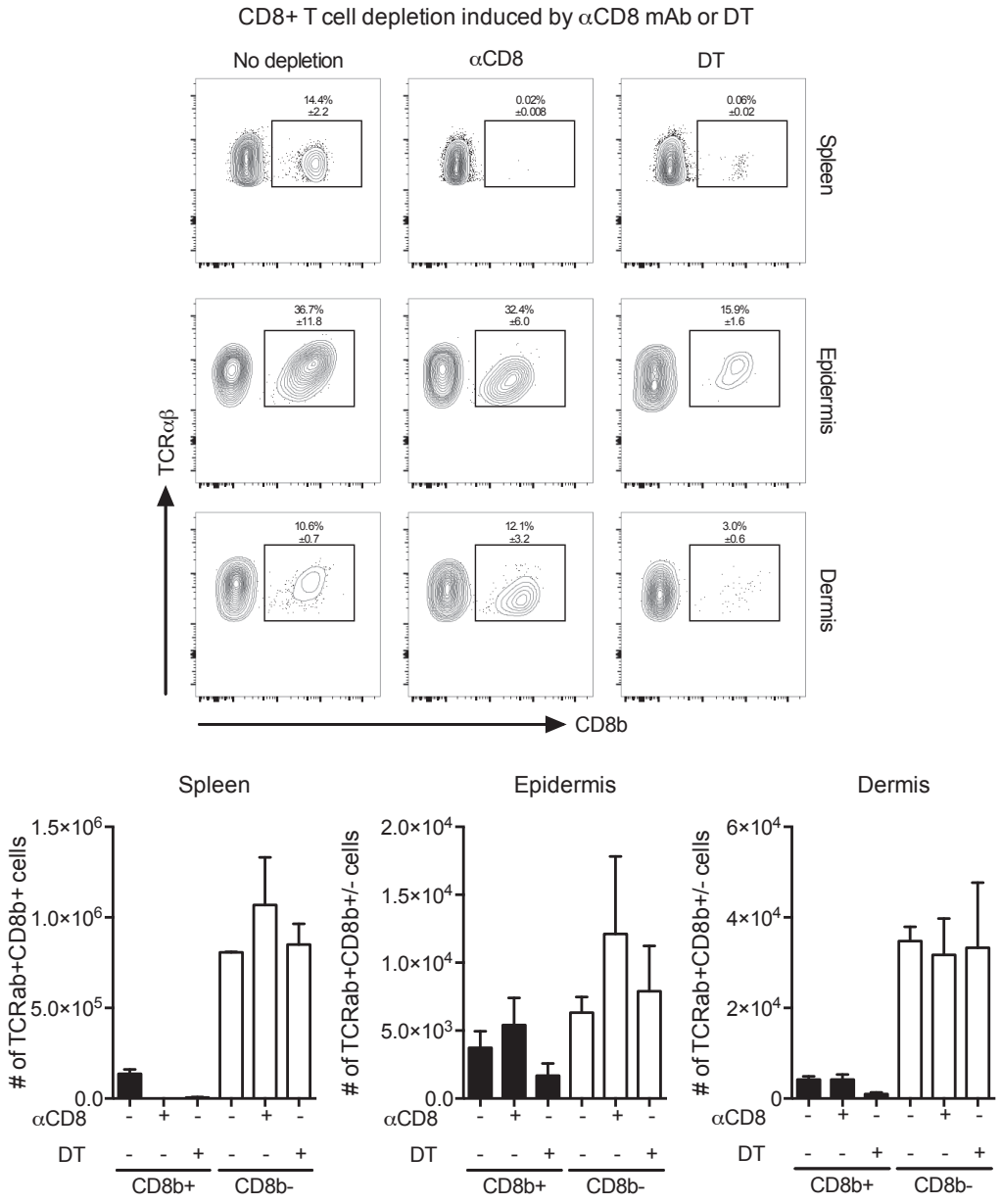

CD8+ T cell depletion induced by αCD8 mAb or DT
No depletion
αCD8
DT
14.4% ±2.2
0.02% ±0.008
0.06% ±0.02
36.7% ±11.8
32.4% ±6.0
15.9% ±1.6
10.6% ±0.7
12.1% ±3.2
3.0% ±0.6
Spleen
Epidermis
Dermis
TCRαβ
CD8b
Spleen
Epidermis
Dermis
# of TCRab+CD8b+ cells
# of TCRab+CD8b+/- cells
# of TCRab+CD8b+/- cells
1.5×10^6
1.0×10^6
5.0×10^5
0.0
2.0×10^4
1.5×10^4
1.0×10^4
5.0×10^3
0.0
6×10^4
4×10^4
2×10^4
0
αCD8
DT
CD8b+
CD8b-

Figure S9

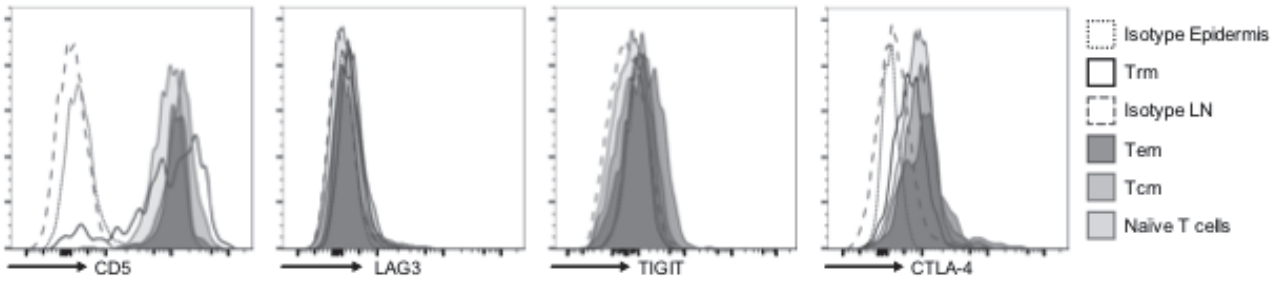


Figure S10

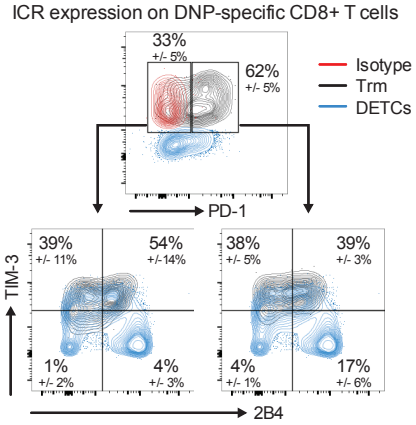


Figure S11

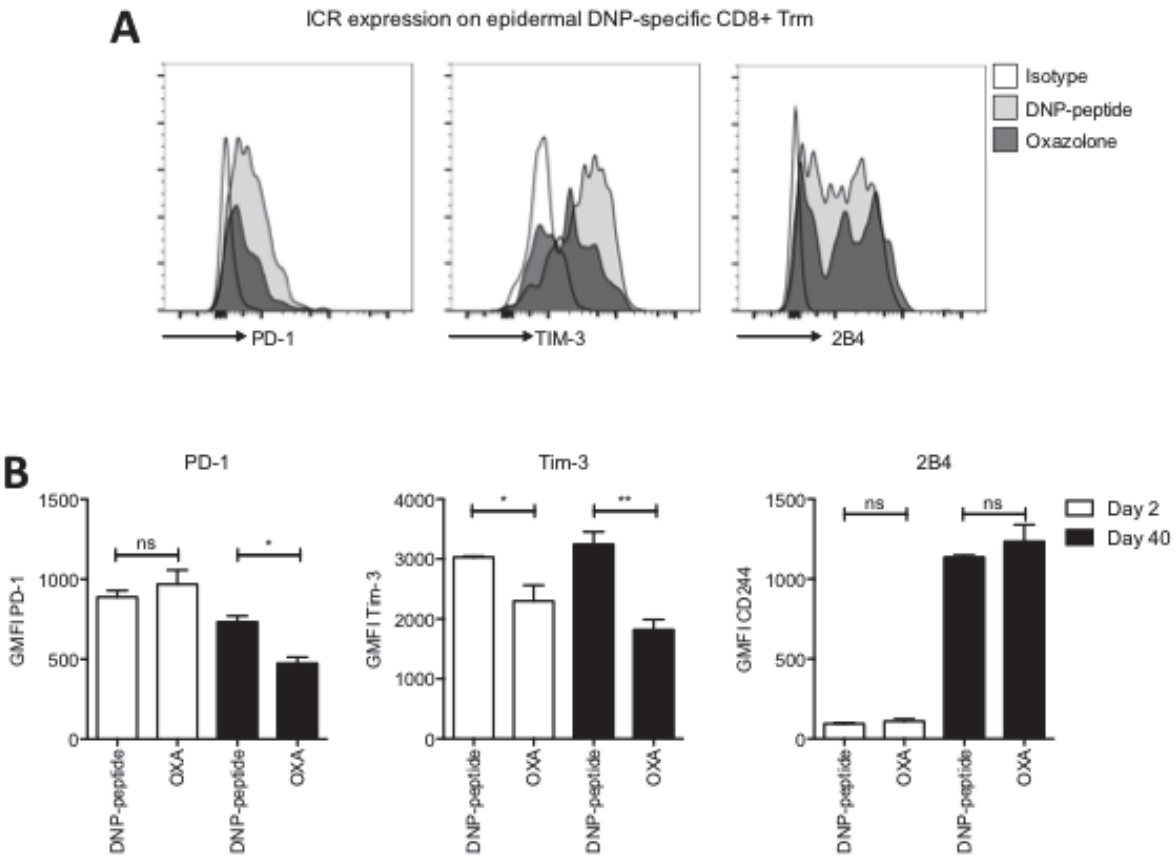

A
ICR expression on epidermal DNP-specific CD8+ Trm
Isotype
DNP-peptide
Oxazolone
PD-1
TIM-3
2B4
B
PD-1
Tim-3
2B4
GMFI PD-1
GMFI Tim-3
GMFI CD244
ns
*
**
Day 2
Day 40
DNP-peptide
OXA

Figure S12

Supplementary references

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Part 4: Complementary data – Trm cells in AD-patients and an AD-like mouse model

In Addition to our work documented in the study „Inhibitory checkpoint receptors PD-1 and TIM-3 control CD8+ Trm reactivation to prevent skin allergy“, certain complementary experiments were performed in samples obtained from clinical AD-patients and in an AD-like mouse model. Following I will present the results from these experiments.

24 Complementary data – AD-like mouse model

24.1 Persistence of CD8+ Trm cells in the skin of healed *Der f*-lesions

Using the CHS model we determined that hapten-specific CD8+ Trm cells are dominantly represented in the epidermis and dermis of DNFB-allergic mice. To confirm their presence in another pre-clinical mouse model of skin allergy, we induced skin inflammation in mice by repeated epicutaneous applications of *Der f* extract once per week for 6-10 consecutive weeks. Approx. 30-60 days after the resolution of this initial skin inflammation the mice were considered to be allergic against *Der f* and we analyzed cells isolated from the epidermis and dermis of the healed skin by fluorescence-activated cell sorting (FACS). Similarly to our findings from the DNFB-allergic mice, we detected a significant accumulation of CD8+ T cells in the epidermis and dermis of healed skin lesions compared to non-lesional skin (Fig. 19A, left). Also no enrichment of CD4+ T cells could be in the epidermis of *Der f*-allergic mice. However, in the dermis of *Der f*-allergic mice a significant accumulation of CD4+ T cells was detected which was not observed in the DNFB model skin (Fig. 19A, right). Of note, due to the down-regulation of CD4 molecules on T cells upon enzymatic separation procedures of dermis and epidermis with dispase II, CD4+ T cells were determined by gating on CD3+, CD45+, CD8b-, Foxp3- cells. Likewise, the expression of the canonical Trm cell markers CD69 and CD103 was detected on CD8+ T cells of *Der f*-allergic mice which was equally high with approx. 80% and 78% in epidermis and dermis, respectively, and almost absent in the dLN skin (Fig. 19B).

