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Differential expression of RANK on Langerhans cells and CD45RA and CD45RO/CLA on T cells in developing human skin after birth

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All truths are easy to understand once they are discovered.
The point is to discover them.

Galileo Galilei

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Zusammenfassung

Die Haut, die Schnittstelle zwischen dem Körper und der Umgebung, ist das größte Organ des Körpers und hat zahlreiche Funktionen. Eine davon ist, dass die Haut die erste Verteidigungsline des Immunsystems darstellt. Wichtige Vertreter des Immunsystems in der Haut, mit denen wir uns in dieser Arbeit befasst haben, sind Antigen-präsentierende Zellen (epidermale Langerhans Zellen) und T Zellen. Interessanterweise, ist relativ wenig über das Immunsystem der Haut von Kindern bekannt. Aus diesem Grund haben wir versucht, ein weiteres Stück des „Kinder Immunsystem Puzzles“ zusammen zu setzen.

Receptor activator of NF-κB (RANK) ist ein Oberflächenrezeptor der auf vielen Zellen exprimiert wird. In menschlicher Haut wurde er auf Langerhans Zellen beschrieben wo er deren Lebensdauer erhöht. Es gibt jedoch Hinweise, dass andere Zellpopulationen innerhalb der Haut ebenfalls RANK exprimieren, allerdings wurde noch nicht gezeigt um welche es sich dabei handelt. Bislang wurde berichtet, dass etwa 95% aller Langerhans Zellen in adulter menschlicher Haut RANK exprimieren. Unsere umfassenden Analysen unter Verwendung mehrerer Methoden haben jedoch ergeben, dass die Zahl der $RANK^+$ Langerhans Zellen in menschlicher Haut wesentlich geringer ist als zuvor beschrieben. Weiters konnten wir zeigen, dass Keratinozyten, nicht aber T Zellen und Melanozyten RANK exprimieren. Die Untersuchung unterschiedlicher Altersgruppen ergab einen Anstieg $RANK^+$ Langerhans Zellen und einen Abfall RANK-exprimierender-Keratinozyten, mit steigendem Alter der Spender, was darauf hindeutet, dass das Immunsystem der Haut nach der Geburt noch nicht dem von Erwachsenen entspricht.

Während man in adulter Haut hauptsächlich Gedächtnis T Zellen findet, gibt es in fötaler Haut fast ausschließlich nur naive T Zellen. Unsere Daten weisen nun daraufhin, dass der Wandel von der predominanten naiven T Zell-Population zur Gedächtnis T Zell-Population bereits unmittelbar nach der Geburt geschieht.

Diese Experimente zeigen eindeutig, dass sich das Immunsystem der Haut von Kindern und Erwachsenen voneinander unterscheidet und tragen somit zur Erweiterung unseres Wissens über das Immunsystem der Haut von Kindern bei.

Abstract

The skin, the interface between the body and the external environment, is the largest organ of the body and has multiple functions. One of them is that the skin represents the first defence line of the immune system. Important representatives of the skin immune system we were working with, were antigen-presenting cells (epidermal Langerhans cells) and T cells. Interestingly, little is known about the skin immune system of children. For this reason we, tried to compile another, yet unknown piece of the “child immune system puzzle”.

RANK is a surface receptor which is expressed on many different cell types including Langerhans cells in adult human skin, where it increases their survival. So far, it is reported that about 95% of all Langerhans cells in adult human skin express RANK. Other epidermal cell populations also express RANK, but the cell type has not been specified yet. Surprisingly, our extensive an analysis using selected methods showed that the number of $RANK^+$ Langerhans cells in human skin is much lower than described. Additionally we showed that keratinocytes, but neither epidermal T cells nor melanocytes express RANK. The analysis of RANK expression in selected age groups revealed an increase of $RANK^+$ Langerhans cells and a decrease of $RANK^+$ keratinocytes with increasing age of the donors, which indicating that the skin immune system after birth differs from the adult one.

While most T cells in adult human skin show a memory phenotype, the majority of fetal T cells are naive. To determine when after birth the switch from naïve to memory T cell phenotype occurs, skin samples from selected age groups after birth were examined for the presence of cells using double immunofluorescence staining on cryostat sections. Our preliminary data show, that the majority of T cells in newborn human skin have already a memory phenotype. The density of memory T cells increased with increasing age.

Our experiments show clearly that the skin immune system from infants and adults is different and expands our knowledge about the skin immune system of children.

