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PhD Thesis

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Epidermal T cells

Studies on distribution and activation

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Preface

This PhD thesis was carried out from 1st October 2016 to 30th September 2019. The experimental work was conducted at the Department of Immunology and Microbiology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark in Professor Carsten Geisler's group and at the Centre for Translational Medicine and Therapeutics, the William Harvey Research Institute, Queen Mary University of London, UK in Dr. Sian Henson's group. Professor Charlotte Menné Bonefeld was the principal supervisor and Professor Jeanne Duus Johansen and Professor Allan Randrup Thomsen were co-supervisors.

Human studies were performed in collaboration with Dr. Malin Glindvad Ahlström and Nurse Anne Marie Topp at the Department of Dermatology and Allergy, Copenhagen University Hospital Gentofte, Denmark, in collaboration with Dr. Beatrice Dyring-Andersen from the Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Denmark and in collaboration with surgeon Torben Norre Rasmussen, Kirurgisk Klinik Christianshavns Torv, Copenhagen, Denmark.

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This PhD thesis is based on the following three studies:

I. **Anne-Sofie Ø. Gadsbøll**, Mia H. Jee, Anders B. Funch, Maria Alhede, Veronika Mraz, Julie F. Weber, Lauren A. Callender, Elizabeth C. Carroll, Thomas Bjarnsholt, Anders Woetmann, Niels Ødum, Allan R. Thomsen, Jeanne D. Johansen, Sian M. Henson, Carsten Geisler, Charlotte M. Bonefeld. Pathogenic $CD8^+$ epidermis-resident memory T cells displace dendritic epidermal T cells in allergic dermatitis. (JID, 2019, in press)

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During my PhD I have furthermore co-authored the following papers, which are not included in my PhD thesis:

- Julie Sølberg, Nina H. Ulrich, Dorriet Krustrup, Malin G. Ahlström, Jacob P. Thyssen, Torkil Menné, Charlotte M. Bonefeld, **Anne-Sofie Ø. Gadsbøll**, Eva Balslev, Jeanne D. Johansen. Skin tape stripping: Which layers of the epidermis are removed? *Contact Dermatitis **80**, 319-321 (2019),* doi: 10.1111/cod.13199

- Trine H. Petersen, Mia H. Jee, **Anne-Sofie Ø. Gadsbøll**, Jonas D. Schmidt, Jens J. Sloth, Gregory F. Sonnenberg, Carsten Geisler, Jacob P. Thyssen, Charlotte M. Bonefeld. Mice with epidermal filaggrin deficiency show increased immune reactivity to nickel. *Contact Dermatitis **80,** 139-148 (2018),* doi: 10.1111/cod.13153

- Mia H. Jee, Jeanne D. Johansen, Terkild B. Buus, Trine H. Petersen, **Anne-Sofie Ø. Gadsbøll**, Anders Woetmann, Niels Ødum, Jacob P. Thyssen, Andrea J. White, Graham Anderson, Carsten Geisler, Charlotte M. Bonefeld. Increased Production of IL-17A-Producing γδ T Cells in the Thymus of Filaggrin-Deficient Mice. *Frontiers in Immunology **9**, 1-11 (2018),* doi: 10.3389/fimmu.2018.00988

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Finally, I would like to thank my friends and family for providing unlimited support and needed breaks during the writing of this thesis. Most important, I would like to thank Claus for all your love, for feeding me delicious food, for encouraging me to work hard and for understanding that sometimes T cells are the most important thing.

Copenhagen, September 2019

Anne-Sofie Østergaard Gadsbøll

Table of contents

Summary in English

The skin forms a barrier to the outside world, protecting us from external exposures to chemicals, allergens and other environmental hazards.

The top layer of the skin is named epidermis and it consists of tightly linked keratinocytes with a network of T cells located in between. The major epidermal T cell subset in unexposed naïve mice is dendritic epidermal T cells (DETC), which belong to the group of γδ T cells. In adult human epidermis γδ T cells are also present, but in less abundant numbers. Instead, the major T cell group is αβ T cells. The reason for this difference in T cell subsets between mice and humans is not clear but is suggested to be a result of species differences.

The main purpose of this PhD thesis is to investigate the distribution and interplay between epidermal T cell subsets in healthy skin and in the T cell-mediated skin disease allergic contact dermatitis (ACD). Moreover, the purpose is to explore the difference between specific and unspecific inflammation upon contact allergen exposure on the skin.

This PhD thesis is based on three studies. In **study I** we investigated the interplay between epidermal T cell subsets using a mouse model of ACD, named the contact hypersensitivity model (CHS). We established that $CD8^+$ tissue-resident memory T cells displace DETC in CHS, potentially due to an increased metabolic fitness. In **study II** we explored the effect of age and antigen-exposure on the epidermal T cell distribution. We found that few epidermal T cells are present in infant human epidermis but that the number increase within the first year of life. We further found that the epidermal T cell distribution was different between infants and adults and that the change was driven by antigen-exposure opposite to age. In **study III** we showed that an unrelated contact allergen could induce inflammation in contact allergen-experienced skin, indicating that an unspecific activation of memory T cells might occur.

Taken together this PhD thesis describes the distribution and interplay between epidermal T cell subsets, it contributes to the understanding of the development of our skins immune system and it investigates a potentially unknown mechanism of unspecific activation in the skin.

Dansk resumé

Huden udgør vores første barriere mod omverdenen, og den beskytter os mod den konstante eksponering fra udefrakommende kemikalier, allergener og andre miljøeksponeringer.

Det øverste lag i huden kaldes epidermis og består af tæt forbundne keratinocytter og et netværk af T-celler. Den største epidermale T-celleundergruppe i ubehandlede mus er dendritiske epidermale T-celler (DETC), som hører til gruppen af γδ T-celler. Epidermis fra voksne mennesker indeholder også γδ T-celler, men langt færre end set hos mus. I stedet hører den største T-celleundergruppe hos voksne mennesker til gruppen af αβ T-celler. Årsagen til denne forskel i T-celleundergrupper mellem mus og mennesker er ikke klar, men det er blevet forslået, at det skyldes artsforskelle.

Det primære formål med denne ph.d.-afhandling er at undersøge fordelingen og samspillet mellem epidermale T-celleundergrupper i rask hud og i den T-celle-medierede hudsygdom allergisk kontakteksem. Derudover er formålet også at undersøge forskellen mellem specifik og uspecifik inflammation efter kontaktallergeneksponering af huden.

Denne ph.d.-afhandling er baseret på tre studier. I **studie I** undersøgte vi samspillet mellem epidermale T-celler ved hjælp af en musemodel for allergisk kontakteksem. Vi viste, at $CD8^+$ hud-iboende-hukommelse T-celler fortrænger DETC i allergisk kontakteksem – potentielt på grund af en stofskiftefordel. I **studie II** undersøgte vi virkningen af alder og antigeneksponering på den epidermale T-cellefordeling. Vi viste, at der kun findes få epidermale T-celler i spædbørn, men at antallet stiger inden for det første leveår. Vi fandt endvidere, at der er forskel på den epidermale T-cellefordeling hos spædbørn og voksne, og at ændringen blev drevet af antigeneksponering i modsætning til alder. I **studie III** viste vi, at et andet kontaktallergen kunne inducere inflammation i tidligere kontaktallergeneksponeret hud, hvilket indikerer, at en uspecifik aktivering af hukommelses T-celler kan forekomme i allergisk kontakteksem.

I denne ph.d.-afhandling beskrives fordelingen og samspillet mellem epidermale T-celleundergrupper. Afhandlingen bidrager herudover til forståelsen af udviklingen af vores huds immunsystem og undersøger en potentielt ukendt mekanisme for uspecifik aktivering i huden.

Abbreviations

Allergic contact dermatitis (ACD)

Antigen presenting cells (APC)

CC-chemokine receptor (CCR)

Central memory T cells (T_{CM} cells)

Circulating memory T cells (T_{CIRCM} cell)

Contact hypersensitivity model (CHS)

Cutaneous lymphocyte antigen (CLA)

Dendritic cells (DC)

Dendritic epidermal T cells (DETC)

Dinitrochlorobenzene (DNCB)

Dinitrofluorobenzene (DNFB)

Draining lymph nodes (dLN)

Effector memory T cells (T_{EM} cells)

Effector T cells (T_E cell)

Fatty acid (FA)

Free fatty acid (FFA)

Herpes simplex virus (HSV)

Innate lymphoid cells (ILC)

Interferon (IFN)

Interleukin (IL)

Keratinocytes (KC)

Knockout (KO)

Langerhans cells (LC)

Major histocompatibility complex (MHC)

NOD-like receptors (NLRs)

Olive oil and acetone (OOA)

Oxazolone (OXA)

Oxidative phosphorylation (OXPHOS)

Pattern recognition receptors (PRRs)

P-phenylenediamine (PPD)

Reactive oxygen species (ROS)

Secondary lymphoid organs (SLO)

Specific-pathogen-free (SPF)

Stratum corneum (SC)

T cell receptor (TCR)

T helper cells (Th cells)

T regulatory cells (T_{reg} cells)

Tissue-resident memory T cells (T_{RM} cells)

Toll-like receptors (TLRs)

Tumor necrosis factor (TNF)

Introduction

1. The skin

The skin is our body's largest organ and it protects us daily from external exposures to pathogens, chemicals and allergens. Besides providing a physical barrier to the outside world, it also provides a microbial, chemical and immunological barrier, which is important to maintain skin homeostasis essential for health[1–3].

The skin is composed of layers. The outermost layer is the epidermis and it is further divided into stratum basale, stratum spinosum, stratum granulosum and stratum corneum (SC), starting from the inner layer (figure 1). The epidermis consists primarily of tightly linked keratinocytes (KC) that differentiate from the basale layer to SC[1–3]. KC are closely connected by tight junctions in the stratum granulosum and by adherence junctions and desmosomes in the lower layers, providing an important inside-out as well as outside-in barrier between our body and the outside world[4]. When the KC reach SC they are dead and surrounded by a cornified envelope, they now become corneocytes. Together with lipids, the corneocytes form an outer physical barrier on the skin surface[1–3]. KC produce structural proteins, including filaggrin, which is produced in it's pro-form in stratum granulosum and later cleaved into its monomer form. The breakdown products of filaggrin is a large proportion of natural moisturizing factor, which is found in SC and is an important mediator of epidermal hydration, essential for the chemical barrier[5,6]. KC also secrete various chemokines and cytokines upon stress or damage, which are important for the immunological barrier. Besides KC, the epidermis also contains other cell types, including melanocytes and immune cells. Underneath the epidermis is the basal membrane, separating the epidermis from the dermis. The dermis is divided into two layers, the upper stratum papillare and the lower stratum reticulare. The dermis has a more complex structure than the epidermis with collagen and elastin fibres, blood vessels and nerve cells and it contains various cell types including fibroblasts and many different immune cells[1,2] (figure 1).

Much of our knowledge about the skin and its immune system has been gathered from mouse studies, and it is therefore important to highlight the differences between mouse and human skin. The overall structure of the skin is the same between the two species, however there are key differences. For example mice are covered in fur and the skin therefore contains closer packed hair follicles than human skin. The follicular hair cycle also differs, with mice having a synchronously

cycle and humans being asynchronous[2,7,8]. Mouse skin is thinner than human skin and has a higher epidermal cell turnover[2,7,8]. This feature, together with a thin muscle layer called the panniculus carnosus, located underneath the skin of mice, provides the mouse with an advantage regarding wound healing. Finally, humans have sweat glands distributed all over the body whereas they are only found on the paws of mice[2,7,8] (figure 1).

1.1 The immune system of the skin

The skin has not always been recognized as an immunological organ, but we now know that it plays an important immunological role in the first line of defence against the environment. The skin itself has an immunological role, with KC expressing pattern recognition receptors (PRRs), which can recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), triggering production and release of immunological mediators[1].

The immunological barrier is also composed of both innate and adaptive mediators. The epidermis contains few innate mediators, only Langerhans cells (LC). While the dermis contains a far more diverse mix of innate immune cells, including mast cells, natural killer cells, innate lymphoid cells, macrophages and various dermal dendritic cells (DC)[1,2]. Adaptive mediators in the skin consist of T cells. Adult skin contains approximately 20 billion T cells, which is close to twice as many T cells as found in the circulation[9]. In unexposed naïve mouse epidermis, the majority of T cells is a subset of γδ T cells, named dendritic epidermal T cells (DETC). In human epidermis, the majority of T cells is αβ T cells, both CD4 and CD8 positive[10–15]. γδ T cells are also present, but in less abundant numbers as compared to mice[13,16–18]. In the dermis, both αβ and γδ T cells are present[1,2] (figure 1).

Little is known about the development of the immune system in human skin, however studying the skin from fetuses obtained from abortions of 18 to 30 week pregnancies has provided some clues. Di Nuzzo and colleagues showed that there are no epidermal T cells present before birth. In contrast, dermal T cells are present at all the investigated gestational ages, with equal $CD4^+$ and $CD8^+$ cells at 18 weeks of gestation age but with predominant $CD4^+$ cells from 23 to 30 weeks of gestation age[19]. The dermal T cells were primarily of a naïve phenotype. Di Nuzzo *et al.* also show that LC were present in the epidermis, already at 18 weeks of gestation age[19]. A study by Akgün

et al. investigated the immune system in foreskin from neonates (1 to 27 days old) and infants (28 days to 24 months old)[20]. They showed that very few T cells are present in skin from both age groups compared to adult skin[20]. They did not investigate CD4 and CD8 expression. The low number of T cells is supported by other studies investigating the immune system in neonate foreskin[10]. Interestingly, Akgün *et al.* showed that neonates primarily have memory T cells in the epidermis and that infants have both naïve and memory T cells in the epidermis[20]. These studies illustrate that immunological development happens in the skin from birth to adulthood.

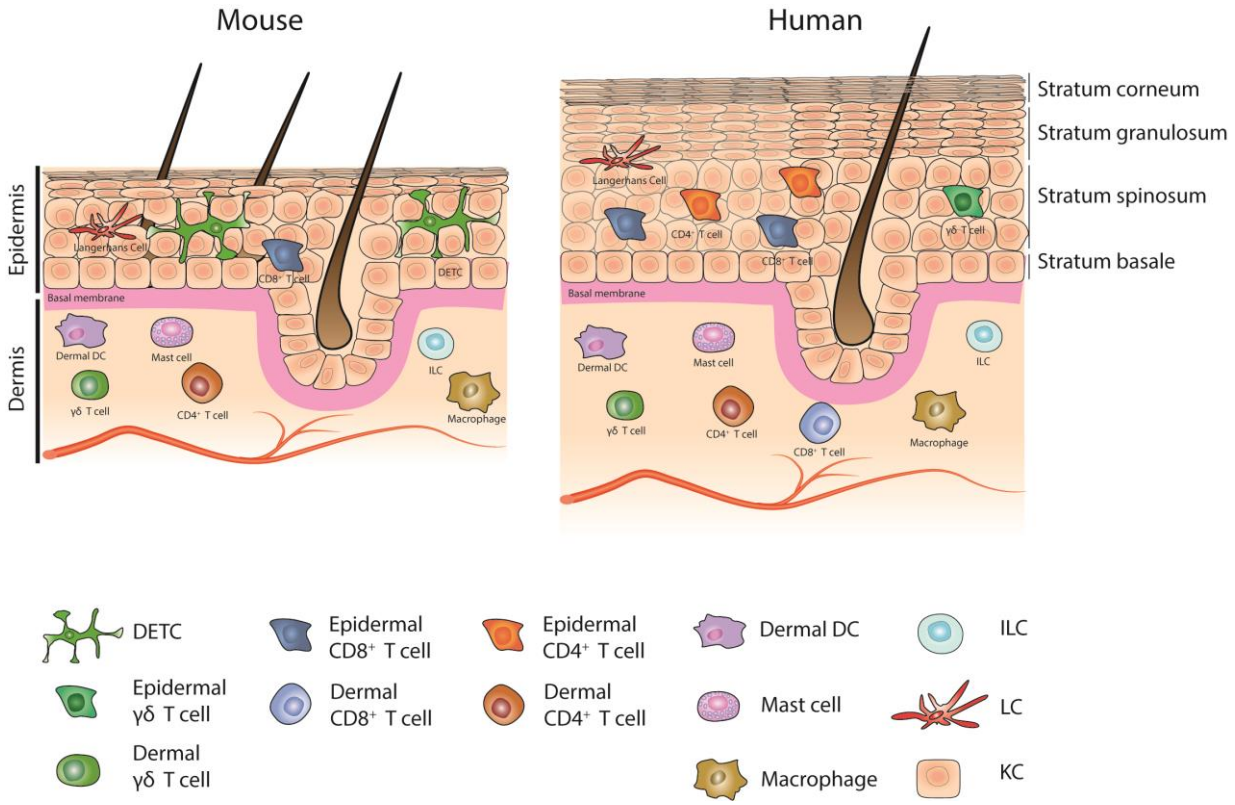

Figure 1: Model of mouse and human skin. *Mouse skin (left) has closer packed hair follicles than human skin (right) and both dermis and epidermis are thinner compared to human skin. Unexposed naïve mouse epidermis contain Langerhans cells (LC) and γδ T cells named dendritic epidermal T cells (DETC). Following antigen exposure $CD8^+$ T cells can also be found in mouse epidermis. Human epidermis also contain LC and γδ T cells, the latter in less abundant numbers compared to mice. Human epidermis furthermore contain $CD8^+$ and $CD4^+$ T cells. The dermis contain a variety of both innate and adaptive immune cells in both mice and humans. Innate lymphoid cells (ILC), keratinocytes (KC).*

1.2 Epidermal T cells

Both the skin barrier and the innate immune system is often sufficient to protect us from external assaults, but if necessary, the highly specific adaptive immune system is activated, including T cells. Skin-resident T cells do not only protect us against invading pathogens, they also respond to damaged and stressed cells and as such, are important for maintaining a healthy skin homeostasis.

In this thesis, epidermal T cell subsets are investigated in healthy skin and in relation to contact allergy, with specific focus on epidermal γδ T cells and $CD8^+$ tissue-resident memory T (T_{RM}) cells (figure 2).

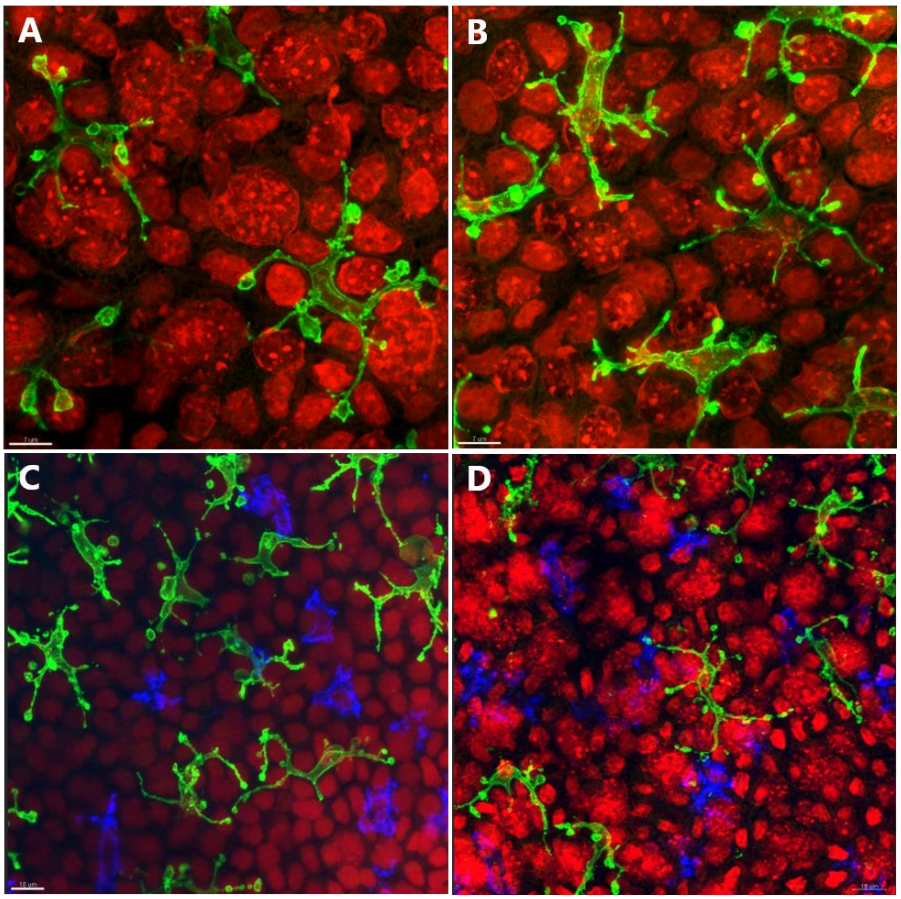

Figure 2: Images of dendritic epidermal T cells (DETC) and $CD8^+$ tissue-resident memory T (T_{RM}) in the epidermis. *Mice were sensitized and challenged twice with (A-B) olive oil and acetone (OOA, vehicle) or (C-D) 0.15% dinitrofluorobenzene (DNFB, contact allergen) in OOA as described in study I figure 1. Epidermal ear-sheets were stained with anti-TCRγδ (green, DETC), anti-CD8α (blue, $CD8^+$ T_{RM} cells) and SYTO™59 Red Fluorescent Nucleic Acid Stain (red). DETC are found in both OOA and DNFB treated skin while $CD8^+$ T_{RM} cells are only found in DNFB treated skin. Scale bars 7 µm (A-B) and 10 µm (C-D).*

1.2.1 Epidermal γδ T cells

γδ T cells only make up 1-5% of the circulating T cell pool, but in epithelial tissues, such as the skin, they make up 5-100% of the total T cells[11,13,16,17,21–23]. DETC were first described in mice in 1983 where they were called Thy-1$^+$ dendritic epidermal cells[22,23]. They were characterized by the expression of the invariant Vγ3Vδ1 T cell receptor (TCR) (Garman nomenclature)[24,25]. Human epidermal γδ T cells express a Vγ1 TCR and are, like their mouse counterpart, distinct from the circulating γδ T cells[26]. Vγ3$^+$ thymocytes develop only in the fetal thymus, from embryonic day 13 to 18, where they are pre-programmed. After this time they migrate to the epidermis where they form a tissue resident population of DETC[24,25,27–30]. To populate the epidermis, DETC express E and P selectin ligands that interacts with E and P selectins on the vascular endothelium allowing homing to the skin[31,32]. They also express the chemokine receptors CCR10 and CCR4, both important for skin-homing through the vascular endothelium and CCR10 in particular for epidermal homing[31,32].

Although γδ T cells have been studied for many years, their TCR ligand is still unknown. Skint1 has been suggested as a DETC TCR ligand. However it exists in mice but not in humans, where the homologues BTNL molecule exists instead[33,34]. Even though the mechanism is not known, it has been shown that DETC recognize self-antigens from KCs and that the two cell types are in close contact with each other[35,36]. It is believed that epithelial cells constantly stimulate the TCR of nearby DETC, putting them in a chronically semi-activated state[37].

Even though the TCR activating signal is unknown, it does not mean than DETC activation is a complete mystery. Several co-receptors are known to contribute to their activation, for mice they include JAML, CD100, NKG2D and CD103, which bind CAR, plexin B2, NKG2D-ligands (H60c, Rae-1, Mult-1) and E-cadherin on keratinocytes, respectively[38–41]. Morphologically when DETC are activated they subtract their dendrites and round up[42,43].

DETC have a dendritic morphology, which enables them to lay in between the KCs and other epidermal cells, monitoring them and help sustain a normal skin homeostasis[36,42,44,45]. The dendrites that point toward the apical portion of the epidermis are immobile, whereas the dendrites positioned toward the basal epidermis are very mobile[37]. Through the secretion of growth factors including insulin-like growth factor I (IGF1)[44] and keratinocyte growth factor (KGF)[45], together with different cytokines (interleukin (IL) 2, IL-3, tumor necrosis factor (TNF)α, interferon (IFN)γ, IL-17A)[43,46,47], and chemokines (CCL3, CCL4, CCL5, XCL1)[47,48], DETC play an important role

in responding to stress and damage in the skin and in the activation and recruitment of other immune cells to the skin.

Much of the knowledge that we have about DETC comes from wound healing studies[42,44,46,49]. Studies in TCRδ$^{-/-}$ mice have revealed that DETC are important for normal wound healing and normal KC renewal, showed by increased KC apoptosis in knockout (KO) mice[42,44]. Studies have also shown that neutrophil recruitment to wound sites are not affected in TCRδ$^{-/-}$ mice, but macrophage homing is[50], underlining the tissue surveillance function of DETC. DETC have also been shown to be important in tumor surveillance and killing of tumor cells[38,51].

1.2.2 Epidermal CD8$^+$ tissue-resident memory T cells

Upon activation, αβ T cells undergo clonal selection and expansion and turn into effector cells. Most of the effector cells undergo apoptosis upon clearing of the antigen, but some turn into memory T cells, which have the ability to make stronger and faster responses if re-activation occurs[52–54]. Memory T cells are divided into different groups, depending on their migration pattern and expression of different surface markers. All memory T cells express the activation marker CD44 (mice and humans) or CD45RO (humans)[55–59]. For many years, memory T cells were assumed to be one subset, but in 1999, it was proposed that memory T cells consist of two subsets, depending on their expression of CC-chemokine receptor 7 (CCR7), which is important for migration to the secondary lymphoid organs (SLO)[60]. The two subsets were named central memory T (T_{CM}) cells and effector memory T (T_{EM}) cells, which both are circulating but in different compartments of the body. T_{CM} cells migrate between the SLO and are beside CCR7$^+$ also CD62L$^+$, also important for SLO homing. T_{EM} cells also migrate, but between the blood and non-lymphoid tissues. They are characterized by being CCR7$^-$ and CD62L$^-$ [60]. Within the last decade, it has become clear that a third group of memory T cells also exists, the T_{RM} cells. Studies have identified T_{RM} cells in many different barrier tissues and they have been shown to dominate the local immune response to re-infections[10,61–65]. In the skin, they are characterized by accumulation at the site of infections with no re-circulation and with minimal migration inside the skin, especially in the epidermis[66–69].

Epidermal CD8$^+$ T_{RM} cells are localized close to the basal membrane and within hair follicles in the epidermis[66]. Like other epidermal immunological cells, CD8$^+$ T_{RM} cells are dendritic and in

contact with the neighbouring epithelial cells, LC and DETC[63,66,70]. $CD8^+$ T_{RM} cells are transcriptionally different from the circulating pool of memory T cells[65,71], but interestingly, a study has shown that after immunization there is a $CD8^+$ T_{RM} cell and a $CD8^+$ T_{CM} cell clone with identical TCRs, indicating a common clonal origin[72].

Epidermal $CD8^+$ T_{RM} cells are characterized using different markers. Two of the commonly used markers are CD69 and CD103. The activation marker CD69 is a lectin that antagonize sphingosine-1-phosphate receptor 1 (S1P1) and thereby limits migration away from the skin. CD103 is the α-chain of the integrin αEβ7 and it binds E-cadherin expressed by epithelial cells[10,56–59,64,65]. Epidermal $CD8^+$ T_{RM} cells are also characterized by expressing other markers, including CD49a and cutaneous lymphocyte antigen (CLA). CD49a is the α-subunit of the α1β1 integrin and it binds collagen[58,59,64]. Interestingly, CD49a has been shown to indicate effector function of $CD8^+$ T_{RM} cells, as human $CD8^+CD49a^+$ T_{RM} cells produce IFNγ, whereas $CD8^+CD49a^-$ cells produced IL-17[73]. CLA is important for entry into the skin and is expressed on the majority of skin T cells in humans[9]. Development and maintenance of $CD8^+$ T_{RM} cells is dependent on TGFβ, IL-15 and CXCR3, the chemokine receptors for CXCL9 and CXCL10, important for entry into the skin[65,74].

The function of $CD8^+$ T_{RM} cells in the skin is to patrol and rapidly recognize antigen-expressing cells and respond to re-infections by a cytokine response or direct lysis of target cells[56,70,75,76]. $CD8^+$ T_{RM} cells are classically activated through TCR stimulation and major histocompatibility complex (MHC) class I expressed antigens. Studies using herpes simplex virus (HSV)-1 infections in mice have shown that activation of $CD8^+$ T_{RM} cells can induce a "pathogen alert state" in the skin, measured by an increased inflammatory signature in the whole skin transcriptome after $CD8^+$ T_{RM} cell activation[70]. It is important to note that the model used for $CD8^+$ T_{RM} cell activation is antigen-specific even though the alerted state is described as antigen-independent.

1.2.3 Epidermal $CD4^+$ T cells

$CD4^+$ T cells are divided into different T helper (Th) subsets, depending on their cytokine profile[77]. Both Th1, Th2, Th17 and T regulatory (T_{reg}) cells can be present in the skin[9,78]. $CD4^+$ T cells are classically activated by MCH class II presented antigens and can respond with both cytokine production and Th1 cells can induce direct lysis of target cells through Fas-FasL mediated killing[79,80].