24.2 Specific T cells in AD

Next, in a preliminary experiment, we analyzed the proportion of *Der f*-specific cells inside the skin of healed lesion. Therefore, extracted T cells from whole skin ear biopsies and draining lymph nodes were re-stimulated *ex vivo* with *Der f*-pulsed bone marrow-derived DCs (BMDCs) or un-pulsed BMDCs as control and the next day the number of spot forming cells

(SFCs) producing IFN-γ (Fig. 20A) or IL-5 (Fig. 20B) was determined by enzyme-linked immuno spot (ELISpot) assay. We detected that approx. 0.02% (200 SFC/10^6 cells) and 0.015% (150 SFC/10^6 cells) of T cells from healed skin lesion and draining lymph node produced IFN-γ and IL-5, respectively, in response to re-stimulation with *Der f*. In comparison to the DNFB model this is only a low enrichment of Ag-specific T cells within the healed skin lesion.

Thus, our results confirm that in *Der f*-lesions similarly to DNFB-lesions allergen-specific T cells are enriched. Although we were not able yet to confirm our results on sorted CD8+ Trm cells, it is most likely that due to their abundance they allocate for most oft the cytokine producing cells. Nevertheless, also in the *Der f* model we detect a vast number of resident cells which did not response to allergen-specific re-stimulation.

24.3 Persisting Trm cells in the skin mediate site- and dose-dependent responses to *Der f*

Confirmative to our results in DNFB-allergic mice, *Der f*-allergic mice that were re-challenged on healed *Der f*-lesions (30-60 days after the resolution of the skin inflammation) developed a robust flare-up. In contrast, a single application of *Der f* extract on non-lesional skin failed to induce any flare-up reaction, since the reaction was similar to the one recorded after *Der f* application on skin of non-allergic mice (Fig. 21A). At this time point we can only speculate whether such response might be allergen-specific, since we do not have a comparative experiment including an irrelevant allergen. Moreover, we observed that the flare-up reactions to *Der f* underlie certain regulations since the repeated application of *Der f* low doses (37.5μg/ear side) once a week for five consecutive weeks failed to induce flare-up reactions comparatively to *Der f* high doses (375μg/ear side) (Fig. 21B).

Here, our results also suggest that *Der f*-specific Trm cell inside healed skin lesions are subjected to regulatory mechanisms and a certain threshold of allergen concentration during the re-challenge has to be overcome in order to be sufficient for their activation.

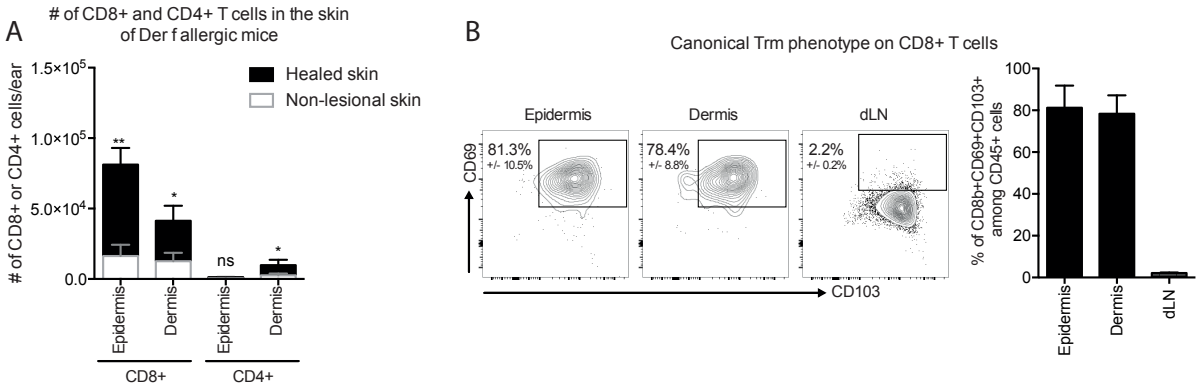


FIGURE 20: CD8+ TRM PERSIST IN THE EPIDERMIS AND DERMIS OF HEALED DER F LESIONS.

Epidermis and dermis from healed lesion or non-lesional skin of Der f-allergic mice were processed mechanic/enzymatic digestion) and analyzed for the persistence and functions of CD8+ or CD4+ Trm by FACS, 30-60 days after induction of skin inflammation.

A) Quantification of skin T cell subsets. Histograms depict mean cell numbers ± SEM of CD8+ (left) and CD4+ (right) T cell subsets present in the epidermis or dermis of healed (black bars) or non-lesional (white bars) skin determined by FACS analysis.

B) Canonical Trm marker expression. Epidermal and dermal CD8+ T cells were analyzed for the expression CD103 and CD69 by FACS analysis. As control, CD8+ T cells from dLN were analyzed. Numbers on the FACS plots and histograms depict mean values (percentage) ± SEM of cells co-expressing the two markers.

*All results are representative of two independent experiments. n.s. – not significant, **P<0.01, Mann-Whitney U test.*

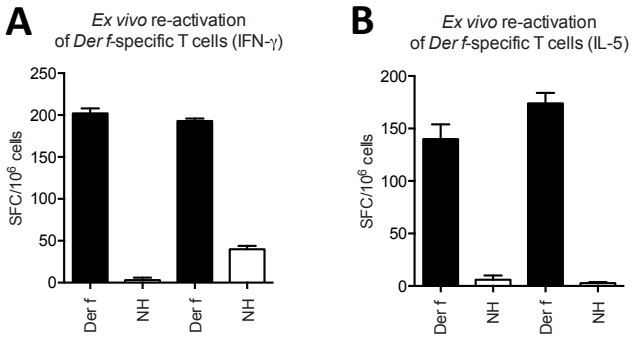


FIGURE 19: DER F-SPECIFIC T CELLS PERSIST IN THE SKIN AND DLN OF DER F-ALLERGIC MICE.

Der f-specific T cell responses were determined in total cells isolated from whole ear biopsies after overnight co-culture with pulsed (Der f; 10mg/ml) or un-pulsed (NH) BMDCs. As control, total cells from dLN were analyzed. The following day, the number of specific T cells producing IFN-g (A) or IL-5 (B) was determined by ELISpot assay. Results are from a preliminary experiment and expressed as mean number of SFC/10^6 T cells ± SEM.

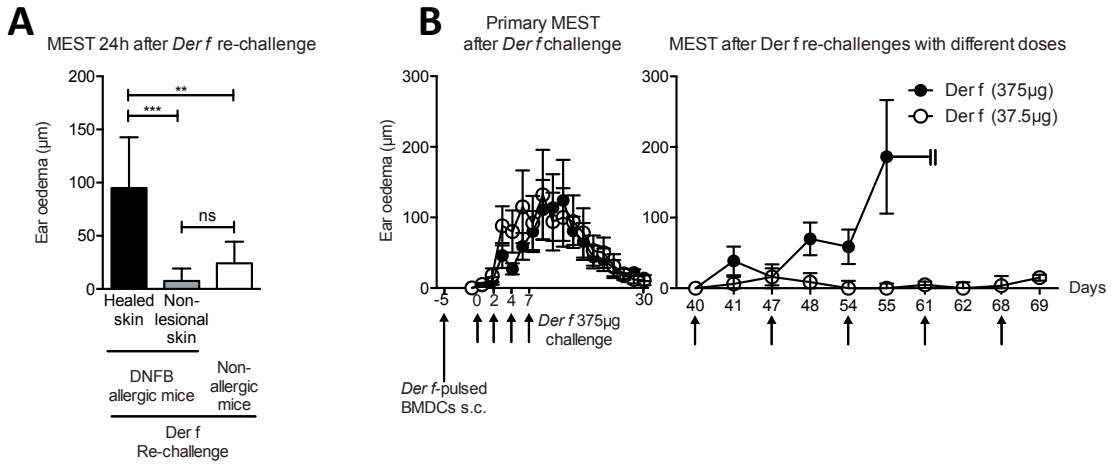


FIGURE 21: CD8+ SKIN TRM MEDIATE A SITE-SPECIFIC FLARE-UP REACTION IN DER F-ALLERGIC MICE.

A) CHS response of DNFB-allergic mice to Der f. Der f-allergic C57BL/6 mice were re-challenged with Der f on healed skin lesions or non-lesional skin 30-60 days after the induction of skin inflammation. As control, Der f was applied on ears of non-allergic mice. The following day, skin inflammation was determined by measuring the mean ear thickness (µm) ±SEM.

B) CHS response of Der f-allergic mice to repeated applications of Der f low doses. Der f-allergic C57BL/6 mice were re-challenged with Der f low doses (37.5µg at day 40, 47, 54, 61, and 68 white circles). As control, Der f-allergic mice were re-challenged with three standard Der f dose (375µg at day 40, 47, and 54, black circles). Skin inflammation was determined by measuring the mean ear thickness (µm) ±SEM on the days following the Der f applications.

*All results are representative of two independent experiments with 4 mice. n.s. = not significant, **P<0.01, ***P<0.001, One way ANOVA.*

25 Complementary data – AD-patients from clinical study

During my thesis I had the opportunity to also analyze skin biopsies and blood samples from allergic AD patients participating in a study to characterize the phenotype, the transcriptomical profile and the Ag-specificity of Trm cells in healed AD-skin lesions. Currently, we have analyzed three patients but since the study is still ongoing, further samples will be collected.

25.1 Outline of the study

Altogether, ten allergic AD patients will be recruited by clinicians associated with the Lyon Recherche Clinique (LYREC) group according to a set of eligibility criteria. Table 5 summarizes the criteria of the first three patients included into the study so far. For instance, selected patients are required to have a clinical history of AD, to be between 18 and 55 years old, and not treated systemically against AD. Also, any topical treatment has to be abrogated at least 4 weeks before any skin biopsies will be obtained. To confirm their allergy, prick tests with *Der f* and *Der p* will be performed. After inclusion of the patients into the study cohort, two punch biopsies (5mm and 3mm) will be obtained from (i) previously healed skin lesions and (ii) from non-lesional skin. From all ten patients, the 3mm biopsies from both healed and non-lesional skin will be analyzed by immunofluorescence microscopy to characterize and localize the cells inside the skin. In addition, from five patients cells from the 5mm biopsies will be isolated by a cell migration assay prior to allergen-specific re-stimulation to determine whether allergen-specific cells persist inside healed lesions compared to non-lesional skin sites. As control, peripheral blood lymphocytes (PBLs) from the patients and skin biopsies from surgical skin explants of unknown donors will be analyzed. From the other five patients, cells from the 5mm biopsies will be isolated by mechanic/enzymatic digestion. After staining with fluorescently labeled mAbs, certain T cell subsets will be isolated by FACS sorting to isolated mRNA that will be subjected to RNA sequencing analysis. As above, PBLs from the patients and skin biopsies from surgical skin explants of unknown donors will be analyzed as control (see FIGURE 22).

Patient criteria		**Patient 1**	**Patient 2**	**Patient 3**
Sex		Female	Female	Male
Age (in years) at time of biopsy		47	30	25
Age at AD onset (in years)		16	0-1	0-1
Atopic co-morbidities	Asthma	Yes; 16-27 years	Yes; 1 year - present	Yes; 1 year - present
	Allergic rhinitis	Yes; 16 years - present	No	Yes, 4 years – present
Topical treatments	Corticosteroids	No	Yes	Yes
	Tacrolimus	Yes	No	No
Time (in weeks) of healed lesions at time of biopsy		4	6	6
Prick test read-out	*Der p*	positive	positive	positive
	Der f	positive	positive	positive

TABLE 5: CLINICAL CRITERIA OF AD PATIENTS INCLUDED INTO THE CLINICAL STUDY.

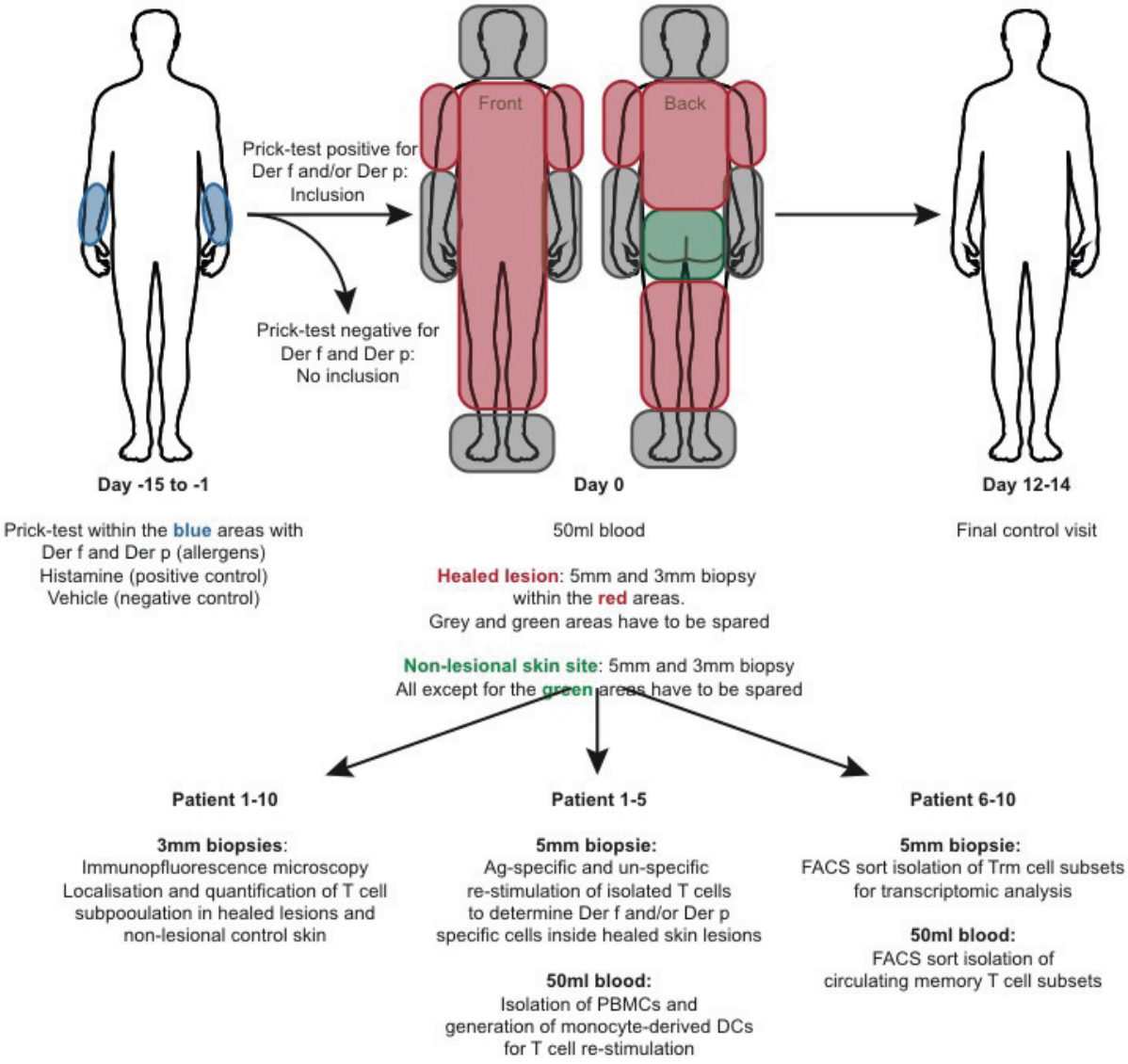


FIGURE 22: LAYOUT OF THE CLINICAL STUDY TO INVESTIGATE THE PERSISTENCE, PHENOTYPE, AG-SPECIFICITY, AND TRANSCRIPTOMICAL SIGNATURE OF 10 AD PATIENTS.

25.2 Patients 1 – 3 Immunofluorescence microscopy analysis

We first aimed to gain information regarding the presence and location of T cell subsets inside the skin at the time when the biopsies are obtained. However, here we were facing the biggest obstacle which escorted us throughout the whole study, namely that only small numbers of cells could be obtained from the skin biopsies by enzymatic/mechanical isolation techniques. Therefore, in order to determined which T cell subsets are localizing the skin of healed AD lesion, we performed immunofluorescence (IF) microscopy to detect CD3+, CD4+/Foxp3-, CD4+/Foxp3+, CD8+, CD103+, and CD69+ T cells (Fig. 23A). However, unlike with FACS analysis we were not able to detect multiple markers at the same time since most of the available and working mAbs suitable for immunofluorescence microscopy are produced in mice limiting their combination potential due to the need of secondary Abs labeled with the fluorophore. Therefore, we plan to establishing the protocol to label purified mAb ourselves with the fluorophores needed in order to obtain multiple color stainings. Overall, similar numbers of CD3+, CD4+Foxp3-, CD4+Foxp3+, and CD8+ T cells could be detected in healed skin lesions and non-lesional skin in both epidermis and dermis. Only in the healed dermis of P02 and healed epidermis from patient P01 and P03, we detected a mild increase of CD3+ T cells compared to non-lesional skin (Fig. 23B). Increased numbers of CD4+/Foxp3- T cells were only observed healed epidermis from patient P03 (Fig. 23C) while CD8+ T cells were found in healed dermis of P02 and healed epidermis of P01 and P03 (Fig. 23D). Noteworthy, the numbers of CD8+ T cells/mm^2 were overall smaller than those of CD4+ cells. Increased number of CD4+Foxp3+ Treg cells could be detected in the healed epidermis of P01 but also in the healed dermis and epidermis of P03 (Fig. 23E). An increased number of CD103+ cell was detected in the healed dermis of patient P03 (Fig. 23F). The expression of CD69 was relatively similar in epidermis and dermis from both healed lesions and non-lesional skin but an increase could be observed in the healed epidermis from patient P03 a difference could be observed between the two different sites (Fig. 23G). Nevertheless, a strong variation between the patients could be observed and some of the cell numbers were also increased in non-lesional skin which might be caused by different immunization/allergy status of each individual patient.

25.1 Cell migration assay and proliferation after allergen-specific re-stimulation

As mentioned above one particular challenge of this study was to obtain a sufficient number of cells from the biopsies that could be analyzed. In order to enlarge the cell yield and obtain enough cell that could be re-stimulated to determine their Ag-specificity, we adapted a migration protocol which was initially presented by Clark et al., 2006, in which the migratory properties of cells are used to isolate a vast number of cells after an incubation time of up to three weeks [393]. While in the initial description of the protocol a three-dimensional growth matrix was used, other investigators were also successful to isolate sufficient cell numbers without it [394]. Therefore, we incubated the biopsies without a growth matrix but in the presence of a stimulation cocktail (composed of IL-4 and IL-2) for up to three weeks. During that time cells migrated into the media from where they could be collected and subjected to proliferation assay. Furthermore, the time delay due to the long incubation time allowed us to generate DCs from the patients' blood needed for re-stimulation of the isolated skin cells.

To determine which T cell subsets had migrated, FACS analysis has been performed on both the migrated cells and on the cells remaining inside the biopsies that were isolated by mechanic/enzymatic digestion after the migration was terminated (Fig. 24). Our results showed that from healed AD lesion mainly CD4+ but not CD8+ T cells accumulated. Only in wells from patient P03 and an increased number of CD8+ T cells could be observed. From non-lesional skin either CD4+ (P02) or CD8+ T cell (P01 and P03) could be collected while incubation of skin from healthy tissue donors resulted in both CD4+ and CD8+ T cells (Fig. 24, top row). Cells isolated from the biopsies mirrored those findings (Fig. 24, bottom row), suggesting that the cells that have migrated out from the skin are representative of the initial landscape, found in the biospes at the time point when they were collected.

Afterwards, the migrated cells from both healed lesions and non-lesional skin were co-cultured for 4-6 days with DCs generated from PBMCs isolated from the patients' blood and *Der f* or *Der p* extract was added to the co-cultures. Also, PBLs from each patient were analyzed to determine whether circulating allergen-specific cells can be detected. Proliferation of the cells in response to allergen-specific re-stimulation was measured by ^{3}H thymidine uptake (Fig. 25). While both patients P01 and P02 showed increased proliferation of PBLs in response to *Der f* and/or *Der p* (Fig. 25C), respectively, no proliferation could be detected in cells isolated from the healed lesion or non-lesional skin biopsy (Fig. 25A+B). For patient P03 we detected less proliferation (and an un-specific response against OVA) among circulating cells but cells from healed skin lesions proliferated strongly in response to

increasing concentrations of the allergens (Fig. 25C+A). Also, low and very low proliferation was found in cells from non-lesional skin of patient P03 and the naïve control donor 3 (Fig. 25B+C).

Our preliminary results demonstrate that even though we obtain sufficient numbers of cells by the migration assay to further detect allergen-specific cells, we might preferentially accumulate certain T cell subsets more responsive to the provided cytokines IL-2 and IL-4. However, in such way isolated T cells can be re-stimulated but more patients have to be analyzed to confirm our findings.

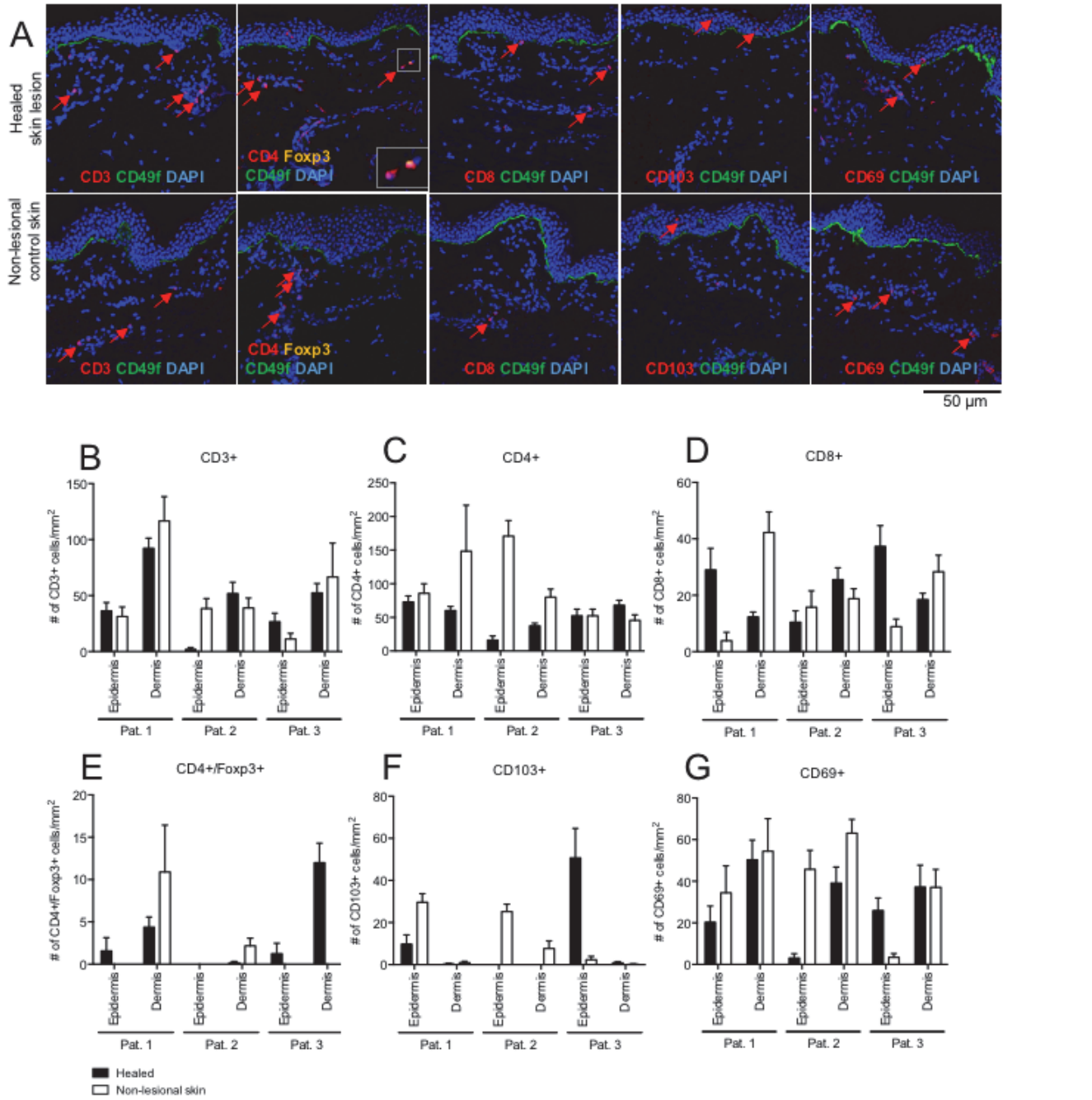


FIGURE 23: QUANTITATIVE ESTIMATION OF T CELL SUBSET IN THE SKIN OF AD PATIENTS.