In unexposed naïve mouse skin, $CD4^+$ cells are mainly located in the dermis but few are also present in the epidermis[81–83]. In humans, a large fraction is seen in the epidermis[10,21,64]. Most of the epidermal $CD4^+$ T cells in adult healthy skin express CD69 and CD103 and are characterized as $CD4^+$ T_{RM} cells[10,64]. The epidermis also contains a smaller fraction of $CD4^+$ $CD69^+$ $CD103^-$ T cells, which are mainly located in the dermis, and a small fraction of recirculating $CD4^+$ T cells[10]. $CD4^+$ $CD103^+$ T cells proliferate less than $CD4^+$ $CD103^-$ T cells, but tend to have an increased effector function[10]. Like the other tissue-resident epidermal T cells, epidermal $CD4^+$ T cells have minimal migration, unlike their dermal counterparts[81,82].

Similar to $CD8^+$ T_{RM} cells, epidermal $CD4^+$ T cells accumulate in the epidermis after inflammation[64]. A human study in psoriasis showed increased numbers of epidermal $CD4^+$ cells with increased transcription of *IL-17A, IL-22, IFNγ, granzyme A* and *B, IL-10* and *FOXP3* in active lesions[64]. The gene profile is to some extend kept in resolved lesions following treatment, illustrated by increased *IL-22* expression, indicating susceptibility to recurrent inflammation in resolved skin[64].

2. T cell metabolism

In order to mount an efficient immune response, T cells need increased amounts of energy to switch from a resting to an active metabolic state. This process requires extensive metabolic changes[84].

Naïve $CD4^+$ T cells have a metabolism that is mainly based on oxidative phosphorylation (OXPHOS). When T cells are activated, they increase their glycolysis and their fatty acid (FA) synthesis[84–87]. Even though there is sufficient oxygen present, the cells still preferentially shift to a glycolytic metabolism, an effect known as the Warburg effect[88]. These metabolic changes induce an increase in biomass, including metabolic intermediates important for membrane formation and other factors involved in cell growth[84,85]. A trigger-factor for this glycolytic switch observed in T cells is hypoxia, a condition that is present in inflamed tissue. It induces both increased proliferation and effector functions in T_{EM} cells, but not T_{CM} cells and naïve T cells because these cells are not glycolytic driven[89,90]. Interestingly, the epidermis is a relative hypoxic tissue, a factor that has the potential to stimulate glycolysis in epidermal T cells[91]. Memory T cells shift their

metabolism back to OXPHOS while retaining an increased fatty acid metabolism[71,92]. Compared to effector T cells, memory T cells have a larger spare respiratory capacity, described as the mitochondria's extra capacity to produce energy under activating conditions, which is important for memory T cells' ability to respond to re-infections (figure 3)[71,93].

Regarding $CD8^+$ T_{RM} cells, a key differentiating factor from other memory subsets is lipid metabolism. A study has shown that long-term maintenance of $CD8^+$ T_{RM} cells in the skin is dependent on free fatty acid (FFA) uptake and that $CD8^+$ T_{RM} cells have an increased FFA uptake compared to circulating memory T cell subsets[71].

The metabolic pathways utilised by DETC are unknown, but a study has shown that different metabolic pathways regulate development of αβ and γδ T cells in the thymus[94]. Since DETC and $CD8^+$ T_{RM} cells have different effector functions, it could be speculated that they also exhibits unique metabolic characteristics. This is yet to be investigated.

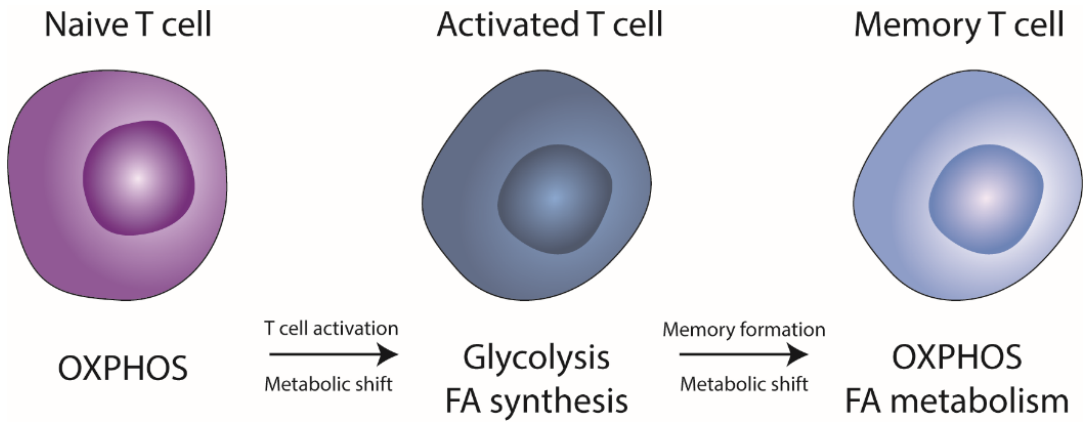

Figure 3: T cell metabolism. *Upon activation T cells switch their metabolism from primarily being based on oxidative phosphorylation (OXPHOS) in naïve T cells toward a glycolytic energy production in activated T cells and with a higher fatty acid (FA) synthesis. The T cells that turn into memory T cells undergo further metabolic changes and go back to OXPHOS as primary energy production pathway, but keep a high FA metabolism.*

3. Allergic Contact Dermatitis

Allergic contact dermatitis (ACD) is an inflammatory skin disease and 20.1% of the general population is sensitized to at least one contact allergen, with a significantly higher prevalence in woman compared to men[95]. More than 4000 contact allergens are known and the list keeps getting longer[96]. Some of the most common contact allergens are metals (nickel, cobalt, chromium), preservatives (methylisothiazolinone), fragrances (limonene) and dyes (p-phenylenediamine (PPD))[95–97].

The symptoms of ACD happens at skin sites that are exposed to the contact allergen, but systemic reactions can also occur[98,99]. The symptoms are redness followed by oedema and vesicle formation. If the contact allergy becomes chronic, it presents with dry and scaling skin. The skin is itchy and the patients can develop wounds at the affected areas. The degree of the disease varies, depending on the contact allergens, the exposure frequency and general skin health. The treatments today are based on identification and avoidance of the contact allergen and topical corticosteroid treatment[100,101].

ACD is characterized as a T cell mediated skin disease, but it is well established that many different cells are involved in the disease, both KCs and circulating and skin-resident immune cells[102–105]. The disease is divided into two phases; the initiating sensitization phase and the later elicitation, or challenge phase.

The bulk of our knowledge about ACD comes from a well-established animal model entitled the contact hypersensitivity model (CHS), which is characterized as a delayed type IV hypersensitivity reaction. The model is typically used on rodents and involves the application of contact allergens on the skin. In this thesis, the CHS model is performed on mice and the contact allergens dinitrofluorobenzene (DNFB) and oxazolone (OXA) are used.

3.1 Sensitization

There are seven important steps in order to elicit sensitization. (1) Contact allergens have to penetrate the skin and (2) modify self-proteins. (3) Both an epidermal and dermal inflammation

must happen. (4) The inflammatory state must lead to DC activation and migration to the draining lymph nodes (dLN) were they (5) present antigen to naïve T cells and (6) activation of T cells occurs with (7) the formation of immunological memory (figure 4).

Sensitization occurs when the skin comes in to contact with contact allergens, also referred to as haptens. Haptens are low molecular weight compounds that have to penetrate the SC to induce sensitization. Previously they were believed to be less than 500 Daltons[106], but recent studies show that larger molecules also have sensitization potential[107,108]. When contact allergens penetrate the skin, they modify self-proteins and form contact allergen-modified self-peptide complexes in the skin. This makes them recognizable for the immune system by looking non-self[109–111]. The ability of the different contact allergens to covalently bind proteins is therefore an important characteristic of contact allergens and it is due to their electrophilic reactivity. Complete contact allergens, e.g. DNFB, are already electrophilic and can directly bind skin proteins. Other contact allergens are harmless and non-reactive until they have been metabolized by enzymes in skin cells (pro-haptens, e.g. urushiol from poison ivy) or otherwise transformed, e.g. by oxidization (pre-haptens, e.g. PPD), which gives them their electrophilic reactivity. Not all contact allergens can make covalent bonds with proteins, e.g. metal salts like nickel forms complexes with the electron-rich parts of proteins[110–112].

Another important feature of contact allergens is their ability to induce inflammation and activate the innate immune system, a crucial step in the sensitization phase[113,114]. After skin penetration, the contact allergen can activate skin cells by inducing danger signals, which activate PRRs, particularly the membrane associated toll-like receptors (TLRs) and cytosolic NOD-like receptors (NLRs). Activation of TLRs, especially TLR2 and TLR4[115], leads to a downstream activation of MyD88, mitogen-activated protein kinases (MAPK) and NF-kB signalling, resulting in the expression of pro-IL-1β, pro-IL-18 and other inflammatory mediators[116–118]. Activation of NLR leads to the activation of the NLR pyrin domain containing 3 (NLRP3) inflammasome and downstream activation of caspase-1, which cleaves pro-IL-1β and pro-IL-18 to their active forms, increasing the pro-inflammatory state in the skin[119–121]. Penetrating contact allergens can furthermore affect skin cells by inducing a state of oxidative stress in KCs with the production of reactive oxygen species (ROS) and ATP release from the affected cells, also leading to inflammasome activation[122,123]. ROS have the ability to break down hyaluronic acids found in the skin, which works as TLR ligands, further contributing to the innate inflammation[124,125].

The inflammatory state generated by the innate immune system, including mast cells and neutrophils[105,126,127], combined with the cellular stress response from the KCs, mature and activate both epidermal and dermal DCs, which migrate to the dLN where they present the contact allergen to naïve T cells through MHC presentation[128–130]. In addition to MHC presentation, T cells also need costimulatory signals from the antigen presenting cells (APC) and local cytokine stimulation to be activated. The activated T cells differentiate into different effector cells that migrate to the inflamed skin area. Crucial for the sensitization phase, a pool of memory T cells is formed from the effector T cells, both locally in the skin but also systemically. This is important for the elicitation response[72].

3.2 Elicitation

Whereas the sensitization phase is normally asymptomatic, the elicitation phase is symptomatic. When the skin encounters the contact allergen again, the elicitation phase begins. This phase includes the unspecific innate response described in the previous section, but it also includes an adaptive immune response, caused by the reactivation of the memory T cells generated in the sensitization phase (figure 4).

Cytotoxic $CD8^+$ T cells secrete inflammatory cytokines in the response to contact allergens and they kill contact allergen-modified cells through both the non-secretory Fas-FasL pathway and the secretory perforin pathway[131–133]. $CD4^+$ Th cells secrete different cytokines depending on their subset and they can also perform direct lysis of target cells through Fas-FasL interaction in the response to contact allergen[78–80,134–139]. Neutrophils and mast cells are also important in the elicitation phase, illustrated in studies showing that antibody depletion of neutrophils prior to contact allergen challenge reduced the ear swelling response[105,140] and from studies which showed that lacking mast cells could also reduce ear swelling[126].

IFNγ and IL-17 are important cytokines in the elicitation phase[78,135,138,141,142], secreted by both γδ and αβ T cells. IFNγ stimulates the production of specific chemokines, e.g. CXCL9 and CXCL10, which increase leukocyte infiltration[143] and it increases Fas and ICAM-1 expression on KCs[133,142]. IL-17 boosts IFNγ-induced upregulation of ICAM-1 on KC[142,144].

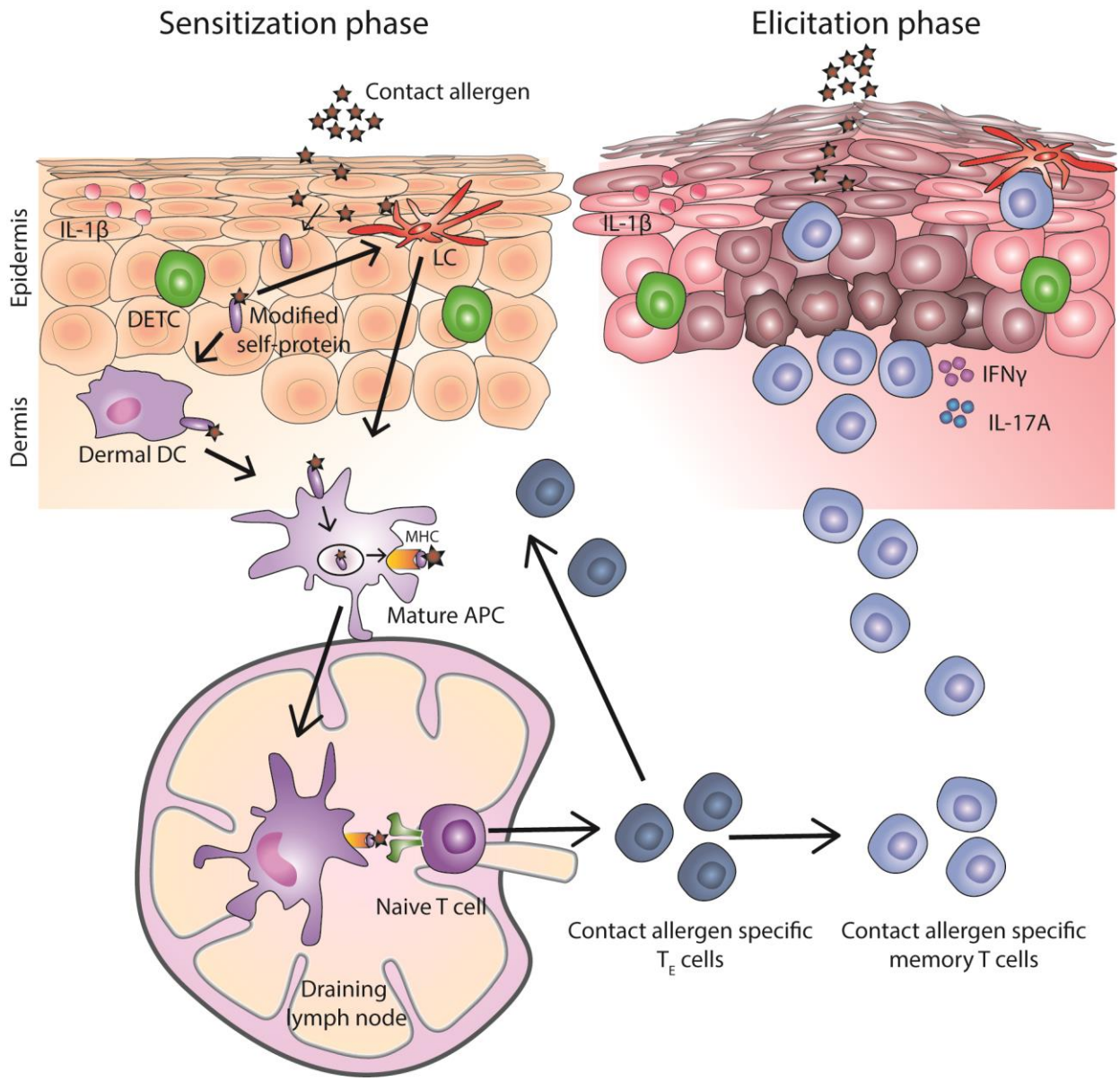

Figure 4: Contact allergy model in mice. *Contact allergy can be divided into the initial sensitization phase (left) and the later elicitation phase (right). In the sensitization phase, the contact allergen must penetrate the skin, modify self-proteins and induce inflammation in the skin. Crucial for the sensitization phase is memory formation, starting with the activation of dendritic cells (DC) which migrate to the draining lymph nodes. Here they present the contact allergen to naïve T cells, which differentiate into effector T (T_E) cells that migrate to the contact allergen-affect skin. Some of the T_E cells later turn into memory T cells. In the elicitation phase, the second encounter with the contact allergen activates the memory T cells and the clinically symptoms of contact allergy appears. Interleukin (IL), interferon (IFN), dendritic epidermal T cells (DETC), Langerhans cells (LC), major histocompatibility complex (MHC), antigen presenting cells (APC).*

3.3 The role of epidermal γδ T cells in contact allergy

The role of DETC in contact allergy is discussed, as DETC have been shown to have both inflammatory and regulatory roles[43,102,140,145–152].

Studies have shown that upon contact allergen exposure of the skin, DETC round up and secrete inflammatory cytokines giving them an active, inflammatory role in the CHS response. Studies from our lab have shown that upon contact allergen exposure, DETC become activated in an IL-1β-dependent manner and secrete IL-17A and IFNγ[43]. Further work has also shown that TCRδ$^{-/-}$ mice have reduced ear swelling after allergen challenge, indicating and inflammatory role for DETC[43,140].

Further evidence exists for an inflammatory role for DETC in CHS. In a study by Huber *et al.*, they showed how DETC are involved in the induction of contact allergy. They demonstrated that epidermal cells treated with contact allergens, directly affect DETC through cell-cell contact by activating the DETC in a non-MHC-restricted way[146]. Studies from our lab have also shown that contact allergens upregulate the NKG2D ligands Mult-1, H60 and Rae-1 on KC which react with DETC that express the NKG2D receptor leading to IFNγ production[145].

A study by Dieli and colleagues revealed how DETC indirectly are important for the CHS response, by helping CD8$^+$ T cells inducing the response. They showed that only CD8$^+$ T cells together with DETC from sensitized mice could transfer the CHS reaction to naïve recipient mice[102]. Other studies support these findings[147,148].

The strongest evidence for a regulatory function of DETC in CHS is KO studies, which show that without DETC in the skin the CHS response is increased. In a study by Girardi and colleagues, they used TCRδ$^{-/-}$ mice on different backgrounds and found that FVB.δ$^{-/-}$ mice, but not C57BL.δ$^{-/-}$ mice, developed increased CHS measured as increased ear thickness after contact allergen painting. The authors argued that DETC down-regulated CHS. They also found that the FVB.δ$^{-/-}$ mice developed spontaneous dermatitis, but that this was only the case when αβ T cells were present. They concluded that the regulatory function of DETC is dependent on the inflammation mediated by the αβ T cells[149].

These findings are supported by a study from Guan *et al.*, who demonstrated an increased ear swelling in TCRδ$^{-/-}$ mice, this time on a C57BL/6 background. They also found that

$CD8^+$ T cells from $TCR\delta^{-/-}$ mice have higher cytolytic activity, from which they conclude that the regulatory function of γδ T cells in CHS is based on limiting $CD8^+$ T cells[150].

Sullivan *et al.* showed that activated contact allergen-conjugated DETC down-regulate CHS. They purified cells from the skin, incubated them with the contact allergen and i.v. delivered them to the mice at day -7. At day 0, the mice were sensitized on the abdomen and challenged on the ear 5 days later. They saw that mice that received the contact allergen-conjugated DETCs had a lower ear swelling response[151]. The study is supported by Welsh *et al.* who showed that a s.c. delivery of a FITC-conjugated DETC cell line induced a lower ear swelling after challenge, further indicating that DETC can down-regulate the CHS response[152].

These findings are opposite to the findings described by our lab and others illustrating the conflicting role of DETCs in CHS[43,102,140,145–148].

Studies have addressed the relationship between human γδ T cells and contact allergy[78,153]. For example, it has been shown that there are increased numbers of IFNγ, IL-17A and IL-22 producing γδ T cells in nickel-challenged skin from nickel-allergic patients[78]. However this study does not specify whether it is dermal or epidermal γδ T cells. Further work has found increased γδ T cell infiltration in dinitrochlorobenzene (DNCB) challenged skin after 48 hours, both in epidermis and dermis, indicating that γδ T cells might not initiate but amplify the response[153]. Studies performed in skin disorders other than ACD have shown that in some cases, an increased number of γδ T cells can be found in the skin. A publication using neoplastic dermatosis samples, specifically Langerhans cell histiocytosis by Alaibac *et al.*, demonstrated increased γδ T cells in dermis and epidermis compared to the number they can detect in heathy skin[154]. However contradictory to this finding a publication by Dupuy *et al.*, investigated many different inflamed skin disorders and found that the number of $CD3^+$ cells varied a lot, with very few or no detectable γδ T cells at all[11].

3.4 The role of $CD8^+$ tissue-resident memory T cells in contact allergy

In the clinic, $CD8^+$ T_{RM} cells have been addressed for years without knowing of their existence, as the T_{RM} cell lineage was not discovered yet. It has been well described that flare-up reactions can occur at previous-affected specific skin sites in patients suffering from ACD when they applied the contact allergen at a different skin location, a now known clear sign of local memory in the skin[98,99].

Though the characterisation of T_{RM} cells were yet to be described, local T cell memory had been observed and reported in contact allergy research for more than 35 years[155]. Scheper and colleagues showed that skin that had already been in contact with contact allergen had an increased and faster reaction to the same contact allergen, compared to skin from mice that only had circulating memory T cells but no local memory[155]. These findings are supported by recent studies, showing how $CD8^+$ T_{RM} cells persist in the skin after CHS induction[72,83,156].

$CD8^+$ T_{RM} cells produce IL-17A and IFNγ in response to contact allergens[70,156,157]. Both T_{RM} cells and circulating memory T cells are important in contact allergy, but $CD8^+$ T_{RM} cells produce a more rapid response in contact allergen-challenged skin[156]. Studies have also shown that multiple exposures to the same contact allergen lead to an increased $CD8^+$ T_{RM} cell pool in the skin and increased ear thickness, indicating an increased inflammatory response[72,83].

3.5 The role of $CD4^+$ T cells in contact allergy

In the response to contact allergens, $CD4^+$ T cells have also been shown to accumulate in the skin[82] and they have both pro- and anti-inflammatory roles as shown in multiple mouse studies using antibody-depletion of $CD4^+$ cells and CD4 KO mice[103,131,134–136,158]. The dual role of $CD4^+$ T cells was demonstrated by Gocinski and colleagues who showed that antibody-depletion of $CD4^+$ T cells prior to DNFB sensitization induced an enhanced ear thickness compared to controls in mice. Thus demonstrating an anti-inflammatory role for $CD4^+$ T cells in CHS. The authors then went on to shown that antibody-depletion of $CD8^+$ T cells decrease the response to DNFB but that depletion of both $CD4^+$ and $CD8^+$ T cells was necessary to completely abolished the response, indicating that $CD4^+$ cells also have an inflammatory role in CHS[103].

Allergen exposure has been shown to induce both Th1 (IL-2, IFNγ, TNFα), Th2 (IL-4), Th17 (IL-17) and T_{reg} (IL-10) responses depending on the contact allergen used [78,134–139,142,144]. However all these studies investigated $CD4^+$ T cells from the dLN or from full skin biopsies and it is therefore difficult to distinguish the cytokine contribution of epidermal $CD4^+$ T cells from that of other $CD4^+$ T cells in the response to allergens.

Thesis objectives

The overall aim of this PhD thesis was to explore the interplay between epidermal T cell subsets and to address specific and unspecific activation upon contact allergen challenge. The specific aims of the studies were:

Study I: To explored the interplay between epidermal T cell subsets in relation to contact allergy in mice

- By characterizing the dynamics between DETC and $CD8^+$ T_{RM} cells in the response to contact allergen
- By examining how different contact allergen doses and multiple exposures affect the dynamics between DETC and $CD8^+$ T_{RM} cells
- By studying possible mechanisms involved in the persistence of $CD8^+$ T_{RM} cells in the epidermis

Study II: To investigate the epidermal T cell subsets in relation to age and antigen exposure in both mice and humans

- By exploring the role of age on the epidermal T cell composition
- By exploring the role of antigen-exposure on the epidermal T cell composition

Study III: To study the contact allergen induced inflammation in sensitized skin in mice

- By investigating if a contact allergen-independent response could be generated in sensitized skin
- By investigating possible inflammatory differences between a contact allergen specific and unspecific response

Study I

Pathogenic CD8$^+$ epidermis-resident memory T cells displace dendritic epidermal T cells in allergic dermatitis

Journal Pre-proof

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Pathogenic CD8$^+$ epidermis-resident memory T cells displace dendritic epidermal T cells in allergic dermatitis

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Abstract

The skin is our interface with the outside world and consequently it is exposed to a wide range of microbes and allergens. Recent studies have indicated that allergen-specific skin-resident memory T (T_{RM}) cells play a role in allergic contact dermatitis (ACD). However, the composition and dynamics of the epidermal T cell subsets during ACD are not known. Here we show that exposure of the skin to the experimental contact allergen 1-fluoro-2,4-dinitrobenzene (DNFB) results in a displacement of the normally occurring dendritic epidermal T cells (DETC) concomitant with an accumulation of epidermal $CD8^+CD69^+CD103^+$ T_{RM} cells in mice. By studying knock-out mice, we provide evidence that $CD8^+$ T cells are required for the displacement of the DETC and furthermore, that DETC are not required for recruitment of $CD8^+$ T_{RM} cells to the epidermis following allergen exposure. We demonstrate that the magnitude of the allergic reaction correlates with the number of $CD8^+$ epidermal T_{RM} cells that again correlates with allergen dose and number of allergen exposures. Finally, in an attempt to elucidate why $CD8^+$ epidermal T_{RM} cells persist in the epidermis, we show that $CD8^+$ epidermal T_{RM} cells have a higher proliferative capability and are bioenergetically more stable, displaying a higher spare respiratory capacity than DETC.

Introduction

Allergic contact dermatitis (ACD) is a major T cell-mediated disease induced by exposure of the skin to contact allergens. Approximately 27% of the European population is sensitized to one or more contact allergen (Diepgen et al., 2016) and estimated 15% of the population develops ACD. ACD can be highly disabling and is characterized by an intensely itching erythema, edema and often vesicles at sites where the allergens contact the skin (Martin et al., 2018). Our knowledge of the immunological mechanisms in ACD in humans has been greatly advanced by studying ACD, often called contact hypersensitivity (CHS) reactions, in mice (Honda et al., 2013). The outmost layer of the skin is called the epidermis and it is mainly composed of keratinocytes. Scattered in between the keratinocytes are epidermal sentinels of the immune system. In mice, the epidermal T cells are dominated by $V\gamma3^{+}V\delta1^{+}$ (Garman nomenclature) (Garman et al., 1986) γδ T cells, termed dendritic epidermal T cells (DETC) (Asarnow et al., 1988, Havran and Allison, 1988, Nielsen et al., 2017, Stingl et al., 1987), whereas in humans both γδ and αβ T cells are found in the epidermis (Toulon et al., 2009). It is known that T cells play important roles in ACD (Gocinski and Tigelaar, 1990, Scheper et al., 1983, Wang et al., 2000, Xu et al., 1996), and that DETC can play both inflammatory and anti-inflammatory roles in response to contact allergens (Dieli et al., 1997, Dieli et al., 1998, Nielsen et al., 2014, Sullivan et al., 1986, Welsh and Kripke, 1990). In addition to resident T cells, activated circulating T cells can enter inflammatory skin sites non-specifically where they produced long-lasting local immunological memory (Mackay et al., 2012, Scheper et al., 1983). We and others have recently shown that exposure of the skin to contact allergens leads to accumulation of allergen-specific $CD8^{+}CD69^{+}CD103^{+}$ memory T cells in the skin, and that these tissue-resident memory T (T_{RM}) cells play a central role in the rapid-onset hypersensitivity reactions to contact allergens (Gaide et al., 2015, Gamradt et al., 2019, Schmidt et al., 2017). However, the composition and dynamics of the various epidermal T cell subsets during CHS are not known. The purpose of

this study was to determine how contact allergens affect the composition and dynamics of epidermal T cell subsets, how the magnitude of the CHS reaction correlates to the presence of various T cell subsets in the epidermis and furthermore, to examine possible mechanisms involved in the persistence of $CD8^+$ T_{RM} cells in the epidermis after exposure of the skin to allergens.

Results

Exposure to allergen leads to generation of CD8$^+$ epidermal T_{RM} cells and a decline of DETC

To determine the effect of allergens on the composition of epidermal T cells, we sensitized mice on their ears with the experimental allergen 1-fluoro-2,4-dinitrobenzene (DNFB) in olive oil:acetone (OOA) or with pure OOA as control for three consecutive days and then challenged them twice with an interval of 21 days (Fig. 1a). After an additional 21 days, we determined the distribution of T cell subsets in the epidermis by flow cytometric analyses (Fig 1b and c and Fig. S1 for gating strategy). Consistent with previous reports (Nielsen et al., 2017), Vγ3$^+$CD69$^+$CD103$^+$ DETC constituted the vast majority of the T cells in the epidermis of control mice, whereas the frequency of CD4$^+$ and CD8$^+$ T cells was < 1%. In contrast, exposure to DNFB resulted in a massive increase in the frequency of CD8$^+$CD69$^+$CD103$^+$ T_{RM} cells rising to approximately 60% of the epidermal T cells with a concomitant decrease in the frequency of DETC to approximately 30% (Fig. 1b and c and Fig. S1 and S2). A minor fraction of epidermal CD4$^+$CD69$^+$ T cells was also generated. In contrast to the CD8$^+$ epidermal T_{RM} cells, only 75% of the CD4$^+$CD69$^+$ T_{RM} cells was CD103$^+$ (Fig. S2) (Gebhardt et al., 2011). To determine whether the DNFB-induced decrease in the frequency of DETC was caused by an actual reduction in the number of DETC or just represented a dilution of the DETC by the incoming CD8$^+$ T_{RM} cells, we determined the actual numbers of DETC and CD8$^+$ T_{RM} cells per mm^2 using 3D images obtained by confocal scanning laser microscopy (CSLM) of epidermal ear sheets (Fig. 1d). In control mice, we found 587 ± 127 DETC per mm^2 epidermis and no CD8$^+$ T_{RM} cells (Fig 1e). In mice exposed to DNFB, we detected an approximately 60% reduction in the number of DETC to 254 ± 175 per mm^2 concomitant with an increase of CD8$^+$ T_{RM} cells to 587 ± 242 per mm^2 (Fig 1e) suggesting that CD8$^+$ T_{RM} cells replace DETC in skin exposed to contact allergens. This was further supported by the inverse relationship observed by plotting the numbers of DETC and CD8$^+$ T_{RM} cells per mm^2 in 18 epidermal ear sheets from mice exposed to

DNFB (Fig. 1f). These experiments demonstrated that exposure to the contact allergen DNFB results in generation of $CD8^+$ T_{RM} cells and a decline in the number of DETC in the epidermis of the exposed area.