A) Localization of T cell subsets inside the skin. Frozen sections obtained from healed skin lesions or non-lesional skin from three AD patients were stained with fluorescent αCD3, αCD4 and Foxp3, αCD8, αFoxp3, αCD103, and αCD69 mAbs prior to analysis by confocal microscopy. Positive cells are indicated by red arrows. The epidermal junction is indicated by staining for CD49f. Representative picture from patient P03 are shown.

C-G) Quantification. The mean numbers (±SEM) of T cell subsets per mm² in healed skin lesions (black bars) and non-lesional skin (white bars) was calculated by determining the total area (mm²) analyzed based on nuclear staining with DAPI and counting of specifically stained cells within this area. For each patients five sections were stained and five pictures of each section were analyzed.

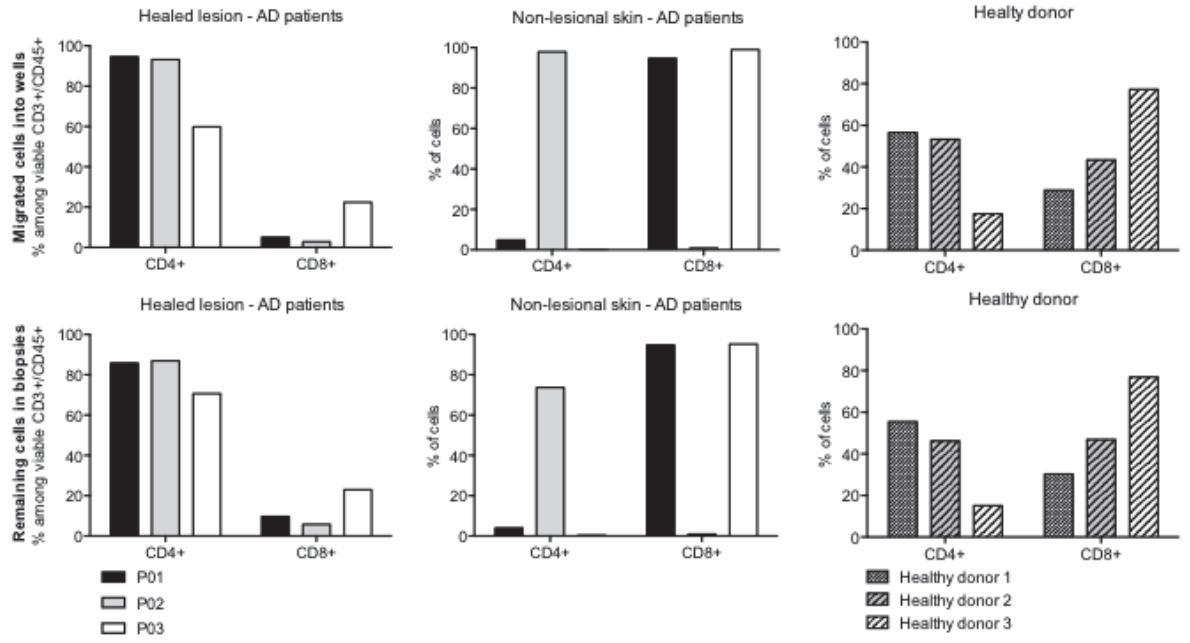


FIGURE 25: FACS ANALYSIS OF CELLS AFTER MIGRATION ASSAY.

Punch biopsies from healed AD skin lesions (left) or non-lesional skin (middle) of three patients were incubated in the presence of IL-4 and IL-2 to stimulate cell migration from the biopies into the media. As control, punch biopsies from three naïve donors (right) were incubated. After 3-4 weeks of incubation, cells that hat migrated into the wells (top row) and cells remaining in the biopsies (bottom row; isolated by mechanic/enzymatic digestion) were analyzed by FACS for the expression of CD4 and CD8 on CD45+ cells.

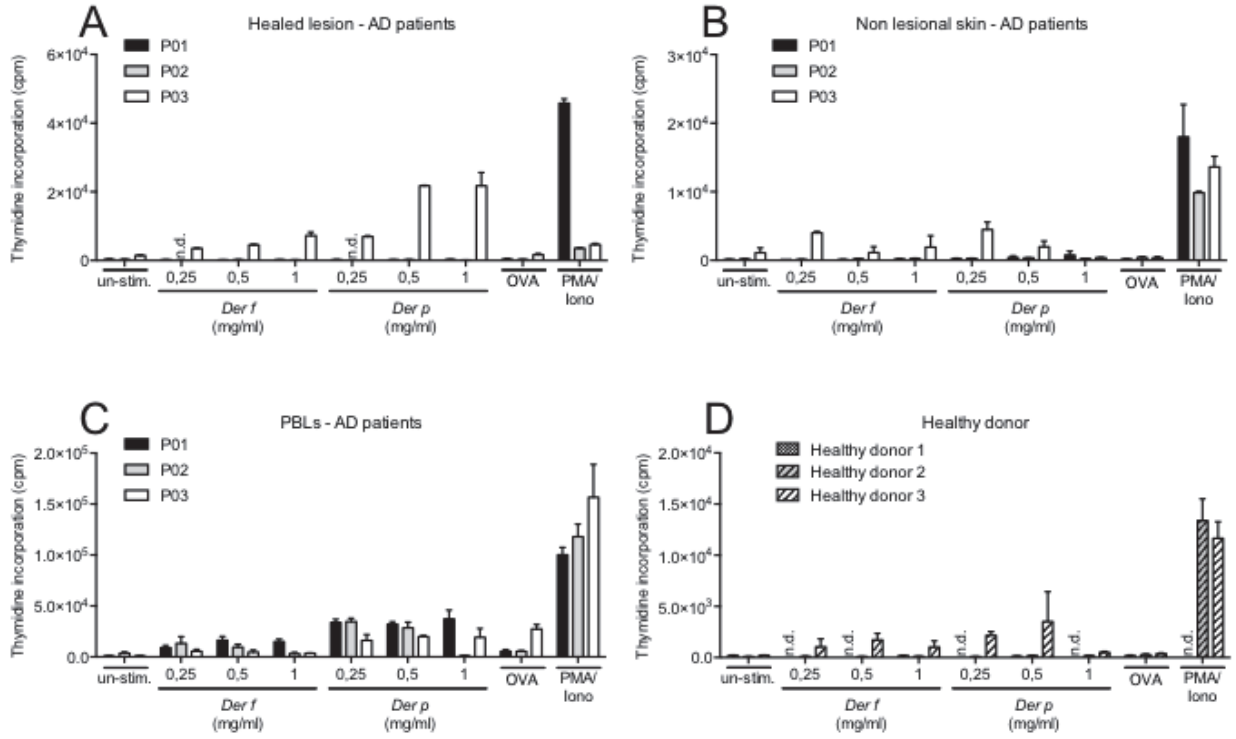


FIGURE 24: ALLERGEN-SPECIFIC RESPONSES OF SKIN AND BLOOD CELLS FROM AD PATIENTS

Cells isolated from biopsies of healed skin lesions (A), non-lesional skin (B), or PBLs (C) from three AD patients were co-cultured for 4-6 days in the presence of different concentrations of Der f or Der p. Un-specific re-stimulation was tested with ovalbumin (OVA) and PMA/Ionomycin. As control, cells isolated from skin of naïve donors were analyzed (D). Allergen-specific proliferation of T cells (mean ±SEM of triplicate wells) was measured by ^{3}H thymidine uptake. n.d. = not determined.

Discussion

The development of CD8+ skin Trm cells appears to be a double-edged sword. While in several studies their protective properties, e.g. during virus re-encounter, have been demonstrated to be beneficial for the host [17], [18], [19], [20], they also have detrimental potential in inflammatory diseases by mediating immune responses against allergens or auto-Ags [24], [25,26].

In addition to our results obtained by using a reference mouse model of ACD that were already discussed in the included study above, we conducted several experiments in an AD-like mouse model induced by *Der f* house dust mite extract and clinical AD-patients.

In the first part of the discussion, I will review those additional AD data in respect to our results from the ACD model. In the second part of the discussion, I will challenge some concepts regarding the benefits of immune memory mediated by Trm cells and potential differences between protective and pathogenic Trm cells.

26 ACD and AD mouse model: Similarities and differences

26.1 Diversity of skin Trm cells between the two models

While we found similar number of CD8+ T cells in the epidermis and dermis of healed AD-lesions compared to ACD lesions, we observed a significant accumulation of CD4+ T cells in the dermis of AD mice that was absent in ACD mice. Even though in human AD skin has been described to harbor both CD8+ and CD4+ T cells [366], [126], in the used mouse model CD8+ T cells infiltrating the skin have been described to be key to induce the initial skin inflammation [129]. Nevertheless, this does not exclude any settling of CD4+ T cells in the skin during the resolution of inflammation. Here, we can only speculate on their pathogenic function since we have excluded them to be mainly Treg cells by testing them for the expression of Foxp3. Moreover, we detected both IFN-γ and IL-5 in cells isolated from both the skin and the draining lymph nodes. The detected of IL-5 indicates a type2 response usually associated with AD [105]. However, in our experiment we lack the information whether CD4+ or CD8+ T cells from the epidermis or dermis are producing IFN-γ and/or IL-5 which clearly needs to be determined to further describe their role in the development of AD-lesions in our model.

26.2 The contribution of skin Trm cells to AD flare-up reactions

Unlike in the CHS model, in the AD model we have not explored the contribution of skin Trm cells to the development of flare-ups yet. However, the conduction of experiments employing the iDTR approach to eliminate skin Trm cells from healed skin lesions is planned and necessary to directly confirm their role in the chronicity of AD. The combination of the AD-like mouse model with our iDTR approach to eliminated certain T cell subsets from the skin would help us to decipher the exact contribution of CD4+ and CD8+ Trm cells in the development of flare-ups.

Although we have not directly demonstrated their role yet, we found that their contribution to AD flare-up reactions might be even more relevant that in the CHS model, since a single application of *Der f* extract only induced skin inflammation in healed AD-lesions and failed to induce any reaction in non-lesional skin. In contrast, a mild reaction was also observed on non-lesional skin in the CHS model after hapten-application on the healed skin.

Such variation could be explained by the different ability of DNFB and *Der f* extract to penetrate the skin barrier and to cause activation of the innate immune response upon a single dose [395], which is probably very important fro the reactivation of Trm cells.

26.3 Cell intrinsic regulation of Trm cells in AD-like lesions

We demonstrated that CD8+ skin Trm cells in the CHS mouse model underlie tight regulation that is most likely implemented by the expression of ICRs and prevents exacerbated immunopathology during the resolution of skin inflammation and flare-up reactions. We do not know yet the underlying mechanisms but several factor could influence to re-activation of Trm cells in the AD model compare to CHS responses. Based on our results in the CHS model demonstrating that the expression of ICRs on CD8+ Trm cells restrains their re-activation, we first tested if their expression is enhanced on Trm cells from AD skin. Here, we have only preliminary data indicating less ICR expression in the AD model compared to the CHS model (data not shown). A detailed analysis for the ICR expression on CD8+ Trm cells from AD skin lesions and complementary data investigating the effect of ICR blocking mAbs in *Der f*-allergic AD mice are still missing but will be necessary to evaluate if they might be a similarly attractive target for new therapeutic approaches in this disease.

Taken together, even though ACD and AD are often condensed under the term eczema and their share certain features in the pathophysiology, the discrepancies in our results between the two models indicate subtle but not negligible differences in the importance of Trm cells

for the development of flare-up reactions and their regulation. Once the complementary experiments in the AD model have been conducted those differences might be addressed by e.g. adapting therapeutical approaches via the Ag-specific Trm cells.

27 Clinical data analysis: First results, ongoing work, and obstacles to overcome

As mentioned above, during this project we also had the opportunity to analyze skin biopsies and blood samples from AD patients to investigate the phenotype, transcriptomic profile, and Ag-specificity of skin Trm cells.

27.1 Is there and accumulation of skin Trm cells in healed AD-lesions?

Quantitative evaluation of T cell subsets inside the skin of healed lesions from *Der f*- and *Der p*-allergic AD patients by immunofluorescence microscopy revealed the presence of both CD4+ and CD8+ T cells. However, the most evident result regarding the phenotype of cells persisting in the skin lesions was the strong variation between the patients. Nevertheless, such variation could be expected in human patients due to their individual status of skin sensitization to allergens, severity of their skin disease, frequencies of experienced flare-ups, co-morbidities etc. Also, we observed that certain T cell subsets were augmented not as expected within the healed skin lesion but within the non-lesional control skin.

However, those results draw the attention to several important points in the layout of our study that have to be considered. Currently, we are depending on the information received from patients and clinicians regarding the site of the biopsies from healed skin and non-lesional control skin.

In a previous study layout, we intended to overcome this issue by applying two identical APTs on each patient to induce skin inflammation under controlled conditions. Here, we would have obtained detailed recordings regarding the exact location and intensity of the skin lesions as well as the duration from active inflammation to healed skin. Moreover, we would have had the opportunity not only to analyze healed but also inflamed skin lesions. Nevertheless, due to the complexity and long duration of the protocol that included multiple visits and two time points of skin biopsies, it was not possible to recruit a sufficient number of AD patients. Also, several times asymmetrical results between the two identical APTs were observed which led to the exclusion of the patients from the study.

In order to pursue with it, we adapted the protocol. To reduce the frequencies of the visits, a

prick test instead of an APT is now performed. More over only healed skin lesions and non-lesional skin site that were both chosen by the clinician depending on the patients' disease history are analyzed while the analysis of inflamed skin lesions is relinquished. Although, we are currently acquiring more AD-patients those study adaptions augment the variability between them e.g. the duration between active and healed lesion or the exact position of a healed lesion, that have to be considered in our analysis.

27.2 Are AD-lesion enriched for *Der f*- and/or *Der p*-specific Trm cells?

Nevertheless, from all skin biopsies we were able to obtained sufficient cell numbers by migration assay in order to perform Ag-specific re-stimulation. Such an approach of cell isolation was necessary, due to too little cell numbers obtained by conventional mechanic/enzymatic cell isolation protocols. Noteworthy, in our migration assay we detected a bias to obtain mainly CD4+ T cells from healed lesions and only very small numbers of CD8+ T cells which was not observed by other investigators employing similar cell migration approaches in order to isolate skin T cells [394]. However, from all three patients we have analyzed, cells from healed skin lesions from only one patient proliferated in response to the Ag. Noteworthy, this was also the only healed lesion from which we isolated the most CD8+ T cells by cell migration. Moreover, in the healed lesion from the same patient we detected the highest numbers of CD103+ and an increase of CD69+ T cells indicating the presence of skin Trm cells. Of note, all patients had positive prick test results indicating the presence of circulating Ag-specific T cells. The prick test results were additionally confirmed by positive proliferation of PBLs in response to the Ags from all patients. Currently, the analysis of cytokine production during the re-stimulation phase is ongoing. So, it is likely that despite active sensitization status to *Der f* and *Der p*, the initial AD lesions in the two non-responding patients was caused by other triggers than these HDM Ags.

27.3 Future plans: deciphering the transcriptional profile of Trm cells from AD lesions and non-lesional skin

As mentioned above more patients have been acquired, who will be analyzed within the near future. Here, we will also include phenotypic analysis by flow cytometry since for some patients we intend to perform FACS sorting of different T cell subsets. In healthy human skin the presence of CD4+ and CD8+ Trm cells that are CD69+CD103+ or CD69+CD103- have been described [246]. Therefore, we intend to analyzed the transcriptomic profile these four

different populations isolated from the skin of healed AD lesions or non-lesional control skin in comparison to their circulating Tem, Tcm, and naïve T cells. While several studies using different virus models demonstrated distinct expression profiles between Trm cells and their circulating counterparts [253], [396], [318] as well as in the genomic signature of healed versus non-lesional AD skin [106], to our knowledge cells isolated from different skin sites of AD patients have not been analyzed in this respect.

While in both the pre-clinical AD model and AD patients several important experiments are missing to confirm their contribution and potential regulation during AD flare-ups, our results in the CHS model are considerably advanced and they are described in the study "Title of the study" included above.

28 Local versus global memory: What's the benefit of Trm cells?

Reflecting previous results demonstrating a protective role of Trm cells for the host [17], [18], [19], [20], in comparison with our results and recent reports from other investigators proposing a pathogenic character for them [24], [25], [26], it becomes clear that we are looking at the two opposing ends of a scale. Having this picture in mind, with the fact that the re-encounter of a given pathogen does not necessarily occur at the same tissue site where Trm cells are usually concentrated [330], [18], we have to ask ourselves: What are the benefits of Trm cell establishment for the host?

One over simplified answer to that question could be, that for an organism it is more reasonable to take the burden of potential pathogenic Trm cells, which in most cases cause unpleasant but not life threatening diseases such as allergies, instead of risking more potentially deadly re-infections with a pathogen. While this might be even partially true, in the following I will discuss further options on the example of skin Trm cells to address the question why Trm cells are of benefit for the host.

28.1 Are Trm cells the guardians of complete Ag clearance?

In both skin infection and our pre-clinical ACD model, skin Trm cells appear quickly after the peak of infiltrating Teff cells and their numbers remain at a stable plateau for at least several weeks ([293] and our own observation). Although, the resolution phase of skin inflammation is associated with the contraction of Teff cell numbers and decreasing amounts of Ag, the elimination of Ag is not immediate but rather progressive. For instance, in our study we were surprised to see that DNFB was present in the skin for at least more than 30 days after the application. Also, other haptens such as FITC have also been reported to remain in the skin for at least 14 days after ear painting and after the skin inflammation subsided [113] and skin Trm cell numbers have reached a plateau. Similarly, viral or bacterial loads are not vanishing instantly but progressively after the arrival of the Teff cells. However, contraction of Teff cells is an important resilience mechanism of the host to avoid exacerbated tissue damage [397] and it might occur even though small amounts of Ag are still present.

Therefore, in the case of protective Trm cells their early tissue-residency might be a back up mechanism to guarantee complete Ag elimination in case of revived Ag-proliferation along the contraction phase of Teff cells. Even though our results demonstrate that skin Trm cells rapidly down-regulate their expression of lytic protein after arrival within the tissue, which has also been reported for e.g. lung Trm cells after influenza infection [398], and corrobates that

the host is protected by the avoidance of exacerbated tissue damage, Trm cells are poised to constitutively express ready-to-use mRNA encoding effector molecules [348]. Taken together, Trm cells could primarily be installed to survey the Ag-elimination and to mediate rapid effector functions in case of insufficiently cleared Ag. Thereby, Trm cells would add another layer of protection for the host in addition to their prolonged persistence and ability to respond to Ag-challenges after long time. In the case of hapten, we wonder whether the organism might promote their skin lodgment in order to avoid or survey potential accumulation of hapten-modified cells. Thus, in the case of skin allergy, Trm cells could play both a detrimental but also protective role for the host.

28.2 Ideal local protection increases distal protection

After T cell priming within the draining lymph node, activated T cells are homed to their respective tissue via the induction of homing molecules [280], [281]. The anatomical region as well as other mediators leads to increased cell infiltration of Teff cells into the infected tissue, which subsequently leads to increased Trm cell numbers within those site [18], [330]. Nevertheless, the rest of the organism is not spared by Ag-specific Trm cells [20], [309]. Moreover, such Trm cell dissemination in the skin can be enhanced by multiple Ag encounters as demonstrated in a recent study using a HSV virus model [336]. Therefore, for the host one beneficial aspect of the optimal establishment of Trm cells at the site of previously affect sites could lie in the simultaneous recruitment of Trm cells to other potential entry site of a given pathogen.

Although the results regarding multiple applications of chemical allergens are controversial stating either no disease exacerbation [399] or transgression towards other forms of skin allergy [400], it remains to be tested whether similar mechanisms of distal lodgment exist for pathogenic Trm cells promoting the spreading of the disease.

28.3 Does optimal skin Trm cell lodgment protects against falsely located pathogenic Trm cells?

Although we know that the number of skin Trm cells can be augmented by multiple application of a pathogen (citation), to my knowledge it has not been demonstrated whether a given skin area is restricted to a maximum amount of skin-resident T cells (including Trm cells, Treg cells, etc.) during homeostasis. Nevertheless, during skin inflammation the displacement of the tissue-resident DETC population upon T cell infiltration has been observed [250], suggesting that also Trm cells generated during subsequent events might replace

each other. However, we can only speculate whether such displacement of skin-resident cell populations underlies any hierarchy or might be random depending on space resources.

Even though mechanism shaping the skin Trm cell landscape are not completely elucidated, it seems attractive to speculate that optimal lodgment of skin Trm cells could be a protective mechanism to avoid the false installation of pathogenic Trm cells. Albeit activated T cells are usually homed according to their anatomical priming site [280], [281], during certain pathologies such as FDE, systemically primed T cells specific for a certain drug are recruited to the skin, were they can induce mild but also severe skin reactions upon re-administration of the same drug [7], [367]. To date, however, there is no demonstration whether in patients suffering from FDE there is a lack of physiologically developed protective skin Trm cells allowing the establishment of pathogenic FDE Trm cells.

In the described scenario, we would assume superior tissue-residency by physiologically formed over pathogenic FDE Trm cells. In the case of skin allergies however, pathogenic Trm cells develop after proper skin challenge indicating that they have the same tissue-residency potential as protective Trm cells formed following infection. If Trm cell displacement is possible by Trm cells formed during subsequent inflammatory events with different Ags, such re-shaping of the skin Trm cell composition maybe could prevent flare-up reactions against allergens simply by replacing pathogenic by protective skin Trm cells.

28.4 Are non-specific skin Trm cells protective against immunopathology?

One of our most striking results was the detection of a significant fraction of non-specific Trm cells after the resolution of CHS responses. This could be either explained by shortcoming of the respective assays that we have used or by potential entrapment of Trm cells with specificity against other Ags than the hapten DNFB which we used to induce the skin inflammation. Noteworthy, skin DCs activated during the sensitization phase are not prevented to engulf other Ag present in the skin aside from the used DNFB e.g. microbiota from the skin flora and present it to naïve T cells. In both skin allergies but also responses to pathogen, such non-specific Trm cells could occupy the skin niche and thereby prevent an excessive accumulation of Ag-specific Trm cells due to possible space restriction as discussed above. Upon Ag re-challenge non-specific Trm cells would prevent exacerbated immune reactions simply by previous restriction of Ag-specific Trm cell accumulation. It is likely that such protective function by non-specific Trm cells has simply been overlooked in previous studies due the use of infection models employing mice with transgenic TCRs specific for certain epitopes expressed by the pathogen e.g. OT-I or gBT-I cells specific for OVA or

glycoprotein B peptide expressed on modified viruses [20], [293]. In contrast with this assumption of protection by non-specific Trm cells is the observation that CHS responses develop in germ-free mice with a lower magnitude than in conventionally-house SPF-mice [401].

Taken together, the potentially beneficial aspects of Trm cell establishment e.g. the protection of distal sites as well as the guarantee of complete Ag elimination, might at least in the case of skin Trm cells, simply out-weight the risk of developing pathogenic Trm cells. Nevertheless, this assumption probably has to be reviewed critically once Trm cells found in non-barrier tissues such as the brain have been demonstrated to actively participate in the pathophysiology of life threatening diseases e.g. multiple sclerosis.

29 Protective versus pathogenic Trm cells: Same same but different?

So far it has been proposed that protective and pathogenic Trm cells engage in the same developmental program in response to pathogens and allergens, respectively [21]. More recently, it has been demonstrated that at least pathogenic skin Trm cells are a rather heterogeneous group of cells with varying phenotype and effector functions in different tissue and in different inflammatory skin diseases [26]. In addition, long-term persistence and functionality of skin Trm cells depends on several factors engaged in their recruitment [20], [335], [336].

29.1 Distinct skin inflammatory diseases are associated with phenotypically different Trm cells – could this true for protective Trm cells?