$CD8^+$ epidermal T_{RM} cells displace DETC in CHS

That exposure to contact allergen lead to recruitment of $CD8^+$ T_{RM} cells concomitant with a decrease in the number of DETC in the epidermis of the exposed skin area could suggest that DETC and $CD8^+$ T_{RM} cells compete for local survival factors in the epidermal niche. Alternatively, the disappearance of DETC and generation of $CD8^+$ T_{RM} cells could be independent events induced by the allergic response. To determine whether $CD8^+$ T_{RM} cells actually displace DETC in CHS, we studied the cell composition in the epidermis after exposure to allergen in B6.129-*B2m*^{tm1jae}N12 mice that do not express MHC I molecules, and hence are deficient in $CD8^+$ T cells but unaffected in their $CD4^+$ and γδ T cells (Koller et al., 1990, Zijlstra et al., 1990). We used the schedule for allergen exposure as depicted in Fig. 1a in these experiments. As in wild-type mice, we found that $CD69^+CD103^+$ DETC completely dominated the epidermal T cells in control B6.129-*B2m*^{tm1jae}N12 mice treated with OOA (Fig. 2a-d). As expected, no $CD8^+$ T_{RM} cells were detected. In contrast to wild-type mice, DETC still constituted the vast majority of the epidermal T cells in B6.129-*B2m*^{tm1jae}N12 mice treated with DNFB (Fig. 2a-d). The small decrease in the fraction of DETC might be due to the generation of a small population of $CD4^+$ T cells (Fig 2a and Fig. S3).

Next, we asked whether DETC are required for the generation of $CD8^+$ T_{RM} cells. To answer this question, we determined the cell composition in the epidermis after exposure to allergen in B6.129P2-*Tcrd*^{tm1Mom}/J mice. B6.129P2-*Tcrd*^{tm1Mom}/J mice have a deletion in the TCRδ gene and consequently do not have any γδ T cells, including DETC (Itohara et al., 1993). As expected, no DETC were found in control B6.129P2-*Tcrd*^{tm1Mom}/J mice treated with OOA. Instead, the epidermal

T cells were dominated by $CD4^-CD8^-CD103^+$ αβ T cells (Fig. 2e and f and Fig. S4) (Itohara et al., 1993, Jameson et al., 2004). Interestingly, exposure to DNFB resulted in the generation of a major population of $CD8^+CD69^+CD103^+$ epidermal T_{RM} cells as seen in wild-type mice (Fig. 2e-h and Fig. S4). Together these data demonstrated that exposure to allergen alone does not lead to displacement of the DETC but that $CD8^+$ T_{RM} cells are required to displace the DETC, and furthermore they show that DETC are not required for the generation of $CD8^+$ T_{RM} cells.

The magnitude of the CHS reaction correlates with the frequency of $CD8^+$ epidermal T_{RM} cells

It has been demonstrated that skin T_{RM} cells play a role in rapid-onset CHS reactions (Gaide et al., 2015, Schmidt et al., 2017). However, how the various epidermal T cell subsets affect the magnitude of the CHS reaction is not known. To determine if the magnitude of the CHS reaction correlated with the number of $CD8^+$ T_{RM} cells, we exposed mice to DNFB as depicted in Fig. 3a. We measured ear thickness at day 0 and 24 hours after the challenge at day 24 and 45. Ear thickness was measured 24 hours after exposure to the allergen to determine the role of T_{RM} cells in rapid-onset CHS reactions and to avoid any contribution of circulating memory T cells to the reaction (Gaide et al., 2015). T cell composition in the epidermis was determined 21 days after the last allergen exposure at day 23, 44 and 65. We found that the magnitude of the CHS reaction, as measured by the increase in ear thickness, directly correlated with the frequency of $CD8^+$ T_{RM} cells and the number of DNFB exposures and inversely correlated with the frequency of DETC (Fig. 3b-d). Next, we wanted to determine how the dose of the allergen affected the epidermal cell composition and the magnitude of the CHS reaction. We treated the mice as depicted in Fig. 1a with increasing doses of DNFB. We found that the magnitude of the CHS reaction and the frequency of $CD8^+$ T_{RM} cells di-

rectly correlated with the dose of allergen, whereas the frequency of DETC inversely correlated with the dose of allergen (Fig. 3e-g).

Expansion of the CD8$^+$ epidermal T_{RM} cell population is mediated by a combination of local proliferation and recruitment from the circulation

The increase in the number of CD8$^+$ T_{RM} cells seen after repeated exposures to allergen might be mediated by recruitment of new allergen-specific memory CD8$^+$ T cells from the circulation, by proliferation of the CD8$^+$ T_{RM} cells in the epidermis or by a combination of the two. To examine these possibilities, we exposed mice to DNFB day 0, 1, 2 and 23 as depicted in Fig. 4a. Half of the mice were given FTY720 in their drinking water from day 22 to the end of the experiment. FTY720 sequesters lymphocytes in lymphoid tissues resulting in a rapid depletion of circulating T cells (Chiba, 2005, Gaide et al., 2015). We noticed a significant reduction in the frequency of CD8$^+$ T_{RM} cells and an increase in the frequency of DETC in mice exposed to DNFB and treated with FTY720 compared to mice exposed to DNFB but not treated with FTY720 (Fig. 4b-d). This indicated that part of the expansion of CD8$^+$ T_{RM} cells normally is caused by recruitment of CD8$^+$ T cells from the circulation. To determine the contribution of CD8$^+$ T cells from the circulation to the expansion of the CD8$^+$ T_{RM} cells, we compared the increase in frequency of CD8$^+$ T_{RM} cells from day 23 (from the experiments shown in Fig. 3d) to day 44 in mice exposed to DNFB either with or without FTY720 treatment. In mice not treated with FTY720, the frequency of CD8$^+$ T_{RM} cells increased approximately 2.5 fold, whereas in mice treated with FTY720 it only increased approximately 1.5 fold (Fig. 4e). These experiments suggested that approximately 40% of the expansion of the CD8$^+$ T_{RM} cells was caused by recruitment of CD8$^+$ T cells from the circulation and 60% of the expansion was due to proliferation of the already existing CD8$^+$ T_{RM} cells. To further investigate this, we exposed mice to DNFB day 0, 1, 2, 21 and 42 as depicted in Fig. 4f. Mice were given bromodeoxyur-

idine (BrdU) i.p. at day 44 and 45, and the frequency of proliferating DETC and $CD8^+$ T_{RM} cells in epidermis were determined at day 46 by Ki-67 and anti-BrdU staining (Fig. 4g and h). DNFB did not induce proliferation of the DETC, whereas it clearly induced proliferation of the $CD8^+$ T_{RM} cells with approximately 30% and 40 % being positive for Ki-67 and BrdU, respectively (Fig. 4g and h). Taken together, these data indicate that the expansion of $CD8^+$ T_{RM} cells is mediated by a combination of recruitment of cells from the circulation and by proliferation of $CD8^+$ T_{RM} cells already present in the epidermis.

$CD8^+$ epidermal T_{RM} cells have a higher spare respiratory capacity than DETC

The composition of the T cell subsets in the epidermis depends on recruitment, proliferation and survival of the individual subsets. The aryl hydrocarbon receptor (AhR) is an important factor in maintenance of both DETC and $CD8^+$ T_{RM} cells (Kadow et al., 2011, Li et al., 2011, Zaid et al., 2014). Another important molecule in the development and maintenance of $CD8^+$ memory T cells is Bcl-2 (Best et al., 2013, Grayson et al., 2000, Kurtulus et al., 2012). To investigate whether the expression levels of AhR and Bcl-2 in DETC and $CD8^+$ T_{RM} cells could explain the apparent advantage of $CD8^+$ T_{RM} cells to exist in the epidermis, we determined the expression level of AhR and Bcl-2 in DETC and $CD8^+$ T_{RM} cells isolated from epidermal sheets a minimum of 21 days after exposure to DNFB. We found that DETC and $CD8^+$ T_{RM} cells expressed similar levels of AhR (Fig. 5a and b) and that DETC surprisingly expressed approximately 2.5-fold higher levels of Bcl-2 than $CD8^+$ T_{RM} cells (Fig. 5c and d). As the expression levels of AhR and Bcl-2 in epidermal T cells 21 days after exposure to DNFB did not readily explain any survival advantage of $CD8^+$ T_{RM} cells over DETC, we next investigate the rapid effect of DNFB on epidermal T cell death. Mice were exposed to DNFB on day 0, 1, 2 and 21, and the number of apoptotic and dead cells were subsequently determined 24 and 48 hours after the last exposure. DNFB induced a small but significant increase in

the number of apoptotic and dead cells in both DETC and $CD8^+$ T_{RM} cells after 24 and 48 hours, respectively (Fig 5e and f). The fraction of apoptotic and dead cells was low between 0.5 – 4% for both DETC and $CD8^+$ T_{RM} cells. However, whereas the total number of DETC decreased slightly from 24 to 48 hours after exposure to DNFB, the number of $CD8^+$ T_{RM} cells slightly increased (Fig. 5g).

Finally, as changes in T cell metabolism are essential for generation and survival of memory T cells, we next wanted to investigate the metabolism of DETC and $CD8^+$ T_{RM} cells (Araki et al., 2009, Pan et al., 2017, Pearce et al., 2009, van der Windt et al., 2012, van der Windt and Pearce, 2012). Consequently, we isolated $CD8^+$ T_{RM} cells and DETC and determined their bioenergetics profiles. We found that oxidative phosphorylation (OXPHOS), as measured by the basal oxygen consumption rate (OCR), and the basal extracellular acidification rate (ECAR) were increased in $CD8^+$ T_{RM} cells compared to DETC (Fig. 5h, i and k). Strikingly, $CD8^+$ T_{RM} cells demonstrated substantially larger spare respiratory capacity than DETC (Fig. 5h and j). As the spare respiratory capacity is the extra capacity available for a cell to produce energy and it is believed to be important for long-term cell survival and function, these data suggested that the advantage of $CD8^+$ T_{RM} cells over DETC for persistence in the epidermis partly could be explained by a superior metabolic fitness of $CD8^+$ T_{RM} cells.

Discussion

In the present study, we show that an accumulation of $CD8^+$ T_{RM} cells concomitant with a reduction in the normally occurring DETC takes place following exposure of the skin to allergen. This relation between $CD8^+$ T_{RM} cells and DETC after exposure to allergen has not been described before, but interestingly, a similar pattern is found during infection of the skin with herpes simplex virus (Zaid et al., 2014). These observations suggest that $CD8^+$ T_{RM} cells and DETC compete for local survival signals. Another possibility could be that inflammatory responses elicited by allergens directly cause the DETC to migrate out of the affected area. This idea is supported by a previous study that found DETC in the draining lymph nodes after exposure of the skin to allergens (Nielsen et al., 2014). However, by studying genetic modified mice, we firmly establish that exposure to allergen does not lead to displacement of the DETC in the absence of $CD8^+$ T cells and that $CD8^+$ T cells are required to displace DETC. This underpins the hypothesis that $CD8^+$ T_{RM} cells and DETC compete for local survival signals within the epidermal niche.

It has been shown that $CD4^+$ T cells are required for the recruitment of $CD8^+$ T cells to virus-infected vaginal mucosa (Nakanishi et al., 2009). As no or very few $CD4^+$ T cells are found in normal mouse epidermis, we asked whether DETC might be required for recruitment and generation of $CD8^+$ T_{RM} cells. By studying genetic modified mice with no γδ T cells, we demonstrated that $CD8^+$ T_{RM} cells are formed in the absence of γδ T cells, including DETC. Since the genetic modified mice had a large population of atypical $CD4^-CD8^-$ T cells in the epidermis, we could not formally exclude that other T cells than γδ T cells might play a role for recruitment of $CD8^+$ T cells to the epidermis. However, we could conclude that γδ T cells, including DETC, are not required in this process. Furthermore, we found that expansion of the $CD8^+$ T_{RM} cell population after re-exposure to allergen was caused both by local proliferation of the $CD8^+$ T_{RM} cells and influx of new $CD8^+$ T

cells from the circulation. Importantly, we confirmed and expanded the pathogenic role of the allergen-specific $CD8^+$ T_{RM} cells by demonstrating that the magnitude of the rapid-onset CHS reaction directly correlated with the number of $CD8^+$ T_{RM} cells and inversely correlated with the number of DETC.

In addition, insight into survival mechanisms determining the balance between the $CD8^+$ T_{RM} cells and DETC is central for understanding how the T cell composition in the epidermis is regulated. The long-time survival of DETC and $CD8^+$ T_{RM} cells is dependent on their expression of the AhR (Kadow et al., 2011, Li et al., 2011, Zaid et al., 2014), and it has been suggested that DETC and $CD8^+$ T_{RM} cells compete for AhR ligands following skin infections (Zaid et al., 2014). We found that DETC and $CD8^+$ T_{RM} cells express the AhR at similar levels following exposure to allergen. Therefore, our finding does not support a hypothesis in which differential AhR expression causes the survival advantage seen in $CD8^+$ T_{RM} cells. Likewise, Bcl-2 expression levels, known to be important for survival of memory T cells (Best et al., 2013, Grayson et al., 2000, Kurtulus et al., 2012), did not explain the survival advantage of $CD8^+$ T_{RM} cells, as DETC surprisingly express higher levels of Bcl-2 than $CD8^+$ T_{RM} cells. $CD8^+$ memory T cells have a larger spare respiratory capacity than naïve and effector T cells (van der Windt et al., 2012). The spare respiratory capacity is the reserve energy-generating capacity of the mitochondria in response to increased work and stress, and it is important for memory T cell development, survival and for their rapid recall ability (Geltink et al., 2018, van der Windt et al., 2012, van der Windt et al., 2013, van der Windt and Pearce, 2012). We found that $CD8^+$ T_{RM} cells have substantial larger spare respiratory capacity than DETC, suggesting that $CD8^+$ T_{RM} cells are more metabolic fit and therefore might have a survival advantage over DETC. Interestingly, a recent study found that $CD8^+$ T_{RM} cells require uptake of free fatty acids for their long-time survival (Pan et al., 2017). These observations suggest that $CD8^+$

T_{RM} cells might outcompete DETC due to their bioenergetic advantage and their ability to take up and metabolize exogenous free fatty acids. This is also supported by our observation that $CD8^+$ T_{RM} cells responded to DNFB by significant proliferation, whereas DETC did not proliferate in response to DNFB. In contrast to cell proliferation, DNFB induced apoptosis and cell death to the same low degree in $CD8^+$ T_{RM} cells and DETC. This argued against a direct cytotoxic effect of the $CD8^+$ T_{RM} cells on the DETC in the acute phase but did not exclude this possibility on a longer time perspective.

In conclusion, we here carefully determined the composition and dynamics of the epidermal T cell subsets during CHS and show that $CD8^+$ T_{RM} cells accumulate in the epidermis and displace the normally occurring DETC following exposure of the skin to contact allergens. We show that the magnitude of the rapid-onset CHS reaction correlates with the frequency of the $CD8^+$ T_{RM} cells and that these pathogenic cells might out-compete the DETC probably due to an increased proliferative capacity, which might be mediated by a superior metabolic fitness. Our results indicate that local immunomodulatory therapy targeting the metabolism of $CD8^+$ T_{RM} cells may provide a novel therapeutic strategy for the treatment of ACD.

Materials and Methods

See the Supplementary Materials online for detailed experimental methods.

Mice

Female C57BL/6 mice were from Taconic (Ry, Denmark) and Janvier Labs (Le Genest-Saint-Isle, France). β2-microglobulin knock-out B6.129-*B2m*^{tm1Jae} N12 (model # B2MN12-F) (Zijlstra et al., 1990) and TCRδ knock-out B6.129P2-*Tcrd*^{tm1Mom}/J (stock number: 002120) (Itohara et al., 1993) mice were from Taconic and The Jackson Laboratory (San Diego, California, USA), respectively. The mice were housed in specific pathogen-free animal facilities at the Department of Experimental Medicine, University of Copenhagen in accordance with the national animal protection guidelines (license number 2018-15-0201-01409). Mice were 8-12 weeks old at the beginning of experiments.

Contact allergy

Mice were painted with 25 µl on the dorsal side of both ears with a solution of the experimental allergen 1-fluoro-2,4-dinitrobenzene (DNFB) (Sigma-Aldrich, Brøndby, Denmark) in olive oil and acetone (OOA (1:4)) or OOA as control. A 0.15% (v/v) solution of DNFB was used unless indicated otherwise in the figures. The mice were sensitized for three consecutive days (day 0-2), challenged twice with 21-day intervals (day 23 and 44) and euthanized a minimum of 21 days after the last exposure to allergen or as otherwise depicted in the figures. Ear thickness was measured at day 0 and 24 hours after exposure to the allergen. For FTY720 treatment, mice received 2.5 µg/ml FTY720 (Sigma-Aldrich) in their drinking water the day before challenge and for the rest of the experiment. In experiments involving fluorescence-activated cell sorting, mice were shaved and exposed to 100 µl 0.15% solution of DNFB on the abdomen for three consecutive days. Cells were

purified after a minimum of 21 days after exposure to DNFB. For BrdU treatment, mice were pulsed with 1 mg BrdU (BD Biosciences, Albertslund, Denmark) diluted in 400ul PBS i.p. 48 hours and 72 hours after the second challenge.

Single-cell suspensions, flow cytometry analysis and confocal scanning laser microscopy

Purification of cells from the skin, antibodies and confocal scanning laser microscopy, as well as standard methods for flow cytometry analysis used in this study, are described in the Supplementary Materials.

Seahorse XF Analysis

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured on a Seahorse XFe96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, Massachusetts, USA). Isolated DETC and $CD8^+$ T_{RM} cells were thawed and resuspended in RPMI-1640 supplemented with 10% FBS, 1% L-glutamin and 1% penicillin-streptamycin and incubated for two hours at 37°C. Subsequently, the cells were plated in buffer-free RPMI supplemented with 143 mM NaCl and 2 mM L-glutamin. Cells were stimulated with 5 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (Sigma-Aldrich) for 15 min. The metabolic stress test was performed using 1 µM oligomycin, 1.5 µM fluorocarbonyl cyanide phenylhydrazone (FCCP), 1 µM antimycin A and 100 nM rotenone (Sigma-Aldrich).

Statistical analysis

GraphPad Prism 7 software was used to perform statistical analysis. Differences between groups were evaluated by two-tailed unpaired Student's *t* test. To evaluate the significant differences in the experiment with different numbers of exposures and cell death, two-way ANOVA followed by Bonferroni's multiple comparisons test was used. Differences were considered significant when *p*-values was <0.05. Statistically significant *p*-values are denoted as: *<0.05, **<0.01, ***<0.001, ****<0.0001.

Conflict of Interest

The authors declare no conflict of interests.

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Author contributions

ASØG designed and performed experiments, analyzed data and wrote the manuscript. MHJ performed experiments and wrote the manuscript. ABF, VM, JFW performed experiments. MA designed and assisted in CSLM imaging and performed the LSM880 images. LAC, EC assisted in metabolic analysis. ART, JDJ designed experiments. SH designed, performed and analyzed the metabolic assays and wrote the manuscript. CG analyzed data and wrote the manuscript. CMB designed experiments, analyzed data and wrote the manuscript. All authors critically proofread, edited and approved the manuscript.

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Figure legends

Figure 1. Exposure to allergen leads to generation of CD8$^+$ epidermal T_{RM} cells and a decline of DETC

(a) Experimental setup. Mice were exposed to 0.15% DNFB in OOA or OOA as control on their ears at day 0, 1, 2, 23 and 44. At day 65, epidermal ear sheets were purifed for further analyses. **(b)** Single-cell suspensions were prepared from epidermal ear sheets from control mice (first column, OOA) and mice exposed to DNFB (second column, DNFB). The CD3ε$^+$ cells were analyzed for their expression of TCRδ and Vγ3 (first row) or TCRβ and CD8a (second row) by flow cytometry. The mean percentage ± SD of cells within each quadrant is indicated. **(c)** Frequency of DETC (TCRδ$^+$Vγ3$^+$ cells) and CD8$^+$ T_{RM} cells (TCRβ$^+$CD8a$^+$ cells) of the total number of epidermal T cells (CD3ε$^+$ cells) from control mice (OOA) and mice exposed to DNFB (DNFB) as determined by flow cytometry. The data were obtained from two independent experiments with five mice in each group. The bars give the mean value and the filled circles represent the individual mice. **(d)** 3D images of epidermal ear sheets from control mice (first row, OOA) and mice exposed to DNFB (second row, DNFB). Earsheets were stained with anti-TCRγδ (green) and anti-CD8a (blue) antibodies and counterstained with SYTO 59 (red) and imaged using CLSM. **(e)** Number of DETC and CD8$^+$ T_{RM} cells per mm^2 in epidermal ear sheets from control mice (OOA) and mice exposed to DNFB (DNFB). The data are obtained from two independent experiments with nine ear sheets in each group. The bars give the mean value and the filled circles represent the individual ear sheets. **(f)** The correlation between the numbers of DETC and CD8$^+$ T_{RM} cells in ear sheets from mice exposed to DNFB. The linear regression line and Pearson's correlation coefficient (r) are shown. Significance was determined by *t*-test, N.D. = non-detectable.

Figure 2. CD8$^+$ epidermal T_{RM} cells displace DETC in CHS

(a) CD8$^+$ T cell deficient B6.129-*B2m*^{tm1Jae}N12 mice were treated as described in figure 1a. Single-cell suspensions were prepared from epidermal ear sheets from control mice (first column, OOA) and mice exposed to DNFB (second column, DNFB). The CD3ε$^+$ cells were analyzed for their expression of TCRδ and Vγ3 (first row) or TCRβ and CD8a (second row) by flow cytometry. The mean percentage ± SD of cells within each quadrant is indicated. **(b)** Frequency of DETC (TCRδ$^+$Vγ3$^+$ cells) and CD8$^+$ T_{RM} cells (TCRβ$^+$CD8a$^+$ cells) of the total number of epidermal T cells (CD3ε$^+$ cells) from control mice (OOA) and mice exposed to DNFB (DNFB) as determined by flow cytometry. **(c)** Fraction of CD69 CD103 double-positive DETC. **(d)** Total number of DETC (TCRδ$^+$Vγ3$^+$ cells). The data were obtained from two independent experiments with five mice in each group. **(e)** B6.129P2-*Tcrd*^{tm1Mom}/J mice lacking γδ T cells were treated as described in figure 1a. Single-cell suspensions were prepared from epidermal ear sheets from control mice (first column, OOA) and mice exposed to DNFB (second column, DNFB). The CD3ε$^+$ cells were analyzed for their expression of TCRδ and Vγ3 (first row) or TCRβ and CD8a (second row) by flow cytometry. The mean percentage ± SD of cells within each quadrant is indicated. **(f)** Frequency of DETC (TCRδ$^+$Vγ3$^+$ cells) and CD8$^+$ T_{RM} cells (TCRβ$^+$CD8a$^+$ cells) of the total number of epidermal T cells (CD3ε$^+$ cells) from control mice (OOA) and mice exposed to DNFB (DNFB) as determined by flow cytometry. **(g)** Fraction of CD69 CD103 double-positive CD8$^+$ cells. **(h)** Total number of CD8$^+$ T_{RM} cells (TCRβ$^+$CD8a$^+$ cells). The data were obtained from two independent experiments with five mice in each group. Significance was determined by *t*-test.

Figure 3. The frequency of CD8$^+$ epidermal T_{RM} cells correlates with the magnitude of the CHS reaction

(a) Experimental setup. Mice were exposed to 0.15% DNFB in OOA or OOA as control on their ears at day 0, 1, 2, 23 and 44. 21 days after the last exposure, at day 23, 44 or 65, epidermal ear sheets were prepared for further analyses. Ear thickness was measured at day 0, 24 and 45. **(b)** Ear thickness of control mice (OOA) and mice exposed to DNFB (DNFB). Frequency of DETC ($TCR\delta^+V\gamma3^+$ cells) and $CD8^+$ T_{RM} cells ($TCR\beta^+CD8a^+$ cells) of the total number of epidermal T cells ($CD3\varepsilon^+$ cells) from **(c)** control mice (OOA) and **(d)** mice exposed to DNFB (DNFB) as determined by flow cytometry. The data in b-d were obtained from two independent experiments with four mice in each group. **(e-g)** Mice were treated as described in figurer 1a with the indicated allergen doses: 0% (control mice, OOA), 0.01%, 0.05%, 0.15% or 0.45% DNFB. **(e)** Ear thickness measured on day 45 on control mice (0% DNFB, OOA) and on mice exposed to different DNFB doses. **(f)** Frequency of DETC ($TCR\delta^+V\gamma3^+$ cells) and **(g)** $CD8^+$ T_{RM} cells ($TCR\beta^+CD8a^+$ cells) of the total number of epidermal T cells ($CD3\varepsilon^+$ cells). The data in e-g were obtained from two independent experiments with 3-4 mice in each group. Significance was determined by two-way ANOVA with Bonferroni correction (b-d) and *t*-test (e-g).

Figure 4. Expansion of the $CD8^+$ epidermal T_{RM} cell population is mediated by a combination of local proliferation and recruitment from the circulation

(a) Experimental setup. Mice were exposed to 0.15% DNFB in OOA or OOA as control on their ears at day 0, 1, 2 and 23. At day 22 and for the rest of the experiment, half of the mice received FTY720 in their drinking water. At day 44, epidermal ear sheets were prepared for further analyses. **(b)** Frequency of DETC ($TCR\delta^+V\gamma3^+$ cells) and **(c)** $CD8^+$ T_{RM} cells ($TCR\beta^+CD8a^+$ cells) of the total number of epidermal T cells ($CD3\varepsilon^+$ cells). **(d)** The ratios of DETC and $CD8^+$ T_{RM} cells between FTY720-treated and untreated groups exposed to DNFB were calculated as [frequency of DETC or $CD8^+$ T_{RM} cells in the FTY720-treated group/frequency of DETC or $CD8^+$ T_{RM} cells in the untreat-

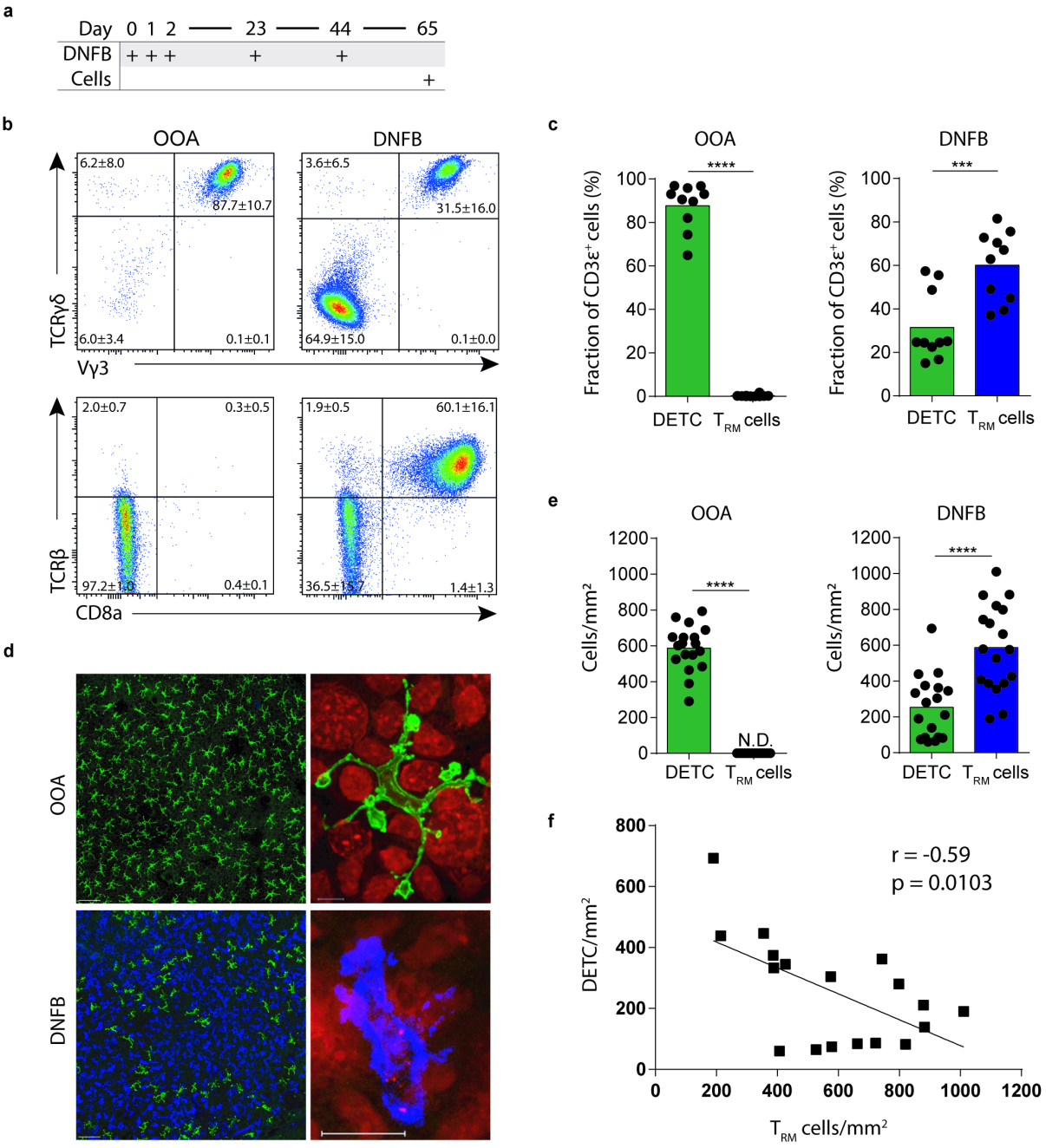
ed group]. **(e)** The increase in $CD8^+$ T_{RM} cells after challenge. The increase was calculated as [frequency of $CD8^+$ T_{RM} cells upon challenge (day 44) (-/+ FTY720 treatment)/frequency of $CD8^+$ T_{RM} cells after sensitization (day 23, data from Fig. 3d)]. **(f)** Experimental setup. Mice were exposed to 0.15% DNFB in OOA or OOA as control on their ears at day 0, 1, 2, 21 and 42. At day 44 and 45 the mice were pulsed with BrdU i.p. At day 45, epidermal ear sheets were prepared for further analyses. **(g)** Frequency of Ki-67^+ DETC and Ki-67^+ $CD8^+$ T_{RM} cells, and **(h)** $BrdU^+$ DETC and $BrdU^+$ $CD8^+$ T_{RM} cells ($TCR\beta^+CD8a^+$ cells) of the total number of epidermal T cells ($CD3\varepsilon^+$ cells). The data were obtained from two independent experiments with 3-4 mice in each group. Significance was determined by *t*-test.