Although CD103+ and CD69+ expression is often used to detect Trm cells, it has become clear that those canonical markers are imperfect since both CD103- and CD69- Trm cells have been described as well [245], [246]. For long it was thought that CD103 expression is mainly involved in the maintenance of Trm cells by binding its ligand E-cadherin which is expressed on epithelial cells [307] but this theory can only be partially true, since CD103 is also expressed by Trm cells within tissue that do not express E-cadherin. Nevertheless, in skin Trm cells CD103 expression is associated with impaired mobility compared to CD103- Trm cells [297]. On the other hand, several studies demonstrated that CD103+ Trm cells have superior effector functions compared to CD103- Trm cells [253], [312], [313]. Since these observation only concern protective Trm cells it would be interesting to investigate if in inflammatory skin diseases a similar discrepancy between CD103+ and CD103- Trm cells could be observed. Interestingly in our DNFB model, we observed a higher frequency of CD103+CD69- Trm cells during the resolution of inflammation than other investigators using a virus model [293]. This observation is

very important to us, since CD69 has been demonstrated to be key for tissue-residency as well as certain metabolic pathways of Trm cells [301].

The phenotype and function of Trm cells might not only be different between protective and pathogenic Trm cells but also vary between different pathologies. For long it has been known that ACD and AD are associated either with type1/17 or type2/22 responses, respectively. Our data confirm that such difference is not only present on the level of recruited Tem and Tcm or secondary Teff cells upon Ag re-encounter but also on the level of skin Trm cells indicated by their different capacity to produce cytokines. Such differences are less surprising, if we considered the diverse nature of allergens in ACD and AD. While in ACD a polyclonal response against a variety of possible hapten-protein complexes can be expected, in AD there are even more triggers involved in shaping the immune response such as the given protein allergen but also self-Ag released by the impaired skin barrier and microbial flora colonizing the skin lesions. More over, a recent study by Cheuk et al. demonstrated the expression of CD49a or the lack of it poises cells to either produce IFN-γ and contribute to the development of vitiligo or to express IL-17a and to drive psoriasis development, respectively [26]. It will be of great interest to further investigate different phenotypes and their associated function of Trm cells in other inflammatory diseases to find targets for new therapeutical approaches.

29.2 Does the initial T cells priming influences the long-term persistence of Trm cells?

One major hallmark of Trm cells which distinguishes them from their circulating counterparts is the long-term persistence inside a certain tissue without re-entering the circulation. In virus models it has been demonstrated that Ag-specific Trm cells can persist for at least one year in the skin without major decrease in their numbers even when skin lodgment was induced by unspecific inflammation [330]. Also in patients with psoriasis, FDE, and vitiligo the long-term persistence of Trm cells has been demonstrated [26], [368], [402]. While in psoriasis and vitiligo the potential cognate Ag of Trm cells is constantly present in the skin and could foster the persistence of Trm cells, in FDE skin Trm cells are rather falsely located after systemic administration of a drug. Nevertheless, skin Trm cells in FDE are know to persist and give rise to the disease even years after drug avoidance. Interestingly, our results in the CHS model against DNFB show that skin Trm cells accumulate rapidly during the resolution of the inflammation. However, preliminary works also demonstrated that they are progressively waning during the following months. One year after the initial inflammation, the number of

skin Trm is not significantly higher anymore than before the DNFB application. The waning of skin Trm cells was even accelerated in case they were pulled by an unspecific stimulus.
Those variations in long-term persistence between the different Ag might suggest a strong influence of the Ag and its potential persistence in priming Trm cells for tissue-residency. For instance in brain Trm cells it has been demonstrated that the strength of TCR stimulation is inversely correlated with their establishment inside the tissue [329].

In summary, protective and pathogenic Trm cells share many steps during their developmental pathway, however, it becomes more and more clear that Trm cells are a rather heterogeneous than homogenous cell population and that they might take different turns along their development. Still, this might not only lead to protective or pathogenic Trm cells but also to distinct Trm cell subpopulation within these two branches depending on the nature of the Ag, its T cell activation stimulation strength, or its persistence.

Conclusion

Pathogenic Trm cells in the skin persist and it has been indirectly demonstrated that they can actively drive inflammatory responses leading to diseases. Our work confirmed this active participation in the development to flare-up reactions against haptens.

Current topical treatments such as corticosteroid used in skin inflammatory diseases have most likely unknowingly targeted pathogenic Trm cells and hampered their re-activation but they do not prevent recurrence of the symptoms upon treatment withdrawal. Nevertheless, several lines of evidence suggest that also cell intrinsic regulatory mechanisms provide protection against exacerbated immunopathology. Transcriptomic analysis of Trm cells showed that even though they are equipped with forceful effector functions, they also express ICRs. Here, we demonstrated that the expression of ICRs on skin Trm cells in DNFB-allergic mice firmly restrains their re-activation and that they can be targeted *in vitro* as well as *in vivo* to release the restrain. In our model, we think that the so far under-estimated persistence of the hapten inside the skin promotes the maintenance of certain ICRs expressed early during the inflammation such as PD-1 and TIM-3. However, we cannot exclude that other ICRs such as 2B4 are up-regulated in response to tissue-derived signals. Currently, we are investigating the possibility whether ICR functions in Trm cells go beyond the restrain of effector functions but might be also involved in other processes e.g. tissue-residency and cell survival.

Future work will also include the demonstration that further restrain can be imposed on skin Trm cells by engaging ICRs with their ligands to avoid their re-activation upon allergen re-encounter. However, in order to switch the treatment approaches from therapies that provide only transient remission, we also must decipher the mechanisms leading to tissue-residency in order to open new strategies targeting the persistence of skin Trm cells in order to re-induce tolerance in allergic patients.

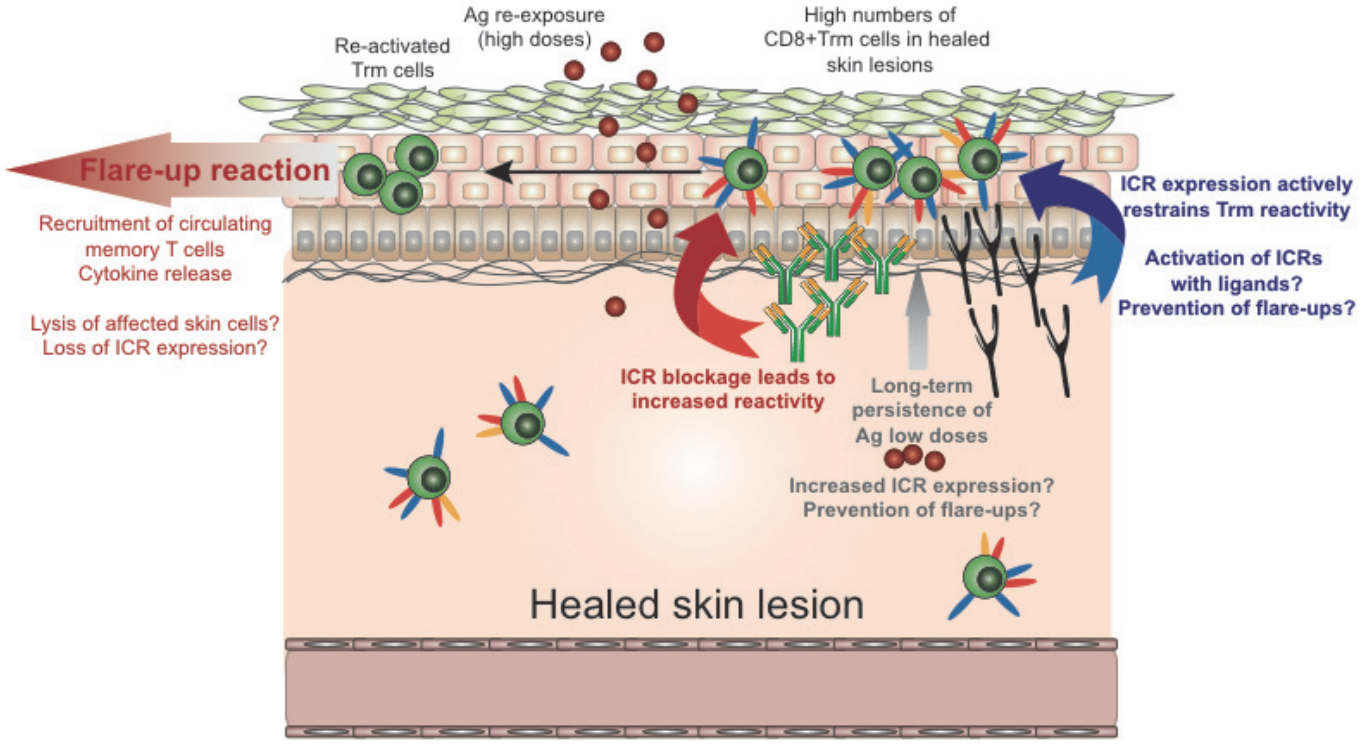


FIGURE 26: CURRENT WORKING MODEL.

Trm cells settle in the skin of healed skin lesions. Upon Ag re-exposure Trm cells actively contribute to the development of flare-up reactions. However, their re-activation is restrained by the expression of ICRs. The restrained can be released in vitro and in vivo by blocking mAbs. It remains to be demonstrated that ICR ligands can impose more restrain on the Trm cells and thereby prevent flare-up reactions. Small doses of hapten-Ag persist long-term in the skin (>30 days after skin painting). It remains to be demonstrated that such small doses of Ag increase the expression of ICRs leading to increased restrain of the reactivity.

Material and Methods

30 Table of Antibodies Mouse

30.1 Flow cytometry

α-CD45 (clone 104), α-TCRαβ (clone H57-597), α-TCRγδ (clone eBioGL3), α-CD4 (clone GK1.5 or RM4-5), α-CD8b (clone H35-17.2), α-CD69 (clone H1.2F3), α-CD103(clone M290), α-granzyme-B (clone NGZB), α-Ki67 (clone B56), α-PD-1 (clone J43), α-TIM-3-PE (R&D systems, clone 215008), α-2B4 (Miltenyi, FITC, clone REA388; or Biolegend, PeCy7, clone m2B4(B6)458.1), α-TIGIT (clone GIGD7), α-CD160 (clone CNX46-3), α-LAG-3 (clone C9B7W), α-BTLA (clone 6F7), α-NKG2A (clone 20D5), α-CD5 (clone 53-7.3), α-MHC-class II (clone 2G9), α-Sca-1 (clone D7), α-CD34 (clone RAM34) and α-EpCAM (clone caa7-9G8) were purchased from BD Biosciences (Le Pont de Claix, France) or Miltenyi Biotech (Bergish Gladbach, Germany) or eBioscience (now ThermoFisher Scientific, Villebon-sur-Yvette, France) or Biolegend (Fell - Germany).

Fluorescent H-2Kb-restricted SIIC^{D}FEKL or SIIK^{D}FEKL dextramers were synthetized by Immudex (Copenhagen, Denmark).

Cell viability was analysed using a Fixable Viability Dye, purchased from eBioscience (San Diego, California, USA). Intracellular staining was performed using BD Cytofix/Cytoperm solutions (BD Biosciences, Franklin Lakes, New Jersey, USA).

30.2 Immunofluorescence microscopy

α-CD3-AF700 (clone 17A2), α-CD4-AF488 (clone RM4-5), α-CD8b-AF647 (clone YST156.7.7), and α-TCRγδ-PE (clone GLE) were purchased from Biolegend (Fell – Germany); α-DNP-purified (polyclonal) and Chicken-α-Rabbit secondary mAb-AF488 were purchased from Life Technologies (Saint-Aubin – France).

31 Table of Antibodies Human

31.1 Flow cytometry

α-CD3- PE-Texas Red (clone 7D6) was purchase from Life Technologies (Villebon-sur-Yvette, France), α-CD4-VioBlue (clone VIT4) was purchased from Miltenyi (Paris – France), α-CD45-FITC (clone HI30) and α-CD8β-APC (clone 2ST8.5H7) were purchased from BD Biosciences (Le Pont de Claix, France).

31.2 Immunoflurescence microscopy

α-CD3-prurified (clone UCHT1) was purchased from Biolegend (Fell – Germany); α-CD4-purified (clone RPA-T4), was purchased from BD Biosciences (Le pont-de-Claix – France); α-CD8a-purified (clone C8/144b), α-Foxp3-purified (clone PCH101), α-CD69-purified (clone FN50), and α-CD103-purified (clone B-Ly7) were purchased from eBioscience (Dardilly – France); Goat-α-Mouse secondary mAb-AF647 and Goat-α-Rat secondary mAb-AF594 were purchased from Life Technologies (Saint-Aubin – France).

32 Animal work

32.1 Breeding and maintenance of mice

Female C57BL/6 mice (6-10 weeks of age) were purchased from Charles Rivers Laboratories (L'Arbresle, France). C57BL/6 mice with a mutation in the CD3ε cluster (CD3ε-/-) lacking T lymphocytes were kindly provided by M. Malissen (Centre d'Immunologie, Marseille – France). Cre-iDTR transgenic mice were kindly provided by A. Waisman (Johannes Gutenber-University, Mainz – Germany). The DTR expression in these mice is prohibited by a STOP cassette which is flanked by two loxP sites that are removable by Cre recombinase [403].

All mice were maintained at the Plateau de Biologie Expérimentale de la Souris (PBES; SFR Biosciences, UMS3444/CNRS, US8/Inserm, ENS de Lyon, UCBL) under SPF conditions and a 12 hours ligh/12hours dark cycle; water and food were available at libitum. In all experiments, mice were sacrificed by cervical dislocation. All experimental procedures were in accordance with the "Comité régional d'éthique pour l'expérimentation animale" guidelines on animal welfare.

32.2 Generation of iDTR/CD4cre mice and adoptive transfer

To generate mice expressing the iDTR on CD4+ and CD8+ T lymphocytes they were crossed to CD4-Cre mice which express the Cre recombinase on both CD4+ and CD8+ lymphocytes due to their double positive state during thymic development [404], [405]. In order to generate mice expressing the iDTR on CD8+ T lymphocytes only, CD8+ T lymphocytes and CD4+ T lymphocytes were isolated by FACS sort from spleen, axillary and inguinal lymph nodes from iDTR and C57BL/6 mice, respectively, and transferred i.v. into lymphopenic CD3ε-/- recipient mice at a ratio of 1 : 2 (CD8+ : CD4+).

32.3 Pre-clinical models

32.3.1 CHS (ACD-like) mouse model

As previously described [112], sensitization of mice was induced by application of 25μl of 2,4-dinitrofluorobenze (DNFB; Sigma-Aldrich, Saint-Quentin Fallavier - France) (0,5%) in Acetone alcohol: Olive oil (AOO, 4:1) on the shaved ventral skin. Control mice were not sensitized. Five days later, sensitized and non-sensitized control mice were anesthetized by intraperitoneal (i.p.) injection of 1.25mg Ketamine (Imalgene®1000) and 0.25mg Xylasin (Rompun®2%; both Alcyon, Miribel - France) in 100μl phosphate-buffered saline (PBS)1x and skin inflammation was induced on the left ear by application of 5μl DNFB (0,13%) in AOO on each side of the ear. On the right ear AOO alone was applied.

In some case, mice were sensitized by s.c. injection of BMDCs pulsed with SIIC^{D}FEKL peptide (BACHEM Distribution, Weil am Rhein – Germany) Six days later, sensitized and non-sensitized control mice were anesthetized as described above and skin inflammation was induced on the left ear by application of 10μl SIIC^{D}FEKL peptide (1mM) in) in DMSO (70% in water; Sigma-Aldrich, Saint-Quentin Fallavier - France) on each side of the ear. On the right ear DMSO (70% in water) alone was applied.

32.3.2 AD-like mouse model

As previously described [129], mice were anesthetized by intraperitoneal (i.p.) injection of 1.25mg Ketamine (Imalgene®1000) and 0.25mg Xylasin (Rompun®2%; both Alcyon, Miribel - France) in 100μl PBS1x and sensitized by applying 10µl *Der f* protein extract (Stallergens, Antony – France) in DMSO (70% in water) once every week on the ear for 6 – 10 consecutive weeks until skin inflammation is observed. The duration of sensitization can be contracted by injecting *Der f* pulsed BMDCs subcutaneously (s.c.). Five days after BMDC injection, *Der f* extract is applied on the ear for 3 – 4 times on every other day until skin inflammation is observed. Noteworthy, the inflammatory dose of *Der f* per ear has to be determined for each newly obtained extract and usually lies between 250 – 400μg for each side of the ear.

32.3.3 Evaluation of skin inflammation by ear swelling

In pre-clinical mouse models described above, the induced skin inflammation was determined by the Mouse ear swelling test (MEST) in which evaluation of the ear thickness was done before and each day after hapten/allergen application with a spring-loaded micrometer (J15, Blet SA, Lyon - France). Ear swelling was calculated by subtracting the initial value from the value of the corresponding day. Mice were allowed to resolve the inflammation for more than 30 after the last application, before they were considered to be allergic against the respective hapten/allergen. In some cases, the mice were analyzed along the resolution of the

inflammation to determine cell kinetics or the allergic mice were re-treated with the respective hapten/allergen.

32.4 Cell depletion in vivo

32.4.1 mAb model

The rat-α-mouse CD4 mAb (clone GK 1.5) was obtained from American Type Culture Collection (ATCC). The rat-α-mouse CD8 mAb (clone H35.17.2 was provided by G. Millon (Institute Pasteur, Paris – France). To deplete CD4+ and CD8+ T lymphocytes sufficiently, mice were given i.p. injections of 100μg of αCD4 and 20μg of αCD8 mAb, respectively, on 200μl PBS 1x at indicated time points before the ear painting. For maintenance of the cell depletion, could be repeated every other day with ¼ of the initial dose of mAbs. Cell depletion was assessed om each mouse by staining for CD4 or CD8 molecules on dLN or spleens, using fluorescence-labeled α-CD4 or α-CD8 mAbs.

32.4.2 iDTR model

In order to deplete cells expressing the iDTR, mice were given a maximum of two i.p. injections of 1μg of Diphtheria Toxin (DT; Calbiochem/Millipore, San Diego – USA) in 100μl PBS 1x at indicated time points before the ear painting. Cell depletion was assessed in each mouse by staining for CD4 and CD8 molecules on dLNs or spleens as well as skin, using fluorescence-labeled α-CD4 or α-CD8 mAbs.

33 Tissue and cell isolation – Mouse

All steps of cell isolation were performed at 4°C unless mentioned otherwise. Once single cell suspensions were prepared, they were kept at 4°C for further use.

33.1 Lymph nodes

LNs were harvested from mice at indicated time points after the ear painting. To obtain single cell suspension the LNs were passed through a 100μm cell strainer using the plunger of a 2ml syringe.

33.2 Skin

33.2.1 Whole skin

Ear biopsies were harvested from mice at indicated time points after the ear painting. The anterior and posterior layers of the skin were separated and diced with sharp scissors into small pieces. The diced skin tissue was incubated for 45min at 37°C with vigorous vortexing every 15min in collagenase type 1 (121,9 CDU/ml; Sigma-Aldrich, Saint-Quentin Fallavier -

France) and DNase I (2 KU/ml; Roche Diagnostics, Meylan - France). To obtain single cell suspension the digested skin tissue was passed through a 100μm cell strainer using the plunger of a 2ml syringe. A consecutive passage over a 70μm cell strainer was performed to reduced debris.

33.2.2 Epidermis and dermis

To isolate epidermal and dermal cells, the anterior and posterior layers of the ear biopsies are separated and incubated 1h at 37° floating with the interior side down on dispase II (1,2 U/ml; Roche Diagnostics, Meylan – France). Epidermal and dermal layers are separated by using forceps and diced into small pieces. Similar to whole skin cell extraction, the diced epidermal and dermal tissue was incubated for 45min at 37°C with vigorous vortexing every 15min in collagenase type 1 (121,9 collagen digestion units (CDU)/ml; Sigma-Aldrich, Saint-Quentin Fallavier - France) and DNase (2 Kunitz units (KU)/ml; Sigma-Aldrich, Saint-Quentin Fallavier - France). To obtain single cell suspension the digested epidermal and dermal tissue was passed through a 100μm cell strainer using the plunger of a 2ml syringe. A consecutive passage over a 70μm cell strainer was performed to reduced debris.

33.3 Cell viability

Cell viability of isolated cells was determined by Trypan blue exclusion using a Malassez counting chamber. Cells were adjusted to an appropriate cell concentration according to following application.

34 Tissue and cell isolation – Human

34.1 Skin

Skin punch biopsies were taken by clinicians and diced with sharp scissors into small pieces. The diced skin tissue was incubated for 2h at 37°C with vigorous vortexing every 15min in collagenase type 1 (31,25 CDU/ml; Sigma-Aldrich, Saint-Quentin Fallavier - France) and DNase (2 KU/ml; Sigma-Aldrich, Saint-Quentin Fallavier - France). To obtain single cell suspension the digested skin tissue was passed through a 100μm cell strainer using the plunger of a 2ml syringe. A consecutive passage over a 70μm cell strainer was performed to reduced debris.

34.1.1 Migrating assay

Cell isolation before re-stimulation was performed by migration. 5mm punch were placed on a 24well plate with the epidermal side up in two ml of Roswell Park Memorial Institute 1640 (RPMI; Life Technologies, Saint-Aubin – France)-SAB medium containing human AB sera

(SAB; 5%; Etablissement Français du Sang (EFS), Décines – France), penicillin/streptomycin (0,1%; Gibco/Thermo Fisher Scientific, Dardilly - France), and gentamycin (0,1%; Invitrogen, Paris - France). In addition, IL-2 (100 U/ml; R&D Systems, Lille - France), and IL-4 (250 U/ml; R&D Systems, Lille - France) were added to the RPMI-SAB and the biopsies were incubate for up to three weeks at 37°C, CO_2 5%. Cell migration was observed daily and media was change every other day by carefully replacing 1ml of old media with 1ml of freshly prepared media. When the desired cell density was obtained, cells were collected and washed two times with RPMI-SAB. The cell number and viability was determined by Trypan blue exclusion as described above and the cells were subjected to the co-culture and proliferation assay. Cells remaining inside the skin biopsy were isolated as described above and FACS staining was performed.

34.1.2 Co-culture of skin cells and proliferation assay

Cells isolated by migration assay as described above were re-suspended in RPMI-SAB without any additional cytokines at a concentration of 1-2x10 cells/ml, plated on a 96well plate, and incubated for 48h at 37°C, CO_2 5%. After starvation, the cells were collected and washed two times with RPMI-SAB. The cell number and viability was determined by Trypan blue exclusion as described above. The cells were re-suspended RPMI-SAB at a concentration of 1-2x10 cells/ml, plated on a 96well plate, and AG-pulsed or un-pulsed DCs were added for co-culture at a ration of 2 : 1 (Skin cell : DC). For unspecific re-stimulation, phorbol 12-myristate 13-acteate (PMA; 0,62ng/ml; Sigma-Aldrich, Saint-Quentin Fallavier - France) and ionomycin (Iono; 62ng/ml; Sigma-Aldrich, Saint-Quentin Fallavier - France) or αCD3 (1μg/ml; eBioscience, Dardilly - France) and αCD28 (2μg/ml; eBioscience, Dardilly - France) were added. The cells were co-cultured for 4 - 6 days. Final volume of each well 200μl. After co-culture 110μl of the supernatant from each well were collected and frozen at -20°C for further use. 5μCi/10μl of ^{3}H thymidine solution (Amersham/Ge Healthcare life Science, Velizy-Villacoublay - France) were added to each well and incubated overnight O/N at 37°C, CO_2 5%. The following day, ^{3}H thymidine uptake by proliferating cells was determined.

35 Flow cytometry

35.1 Surface and intracellular staining

All stainings were carried out in V-bottom 96-well plates and centrifugation was performed at 515rcf at 4°C unless mentioned otherwise. During the whole staining process unnecessary

light exposure was avoided to protect the fluorescent labeled cells. Isolated cells were pelleted by centrifugation and washed twice with 200μl of FACS buffer. Cells were re-suspended in 50μl FACS buffer containing rat-α-mouse CD16/CD32 antibodies (1:4) and incubated for 15min to block the Fc receptors, followed by two washes with 200μl of FACS buffer. In case CCR7 staining was performed, the cells were re-suspended in 50μl of FACS buffer containing the antibody in an appropriate dilution determined by titration and incubated for 30min at 37°C before the remaining antibodies were added and incubated at 4°C. If no CCR7 staining was performed, cells were directly re-suspended in 50μl FACS buffer containing the desired antibodies for the surface staining in an appropriate dilution determined by titration. After 20min incubation, cells were washed twice with 200μl of FACS buffer. The staining process was repeated, in case biotin-labeled antibodies were included and staining with streptavidin conjugates was necessary. To prepare the cells for viability staining, they were washed two times with 200μl of PBS 1x to remove all remains of bovine serum albumin (BSA) from the FACS buffer before they were re-suspended in 200μl PBS 1x containing fixable viability stain (1:1000) and incubated for 30min.