Figure 5. $CD8^+$ epidermal T_{RM} cells have a higher spare respiratory capacity than DETC

(a-d) Mice were exposed to 0.15% DNFB in OOA on their ears at day 0, 1 and 2. After a minimum of 21 days, single-cell suspensions were prepared from epidermal ear sheets. **(a-b)** Expression of AhR and **(c-d)** Bcl-2 in DETC ($TCR\delta^+V\gamma3^+$ cells) and $CD8^+$ T_{RM} cells ($TCR\beta^+CD8a^+$ cells). Dashed lines illustrate fluorescence-minus-one (FMO) controls. All MFI values are subtracted the FMO value. The data were obtained from two independent experiments with four mice in each experiment. **(e-f)** Mice were exposed to 0.15% DNFB in OOA on their ears at day 0, 1, 2 and 21. At day 22 (24h) and 23 (48h), single-cell suspensions were prepared from epidermal ear sheets. **(e)** Number of apoptotic cells ($AnnexinV^+$) and **(f)** number of dead cells ($Viadye^+Annexin^{-/+}$) **(g)** Ratio of total number of DETC and $CD8^+$ T_{RM} cells at 48 hours versus at 24 hours. **(h-k)** DETC and $CD8^+$ T_{RM} cells were sorted from single-cell suspensions from epidermal sheets from the abdomen of untreated mice and mice exposed to DNFB, respectively, as described in figure 1a. Oxygen consumptions rates (OCR) and extracellular acidification rates (ECAR) of sorted DETC and T_{RM} cells were measured in real-time ex vivo on a Seahorse XFe96 Extracellular Flux Analyzer. **(h)** Cells

were stimulated with 5 ng/ml PMA and 500 ng/ml ionomycin and mitochondrial inhibitors were added as indicated (1µM oligomycin, 1.5 µM fluorocarbonyl cyanide phenylhydrazone (FCCP), 1µM antimycin A and 100nM rotenone). **(i)** Basal OCR, **(j)** spare respiratory capacity (SRC) and **(k)** Basal ECAR. The data were obtained from four individual sorts with 61-68 mice in each sort and two independent Seahorse experiments. Significance was determined by *t*-test.

Figure 1
ASØG et al.



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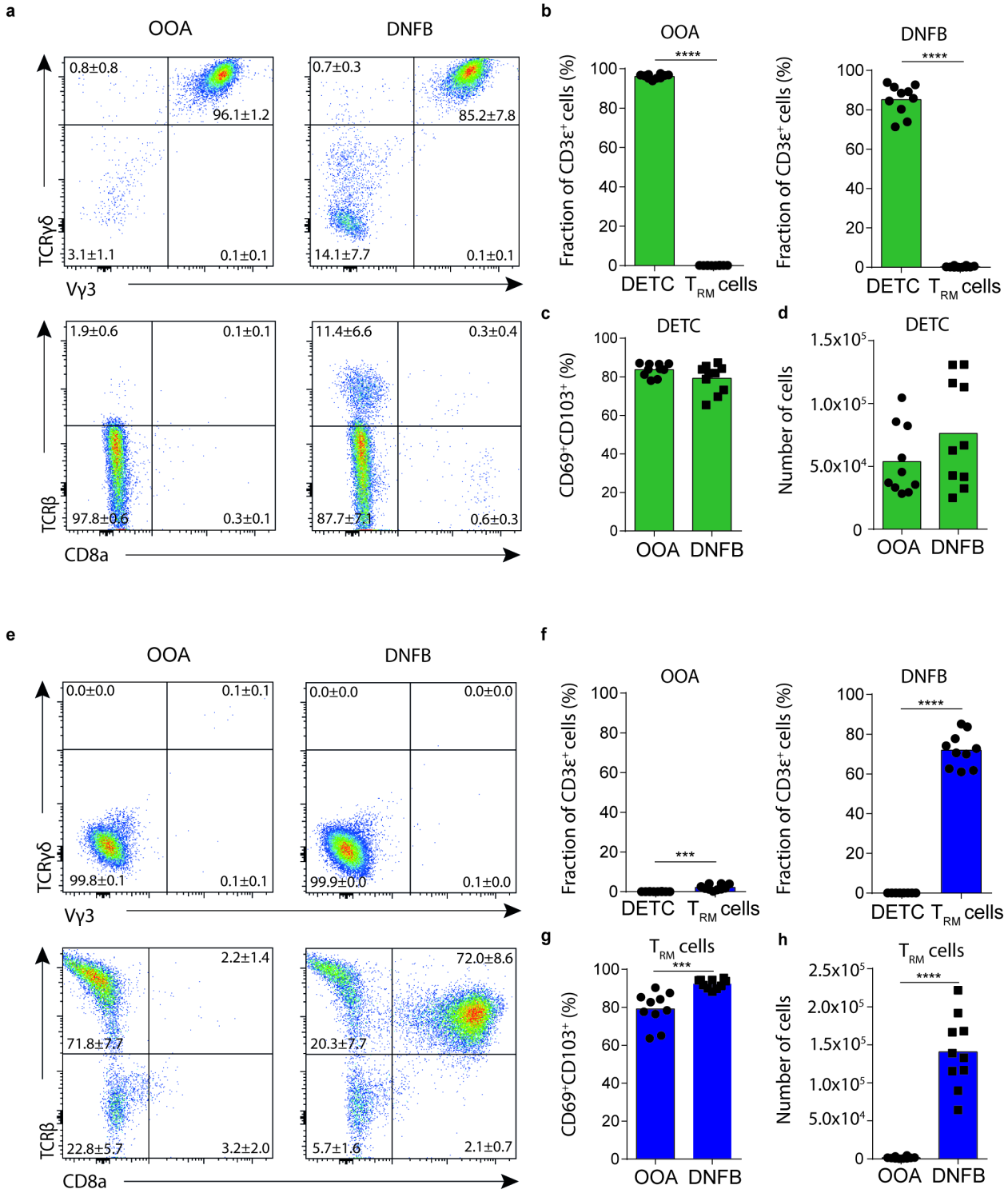

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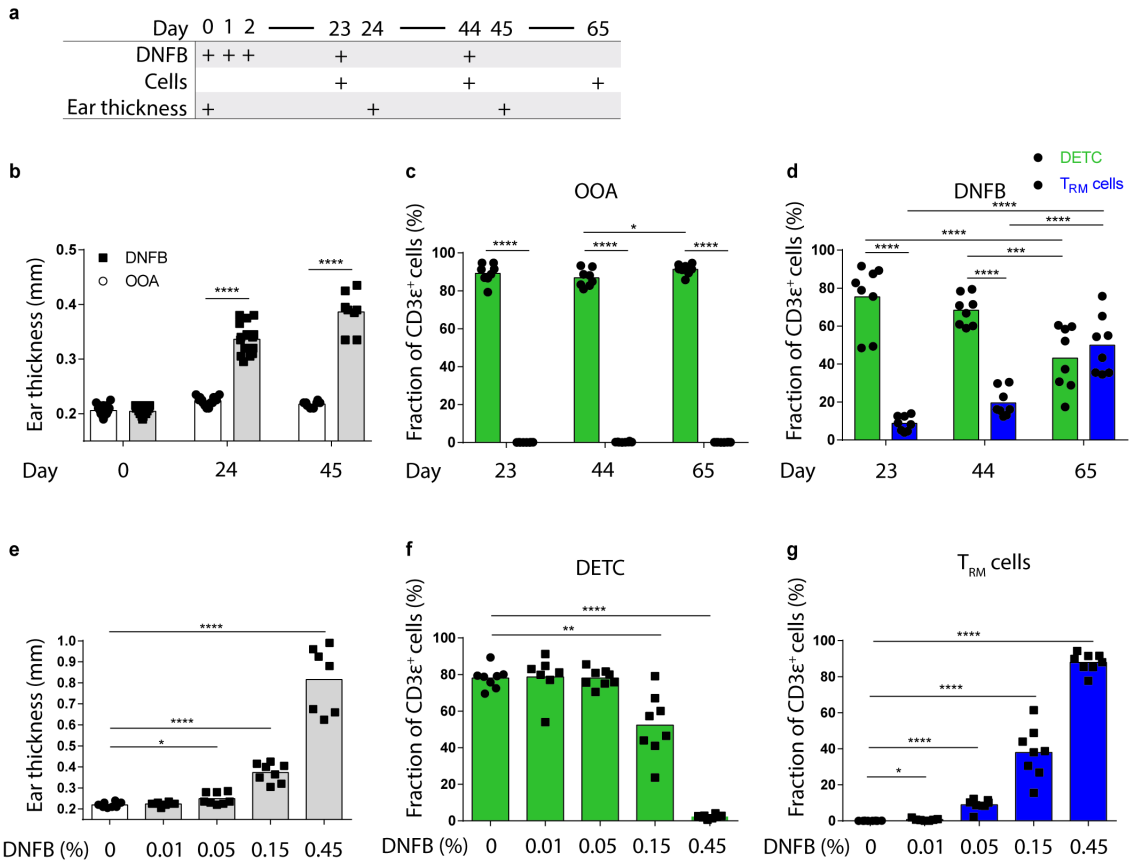

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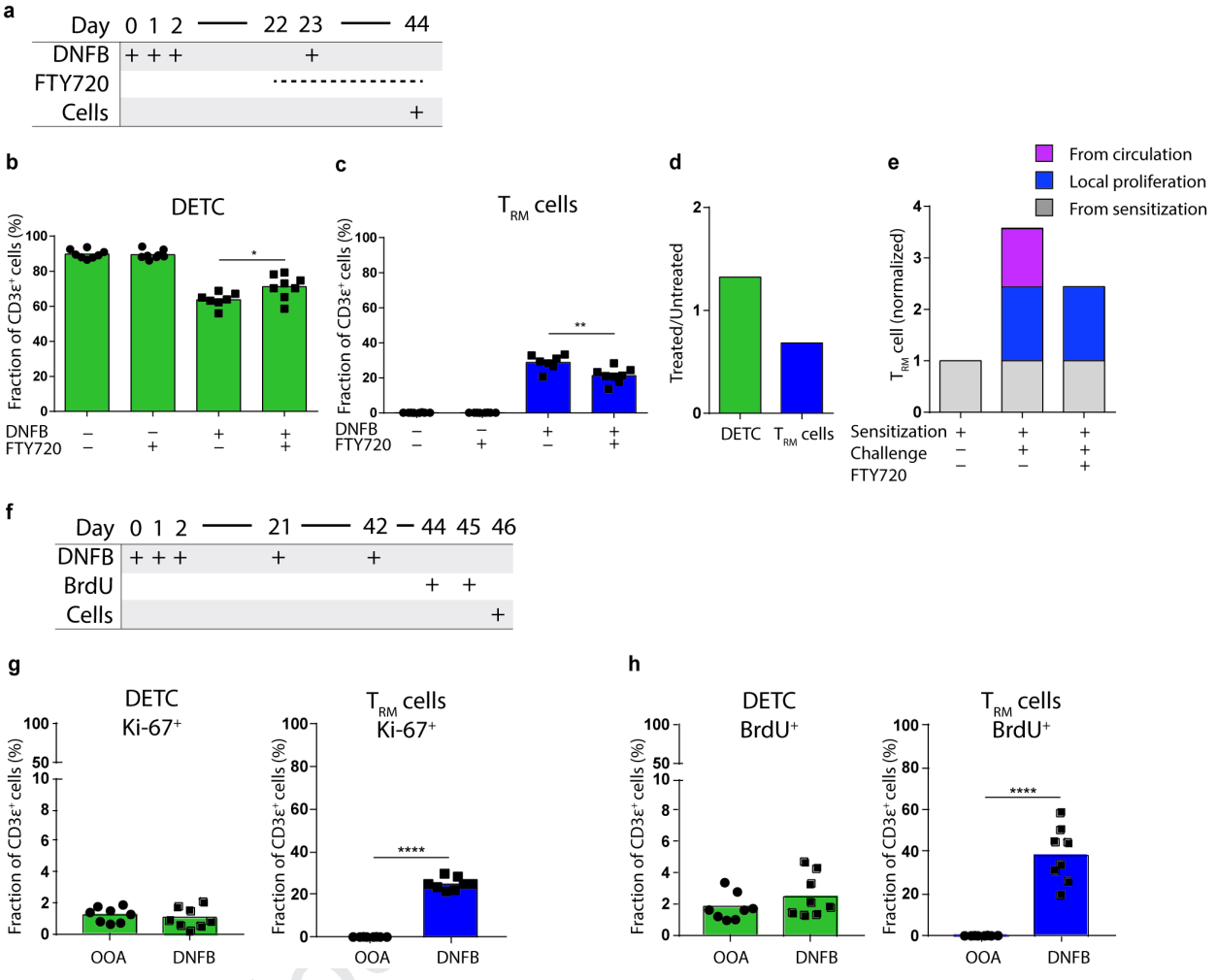

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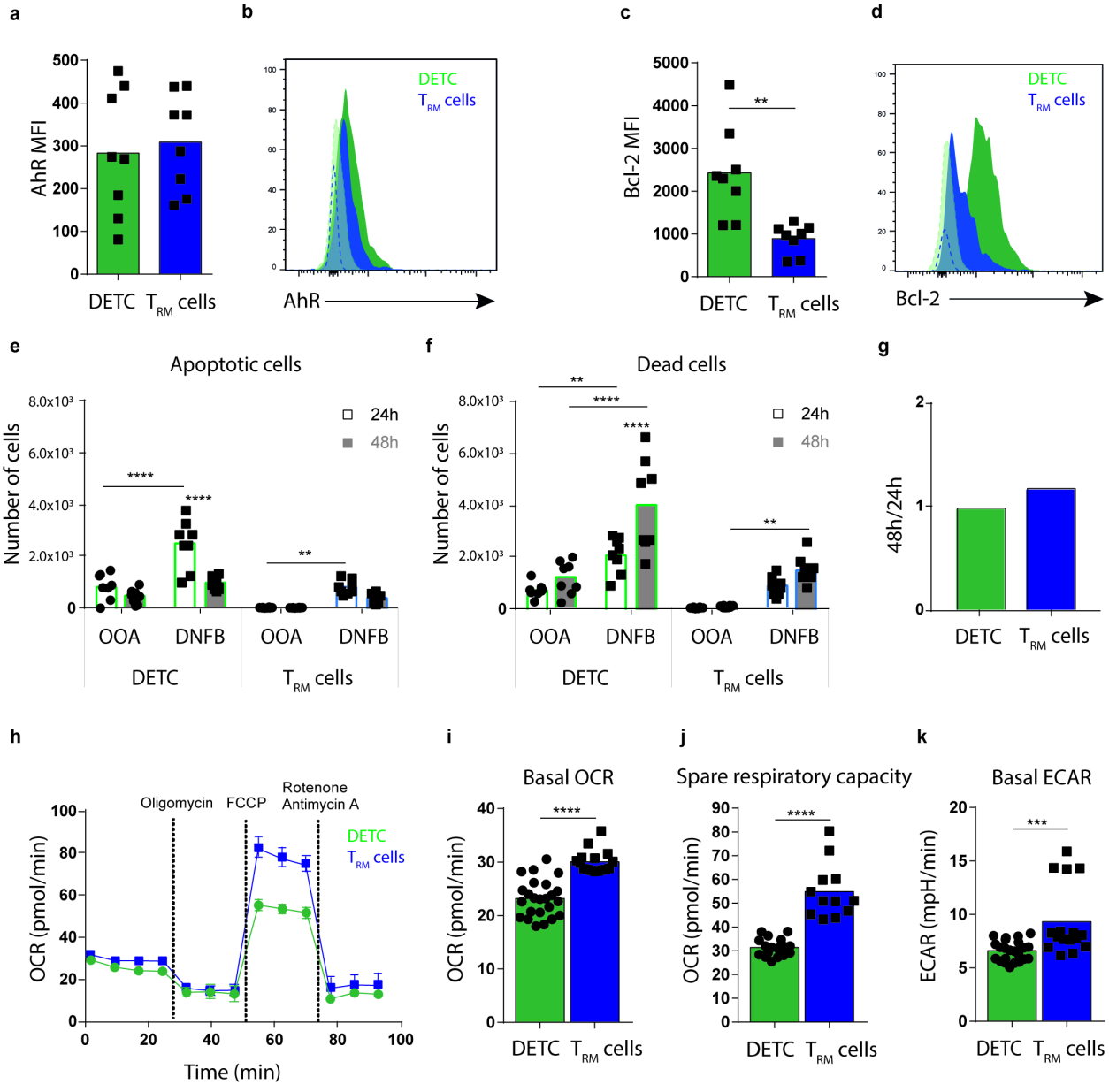

Figure S1
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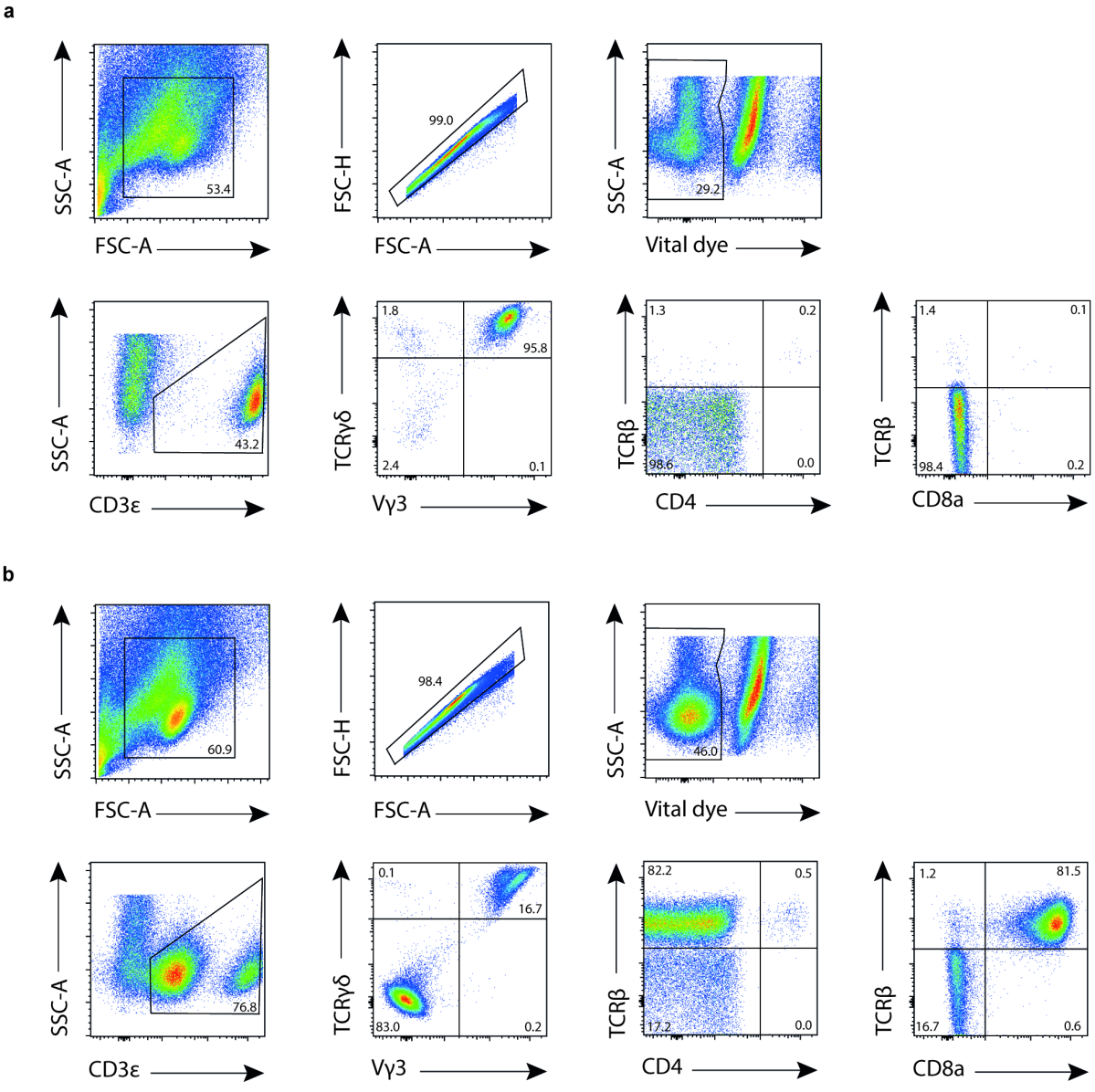

Figure S2
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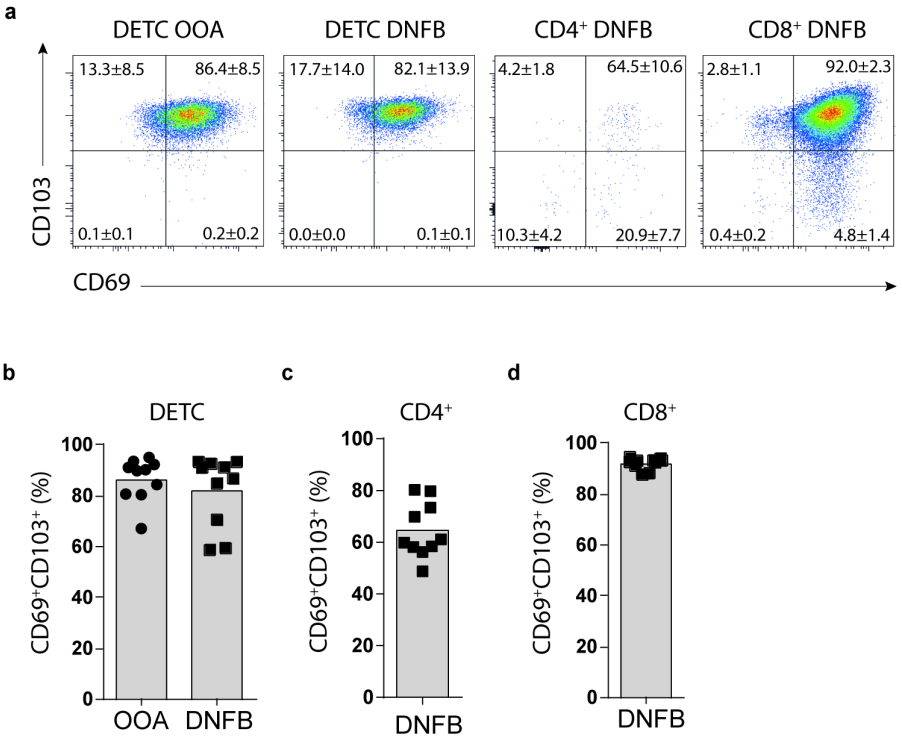

Figure S3
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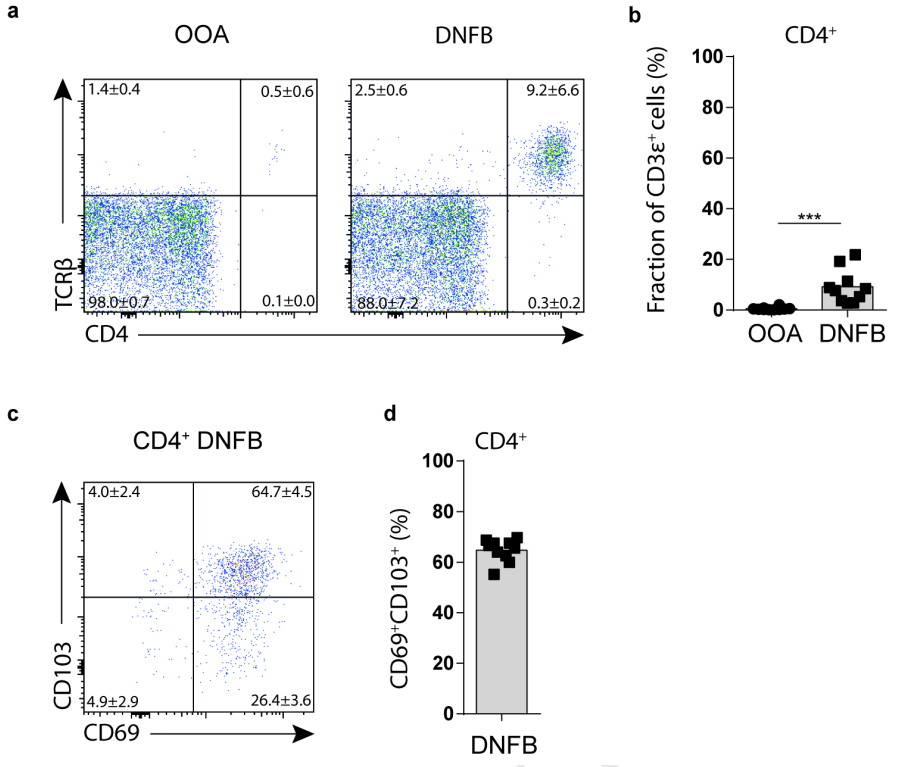

Figure S4
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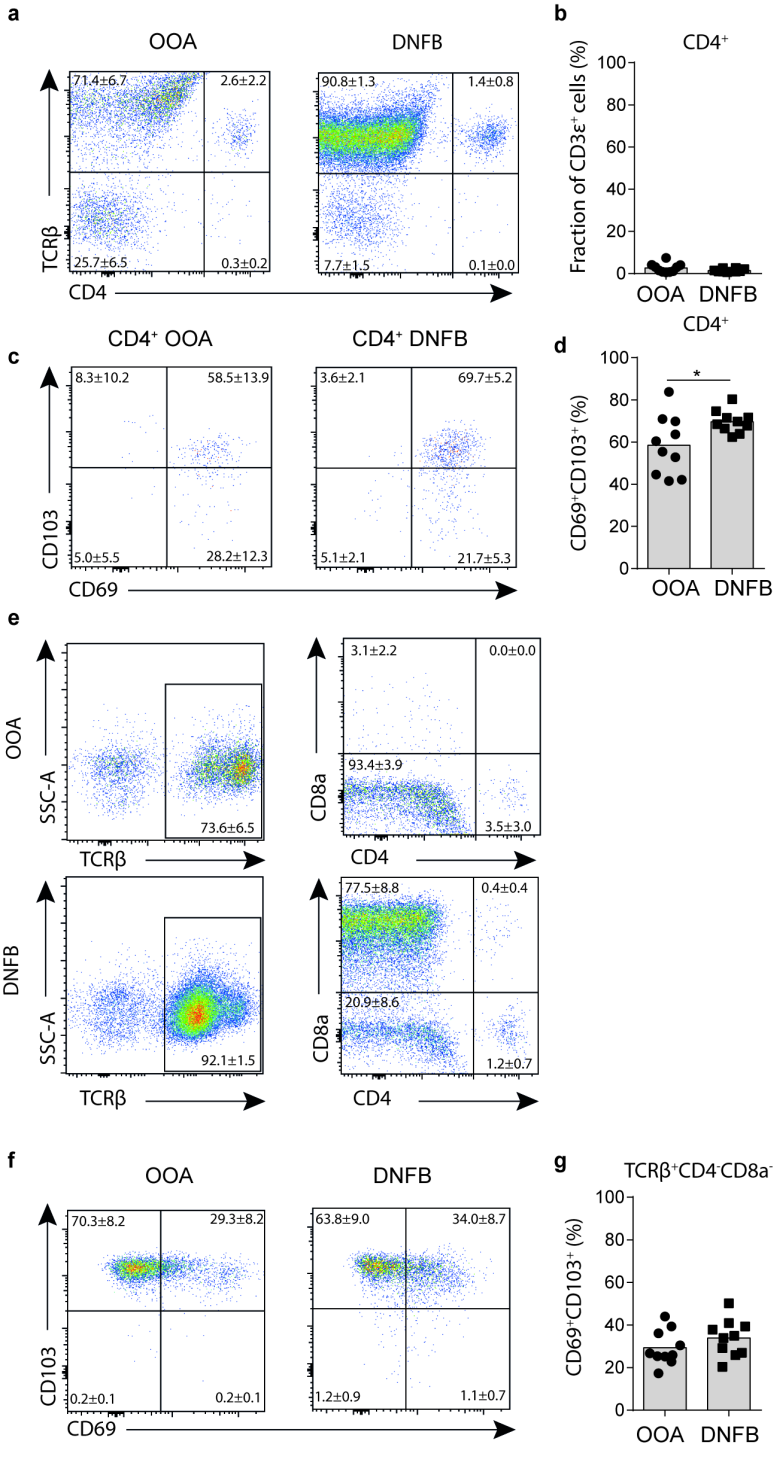

Figure S5
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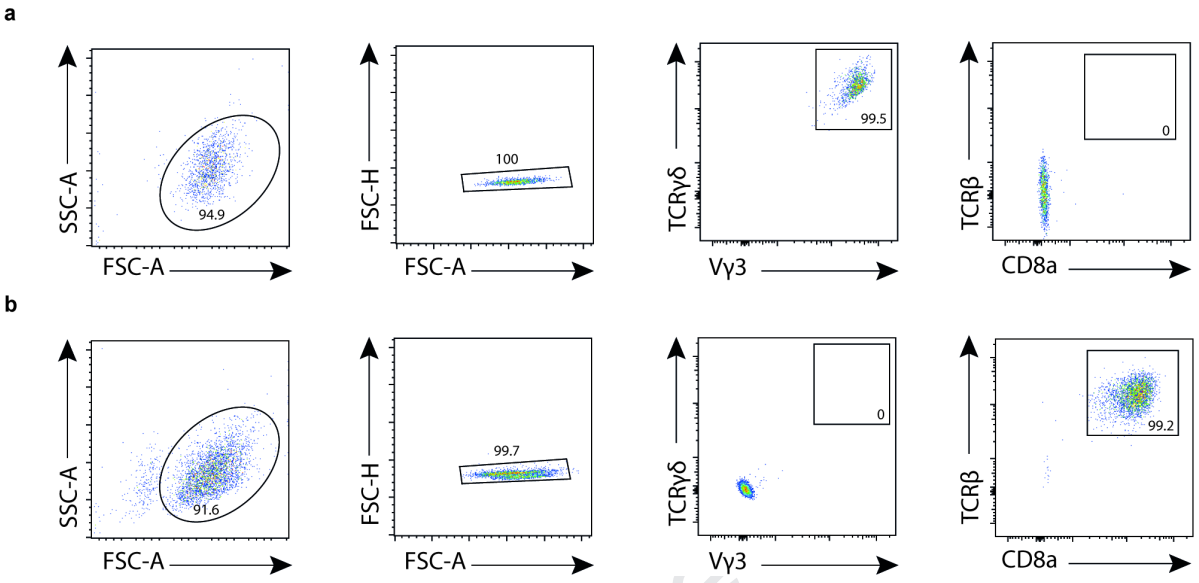

Supplementary Materials

Single-cell suspensions and flow cytometry

Ears were split in two and the dorsal side was placed with the dermis-side down in a 0.3% trypsin (Sigma-Aldrich) solution, pH 7.6 supplemented with 149 mM NaCl, 5 mM KCl and 5 mM dextrose and incubated for 1 hour at 37°C. Subsequently, the epidermis was peeled from the dermis and further degraded into a single-cell suspension with 0.3% trypsin and 0.1% DNase (Sigma-Aldrich) by incubating the cells for 10 min at 37°C. To allow re-expression of surface markers potentially cleaved by trypsin, cells were incubated over-night at 37°C in complete medium containing RPMI-1640 (Sigma-Aldrich), 10% FBS (Biological Industries), 1% L-glutamin, 1% penicillin-streptamycin, 50 µM 2-Mercaptoethanol, 25 mM hepes, 100 mM Na Pyruvat and 100 µM non-essential amino acids. The next day, Fc-receptors were blocked with anti-CD16/CD32 (2.4.G2) (BD Biosciences, Albertslund, Denmark) and surface markers were stained with anti-CD3ε-PE/Cy7 (145-2C11), anti-CD8a-BV421 or APC (53-6.7), anti-Vγ3-PE or APC (536), anti-CD103-PerCP/Cy5.5 (2E7) (BioLegend, San Diego, California, USA), anti-TCRβ-BV711 (H57-597), anti-CD4-BV786 (RM4-5), anti-TCRγδ-PE-CF594 (GL3) and anti-CD69-FITC (H1.2F3) (BD Biosciences) diluted in Brilliant Stain Buffer (BD Biosciences,). Fixable Viability Dye (eFlour® 780) (eBioscience, San Diego, California, USA) was used to assess the viability of the cells. For intracellular staining, cells were fixed and permeabilized with the Transcription Factor Buffer Set (BD Biosciences) and stained with anti-AhR-PE (clone T49-550) (BD Biosciences) or fixed with Fixation/Permeabilization Solution Kit (BD Biosciences) and stained with anti-Bcl2-PE (clone 3F11) (BD Biosciences) (Bonefeld et al., 2008). For Ki-67 staining, cells were fixed and permeabilized with the Foxp3 Transcription Factor Staining Buffer Set (eBioscience) and stained with anti-Ki-67-BV421 (clone B56) (BD Biosciences). BrdU staining was performed accordingly to

manufactures protocol (BD Biosciences) and stained with anti-BrdU-APC. For Annexin V staining, cells were diluted in 100ul 10x diluted Annexin V binding buffer (Nordic Biosite, Täby, Sweden) and added anti-Annexin V-PE (BioLegend). The cells were stained for 10min, added an extra 100ul of 10x diluted Annexin V binding buffer and analyzed immediate after. Samples were analyzed on a BD LSRFortessa and data were processed with FlowJo (Treestar).

Ear sheets and confocal scanning laser microscopy

Hair was removed from the ears with Veet™ hair removal cream (Reckitt Benckiser, Slough, UK). The ears were split in two and placed in ammonium thiocyanate for 13 minutes at 37°C and the epidermal sheet was peeled from the dermis. The sheets were fixed with 3% paraformaldehyde, incubated with anti-CD8a-BV421 and anti-TCRγδ-AF488 (GL3) (BioLegend) anibodies and counterstained with SYTO 59 (Thermo Fisher Scientific). Sheets were mounted on slides with DAKO fluorescent mounting medium (DAKO, Carpinteria, California, USA). Image acquisition were performed using a confocal scanning laser microscope (LSM 710 or LSM 880; Carl Zeiss GmbH, Germany). High resolution image acquisition was performed using Airy Scan mode on LSM880. Images were obtained using an 63 x oil objective lens and scanned at 405 nm (blue), 488 nm (green), 561 nm (red) and 594 nm (red). Images were generated using Imaris software (version 8.4, Bitplane AG) and were processed for display using Power Point (Microsoft). For estimation of cells per mm^2, three 3D pictures were obtained per slide and the cells were counted blinded.

Fluorescence-activated cell sorting analysis

Hair was removed with Veet™ hair removal cream and abdominal skin was harvested, cut into stripes and placed with the dermis-side down in a 0.3% trypsin solution as described for single-cell

suspensions for flow cytometry. The skin was incubated for 2 hours at 37°C and the epidermis was subsequently peeled from the dermis and further degraded into a single-cell suspension by incubating the cells for 20 min at 37°C in 0.3% trypsin and 0.1% DNase. Lymphocytes were purified using Lympholyte®-M (Cedarlane, Burlington, Canada) and the cells were incubated over-night at 37°C in complete medium supplemented with 10 U/ml recombinant mouse IL-2 (R&D Systems, Minneapolis, Minnesota, USA). The next day, Fc-receptors were blocked with anti-CD16/CD32 and the cells were stained with anti-TCRβ-BV711, anti-CD8a-BV421, anti-TCRγδ-PE-CF594, and anti-Vγ3-PE antibodies. Cells were sorted on a BD FACS Aria-II to a purity of approximately 99% (Fig. S5). The isolated cells were incubated over-night at 37°C in complete medium supplemented with IL-2 (10 U/ml) and frozen the following day.

Supplementary figure legends

Fig. S1. Flow cytometry gating strategy and analyses of epidermal T cell subsets

Gating strategy used for identification of epidermal T cell subsets in single-cell suspensions from epidermal ear sheets by flow cytometry. A representative example of cells obtained from mice treated with **(a)** OOA and **(b)** DNFB as depicted in Fig. 1a at day 65. The percentage of cells within each gate and quadrant is indicated.

Fig. S2. CD69 and CD103 expression on epidermal T cell subsets

(a) CD69 and CD103 expression on $V\gamma3^{+}TCR\gamma\delta^{+}$ cells (DETC) from epidermal ear sheets after exposure of the mice to OOA or DNFB and on $CD8^{+}TCR\beta^{+}$ ($CD8^{+}$) and $CD4^{+}TCR\beta^{+}$ ($CD4^{+}$) cells from epidermal ear sheets after exposure of the mice to DNFB. The mean percentage ± SD of cells within each quadrant is indicated. Fraction of $CD69^{+}CD103^{+}$ **(b)** DETC, **(c)** $CD4^{+}$ and **(d)** $CD8^{+}$ cells. The bars give the mean value and the filled symbols represent individual mice.

Fig. S3. Flow cytometric analyses of $CD4^{+}$ epidermal T cells from β2-microglobulin knock-out B6.129-*B2m*^{tm1Jae} N12 mice

(a) Single-cell suspensions were prepared from epidermal ear sheets from control B6.129-*B2m*^{tm1Jae} N12 mice (OOA) and B6.129-*B2m*^{tm1Jae} N12 mice exposed to DNFB (DNFB). The $CD3\varepsilon^{+}$ cells were analyzed for their expression of TCRβ and CD4 by flow cytometry. **(b)** Fraction of $CD4^{+}$ T cells of the total number of epidermal T cells. **(c)** CD69 and CD103 expression on $CD4^{+}$ T cells from epidermal ear sheets after exposure of the mice to DNFB. In a and c, the mean percentage ± SD of cells within each quadrant is indicated. **(d)** Fraction of $CD69^{+}CD103^{+}$ $CD4^{+}$ T cells. The data in b and d were obtained from two independent experiments with five mice in each group. The bars give the mean value and the filled symbols represent the individual mice. Significance was determined by *t*-test.

Fig. S4. Flow cytometric analyses of epidermal T cells from TCRδ knock-out B6.129P2-*Tcrd*^{tm1Mom}/J mice

(a) Single-cell suspensions were prepared from epidermal ear sheets from control B6.129P2-*Tcrd*^{tm1Mom}/J mice (OOA) and B6.129P2-*Tcrd*^{tm1Mom}/J mice exposed to DNFB (DNFB). The $CD3\varepsilon^{+}$ cells were analyzed for their expression of TCRβ and CD4 by flow cytometry. **(b)** Fraction of $CD4^{+}$ T cells of the total number of epidermal T cells. **(c)** CD69 and CD103 expression on $CD4^{+}$

epidermal T cells from mice exposed to OOA or DNFB. **(d)** Fraction of $CD69^{+}CD103^{+}$ $CD4^{+}$ epidermal T cells. **(e)** Analyses of the expression of CD4 and CD8 on $TCR\beta^{+}$ epidermal T cells from control B6.129P2-*Tcrd*^{tm1Mom}/J mice (OOA, first row) and B6.129P2-*Tcrd*^{tm1Mom}/J mice exposed to DNFB (DNFB, second row). **(f)** CD69 and CD103 expression on $CD4^{-}CD8^{-}TCR\beta^{+}$ epidermal T cells after exposure of the mice to OOA or DNFB. In a, c, e and f the mean percentage ± SD of cells within each quadrant is indicated. **(g)** Fraction of $CD69^{+}CD103^{+}$ $CD4^{-}CD8^{-}TCR\beta^{+}$ epidermal T cells. The data in b, d and g were obtained from two independent experiments with five mice in each group. The bars give the mean value and the filled symbols represent the individual mice. Significance was determined by *t*-test.

Fig. S5. Flow cytometric analysis of isolated T cell subsets

(a) $V\gamma3^{+}TCR\gamma\delta^{+}$ and **(b)** $CD8^{+}TCR\beta^{+}$ cells were isolated from single-cell suspension from epidermal sheets by fluorescence-activated cell sorting and tested for purity by staining with anti-Vγ3, anti-TCRγδ, anti-CD8a and anti-TCRβ antibodies. The percentage of DETC ($V\gamma3^{+}TCR\gamma\delta^{+}$ cells) and $CD8^{+}$ T_{RM} cells ($CD8^{+}TCR\beta^{+}$ cells) is given in the relevant squares.

Study I summary figure

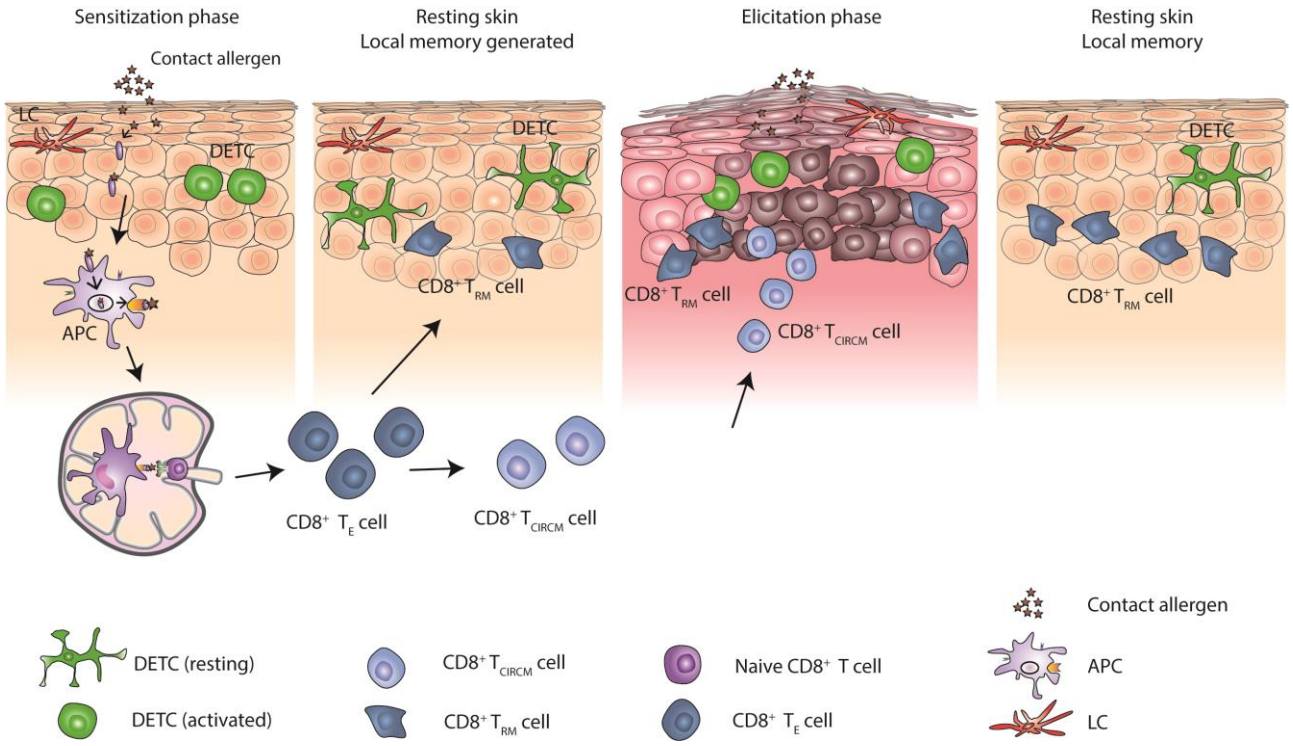

Figure 5: Study I summary. Epidermal CD8$^+$ tissue-resident memory T (T_{RM}) cells displace dendritic epidermal T cells (DETC) in contact allergy. *Exposure of the skin to the contact allergen dinitrofluorobenzene (DNFB) lead to the accumulation of CD8$^+$ T_{RM} cells, which displace DETC in the epidermis of mice. Increased dose and multiple exposures to DNFB increase this inverse correlation between the two epidermal T cell subsets. The increased pool of CD8$^+$ T_{RM} cells is mediated both by local proliferation of CD8$^+$ T_{RM} cells and by recruitment from the circulation. We further elucidated the mechanism behind the inverse relationship and showed that CD8$^+$ T_{RM} cells have a metabolic advantage over DETC, having a larger spare respiratory capacity and are therefore bioenergetically more stable. Langerhans cells (LC), Antigen presenting cells (APC), effector T (T_E) cells, circulating memory T (T_{CIRCM}) cells.*

Study II

Epidermal T cell subsets - effect of age and antigen exposure in humans and mice

Epidermal T cell subsets - effect of age and antigen exposure in humans and mice

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Abstract

Epidermal T cells play a central role in the immune surveillance of the skin. The majority of epidermal T cells found in adult human skin are αβ T cells. In contrast, γδ T cells constitute the majority of epidermal T cells in naïve specific pathogen free (SPF) mice. This discrepancy in T cell subsets between humans and mice have contributed to the discussion of the usefulness of mouse models for human skin diseases. However, the epidermal T cell composition in skin from infants is poorly described and the discrepancy between epidermal T cell subsets in adult humans and naïve mice might be due to age and/or exposure to antigens. Here we show that very few epidermal T cells are found in human neonates and that this number gradually increases during the first year of life. We demonstrate that during infancy the majority of epidermal T cells are $CD4^+$ αβ T cells. The frequency of epidermal $CD8^+$ αβ T cells increases with age constituting 13% of the αβ T cells in infants (1 – 12 months) and 50% in young adults (21 – 29 years). A small decrease in the $CD8^+$ frequency to 40% of the αβ T cells is seen in aged adults (60 – 70 years). The distribution of the epidermal T cell subsets did not vary between different anatomic locations. In contrast, we found that the epidermal T cell composition in SPF mice did not change with age. Interestingly, epidermal $CD4^+$ and $CD8^+$ αβ T cells were found in humans and antigen-experienced mice purchased from pet shops, but not in SPF mice. Our study suggests that the level of antigen exposure, as opposed to age, is the major factor determining the composition of the epidermal T cell subsets and that some of the discrepancy between the epidermal T cell subsets found in adult humans and naïve SPF mice might be explained by the different scale of exposure to antigens.

Introduction

The epidermis is the outermost layer of the skin and it provides an important physical, chemical and an immunological barrier to the outside world. Epidermal T cells play a central role in the immunological barrier. The epidermal T cells act as sentinels that are patrolling the epidermis and they can respond very rapidly when they encounter antigens[1–3]. The first evidence that T cells are present in the epidermis of rodents and humans was published 70 years ago[4]. Later it became clear that there are major differences in the composition of epidermal T cells between unexposed naïve mice and adult humans.

In naïve mice housed under specific pathogen free (SPF) conditions, the vast majority (> 90%) of the epidermal T cells is constituted by a subset of γδ T cells termed dendritic epidermal T cells (DETC)[5,6]. DETC develop exclusively during the embryonic stages and they migrate to the epidermis at embryonic day 14-17[7–9]. Thus, mice have a network of epidermal T cells ready to provide protection already at birth. Only few studies have investigated epidermal T cells in early human life, and they indicate that no T cells are present in the epidermis of human fetuses and very few T cells are present in the epidermis of human new-borns (3 days)[10,11]. In contrast, a network of T cells similar to that found in unexposed naïve mouse epidermis is found in the epidermis of adult humans[1]. However, in contrast to mice, the majority of epidermal T cells in humans are $CD4^+$ and $CD8^+$ αβ T cells and only a minor fraction is γδ T cells[12–15]. These observations could indicate that the composition of epidermal T cells is species dependent. However, we and others have shown that exposure of the skin to antigens leads to the generation of epidermal $CD8^+$ memory T cells that displace the DETC from the mouse epidermis[16] (Gadsbøll et al, JID, 2019, in press). This shows that exposure to antigen plays a role in the composition of the epidermal T cells in mice. The majority of T cells found in human epidermis is

memory T cells[11,12,17], suggesting that exposure to antigens might also play an important role in regulation of the T cell composition in the epidermis of humans. The purpose of this study was to determine the effect of age and exposure to antigens on the composition of epidermal T cells in humans and mice.

Results

CD4$^+$ T cells are the major T cell subset in infant human epidermis

Whereas mice are born with DETC in the epidermis, studies have suggested that T cells first migrate to the epidermis after birth in humans[7,9–11]. However, the composition of epidermal T cells in the epidermis of infants is poorly described. To investigate this, foreskin samples were collected from 18 infants that we divided into four age-groups: 0-1 months (n=2), 1-2 months (n=6), 2-4 months (n=5) and 4-12 months (n=5) (Table 1). Single-cell suspensions were made from the epidermis and the T cell composition was determined by flow cytometry. In accordance with a previous study, we found very few (160 per 6 mm biopsy) T cells in the epidermis of neonates (0-1 month) (Fig. 1A and Table 1)[11]. An approximate 7-fold increase in the number of epidermal T cells (1168 per 6 mm biopsy) was found in the 1-2 month group compared to the 0-1 month group and an additional 4-fold increase was observed in the two oldest age-groups (4410 and 4790 per 6 mm biopsy, respectively) compared to the 1-2 month group (Fig. 1A and Table 1). In contrast to mouse epidermis, the majority (75 – 92%) of epidermal T cells were αβ T cells and only a minor fraction (7 – 21%) was γδ T cells (Fig. 1B-E). Furthermore, the majority of epidermal T cells found in infant foreskin was CD4$^+$ T cells (Fig. 1B-G). Despite the low number of infants in each group, it appeared that the youngest group had a larger fraction of γδ T cells and non-conventional αβ T cells, possibly NKT cells, compared to the older age groups (Fig. 1G). After the first month, the epidermal T cell composition did not seem to change, even though the total number of epidermal T cells increased with age (Fig. 1).

An increased frequency of CD8$^+$ T cells is found in human adult versus infant epidermis

It has previously been shown that the ratio between CD4$^+$ T cells and CD8$^+$ T cells in human epidermis is 2:3 [12]. In contrast, we found that CD4$^+$ T cells account for 60-70% of the epidermal T cells in

infants. This discrepancy could be due to differences in age as Foster *et al.* examined the T cell composition in samples from adult humans[12]. Another possibility could be that the epidermal T cell composition differs depending on the anatomic locations as previously suggested[12]. To investigate this further, we analyzed epidermal foreskin samples from adult men (Table 1) and from epidermal samples collected from the dorsal lower part of the forearm close to the wrist and from the upper buttock of adult healthy volunteers (21 – 29 years old, Table 2). Single-cell suspensions were prepared from the epidermal samples and the epidermal T cell composition was determined by flow cytometry. A 2-fold increase in the number of T cells was found in adult foreskin compared to the number found in foreskin from 4-12 month old infants (Fig. 1A and 2A). Equal numbers of T cells were found in the epidermis from hand and buttock (Fig. 2A). It was not possible to compare the T cell numbers in epidermis from foreskin, hand and buttock because the samples were collected with different techniques. However, the frequency of the various epidermal T cell subsets was similar in epidermis from adult foreskin, hand and buttock with approximately 40% $CD4^+$ T cells, 40% $CD8^+$ T cells, 10% γδ T cells and 10% other T cells (Fig. 2B-G). Compared with the infants (0 – 12 months), the adults (21 – 29 years old) had a higher frequency of $CD8^+$ T cells in the epidermis. To determine whether the composition of the epidermal T cells further changed with age, we next determined the T cell subsets in the epidermis from elderly healthy donors (60 – 70 years old, Table 2). Surprisingly, we found that the elderly adults had a slightly lower frequency of $CD8^+$ T cells in their epidermis than the young adults (Fig. 2). Taken together, these data suggest that the human epidermal T cell composition changes from infanthood to adulthood and that the composition of the epidermal T cells does not vary with the anatomical location in adults.

Exposure to antigen drives the epidermal T cell composition in mice

The age-related change in the epidermal T cell composition in humans could either be mediated by age-related changes in the immune system or by an increased cumulated antigen exposure with age. To address this question, we turned to analyses in mice as it cannot be done in humans.

We examined age-related changes in the epidermal T cell composition to determine the different T cell subsets in the epidermis of young (8 weeks old) and old (1.5 years old) C57BL/6 mice housed under SPF conditions by flow cytometry. A similar T cell composition was found in the epidermis from young and old mice with the vast majority of T cells being DETC (Fig. 3A-C). This indicated that the changes in the epidermal T cell composition is not simply mediated by age. We and others have shown that exposure of the skin to antigen results in the generation of epidermal $CD8^+$ T cells that displace the DETC from the epidermis in mice (Gadsbøll et al, JID, 2019, in press)[16]. Therefore, another possibility is that the changes seen in human epidermis with age are caused by repeated exposure to antigens. To further elucidate the effect of repeated antigen exposure on the epidermal T cell composition, we investigated the epidermis from antigen-experienced mice. We used mice that had been housed under pet shop conditions as a model for antigen-experience. Even though DETC still constituted the majority of T cells, a substantial number of both $CD8^+$ and $CD4^+$ T cells were found in the epidermis from these antigen-experienced mice (Fig. 3A-C), supporting our hypothesis that the epidermal T cell composition reflects the level of antigen experience. To further investigate the effect of antigens on the epidermal T cell composition in young and old mice, we sensitized mice with DNFB for three consecutive days and determined the epidermal T cell composition after a minimum of 21 days by flow cytometry. In accordance with our previous studies, we found that sensitization with DNFB resulted in the generation of epidermal $CD8^+$ T cells and a displacement of DETC in both young and old mice (Fig. 3D and E). Interestingly, an increased fraction of epidermal $CD4^+$ T cells were generated after exposure to DNFB in the old compared to the young mice (Fig. 3F).

Taken together, these data support the idea that the epidermal T cell composition reflects the level of antigen exposure and not simply age.

Discussion

In the present study, we show that very few T cells are present in the epidermis of human neonates and that the number of T cells in the epidermis gradually increases during the first year of life. The majority of T cells found in epidermis of infants were $CD4^+$ T cells. Furthermore, the composition of the epidermal T cell subsets changed with age with an increase in the frequency of $CD8^+$ T cells. The epidermal T cell composition did not depend on the anatomic site as we found a similar T cell composition in the epidermis of foreskin, hand and buttock samples from healthy human adults. In mice, the epidermal T cell composition is primarily driven by antigen exposure, and to a lesser extent by age.

Our findings support previous studies showing that T cells first migrate to the epidermis after birth in humans[10,11]. We found a gradual increase in the number of epidermal T cells in foreskin during the first months of life and an additional 2-fold increase in the number of epidermal T cells found in adult foreskin compared to infant (4 - 12 month) foreskin. Compared with neonates (0 - 1 month), we found an approximate 60-fold increase in the number of epidermal T cells in the foreskin from healthy adults. This is in agreement with a recent study that found very few epidermal T cells in neonatal human foreskin and a 45-fold increase in the numbers of epidermal T cells from neonatal foreskin to adult skin[18].

Little is known about the phenotype of the epidermal T cells in infants. In this study, we show that the majority of epidermal T cells in infant foreskin is $CD4^+$ T cells, whereas $CD8^+$ T cells only accounts for 11-15% of the total number of epidermal T cells. However, the frequency of $CD8^+$ epidermal T cells increases with age and an equal number of epidermal $CD4^+$ and $CD8^+$ T cells was ob-

served in the adult group. In accordance, previous studies on adult human epidermis describe a comparable CD4:CD8 T cell ratio, although with a tendency of a higher frequency of $CD8^+$ than $CD4^+$ T cells[12,19,20]. In contrast, Watanabe *et al.* found that approximately 70% of the epidermal T cells were $CD4^+$ T cells[18]. The reason for this discrepancy is not clear.