If no further staining was performed, cells were washed twice with 200μl of PBS 1x and fixed in 200μl of paraformaldehyde (PFA; Sigma-Aldrich, Saint-Quentin Fallavier – France) for at least 20min or O/N followed by two washes with 200μl of FACS buffer. The surfaced stained and fixed cells were re-suspended in 200μl of FACS buffer and acquired at the flow cytometer.

In case intracellular staining was performed after live/dead staining, cells were fixed for at least 20min or O/N in 200μl of 1x Fixation/Permeabilisation solution (eBioscience, Dardilly - France), followed by two washes 200μl of 1x Permeabilisation solution (eBioscience, Dardilly - France). Cells were re-suspended in 200μl of 1x Permeabilisation solution and incubated for 20min. Afterwards, cells were pelleted by centrifugation, re-suspended in 50μl of 1x Permeabilisation solution containing rat α-mouse CD16/CD32 antibodies (1:4), and incubated for 15min to block the Fc receptors. The cells were washed twice with 200μl of 1x Perm solution and re-suspended in 50μl of 1x Permeabilisation solution containing the desired antibodies for intracellular staining in an appropriate dilution determined by titration. After 20min incubation, cells were washed two times with 200μl of FACS buffer (FB), re-suspended in 200μl of FB, and acquired at the flow cytometer.

Stained cells were analyzed on a LSRFortessa flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA), and data were analyzed using FlowJo software (Treestar, Ashland,

Oregon, USA). Total cell numbers were calculated based on the numbers of viable cells determined by Trypan blue exclusion during the isolation process. In some experiments, the number of skin Trm cell subsets present in the epidermis or the dermis of allergic animals were estimated using AccuCheck Counting Beads (Invitrogen, Cergy Pontoise - France) according to the manufacturers protocol.

36 Generation of BMDCs – Mouse

BMDCs were generated *in vitro* from bone marrow (BM) progenitors from naïve C57BL6/mice. Briefly, femur and tibia were harvested and flushed with RPMI-complete (RPMI-c). Lysis of the red blood cells (RBCs) was performed with Ammonium-Chloride-Potassium (ACK) lysis buffer (Gibco/Thermo Fisher Scientific, Dardilly - France) and the cells were washed two time with RPMI-c to remove all remaining ACK lysis buffer. The obtained cells were plated for 6 days on a 6well plate at a concentration of $0,5x10^6$cells/ml starting with $1,5x10^6$cells/well in RPMI-c containing GM-CSF at a previously determined optimal concentration at 37°C, CO_2 5%. Every other day, 3ml fresh RPMI-c and GM-CSF were added. Generated BMDCs were pulsed in RPMI media containing *Der f* extract (1mg/ml) or DNFB (1,4mM) at a concentration of $10x10^6$cell/ml for 2h (*Der f*) or 20min (DNFB) at 37°C in the dark. The cell viability is determined by Trypan blue exclusion and the cell concentration is adjusted for further use of the DCs in co-culture with isolated murine skin or LN cells. In order to pulse BMDCs with the $SIIC^DFEKL$ peptide, on day 5 of incubation $SIIC^DFEKL$ peptide was added to the BMDCs at a final concentration of 0,5mM and incubated O/N at 37°C, CO_2 5%.

37 Generation of PBMCs/DCs – Human

Peripheral blood mononuclear cells (PBMCs) were prepared from whole blood from patients by Ficoll (Eurobio, Les Ulis - France) gradient centrifugation. Briefly, whole blood was diluted with PBS 1x (1:3) and 30ml of diluted blood suspension were carefully covered over 15ml of Ficoll solution in a 50ml tube followed by 20min centrifugation at 460rcf at room temperature (RT) without acceleration or deceleration. PBMCs can be collected at the interface between Ficoll solution and blood suspension. PBMCs are washed two times with RPMI-SAB to remove remaining Ficoll solution and the cell number is determined by Trypan blue exclusion. Monocytes were further isolated by magnetic positive selection using CD14 microbeads (Miltenyi, Paris - France) and AutoMACS instrument (Miltenyi, Paris - France) according to the manufacturer's protocol into CD14+ monocytes and CD14- PBLs. Immature

DCs were generated from CD14+ monocytes cultured for 6 days on a 6well plate at a concentration of $1x10^6$cells/ml in RPMI-SAB containing GM-CSF (100u/ml; Miltenyi, Paris - France) and IL-4 (10u/ml; R&D Systems, Lille - France). Generated DCs were pulsed in RPMI media containing *Der f* (1mg/ml) or *Der p* extract (1mg/ml; ENZO Life Science, Villeurbanne - France) at a concentration of $10x10^6$cells/ml for 2h at 37°C in the dark. The cell viability is determined by Trypan blue exclusion and the cell concentration is adjusted for further use of the DCs in co-culture with isolated cells from the patient's skin biopsy or PBLs.

38 ELISPOT assay Mouse

For IFNγ ELISpot assay, 96well nitrocellulose plates were (Millipore, Molsheim - France) O/N at 4°C or for 2 hours at 37°C, respectively, with α-IFN-γ Ab (BD Biosciences, Le Pont de Claix – France) and blocked with RPMI-c for 1 hour at 37°C. The plates were washed five times with 0,05% PBS 1x/Tween 20 (Sigma-Aldrich, Saint-Quentin Fallavier - France) before use. Isolated skin or LN cells were dispensed in the plates and co-cultured with DNFB- or *Der f*-pulsed BMDCs for 18 hours. SPCs were developed using avidin horseradish peroxidase (HRP) (eBioscience, Dardilly – France)) incubated for 20min at RT, and extensively washed before adding the amino ethyl carbazole (Sigma-Aldrich, Saint-Quentin Fallavier - France).

39 Immunofluorescence microscopy

39.1 Frozen sections

Samples of human skin or mouse ear biopsies were embedded in Optimal Cutting Temperature (OCT) medium (Sakukar, Leiden - Netherlands) and snap-frozen in liquid nitrogen. Frozen sections (5μm) were prepared, allowed to dry, and stored at -80°C until further use. Sections were fixed for 20min with Paraformaldehyde (PFA; 2%; Sigma-Aldrich, Saint-Quentin Fallavier - France), rehydrated with PBS1x, and permeabilized with TritonX100 (0.1%; Sigma-Aldrich, Saint-Quentin Fallavier - France) before blockage with a solution of BSA (1%; Sigma-Aldrich, Saint-Quentin Fallavier - France) and normal goat serum (10%; Sigma-Aldrich, Saint-Quentin Fallavier - France). Following, the sections were incubated for 2h at room in a wet chamber with a mixture of primary mAbs (see list above) diluted in blocking solution at a previously determined optimal concentration. After thoroughly washing with PBS1x, fluorescently labeled secondary Abs diluted in blocking solution at a previously determined optimal concentration were added on to the sections and incubated for 1.5h at room in a wet chamber. Sections were washed thoroughly washed with PBS1x and Fluoromount medium (DAKO, Les Ulis - France) containing 4',6-Diamidino-2-

phenylindole dichloride (DAPI, 1μg/ml; Sigma-Aldrich, Saint-Quentin Fallavier - France) was added before placing cover slips.

39.1.1 Acquisition and processing of frozen section images

Image acquisition of whole frozen sections was performed on a LSM710 confocal microscope (Carl Zeiss Microscopy GmbH, Jena – Germany) equipped with an inverted stand with 20X/NA=0.5 air objective and numerical zoom 0.6, Acquired images have a size of 708.3x708.3μm. All acquired raw data images were processed using ZEN 2011 imaging software (Carl Zeiss Microscopy GmbH, Jena – Germany).

Acquired images were analyzed using ImageJ software (NIH Image, Bethesda, Maryland – USA). Briefly, epidermis and dermis surface (μm^2) were measured using the roi manager based on nuclear staining with DAPI,. Thresholds were applied on splitted channels (DAPI, AF594, and AF647) and converted into binary pictures. The number of DAPI+, AF594+, and AF647+ cells were determined by using the automated "Analyze particle" method for events bigger that 10μm^2.

39.2 Epidermal sheets

Mice were anesthetized by i.p. injection of 1.25mg Ketamine (Imalgene®1000) and 0.25mg Xylasin (Rompun®2%) in 100μl PBS1x (Alcyon, Miribel - France). To remove any fur, depilatory cream (Veet-Reckitt Benckiser, Massy – France) was applied on the ears for 1 minute and thoroughly washed with distilled water. Following hair removal, the mice were sacrificed by cervical dislocation and the ears were cut proximal. The ventral and dorsal leaves were separated along the cartilage and incubated for two hours at 37°C floating on ethylenediaminetetraacetic acid (EDTA, 2mM; Sigma, Saint-Quentin Fallavier – France) with the epidermal site up. Following incubation, the epidermal sheets were harvested, washed with 1X PBS, and fixed in ice-cold acetone for 20 minutes followed by 2x5min washes in 1x PBS. To block un-specific binding sites, the epidermal sheets were placed in blocking solution containing BSA (2%; Sigma, Saint-Quentin Fallavier – France) and incubated for 1h at 37°C. After incubation, the blocking solution was removed and the epidermal sheets were incubated with anti-DNP (1/50; Life- Technologies, Saint-Aubin – France) diluted in fresh blocking solution for 2 hours at 37°C followed by 3x5min washes in 1X PBS. Next, the epidermal sheets were incubated for 2h at 37°C in fresh blocking solution containing fluorescent labeled secondary Abs, followed by 3x5min washes in 1X PBS. Finally, the epidermal sheets were incubated O/N at 4°C in fresh blocking solution containing anti-CD8b (1/150; Biolegend, Fell - Germany) and anti-TCRγδ (1/150; Biolegend, Fell - Germany)

directly fluorescently labeled. The next day, the epidermal sheets were washed 3x5min in 1X PBS for 5 before counter staining with DAPI (1μg/ml, Sigma-Aldrich, Saint-Quentin Fallavier - France) diluted in Fluoromount medium (DAKO, Les Ulis - France). Epidermal sheets were stored at 4°C in the dark before images were acquired.

39.2.1 Acquisition and processing of epidermal sheet images

Image acquisition of the epidermal sheet stained with fluorescent labeled antibodies was perform on a Lightsheet Z.1 microscope (Carl Zeiss Microscopy GmbH, Jena – Germany) with 20×/1.0NA water-immersion detection optics and two-sided 10×/0.2 illumination optics, equipped with two PCO EDGE 4.2 cameras with scientific complementary metal-oxide-semiconducter (sCMOS) sensor (square pixels of 6.5×6.5µm side length, 1920×1920-pixel resolution, 4-channel images, 16-bit dynamic range) (PCO AG, Kelheim – Germany). For all 3D datasets, a *z*-interval of 0.5µm with a zoom of 0.80 was applied. Volumetric images have a size of 553.2*553.2*1000µm (1920×1920×500 pixels). Directly before the acquisition, the epidermal sheets were immersed in 1% low-melting-point agarose 1% (Sigma-Aldrich, Saint-Quentin Fallavier - France) pre-warmed at 37°C and immediately loaded into a 1 mL syringe. Following solidification of the agarose, the syringe was introduced into the acquisition chamber containing 1X PBS and the images were acquired. Image acquisition was performed in triplicate. All acquired Lightsheet fluorescence microscopy (LSFM) raw data images were processed using ZEN 2011 imaging software (Carl Zeiss, Oberkochen – Germany).

Images were de-convolved using classic maximum likelihood estimation (CMLE) with 20 iterations and theoretical point spread function (PSF) on Huygens software (Scientific Volume Imaging B.V., Hilversum – Netherlands). Threshold segmentation was performed using Imaris software (Bitplane, Zurich – Switzerland) for each fluorescence channel. The total volume of the imaged tissue was calculated on based on the DAPI channel. In all images we determined the mean fluorescence intensity (MFI) and the volume of DNP patterns as well as the number of CD8b+ and TCRγδ+ T cells.

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