DETC are the major subset of epidermal T cells found in naïve SPF mice[5]. In contrast, γδ T cells only account for 10-20% of the epidermal T cells found in adult humans[13,20,21]. Interestingly, we found that γδ T cells constitute approximately 20% of the epidermal T cells in neonates and that their frequency rapidly falls and levels out at 6-13% for the rest of the life. This reduction in frequency of γδ T cells could likely be mediated by the influx of αβ T cells as also seen in mice exposed to antigens (Gadsbøll et al, JID, 2019, in press)[16].

The differences in the immune system of laboratory mice that live in SPF facilities and human adults have generally been ascribed to species differences. However, a recent study elegantly showed that at least some of the differences are due to the level of exposure to antigens[22]. By immune profiling of laboratory and pet store mice and newborn and adult humans, the authors found that laboratory mice resembled human newborns by the lack of effector and memory T cells whereas the pet store mice more closely reflected the immune status of adult humans than of neonates[22]. Furthermore, they showed that co-housing laboratory mice with pet store mice induced at change in the T cell composition of the skin of laboratory mice with an increased number of both $CD4^+$ and $CD8^+$ T cells and a small increase in γδ T cells[22]. In accordance with this, we found an increased frequency of epidermal $CD4^+$ and $CD8^+$ T cells in both pet shop mice and antigen-experienced laboratory mice compared to both young and old laboratory mice. Furthermore, we found a reduced frequency of DETC in the epidermis of both pet shop mice and of antigen-experienced laboratory mice compared to both young

and old laboratory mice. This is in agreement with studies showing that exposure to antigens results in the generation of epidermal $CD8^+$ T cell that displace the DETC from the epidermis (Gadsbøll et al, JID, 2019, in press)[16]. Surprisingly, we found that the frequency of epidermal $CD8^+$ T cells slightly decreased from the young to the elderly adults concomitant with an increase in the frequency of $CD4^+$ epidermal T cells. Interestingly, in parallel we observed that old mice recruited more $CD4^+$ T cells to the epidermis than young mice following exposure to antigens.

In conclusion, this study suggest that the composition of epidermal T cell subsets may mainly be determined by the level of antigen exposure as opposed to age, and that some of the discrepancy between the epidermal T cell subsets found in adult humans and laboratory mice may be explained by different levels of antigen exposure.

Materials and Methods

Foreskin samples

Discarded, non-inflammatory tissues were collected from healthy volunteers that were being circumcised for other reasons than this study. The volunteers were divided into five age groups: 0-1 months (n=2), 1-2 months (n=6), 2-4 months (n=5), 4-12 months (n=5) and 18-70 years old (n=1) (Table 1). All volunteers had a skin type from 1 to 4 on the Fitzpatrick skin type scale[23]. Written consent was obtained in accordance with the Declaration of Helsinki principles for research involving human subjects. The ethics committee of Copenhagen (H-18041841) and the Danish Data Protection Agency approved the study. After removal, the foreskin was placed in 20 ml RPMI medium supplemented with 10% FBS, 1% L-glutamin, 1% penicillin-streptomycin and kept on ice until analysis.

Curettage skin samples

Eighteen 20-30 year old and five 60-70 year old healthy volunteers having a skin type from 1 to 4 on the Fitzpatrick skin type scale[23] were enrolled (Table 2). None of the subjects had a history of skin disease, arthritis or diabetes and none of the subjects used regular medication. Prior to sample collection, the subjects were asked not to apply any lotion or oil to the skin test areas and all subject were asked to fill out a questionnaire regarding skin care habits. Written consent was obtained in accordance with the Declaration of Helsinki principles for research involving human subjects. The ethics committee of Copenhagen (H-17017536) and the Danish Data Protection Agency approved the study. Epidermal samples, approximately 4x4 mm, were obtained with curettage from the dorsal lower part of the forearm close to the wrist (all subjects) and from the buttock at a non-sun exposed area (only the group of 20-30 years old). Each sample were placed in 1.5 ml RPMI medium supplemented with 10% FBS, 1% L-glutamin, 1% penicillin-streptomycin and kept on ice until further analysis.

Single-cell suspension and flow cytometry of human skin samples

From foreskin samples, 6 mm biopsies were made and placed in 2.4 Units/ml Dispase II (Roche, Sigma) dissolved in hepes and incubated for 2 hours at 37°C followed by peeling of the epidermis from the dermis. The peeled epidermis or curettage samples were placed in 3 ml 0.5 mg/ml Collagenase D (Roche, Sigma-Aldrich, Brøndby, Denmark) and the samples were incubated for 1 hour at 37°C. Subsequently, the samples were filtered through a cell strainer and washed with DMEM medium supplemented with 20% FBS, 1% DNase. To allow re-expression of surface markers, cells were incubated overnight at 37°C in complete epidermal and dermal cell culture medium containing RPMI, 10% FBS, 1% L-glutamin, 1% penicillin-streptamycin, 50 µM 2-Mercaptoethanol, 25 mM HEPES, 100 mM Na Pyruvat and 100 µM non-essential amino acids. At day two, Fc-receptors were blocked with human BD Fc Block (BD Biosciences, Albertslund, Denmark) and surface markers were stained with anti-CD3-BUV395 (UCHT1), anti-CD8-FITC (RPA-T8), anti-CD4-APC (RPA-T4), anti-TCRγδ-PE (11F2) (BD Biosciences) and anti-TCRαβ-BV421 (IP26) (BioLegend, San Diego, California, USA). Fixable Viability Dye (eFluor® 780) (eBioscience, San Diego, California, USA) was used to asses viability of the cells. Samples were analyzed on a BD LSRFortessa, and with FlowJo Software (Treestar).

Mice

Female C57BL/6 mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Mice were 8 weeks or 1.5 years old at the beginning of experiments. The mice were housed in specific pathogen-free animal facilities at the Department of Experimental Medicine, University of Copenhagen in accordance with the national animal protection guidelines (license number 2018-15-0201-01409). As antigen-experienced mice, we used mice bred for snake feed that had been housed under pet shop

conditions. The antigen-experienced mice were approximately 12 weeks old and on an unknown background. The ears were harvested from the euthanized mice, before they were used as feed.

Mouse model for contact hypersensitivity (CHS)

To induce CHS in the mice, we used a previously described model[24]. In short, mice were sensitized for three consecutive days with 25 µl on the dorsal side of both ears with 0.15% (v/v) of 1-fluoro-2,4-dinitrobenzene (DNFB) (Sigma-Aldrich, Brøndby, Denmark) in olive oil and acetone (OOA (1:4)). Control mice were exposed to OOA. The epidermal T cell composition was determined a minimum of 21 days after the last exposure to allergen.

Single-cell suspension and flow cytometry of mouse samples

Single-cell suspensions were made from mice ears as previously described (Gadsbøll et al, JID, 2019, in press). In short, ears were split and the dorsal side was placed with the dermis-side down in a 0.3% trypsin (Sigma-Aldrich) solution, pH 7.6 supplemented with 149 mM NaCl, 5 mM KCl and 5 mM dextrose and incubated for 1 hour at 37°C. After peeling off the epidermis, it was further degraded into a single-cell suspension with 0.3% trypsin and 0.1% DNase (Sigma-Aldrich) by incubating the cells for 10 min at 37°C. Cells were incubated overnight at 37°C in complete medium containing RPMI-1640 (Sigma-Aldrich), 10% FBS (Biological Industries), 1% L-glutamin, 1% penicillin-streptomycin, 50 µM 2-Mercaptoethanol, 25 mM HEPES, 100 mM Na Pyruvat and 100 µM non-essential amino acids to allow re-expression of surface markers. The next day, cells were stained using anti-CD16/CD32 (2.4.G2) (BD Biosciences, Albertslund, Denmark) to block Fc-receptors and anti-CD3ε-PE/Cy7 (145-2C11), anti-CD8a-BV421 (53-6.7), anti-Vγ3-PE (536), anti-CD103-PerCP/Cy5.5 (2E7) (BioLegend, San Diego, California, USA), anti-TCRβ-BV711 (H57-597), anti-CD4-BV786

(RM4-5), anti-TCRγδ-PE-CF594 (GL3) and anti-CD69-FITC (H1.2F3) (BD Biosciences) diluted in Brilliant Stain Buffer (BD Biosciences,). Viability of the cells were assessed using Fixable Viability Dye (eFluor® 780) (eBioscience, San Diego, California, USA). Finally, samples were analyzed on a BD LSRFortessa and followed by data processing with FlowJo (Treestar).

Statistical analysis

Two-tailed unpaired Student's *t*-test was used to assess significant differences. *p*-values <0.05 were considered significant and denoted *<0.05, **<0.01, ***<0.001. Outliers were identified in figure 3 using the Robust regression and Outlier removal test (ROUT, Q = 1%). GraphPad Prism 7 software was used to perform statistical analysis.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

Anne Marie Topp

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Figure legends

Figure 1. Age-related changes in the epidermal T cell composition in human infants.

The epidermal T cell composition was determined in foreskin samples from infant humans by flow cytometry. The samples were divided into four groups according to age: 0-1 months (n=2), 1-2 months (n=6), 2-4 months (n=5) and 4-12 months (n=5). (A) Total number of $CD3^+$ cell/6 mm biopsy. (B) The fraction of $TCR\gamma\delta^+$ T cells of all $CD3^+$ cells. The fraction of $CD8^+$ T cells (C) and $CD4^+$ T cells (D) of all $CD3^+TCR\alpha\beta^+$ cells. (E) Epidermal single cells were gated for CD3 and for viability and were further gated for TCRαβ and TCRγδ. Plots are representative and the numbers indicate mean±SD percentages. (F) Epidermal viable single $CD3^+TCR\alpha\beta^+$ cells were gate for CD4 and CD8. Plots are representative and the numbers indicate mean±SD percentages. (G) Epidermal T cell distribution diagrams. The numbers within the diagrams represent the mean percentages of each T cell subset of all $CD3^+$ cells. Significance was determined by *t*-test.

Figure 2. Epidermal T cell composition in adult human epidermis.

The epidermal T cell composition was determined in skin samples from adult humans by flow cytometry. The epidermal T cell composition was determined in two types of samples from different anatomic locations: 1) skin biopsies (foreskin (n=1)) and 2) curettage skin samples (hand (n=18), buttocks (n=18) and aged hand (≥60 year old, n=5). (A) Total number of $CD3^+$ cell/6 mm biopsy or total number of $CD3^+$ cells/4 mm curettage scrape. (B) The fraction of $TCR\gamma\delta^+$ T cells of all $CD3^+$ cells. The fraction of $CD8^+$ T cells (C) and $CD4^+$ T cells (D) of all $CD3^+TCR\alpha\beta^+$ cells. (E) Epidermal single cells were gate for CD3 and for viability and were further gated for TCRαβ and TCRγδ. Plots are representative and the numbers indicate mean±SD percentages. (F) Epidermal viable single

$CD3^+TCR\alpha\beta^+$ cells were gate for CD4 and CD8. Plots are representative and the numbers indicate mean±SD percentages. (G) Epidermal T cell distribution diagrams. The numbers within the diagrams represent the mean percentages of each T cell subset of all $CD3^+$ cells.

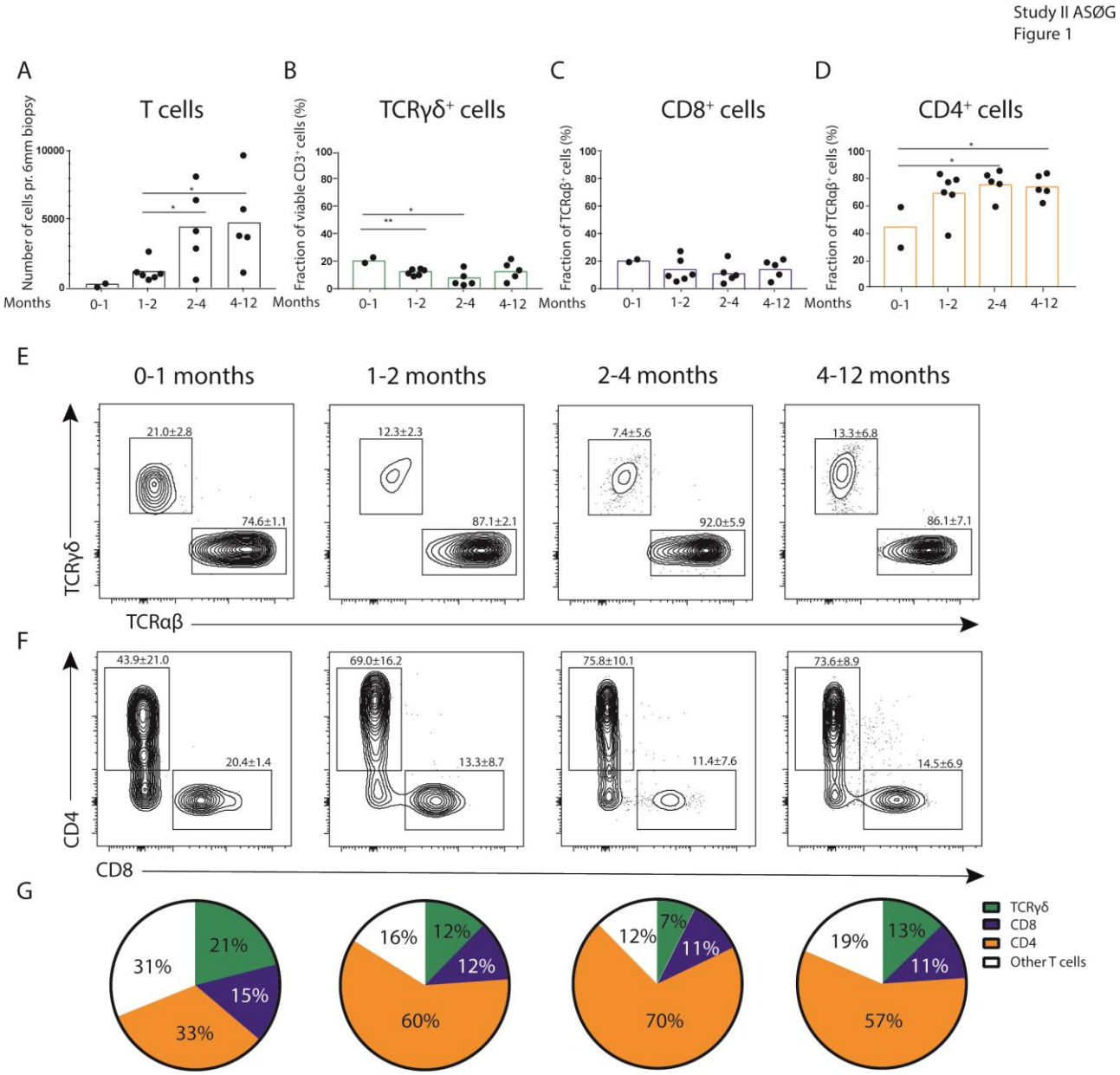
Figure 3. Antigen-exposure induce a change in the epidermal T cell composition.

The epidermal T cell composition was determined in young (8 weeks, n=3-4) and old (1.5 years, n=2-5) C57BL/6 mice and in mice breed under pet shop conditions (antigen experienced, n=10). Single-cell suspensions were prepared from epidermal ear sheets and $CD3\varepsilon^+$ cells were analyzed for their expression of TCRγδ and Vγ3 (DETC) or TCRβ and CD8a ($CD8^+$ T cells) or TCRβ and CD4 ($CD4^+$ T cells) by flow cytometry. (A) Frequency of DETC ($TCR\gamma\delta^+V\gamma3^+$ cells). (B) Frequency of $CD8^+$ T cells ($TCR\beta^+CD8a^+$ cells). (C) Frequency of $CD4^+$ T cells ($TCR\beta^+CD4^+$ cells). The effect of DNFB on the epidermal T cell composition were determined in young (8 weeks, n=4) and old (1.5 years, n=3-5) C57BL/6 mice. Mice were exposed to 0.15% DNFB in OOA or OOA as control on their ears at day 0, 1, 2. At day 23 or later, epidermal ear sheets were purified for further analyses. (D) Frequency of DETC, (E) $CD8^+$ T cells and (F) $CD4^+$ T cells of the total number of epidermal T cells ($CD3\varepsilon^+$ cells) from control mice (OOA) and mice exposed to DNFB (DNFB) as determined by flow cytometry. The data were obtained from two independent experiments in A-C and one (young mice) and two (old mice) independent experiments in D-F. The bars give the mean value and the filled circles represent the individual mice. Significance was determined by *t*-test.

Fig. S1. Flow cytometry gating strategy and analyses of epidermal T cell subsets

Gating strategy used for identification of epidermal T cell subsets in single-cell suspensions from epidermal samples by flow cytometry. A representative example of cells obtained from (A) foreskin, (B) curettage and (C) mouse epidermal ear sheets. The percentage of cells within each gate is indicated.

Figure 1


Study II ASØG
Figure 1
A
T cells
Number of cells pr. 6mm biopsy
Months
0-1
1-2
2-4
4-12
B
TCRγδ⁺ cells
Fraction of viable CD3⁺ cells (%)
C
CD8⁺ cells
Fraction of TCRαβ⁺ cells (%)
D
CD4⁺ cells
E
0-1 months
1-2 months
2-4 months
4-12 months
21.0±2.8
74.6±1.1
12.3±2.3
87.1±2.1
7.4±5.6
92.0±5.9
13.3±6.8
86.1±7.1
TCRγδ
TCRαβ
F
43.9±21.0
20.4±1.4
69.0±16.2
13.3±8.7
75.8±10.1
11.4±7.6
73.6±8.9
14.5±6.9
CD4
CD8
G
21%
15%
33%
31%
12%
12%
60%
16%
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70%
12%
13%
11%
57%
19%
TCRγδ
CD8
CD4
Other T cells

Figure 2

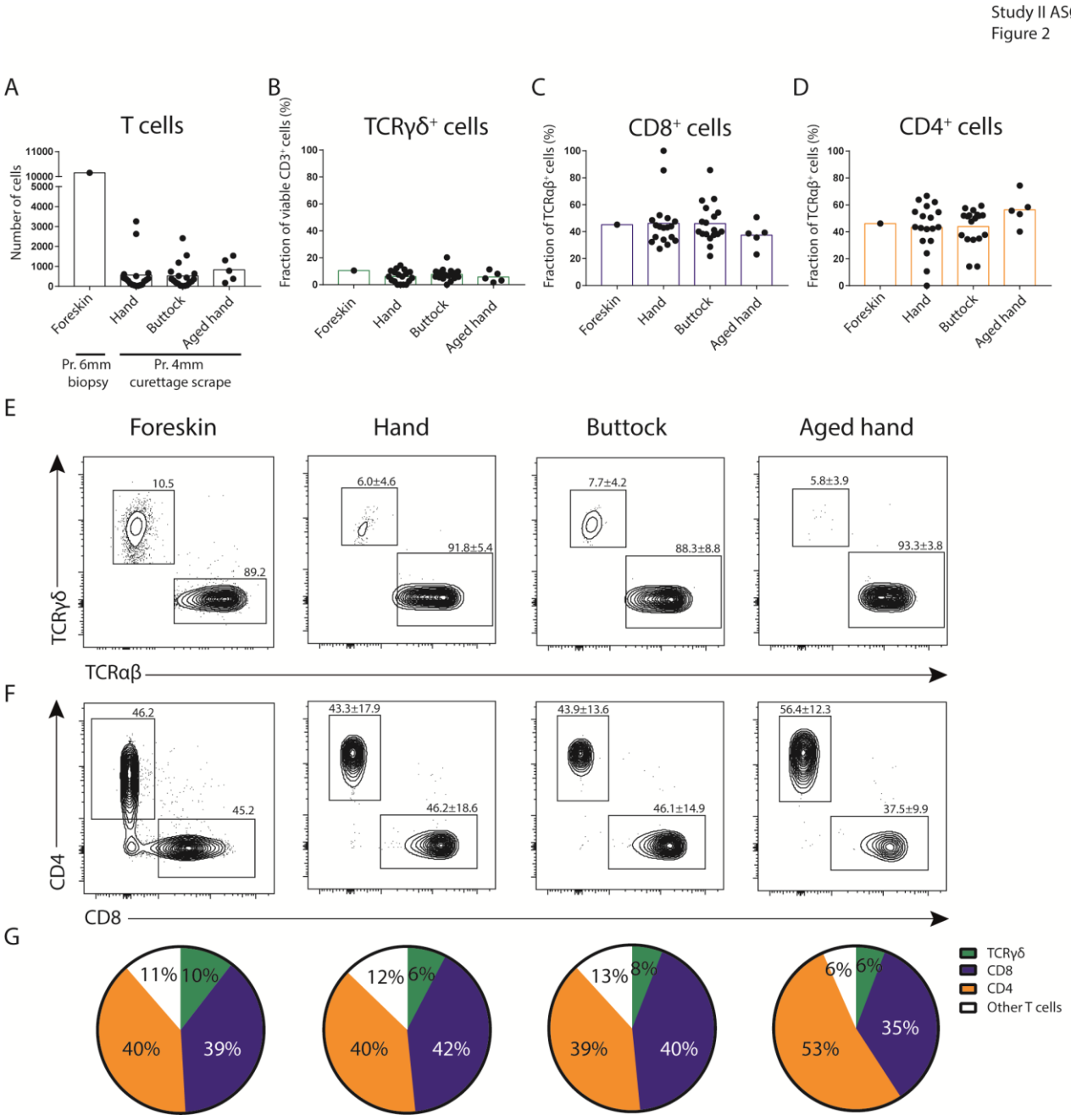
Study II ASØG
Figure 2
A
T cells
Number of cells
Foreskin
Hand
Buttock
Aged hand
Pr. 6mm biopsy
Pr. 4mm curettage scrape
B
TCRγδ⁺ cells
Fraction of viable CD3⁺ cells (%)
C
CD8⁺ cells
Fraction of TCRαβ⁺ cells (%)
D
CD4⁺ cells
E
Foreskin
Hand
Buttock
Aged hand
TCRγδ
TCRαβ
10.5
89.2
6.0±4.6
91.8±5.4
7.7±4.2
88.3±8.8
5.8±3.9
93.3±3.8
F
CD4
CD8
46.2
45.2
43.3±17.9
46.2±18.6
43.9±13.6
46.1±14.9
56.4±12.3
37.5±9.9
G
11%
10%
40%
39%
12%
6%
40%
42%
13%
8%
39%
40%
6%
6%
53%
35%
TCRγδ
CD8
CD4
Other T cells

Figure 3

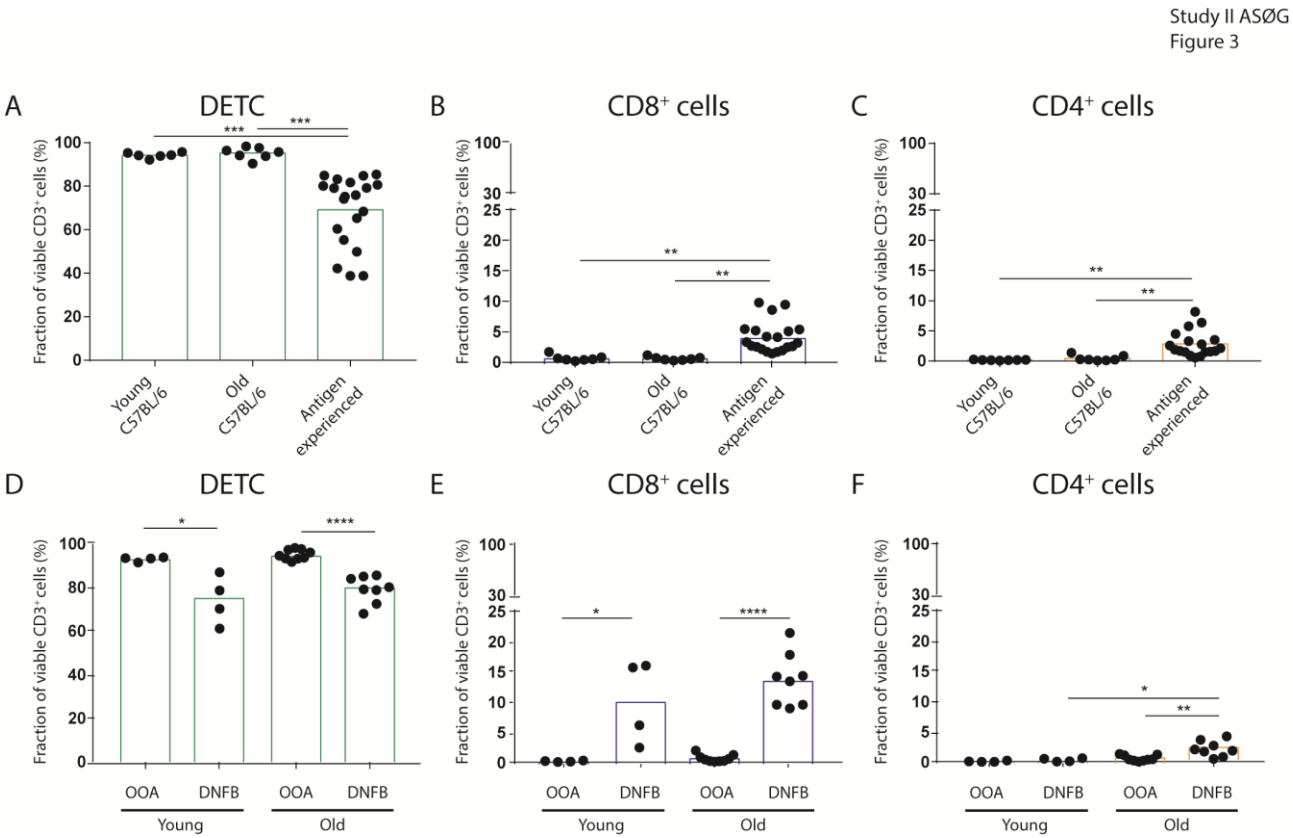
Study II ASØG
Figure 3
A
DETC
B
CD8⁺ cells
C
CD4⁺ cells
D
DETC
E
CD8⁺ cells
F
CD4⁺ cells
Fraction of viable CD3⁺ cells (%)
Young C57BL/6
Old C57BL/6
Antigen experienced
OOA
DNFB
Young
Old

Figure S1

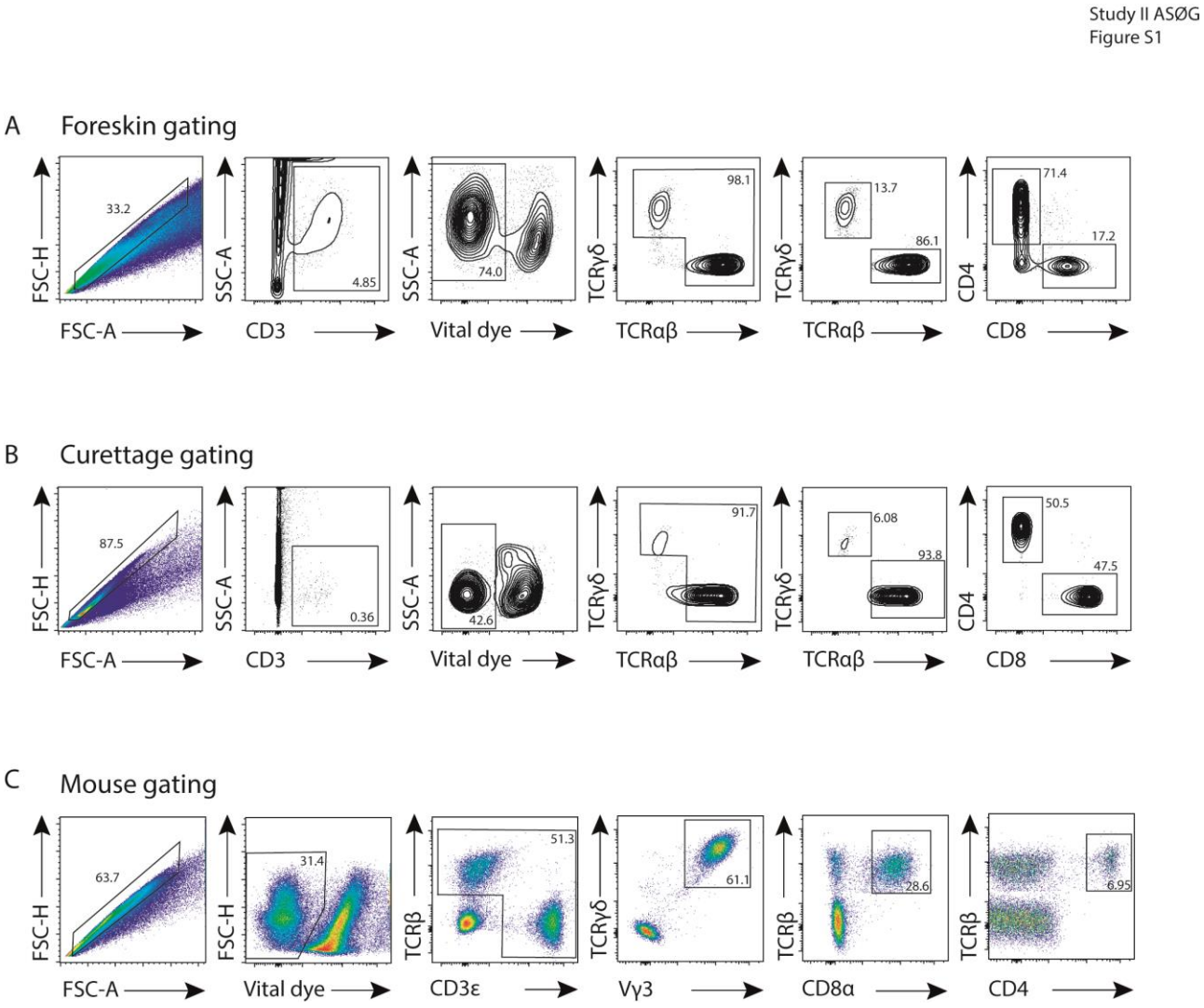
Study II ASØG
Figure S1
A Foreskin gating
FSC-H
FSC-A
33.2
SSC-A
CD3
4.85
SSC-A
Vital dye
74.0
TCRγδ
TCRαβ
98.1
TCRγδ
TCRαβ
13.7
86.1
CD4
CD8
71.4
17.2
B Curettage gating
FSC-H
FSC-A
87.5
SSC-A
CD3
0.36
SSC-A
Vital dye
42.6
TCRγδ
TCRαβ
91.7
TCRγδ
TCRαβ
6.08
93.8
CD4
CD8
50.5
47.5
C Mouse gating
FSC-H
FSC-A
63.7
FSC-H
Vital dye
31.4
TCRβ
CD3ε
51.3
TCRγδ
Vγ3
61.1
TCRβ
CD8α
28.6
TCRβ
CD4
6.95

Table 1 – participant in foreskin biopsies from infants and adults

Partici-pants	Age (days)	Age (months)	Number of T cells pr. 6mm biopsy	TCRγδ$^+$ cells from the CD3$^+$ pool (%)	CD8$^+$ cells from the CD3$^+$ pool (%)	CD4$^+$ cells from the CD3$^+$ pool (%)
1	6	0-1	28	19.0	14.3	21.4
2	23	0-1	293	23.0	16.1	44.2
3	40	1-2	2632	14.8	8.7	66.7
4	41	1-2	572	10.2	4.6	68.6
5	47	1-2	906	13.7	6.2	71.1
6	51	1-2	1123	14.3	23.4	32.4
7	60	1-2	765	9.3	18.3	62.4
8	62	1-2	1008	11.4	8.1	59.4
9	67	2-4	8101	9.3	7.1	69.9
10	68	2-4	574	16.4	19.6	48.6
11	71	2-4	4131	2.9	11.4	72.9
12	82	2-4	2857	4.2	9.3	78.2
13	101	2-4	6385	4.3	3.5	81.2
14	177	4-12	3790	17.3	17.4	57.5
15	181	4-12	9658	4.3	9.8	79.6
16	183	4-12	5721	13.7	14.8	61.5
17	245	4-12	3686	9.4	4.3	73.4
18	258	4-12	1095	21.9	14.8	47.6

Partici-pants	Age (years)	Number of T cells pr. 6mm biopsy	TCRγδ$^+$ cells from the CD3$^+$ pool (%)	CD8$^+$ cells from the CD3$^+$ pool (%)	CD4$^+$ cells from the CD3$^+$ pool (%)
19	25	10152	10.5	40.3	41.2

Table 2 – participant in curettage scraping on adult hand and buttocks

Par-ticipants	Age (years)	Sex	Skin type Fitzpatrick scale	Number of T cells (hand)	Number of T cells (buttocks)	TCRγδ$^+$ cells from the CD3$^+$ pool (%) (hand)	TCRγδ$^+$ cells from the CD3$^+$ pool (%) (buttocks)	CD8$^+$ cells from the CD3$^+$ pool (%) (hand)	CD8$^+$ cells from the CD3$^+$ pool (%) (buttocks)	CD4$^+$ cells from the CD3$^+$ pool (%) (hand)	CD4$^+$ cells from the CD3$^+$ pool (%) (buttocks)
1	25	M	3	318	244	8.1	2.4	43.3	34.2	37.8	48.8
2	23	M	3	662	521	5.1	6.1	34.2	37.6	49.0	49.1
3	22	M	4	142	733	12.1	9.8	31.0	36.6	36.2	42.4
4	29	F	3	255	125	6.2	6.7	76.7	46.6	9.6	31.1
5	24	M	4	23	28	0.0	5.9	42.9	35.3	42.9	35.3
6	22	M	3	365	188	2.9	7.3	31.5	48.8	32.3	34.1
7	24	F	1	394	288	10.4	8.5	26.9	35.1	56.7	47.9
8	27	M	3	18	52	9.1	11.1	27.2	19.5	54.6	52.8
9	25	F	3	90	176	0.0	7.4	100.0	37.0	0.0	48.2
10	22	F	3	51	21	0.0	9.1	26.1	54.5	56.6	9.1
11	23	M	3	279	436	9.4	5.5	45.3	27.2	30.2	49.0
12	24	M	3	54	36	0.0	0.0	48.0	50.0	40.0	11.1
13	23	F	3	608	1188	3.4	4.9	42.0	44.3	52.4	50.1
14	27	F	2	518	358	14.4	20.3	37.4	29.9	46.7	45.2
15	21	M	3	81	369	11.4	10.2	37.1	43.2	20.0	25.9
16	27	F	4	413	632	2.6	4.6	41.9	45.1	42.3	49.0
17	26	F	4	2624	2415	7.5	8.7	31.3	34.7	57.2	50.9
18	24	M	3	3258	1553	6.1	9.8	44.6	57.0	47.4	31.6
			Mean (age)						24		
			SD						**2**		
			Female						**8**		
			Male						**10**		

Partici-pants	Age (years)	Sex	Skin type Fitzpatrick scale	Number of T cells (hand)	TCRγδ$^+$ cells from the CD3$^+$ pool (%) (hand)	CD8$^+$ cells from the CD3$^+$ pool (%) (hand)	CD4$^+$ cells from the CD3$^+$ pool (%) (hand)
19	62	M	4	372	8.2	35.7	53.0
20	63	M	4	782	4.7	48.3	38.3
21	60	M	3	1304	11.4	31.5	46.9
22	64	F	4	1534	1.7	22.6	72.8
23	70	F	4	171	3.2	36.5	52.7
			Mean (age)				64
			SD				**4**
			Female				**2**
			Male				**3**

Study III

Antigen-independent contribution to the allergic response induced by challenge with contact allergens

Antigen-independent contribution to the allergic response induced by challenge with contact allergens

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Abstract

Many people are sensitized to one or more contact allergens. Upon re-exposure to the same contact allergen, the memory T cell generated in the sensitization phase gets activated and the clinical symptoms of allergic contact dermatitis (ACD) occur. Interestingly, studies have indicated that memory T cells can be antigen-independently activated. However, whether an unrelated contact allergen can induce inflammation in previously sensitized skin is not clear. In this study we show that allergen-challenge can induce a response in a TCR-independent way. By using the two contact allergens dinitrofluorobenzene (DNFB) and oxazolone (OXA), we demonstrate that an unrelated contact allergen induce increased ear thickness in sensitized mice. We further show that sensitization with DNFB and challenge with OXA induce an altered expression of immune related genes, which is different from the expression seen upon sensitization and challenge with DNFB. Further studies are needed to elucidate the unique inflammatory profile of the contact allergen unspecific inflammatory response in sensitized skin. Additional studies are moreover needed to clarify if the inflammatory profile is an allergic or irritant response.

Introduction

Allergic contact dermatitis (ACD) is a T cell-mediated immune reaction and one of the most common inflammatory skin diseases in the European population. It has recently been shown that 20% of the general population are sensitized to one or several contact allergens[1]. Contact allergens are low molecular weight chemicals e.g. metals, fragrances and preservatives that can modify self-proteins in a way that they become immunogenic[2–4]. The immune response induced by contact allergens can be divided into two phases: the sensitization phase and the challenge phase. During the sensitization phase, the contact allergen will modify self-protein and induce an inflammatory response[2–4] The modified protein will be taken up and presented by skin resident dendritic cells that due to the inflammation will be activated and migrate to the draining lymph node[5–7]. Here the dendritic cells will present the allergen to allergen-specific naïve T cells and induce T cell activation[5–7]. Some of the activated T cells will differentiate into memory T cells and the allergen-exposed individual is now sensitized[8]. The challenge phase is induced upon re-exposure to the specific allergen, which results in a reactivation of the memory T cells. These will mediate their effector function in the skin at the site of allergen exposure leading to destruction of allergen-modified keratinocytes and the clinical symptoms of ACD[9–19]. Thus, the allergen specific activation and re-activation of naïve T cells and memory T cells, respectively, is believed to be the key-point in the immune response to contact allergens.

Activation of the adaptive immune response is classically described to be induced upon antigen recognition via highly specific antigen receptors. T cells recognise antigens in the form of peptides presented in major histocompatibility complex (MHC) via the T cell receptor (TCR)[20–22]. Interestingly, this classic dogma has been challenged as it has been shown that both effector and memory T cells can be activated in an antigen-independent way in various infectious models[23–27]. This activation can be mediated by various stimuli, normally ascribed to activation of cells within the innate system, like

TLR stimulation, cytokine stimulation (e.g. IL-12 and IL-18) and engagement of NKG2D[26–29]. However, whether all subsets of memory T cells can be activated via an antigen-independent way is still not clear. It has been suggested that $CD8^+$ tissue-resident memory T (T_{RM}) cells require antigen-recognition to be activated[30]. In agreement with this, Gamradt *et al.* recently showed using a mouse model for ACD that the challenge response induced by local memory is allergen-specific[31]. In contrast, our previous results indicate that a challenge response can be induced in an allergen-independent way[32]. The reason for this discrepancy is not clear. In this study, we therefore wanted to investigate if challenge response to contact allergens can be induced in an allergen-independent way. To this end, we used a mouse model for ACD where mice were either sensitized with dinitrofluorobenzene (DNFB) or Oxazolone (OXA) and challenged with DNFB or OXA. By measuring changes in ear-thickness, we found that allergen-challenge can induce a response in an allergen-independent way. Furthermore, we found altered expression of immune related genes in mice sensitized with DNFB and challenged with OXA compared to the response seen in control mice. However, when we examined the most differentially expressed genes on the protein level, no difference was found between the mice sensitized with DNFB and challenged with OXA and mice sensitized to OOA and challenged with OXA.

Results

Skin inflammation can be induced in an allergen-independent way in sensitized mice upon challenge

To investigate if allergen challenge can induce skin inflammation in an allergen-independent way in sensitized mice, we sensitized mice on the ears for three consecutive days with either 0.15% DNFB or with the vehicle, olive oil and acetone (OOA) (1:4). After a minimum of 21 days, groups of mice were challenged with either 0.15% DNFB or 0.5% OXA as shown in figure 1A. The control mice were either exposed to OOA or 0.5% OXA (Fig. 1A). Ear thickness was measured in all four groups of mice at various time points after challenge. A time-dependent increase in ear thickness was observed in mice challenged with either DNFB or OXA (Fig. 1B). Interestingly, at the early time point, OXA challenge and DNFB challenge induce comparable increases in ear-thickness, but 24 hours after challenge, a larger response was induced by DNFB challenge compared to OXA challenge (Fig. 1B-C). The OXA induced increase in ear thickness seems to depend on the local memory formed during sensitization, as no difference in ear-thickness was seen in control mice exposed to either OOA or 0.5% OXA (Fig. 1B). To investigate if the allergen-independent skin inflammation was allergen-dependent, we sensitized mice on the ears for three consecutive days with either 0.5% OXA or with OOA. After a minimum of 21 days, groups of mice were challenged with either 0.5% OXA or 0.15% DNFB as shown in figure 1D. The control mice were exposed to either OOA or 0.15 % DNFB (Fig. 1D). Again, changes in ear thickness were measured at various time points after challenge. In contrast to mice sensitized and challenged with DNFB (Fig. 1B-C), mice sensitized and challenged with OXA exhibited a much stronger response (Fig. 1E-F) and mice sensitized with DNFB and challenged with OXA induced a stronger response than seen when mice were sensitized with OXA and challenged with DNFB (Fig. 1C-F). Still, it seems that the presences of local memory could mediate an allergen-

independent response, as an increased ear thickness was seen upon DNFB challenge in OXA sensitized mice compared to the response seen in mice sensitized to OOA (Fig. 1E).

Challenge with OXA induces expression of immune-related genes in the skin from DNFB-sensitized mice

To further investigate the allergen-independent response described above, we determined how challenge with OXA affects expression of immune-related genes in the ear from DNFB sensitized mice compared to the expression induced by DNFB challenge and in control mice. To do this, gene expression was analysed 24 hours after challenge using nanoString technology containing a panel of 575 immune-related genes. Compared to the controls, challenge with OXA and DNFB significantly affected the expression of immune-related genes in DNFB sensitized mice (Fig. 2A). The expression profile of the 125 most variable, immune related genes (each gene is represented by a row in the heat map) across all samples is shown in Fig. 2A, which illustrates that DNFB-DNFB treatment induces a stronger change in gene expression compared to that of DNFB-OXA vs. OOA-OOA. A principal component analysis (PCA) plot based on the same 125 most variable genes revealed 3 sample clusters (Fig. 2B). The mice sensitized and challenge with DNFB appear in a clearly distinct cluster. The DNFB sensitized and OXA challenged mice formed another cluster, except for mouse #7, which had an expression profile more similar to that of the control mice (Fig. 2B). Most of the differentially expressed genes (122 out of 124) following OXA challenge were part of the larger DNFB challenge-induced 228-gene signature (Fig. 2C). 116 genes were unique to the mice challenged with DNFB compared to controls and 12 genes were unique to mice challenged with OXA compared to controls (Fig. 2C).

CCL20 is uniquely up-regulated in the unspecific response

To further investigate the genes differentially expressed upon the different treatments, we compared the expression profiles of control mice and mice sensitized with DNFB and challenged with OXA (Fig. 3A), control mice and mice sensitized and challenged with DNFB (Fig. 3B), and mice sensitized and challenged with DNFB and mice sensitized with DNFB and challenged with OXA (Fig. 3C). *IL1β* was the most up-regulated gene (41-fold upregulation) in skin from mice sensitized with DNFB and challenged with OXA compared to skin from control mice, and it was up-regulated even more in skin from mice sensitized and challenged with DNFB (221-fold upregulated) (Fig. 3D). At the protein level, even though an increased level of IL-1β was found in skin from mice sensitized with DNFB and challenged with OXA compared to skin from control mice, the level was not higher than in skin from mice only exposed to OXA during challenge (Fig. 3E). This suggests that the increased level of IL-1β found in the skin from mice sensitized with DNFB and challenged with OXA simply is part of the inflammatory response induced by allergen stimulation of the innate immune system. The most up-regulated gene in skin from mice sensitized and challenged with DNFB compared to skin from control mice was *Ccl4* (223-fold upregulated), which was also up-regulated in skin from mice sensitized with DNFB and challenged with OXA (14-fold upregulated) compared to skin from control mice but to a lower degree as compared to mice sensitized and challenged with DNFB (Fig. 3D). However, an up-regulation of CCL4 at protein level was only found in mice sensitized and challenged with DNFB compared to controls (Fig. 3E). The genes that were most up-regulated in skin from mice sensitized and challenged with DNFB compared to skin from mice sensitized with DNFB and challenged with OXA were the IFNγ-induced chemokines *Cxcl9* and *Cxcl10* (44 and 30 fold upregulated, respectively) (Fig. 3D), which was confirmed at a protein level for CXCL9 (Fig. 3E). In accordance, IFNγ was only found to be up-regulated in skin from mice sensitized and challenged with DNFB (3.3 fold upregulated) and not in skin from mice sensitized with DNFB and challenged with OXA (Fig.

3D-E). 6 genes were found to be down-regulated in the skin from mice sensitized and challenged with DNFB compared to the expression level found in skin from mice sensitized with DNFB and challenged with OXA. The most differentially expressed of these 6 genes was *Ccl20* that was found to be 10-14-fold up-regulated in the skin from mice sensitized with DNFB and challenged with OXA compared to the two other groups of mice (Fig. 3D). We found that CCL20 was also up-regulated at the protein level in skin from mice sensitized with DNFB and challenged with OXA compared to skin from mice sensitized and challenged with DNFB as well as control mice (Fig 3E). However, as CCL20 was also up-regulated in skin from mice exposed to OOA during sensitization and OXA during challenge (Fig. 3E), CCL20 seems to be a marker for unspecific inflammation.

Discussion

In this study, we found an increased response upon allergen challenge on skin areas previously sensitized with an unrelated allergen compared to the response seen upon challenge of allergen-naïve skin when measured as changes in ear thickness. This indicates that allergen-specific T cells might be activated in an antigen-independent way as shown in several infectious models[23–27]. Furthermore, we found that challenge with OXA induced a differential expression of immune related genes in the skin from mice sensitized with DNFB compared to the expression found in skin from mice sensitized and challenged with DNFB as well as in skin from control mice. Surprisingly, no differences were seen in the expression of most differentially expressed genes at the protein level between mice sensitized with DNFB and challenged with OXA and mice exposed to OOA during sensitization and challenged with OXA. Therefore, it could seem as if the altered expression profile seen in the skin from mice sensitized with DNFB and challenged with OXA compared to the two other groups simply is reflecting an irritant response induced by OXA. However, as we only see an increased ear thickness in mice sensitized with DNFB, and not in mice sensitized with OOA, upon DNFB or OXA challenge we find it likely that an antigen-unspecific T cell activation is involved in the immune response to contact allergens but that the mechanisms mediating this do not involve the most differentially expressed genes.

Recently, Gamradt *et al.* showed that challenge on the ears with OXA did not induce skin inflammation in mice previously sensitized with DNFB[31]. The reason for this discrepancy to our findings is not clear. However, one major difference in the experimental conditions is that whereas we use 0.5% OXA as challenge dose, Gamradt *et al.* use 1.0% OXA[31]. Challenge with 1.0% OXA seems to induce an irritant response seen as increased ear thickness compared to the response induced by challenge

with 0.13% DNFB[31]. In contrast, our results with 0.5% OXA did not seem to induce an irritant response in allergen-naïve mice. Taken together, this suggests that the allergen-dose in the challenge response could explain the difference to our results. In connection with this, we found that the unspecific response differs depending on whether mice were sensitized with DNFB and challenged with OXA or sensitized with OXA and challenged with DNFB, as illustrated by a higher relative ear thickness for DNFB-OXA mice compared to OXA-DNFB mice. This could indicate that the unspecific response is allergen-dependent but might also be due to different dose and potency of DNFB and OXA. This needs to be further investigated.

Materials and Methods

Mice

Female C57BL/6 mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Mice were 8 weeks old at the beginning of experiments. The mice were housed in specific pathogen-free animal facilities at the Department of Experimental Medicine, University of Copenhagen in accordance with the national animal protection guidelines (license number 2018-15-0201-01409).

Mouse model for contact hypersensitivity (CHS)

To induce CHS mice were sensitized for three consecutive days with 25 µl on the dorsal side of both ears with 0.15% (v/v) of 1-fluoro-2,4-dinitrobenzene (DNFB) (Sigma-Aldrich, Brøndby, Denmark) or 0,5% (v/v) of 4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (OXA) (Sigma-Aldrich) in olive oil and acetone (OOA (1:4)). Control mice were exposed to OOA. Three weeks later, mice were challenged once with 25 µl on the dorsal side of both ears with DNFB, OXA or OOA. Ear thickness was measured before challenge and 6 hours, 12 hours and 24 hours after challenge. Mice were euthanized 24 hours after challenge.

Gene-expression of immune-related genes

Mouse ears were split in two and the dorsal sides were snap frozen with liquid nitrogen in 1ml of TRI-reagent (Sigma-Aldrich). QIAzole Lysis Reagent (Qiagen, Germany) and Precellys Tissue Homogenizer (Bertin Technologies, France) was used to disrupt the tissue and RNeasy Micro kit (Qiagen) was used to purify RNA, both according to manufactures protocol. Gene expression analysis was performed using nanoString Mouse Immunology Panel (BioXpedia, Aarhus, Denmark) including 561 genes and 14 references genes.

Protein purification and ELISA

Ears were homogenized using a Precellys Evolution Tissue Homogenizer (Bertin Technologies, France) and lysis buffer containing 50 mM Tris Base, 250 mM NaCl, 5 mM EDTA and 1% Triton X-100. Subsequently, samples were spun down, and the supernatant containing protein was recovered. Bradford assay was used to determine protein concentration and lysates were adjusted to a concentration of 1 µg/µl. Concentrations of IL-1β, IFNγ, CXCL-9, CXCL-10, CCL4 and CCL20 were determined using Mouse IL-1β/IL-1F2, Mouse IFNγ, Mouse CXCL-9/MIG, Mouse CXCL-10/IP-10/CRG-2, Mouse CCL4/MIP-1β and Mouse CCL20/MIP-3α DuoSet ELISA kits (R&D Systems, Minnesota, USA) according to the manufacturer's specifications.

Statistics

Statistically significant differences between groups were evaluated with two-way ANOVA (for ear thickness) or one-way ANOVA (for ELISA and boxplots), with Tukey's multiple comparisons test (Fig. 1 B and E and Fig. 3 D and E) or Sidak's multiple comparisons test (Fig. 1 C and F). GraphPad Prism 7 software was used for the analysis. The NanoString nSolver software (version 4.0, 2016) was used to normalize each sample based on the geometric mean of positive controls and the geometric mean of selected housekeeping genes. Differentially expressed genes (DEG) were identified by ANOVA (cut-off: 2-fold change and $P<0.05$), and significance was adjusted for multiple testing by estimating False Discovery Rate (FDR). Data were visualized in Qlucore Omics Explorer version 3.4 (Qlucore AB, Lund, Sweden), including principal component analysis (PCA), heat maps, unsupervised hierarchical clustering, and box plots. Statistically significant *p*-values are denoted as: *<0.05, **<0.01, ***<0.001, ****<0.0001. ns = non-significant.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Figure legends

Figure 1. Ear thickness at different time points after challenge with contact allergens

(A and D) Mouse models. Mice were either sensitized with 0.25ul of OOA (control), 0.15% DNFB in OOA or 0.5% OXA in OOA on both ears for three consecutive days. Mice were challenged a minimum of 21 days later with either 0.25ul of OOA, 0.15% DNFB or 0.5% OXA on both ears. (B) Ear thickness of control mice (OOA-OOA), allergen-naïve mice challenged with OXA (OOA-OXA), mice sensitized to DNFB and challenged with OXA (DNFB-OXA) and mice sensitized and challenged with DNFB (DNFB-DNFB) measured before challenge (0 hours) and 6 hours, 12 hours and 24 hours after challenge. (C) Relative ear thickness of DNFB-OXA and DNFB-DNFB treated mice. (E) Ear thickness of control mice (OOA-OOA), allergen-naïve mice challenged with DNFB (OOA-DNFB), mice sensitized to OXA and challenged with DNFB (OXA-DNFB) and mice sensitized and challenged with OXA (OXA-OXA) measured before challenge (0 hours) and 6 hours, 12 hours and 24 hours after challenge. (F) Relative ear thickness of OXA-DNFB and OXA-OXA treated mice. Data were obtained from two independent experiments with 4 mice/group. Significance was determined with 2-way ANOVA with Tukey's (B and E) or Sidak's (C and F) multiple comparisons test.

Figure 2. Difference in transcription profile in DNFB-sensitized mice challenged with OXA or DNFB

Gene expression analysis using nanoString technology was used to determine the immunological transcriptional profiles of OOA-OOA, DNFB-OXA and DNFB-DNFB treated mice (n=4 samples/group). (A) Heat map and unsupervised hierarchical clustering based on the 125 most variable genes across samples (B) Principal component analysis based on the same 125 most variable genes (applying a variance filter of 0.275 sigma/sigma_max (s/smax) to maximize the projection score (PS) to 0.39). (C) Venn diagram illustrating the overlap (122 DEG) in differentially expressed genes

(DEG), when comparing DNFB-DNFB to OOA-OOA (228 DEG) and DNFB-OXA to OOA-OOA (134 DEG).

Figure 3. Transcriptional and translational profiles of DNFB-sensitized mice challenged with OXA or DNFB

Heat maps and unsupervised hierarchical clustering (cut-off: 4-fold change and $p<0.05$) based on the (A) 44 genes (all upregulated) that differ the most between DNFB-OXA and OOA-OOA, (B) 120 genes (all upregulated) that differ the most between DNFB-DNFB and OOA-OOA, (C) 54 genes (53 upregulated, 1 downregulated) that differ the most between DNFB-OXA and DNFB-DNFB. (D) Box-plots illustrating expression of selected genes (log2 transformed data) and their corresponding proteins (E). Data were obtained from one independent experiment with 4 mice/group in A-D and two independent experiments with 4 mice/group in E. Significance was determined with 1way ANOVA with Tukey's multiple comparisons test for D and E.

Figure 1

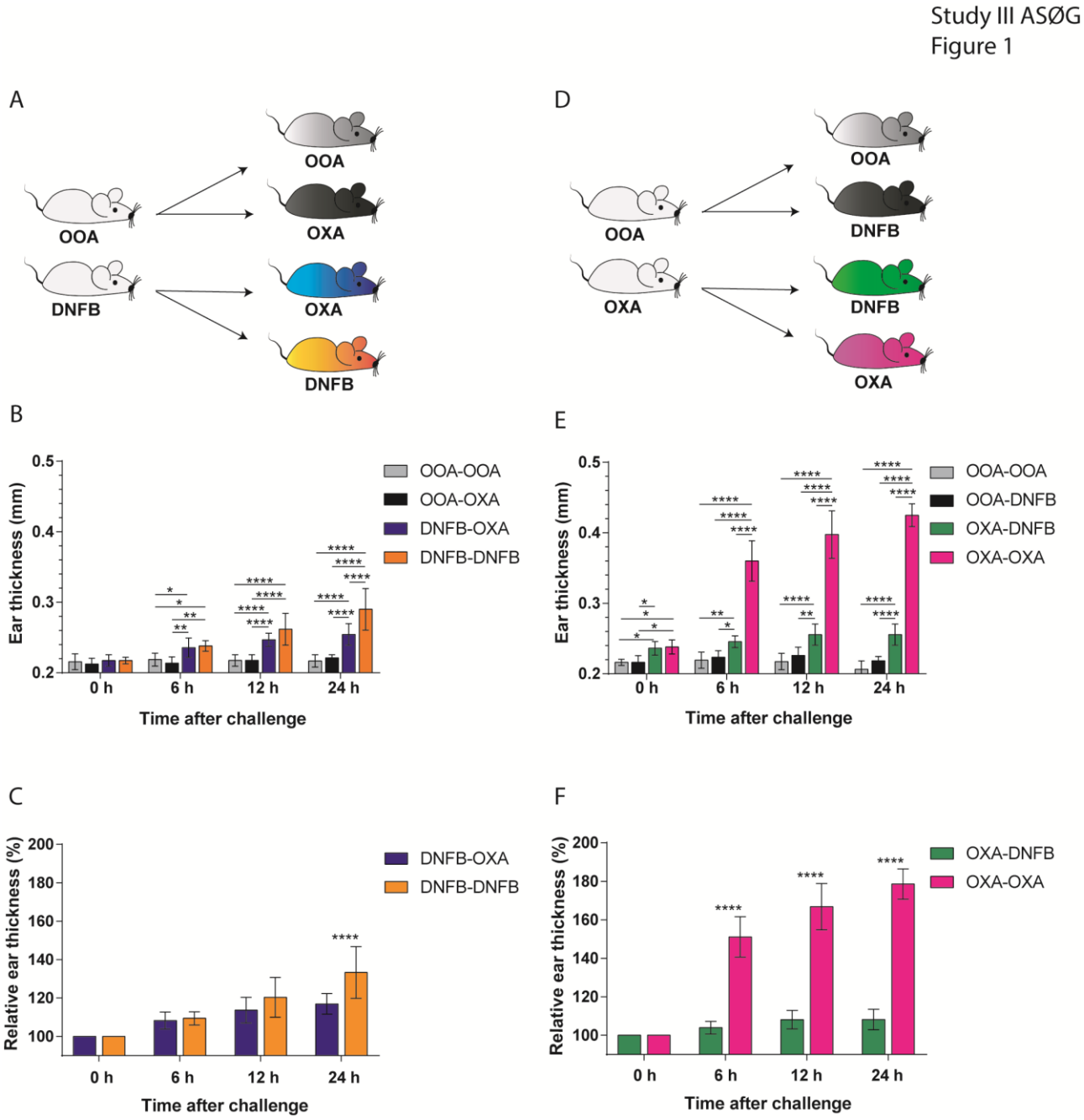

Study III ASØG
Figure 1
A
OOA
OOA
OXA
DNFB
OXA
DNFB
D
OOA
OOA
DNFB
OXA
DNFB
OXA
B
Ear thickness (mm)
0.5
0.4
0.3
0.2
0 h
6 h
12 h
24 h
Time after challenge
OOA-OOA
OOA-OXA
DNFB-OXA
DNFB-DNFB
E
Ear thickness (mm)
0.5
0.4
0.3
0.2
0 h
6 h
12 h
24 h
Time after challenge
OOA-OOA
OOA-DNFB
OXA-DNFB
OXA-OXA
C
Relative ear thickness (%)
200
180
160
140
120
100
0 h
6 h
12 h
24 h
Time after challenge
DNFB-OXA
DNFB-DNFB
F
Relative ear thickness (%)
200
180
160
140
120
100
0 h
6 h
12 h
24 h
Time after challenge
OXA-DNFB
OXA-OXA

Figure 2

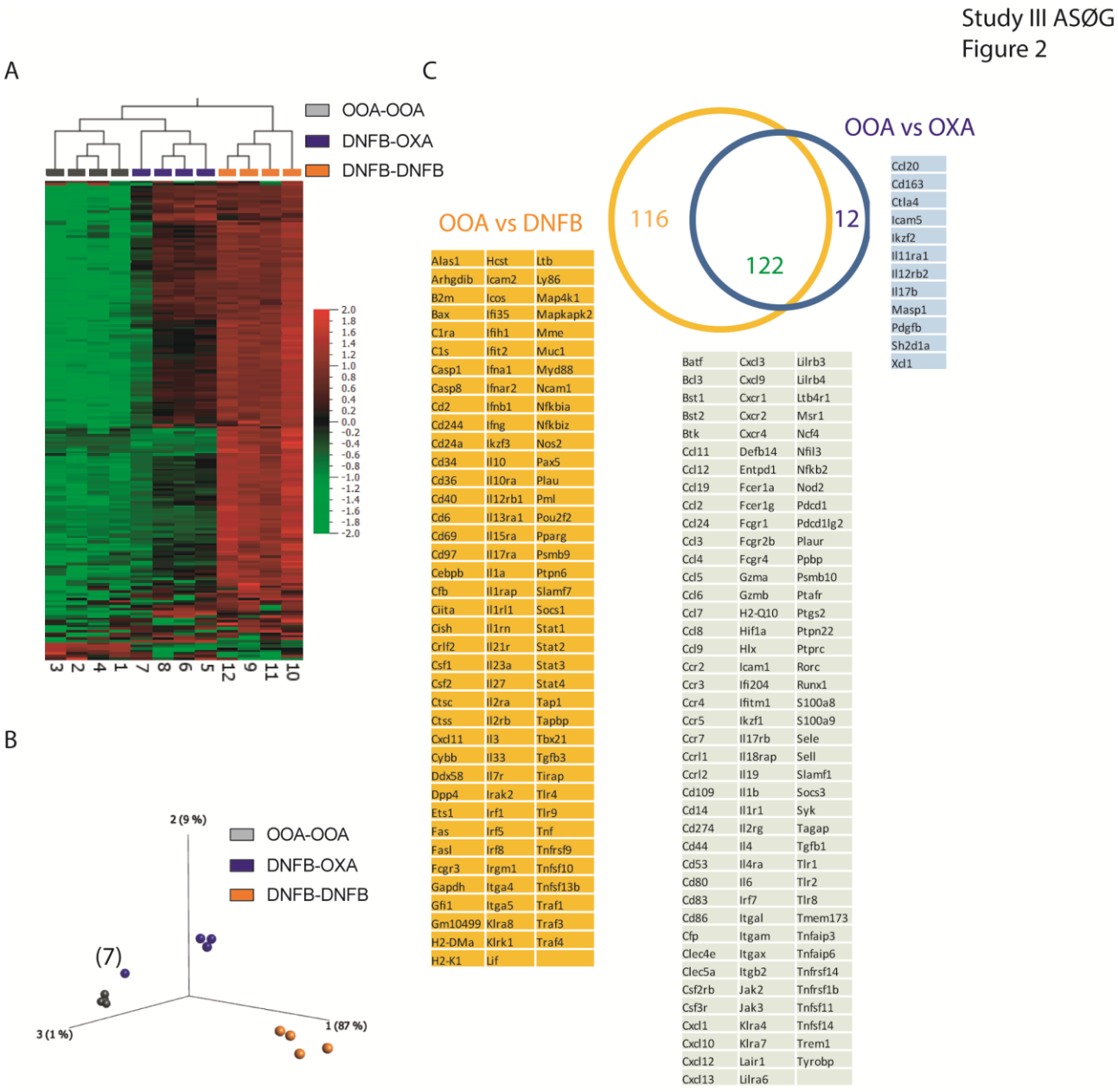
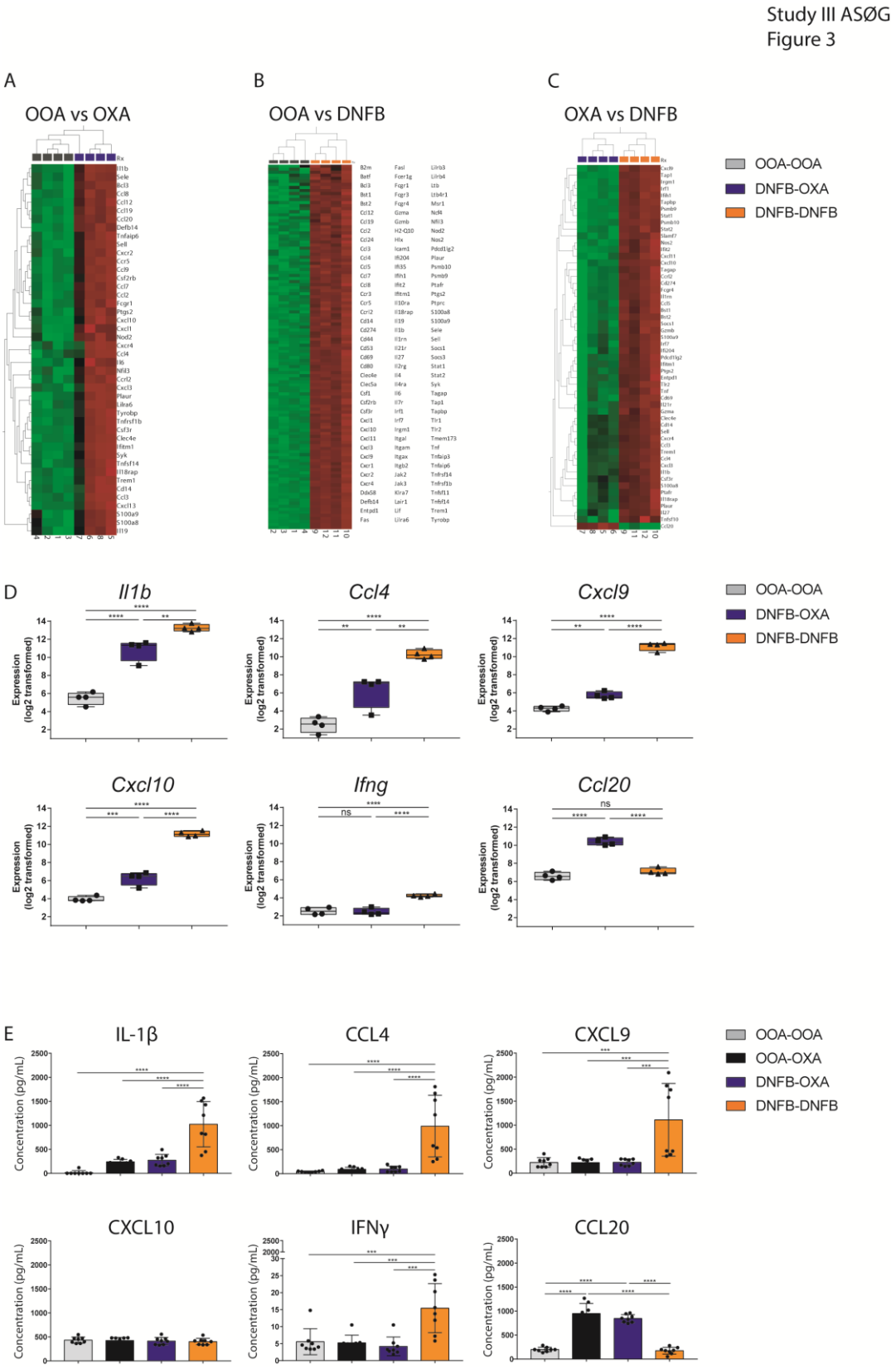

Study III ASØG
Figure 2
A
OOA-OOA
DNFB-OXA
DNFB-DNFB
3 2 4 1 7 8 6 5 12 9 11 10
B
2 (9 %)
OOA-OOA
DNFB-OXA
DNFB-DNFB
(7)
3 (1 %)
1 (87 %)
C
OOA vs DNFB
Alas1 Hcst Ltb
Arhgdib Icam2 Ly86
B2m Icos Map4k1
Bax Ifi35 Mapkapk2
C1ra Ifih1 Mme
C1s Ifit2 Muc1
Casp1 Ifna1 Myd88
Casp8 Ifnar2 Ncam1
Cd2 Ifnb1 Nfkbia
Cd244 Ifng Nfkbiz
Cd24a Ikzf3 Nos2
Cd34 Il10 Pax5
Cd36 Il10ra Plau
Cd40 Il12rb1 Pml
Cd6 Il13ra1 Pou2f2
Cd69 Il15ra Pparg
Cd97 Il17ra Psmb9
Cebpb Il1a Ptpn6
Cfb Il1rap Slamf7
Ciita Il1rl1 Socs1
Cish Il1rn Stat1
Crlf2 Il21r Stat2
Csf1 Il23a Stat3
Csf2 Il27 Stat4
Ctsc Il2ra Tap1
Ctss Il2rb Tapbp
Cxcl11 Il3 Tbx21
Cybb Il33 Tgfb3
Ddx58 Il7r Tirap
Dpp4 Irak2 Tlr4
Ets1 Irf1 Tlr9
Fas Irf5 Tnf
Fasl Irf8 Tnfrsf9
Fcgr3 Irgm1 Tnfsf10
Gapdh Itga4 Tnfsf13b
Gfi1 Itga5 Traf1
Gm10499 Klra8 Traf3
H2-DMa Klrk1 Traf4
H2-K1 Lif
OOA vs OXA
116
122
12
Batf Cxcl3 Lilrb3
Bcl3 Cxcl9 Lilrb4
Bst1 Cxcr1 Ltb4r1
Bst2 Cxcr2 Msr1
Btk Cxcr4 Ncf4
Ccl11 Defb14 Nfil3
Ccl12 Entpd1 Nfkb2
Ccl19 Fcer1a Nod2
Ccl2 Fcer1g Pdcd1
Ccl24 Fcgr1 Pdcd1lg2
Ccl3 Fcgr2b Plaur
Ccl4 Fcgr4 Ppbp
Ccl5 Gzma Psmb10
Ccl6 Gzmb Ptafr
Ccl7 H2-Q10 Ptgs2
Ccl8 Hif1a Ptpn22
Ccl9 Hlx Ptprc
Ccr2 Icam1 Rorc
Ccr3 Ifi204 Runx1
Ccr4 Ifitm1 S100a8
Ccr5 Ikzf1 S100a9
Ccr7 Il17rb Sele
Ccrl1 Il18rap Sell
Ccrl2 Il19 Slamf1
Cd109 Il1b Socs3
Cd14 Il1r1 Syk
Cd274 Il2rg Tagap
Cd44 Il4 Tgfb1
Cd53 Il4ra Tlr1
Cd80 Il6 Tlr2
Cd83 Irf7 Tlr8
Cd86 Itgal Tmem173
Cfp Itgam Tnfaip3
Clec4e Itgax Tnfaip6
Clec5a Itgb2 Tnfrsf14
Csf2rb Jak2 Tnfrsf1b
Csf3r Jak3 Tnfsf11
Cxcl1 Klra4 Tnfsf14
Cxcl10 Klra7 Trem1
Cxcl12 Lair1 Tyrobp
Cxcl13 Lilra6
Ccl20
Cd163
Ctla4
Icam5
Ikzf2
Il11ra1
Il12rb2
Il17b
Masp1
Pdgfb
Sh2d1a
Xcl1

Figure 3


Study III ASØG
Figure 3
A
OOA vs OXA
B
OOA vs DNFB
C
OXA vs DNFB
OOA-OOA
DNFB-OXA
DNFB-DNFB
D
Il1b
Ccl4
Cxcl9
Cxcl10
Ifng
Ccl20
Expression (log2 transformed)
OOA-OOA
DNFB-OXA
DNFB-DNFB
E
IL-1β
CCL4
CXCL9
CXCL10
IFNγ
CCL20
Concentration (pg/mL)
OOA-OOA
OOA-OXA
DNFB-OXA
DNFB-DNFB

Manuscript improvements

Study II

To improve study II for publication, we wish to include more foreskin samples from participants in the age group 0-1 month (figure 1) and adults (figure 2). Both of these age groups are rarely circumcised in the clinic that we collaborate with. We furthermore wish to repeat the aged hand experiment (figure 2) and the young OOA/DNFB sensitisation experiment (figure 3D-F).

Study III

Study III indicate that an antigen-unspecific inflammation occurs in sensitized skin. This is shown by increased ear thickness in mice sensitized with DNFB and challenged with OXA (DNFB-OXA) compared to mice exposed to OOA during sensitization and OXA during challenge (OOA-OXA) (figure 1B-C).

To further elucidate the unique inflammatory profile for the antigen-specific (DNFB-DNFB) and antigen-unspecific (DNFB-OXA) inflammation, we performed transcriptome analysis using nanoString analysis. Based on the transcriptome data, we performed protein measurements on the most upregulated genes, when DNFB-DNFB (*Ccl4*) or DNFB-OXA (*Il1β*) is compared to the control group (OOA-OOA). We also performed protein measurements on the most upregulated genes (*Cxcl9* and *Cxcl10*) and the most downregulated gene (*Ccl20*) when the two inflammation groups were compared. We moreover measured the protein level of IFNγ, as this cytokine is produced by $CD8^+$ T_{RM} cells in CHS as response to cognate contact allergen[156] (figure 3).

The protein measurement results call for alternative strategies, as the DNFB-OXA group do not show any significant difference from the OOA-OXA group and we therefore cannot exclude that the observed changes are due to an irritant response, generated by OXA, instead of a memory response in the skin. We therefore wish to further investigate if the DNFB-OXA group is different from the OOA-OXA group, to clarify if the observed differences in DNFB-OXA is caused by an allergen-unspecific memory response or an innate irritant response.

To explore this, we wish to: (1) Include the group OOA-OXA in an additional nanoString analysis to verify new target genes that differ between the OOA-OXA group and the DNFB-OXA group. (2) Verify the possible findings by ELISA. (3) Perform kinetic studies (6

hours, 12 hours and 24 hours) on the chosen target genes and (4) perform the ear thickness measurements using different allergen doses, to elucidate if there is a threshold for the antigen-unspecific activation.

Hopefully, these further investigations will clarify if an unrelated contact allergen can induce a memory driven inflammatory response in sensitized skin.

Considerations on methodology

This section focuses on strengths and limitations of the method used in this PhD thesis.

We have studied contact allergy in mice by sensitizing and challenging on the same skin area, primarily the ears. We sensitized for three consecutive days and challenged a minimum of 21 days thereafter. This timeframe was used because we want to ensure that the primary response has declined and that a memory response had enough time to be formed.

Other studies often use a CHS model where they sensitized on the flank or abdomen and challenge on the ears approximately 5 days later[83,102,123,149,150,157,159]. Because this timeframe between sensitization and challenge is so short, we speculated that memory formation after sensitization could not have occurred in this model. We also speculated that the challenge response could not be due to the presence of T_{RM} cells, as the challenged skin has not seen the contact allergen before, and therefore must recruit circulating memory T cells to the challenged skin to induce the memory-generated response. It is important to highlight that studies have shown that skin infections can induce a tissue-wide protection with the formation of $CD8^+$ T_{RM} cells at previous unexposed skin sites, showed by Jiang and colleagues who used a vaccinia virus skin infectious model[62]. We do not expect these distal skin areas to have as large a T_{RM} cell pool as compared to contact allergen-exposed skin.

In our studies the epidermal T cell subsets were investigated a minimum of 21 days after the last allergen exposure, with the exception of the investigations into proliferation (study I figure 4) and apoptosis (study I figure 5) after contact allergen application. We chose this time point because we wished to examine the T cell subsets in resting skin.

To further ensure that we were investigating the tissue-resident subsets, we included CD69 and CD103 in our mouse antibody panels[65]. These markers are currently thought to best discriminate tissue-resident skin cells. However some studies have shown that not all skin T cells expressing both markers[10,160]. An additional benefit of investigating the resting skin is therefore that we can ensure we are looking at the tissue-resident cells and not the infiltrating circulatory cells. However we have not investigated the epidermal subsets later than approximately a month after the last contact allergen application and therefore cannot exclude that the T cell subset distribution can vary over time.

We used C57BL/6 mice to study the response to contact allergens. These mice are known to have more DETC than for example C3H and BALB/c mice[22] which could be expected to influence our results as the suggested competition (study I and Zaid *et al.*[66]) between the epidermal T cell subsets would be affected.

We used antigen-experienced or "dirty" mice to address the effect of antigen-exposure on the epidermal T cell distribution in study II. The mice were bread for snake feed by two different venders under pet store conditions. The mice were on an unknown background but with different colour fur and we therefore expect them to be genetically different. Both male (n=14) and female (n=6) mice were used and they were approximately 12 weeks old. Further investigations using "dirtier" mice, meaning mice caught in the wild or co-housed as described by Beura *et al*. and Huggins *et al.*[55,161], would be interestingly to use as a mice model to address if the inverse correlation between DETC and $CD8^+$ T_{RM} cells is stronger in more antigen-exposed skin compared to the results we found in pet store mice.

We have predominantly used flow cytometry to investigate the epidermal T cell subsets. This method is reliable for distribution studies, but it has been criticized when it comes to quantification of cells, as studies have shown that other methods are better at estimating the exact number of cells[9,162]. For this reason we have used microscopy in study I (figure 1) to quantify the epidermal T cells and confirm our flow cytometry data.

We also performed Seahorse XF analysis, however we had to freeze the cells. This was necessary, as the cells were generated, purified and sorted in Copenhagen and the analysis performed in London. It was also necessary, because more than one purification was required to insure enough cells per Seahorse experiment. It could be speculated that freshly isolated cells would have shown different results, however freezing has been shown not to change the metabolic profile of conventional T cell subsets (unpublished data Henson Lab) and we therefore did not expect it to influence our skin-resident T cell subsets.

We investigated the T cell subsets in human epidermis using two different skin-harvesting techniques, peeled biopsies and epidermal curettage scraps, respectively (study II).

We chose to use curettage because the method was less invasive for the participants and because fewer enzyme treatments were required for the downstream skin degradation and

processing when compared to biopsies. We used biopsies on foreskin samples, because these samples were from discarded skin where curettage scraps could not be performed.

Based on our results (study II figure 2A) we can see that the two skin harvesting methods gives different T cells numbers, and we speculate this to be caused by the methods and not the anatomically different skin sites. As the distribution of cells does not vary between the two techniques, we believe that both methods are reliably.

A possible limitation in study II is that the adult foreskin samples were not from the same participants as the hand and buttock sample. This could potentially increase sample variation.

We find a different CD4:CD8 ratio compared to Watanabe *et al.* as discussed in study II[10]. The reason for the discrepancy could be explained by Watanabe *et al.* having obtained skin from patients having cosmetic surgery. A study in mice showed that obesity affects the number and function of epidermal T cells and as Watanabe *et al.* obtained skin from patients having cosmetic surgery, this might explain the different results[10,36].

Discussion and future perspectives

Understanding the interplay between DETC and $CD8^+$ T_{RM} cells is important, because both cells are essential for healthy skin homeostasis and the CHS response [13,43–45,70,72,83,102,140,145–148,156,157]. αβ T_{RM} cells constitute the largest T cell group in adult human skin. In unexposed naïve mice, the DETC constitutes the largest group[10–15]. Both subsets are dendritic cells located in the epidermis and they are both capable of making rapid response against infections and other skin assaults, making them an essential part of the skins function as first line of defence[66,70,75].

A main difference between the two cells are their activation profile. Full αβ T cells activation depends on three signals; TCR-MHC signal, co-receptor signal and cytokine signaling[163–165]. γδ T cells activation is still not fully understood and as mention in the introduction, they have a limited TCR repertoire. It has therefore been speculated that they react to self-proteins from stressed cells more than to invading pathogens[166]. Another difference is their dendrites. Dendrites from DETC point upward toward stratum corneum, whereas dendrites from $CD8^+$ T_{RM} cells points more parallel with the epidermis. This has been suggested to affect how the two subsets patrol the skin[75]. Mackay and colleagues performed transcriptional profiling and compared DETC and $CD8^+$ T_{RM} cells after HSV infection and found, not surprisingly, that the two cell types were distinct from each other and that both subsets where distinct from the circulating T cell pool[65].

Zaid and colleagues have shown how an inverse relationship between the two cell types are found in mice after HSV-infection in the skin, comparable to what we show in study I with CHS, suggesting local competition between the two cell types[66]. Other studies have shown how DETC helps αβ T cells in CHS, suggesting a cross-talk between the two cell types[48,102,147,148]. Taken together, these studies highlight that an interplay occurs between the two cell types, maybe both a competitive and a supportive interplay, and that there is a need for both cell types in the epidermal niche.

When we discuss the epidermal niche and the possible competition between the cells in it, it is important to address for how long the allergen is on the skin, as this might potentially define the tissue-residency of the epidermal T_{RM} cells and therefore their ability to push DETC away from the epidermis. Gamradt *et al.* claims, using DNP-staining, that 5% of the epidermal compartment stains positive for DNFB 30 days after DNFB challenge. They also claim that long-term persistence of the allergen is important for the $CD8^+$ T cells tissue-residency[83]. Schmidt *et al.*

also used DNP-staining and found that DNFB could not be detected in the skin 21 days after sensitization[156]. Further investigation is needed to clarify this matter.

We established in study I that $CD8^+$ T_{RM} cells were able to displace DETC in contact allergy. It would therefore be interesting to see if a different antigen or infection could give rise to $CD8^+$ T_{RM} cells with another TCR specificity that could replace the $CD8^+$ T_{RM} cells already present in the skin. This hypothesis is justified as there must be limited space in the epidermis, even though studies have shown that the number of $CD8^+$ T_{RM} cells decline over time in the skin, potentially making room for $CD8^+$ T_{RM} cells with another TCR specificity[83]. Interestingly, Park *et al.* demonstrate that there is only space for a limited number of $CD8^+$ T_{RM} cells in the skin, but that newly generated T_{RM} cells did not displace the pre-existing once[167].

Further reflecting on the interplay between DETC and $CD8^+$ T_{RM} cells, one could speculate that DETC act as the first line of defense until the $CD8^+$ T_{RM} cells take over. This hypothesis could make sense in mice, because they are born with DETC in the skin and later can acquire $CD8^+$ T_{RM} cells. In this setting, it would be interesting to investigate if $CD8^+$ T_{RM} cells take over the known DETC functions. Are they for example just as efficient in wound repair and barrier protection as the DETCs? In humans, epidermal γδ T cells are not present at birth, so it is difficult to say that they are the first line of defense. It could be argued that they are still important in the beginning of life, because we see a stable pool arising early after birth in the epidermis and that they therefore protects together with the $CD4^+$ cells.

Because $CD8^+$ T_{RM} cells replace DETC in mice, we hypothesized that neonatal human epidermis contains a higher fraction of γδ T cells as compared to adults, based on the idea that the skin would have had less antigen exposure. We did see a higher frequency in the youngest group compared to older groups, but because the epidermal T cell count overall was so low in the youngest group, we conclude that the human epidermis is not covered with a protective T cell network from birth as seen in mice. On the other hand, both LC and dermal T cells are present before birth[19], showing that human skin is also born with an immune system. The quick increase in epidermal T cell numbers seen within the first year of life in study II underline the importance of epidermal T cells as first line of defence in our skin.

We have not further investigated the phenotype of the $CD4^+$ T cells seen in infant epidermis, but we speculate that these cells are possibly T_{reg} cells. A study by Thome *et al.* investigate the immune system of infants (2 months) and found that naïve T cells dominate in the circulation and in lymphoid and mucosal tissues; and that lungs and intestines are the first organs

to contain T_{EM} cells[57]. They saw that 2 year old children had very few T_{RM} cells ($CD69^+CD103^+$ phenotype) but that 20 year old adults had more, in line with our findings in adult skin in study II. Interestingly, they saw that $CD4^+$ T_{reg} cells are very prevalent in infants and they speculate that pediatric tissue have an increased suppression of T cell activation that potentially could be targeted early in life to ensure a bigger memory response in the tissues[57].

As described in the introduction, mice and humans have both similarities but also differences regarding their skin immunity. Some of this is undoubtedly caused by species differences, but some could also be caused by differences in antigen exposure, as addressed in study II. Clean specific-pathogen-free (SPF) mice have been used for many years in research, providing a model with high consistency. Interestingly, studies using "dirty" models are increasing, to mimic a more developed immune system as seen in adult humans[55,161,168,169]. Due to the immunological differences regarding epidermal γδ T cells between human and mice, it can be argued that mice are not a suitable model for the investigations of immunological skin diseases. Even though mouse research cannot be directly translated to human studies, we argue that it is relevant, as γδ T cells have also been shown to play a role in ACD, as there is an increase in the number of γδ T cells in the skin of nickel-allergic patients and DNCB treated skin[78,153].

As addressed in the introduction, DETC are described as innate-like cells (skin homeostasis and wound repair) and memory T cells are described as classically adaptive cells (immune memory and pathogen clearance). Interestingly, memory T cells have also been shown to have an innate-like nature, responding to antigen-unrelated stimuli as addressed in study III [170–174]. A study by Mackay and colleagues showed how in vitro activated virus-specific memory T cells migrated to DNFB treated skin. The contact allergen treated skin acts as a non-specific skin inflammation without local antigen presentation[67]. They showed that the recruited memory T cells formed into a tissue resident population and that they protected against local HSV infection[67]. These findings illustrate that circulating virus-specific memory T cells can be recruited by unspecific inflammatory signals and not solely by antigen recognition. Whether the recruited cells, now located in the skin will become activated by an unrelated antigen requires further investigations.

In study III we therefore set out to investigate whether sensitized skin would react with an inflammatory response to an unrelated contact allergen challenge. As the sensitized skin contains $CD8^+$ T_{RM} cells, we speculated that the potentially antigen-unspecific inflammation would be generated by the $CD8^+$ T_{RM} cells. Larsen *et al.* showed that challenge with a different contact allergen than used for the sensitization was able to induce a small increase in the number

of $CD8^+$ T cells in the dLN, indicating that there is a contact allergen-unspecific activation of circulating T cells in CHS[175]. The increase in cell number was not seen for $CD4^+$, B and NK cells and the proliferation of $CD8^+$ T cells was larger when the same contact allergen was used for both sensitization and challenge compared to when two different contact allergens were used[175]. Larsen *et al.* harvest the dLN two days after challenge[175]. It would be interestingly to investigate whether a possible contact allergen-unspecific activation potentially presents at an earlier time point. It is likely that the kinetics would vary between the circulating memory response, as investigated by Larsen *et al.*, and the tissue-resident response, generated by skin resident T cells. Interestingly, based on study III we can see that an unrelated contact allergen can induce inflammation in the skin. Further investigation into whether it is mediated by $CD8^+$ T_{RM} cells would be interesting to explore.

Conclusions

In this PhD thesis, we have investigated the interplay between epidermal T cells in contact allergy and in healthy skin at different ages. We have further addressed the possibility that contact allergens can lead to an antigen-unspecific memory response in the skin.

In **Study I** (Gadsbøll et al, JID, 2019, in press) we found that $CD8^+$ T_{RM} cells replace DETC in contact allergy, potentially due to a higher spare respiratory capacity, indicating that $CD8^+$ T_{RM} cells are more metabolically fit than DETC. The study show that an increase in $CD8^+$ T_{RM} cells is generated by recruitment from the circulation and by local proliferation of the already existing $CD8^+$ T_{RM} cells in the response to contact allergens. DETC did not proliferate in the response to contact allergen, indicating another possible explanation for the inverse relationship between the two epidermal cells in contact allergy. The inverse relationship is both exposure and dose dependent. In **Study II** (manuscript) we investigated epidermal T cells in relation to age and antigen exposure. We confirm previous findings showing that human infant skin, opposite adult skin, contains very few epidermal T cells. We show that the number of epidermal T cells increase within the first year of life and that it is dominated by $CD4^+$ T cells, but that $CD8^+$ T cells and γδ T cells are also present. We further show that the epidermal T cell composition change with age, with an increase in the frequency of $CD8^+$ T cells. Using mice, we show that antigen exposure and not age gives the observed changes in T cell composition. In **Study III** (manuscript) we showed that an antigen-unspecific inflammation occurs in sensitized mice upon challenge with a different contact allergen than the mice were sensitized with. We show that there is a difference in the transcriptional profile between the contact allergen-specific and contact allergen-unspecific inflammation. Additional work has yet to be done to elucidate the unique inflammatory profile for the contact allergen-unspecific inflammation.

This PhD thesis has contributed to the understanding of epidermal T cell dynamics and the development of our immune system in the skin. Further work in this field will potentially lead to the development of treatments against ACD and other T cell driven skin diseases.

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