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1 Abbreviations

7-AAD	7-amino-actinomycin D
APC	Allophycocyanin
BDCA	Blood dendritic cell antigen
BSA	Bovine serum albumin
CCL	CC-chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CLA	Cutaneous lymphocyte-associated protein
CXCL	CXC-chemokine ligand
DC	Dendritic cell
E-selectin	Endothelial-cell selectin
EDTA	Ethylenediaminetetraacetic acid
EGA	Estimated gestational age
FACS	Fluorescence-activated cell sorter
FasL	Fas ligand
FCS	Fetal calf serum
FITC	Fluorescein-isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA-DR	Human leucocyte antigen-DR
ICAM	Intercellular adhesion molecule
IDO	Catabolizing enzyme ideolamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LC	Langerhans cell
K	Keratin
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MADCAM1	mucosal vascular addressin cell-adhesion molecule 1
MHC	Major histocompatibility complex
mm	Millimeter
NF-κB	Nuclear factor-κB
NK cell	Natural killer cell

NKT cell	Natural killer T-cell
NLR	Nucleotide-binding domain, Leucine-Rich repeat containing protein
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PRR	Pattern recognition receptor
P-selectin	Platelet selectin
RANK	Receptor activator of NF-κB
RANKL	Receptor activator of NF-κB ligand
RPMI	Roswell Park Memorial Institute
TCR	T-cell receptor
TGF	Transforming growth factor
T_H cell	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
T_{reg} cell	Regulatory T-cell
UV	Ultraviolet
VLA-4	Very late antigen

2 Introduction

2.1 Skin

The skin is the largest organ of the body and represents an interface between the body and the external environment. It is a multifunctional organ, which represents a barrier against ultraviolet (UV) light, as well as biological, chemical, and mechanical insults. The skin protects the body from dehydration or massive absorption of water, functions as a thermo regulator and sense organ, and it also represents the first defence line of the immune system.

Human skin is organised in three layers, including epidermis, dermis and hypodermis (**Figure 1**). The epidermis has an ectodermal origin, while the dermis and the hypodermis are of mesenchymal origin[1-3]. The epidermis and dermis are connected by the dermal-epidermal junction, which is synthesised by basal keratinocytes and dermal fibroblasts[4].

The skin is not built up equally; variations like thickness (varying from 1 to 4 mm), density of melanocytes and distribution of epidermal appendages are just few regional differences[4]. Palms, soles, and foreskin are hairless, whereas the rest of the body is covered by hair-bearing skin.

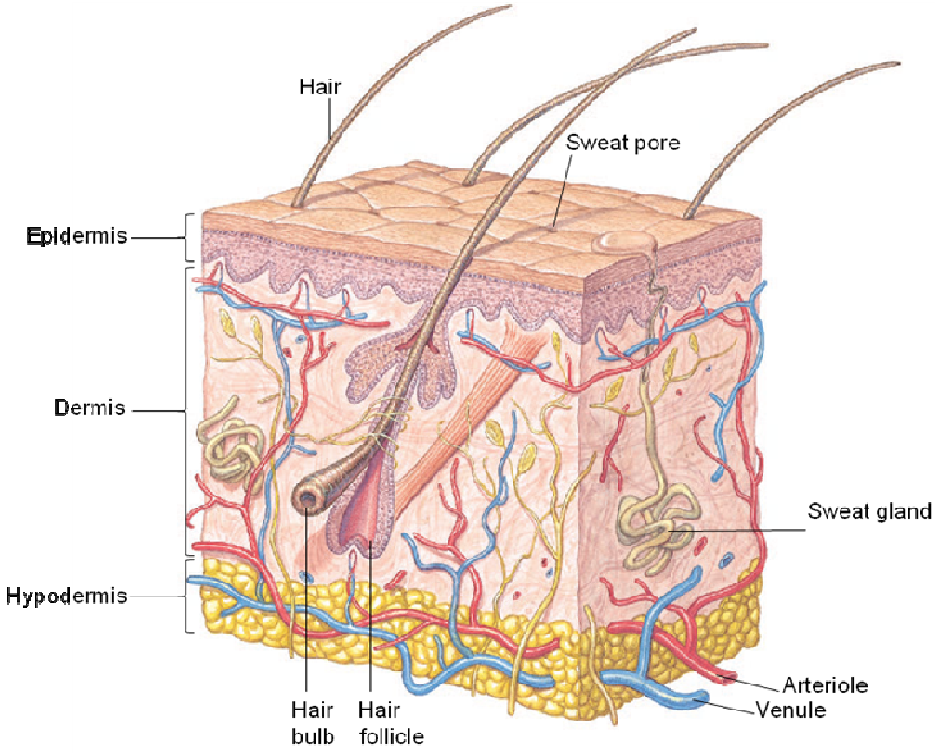

Figure 1. A diagram of the skin[5]

2.1.1 Epidermis

The epidermis is a stratified, non-vascularized epithelium that renews itself continuously; its thickness varies between 75 and 600 µm (palms and soles). Approximately 90-95% of the epidermal cell types are keratinocytes, which undergo the specific differentiation progress of keratinization, resulting in the production of corneocytes which shed from the skin surface within time. Other epidermal cell types are melanocytes, Merkel cells, T cells and bone marrow-derived Langerhans cells (LCs)[4;6].

The epidermis is arranged in four layers (**Figure 2**):

a. The **stratum basale** is the deepest layer of the epidermis and consists of keratinocytes, melanocytes, Merkel cells and T cells[7]. The keratinocytes in this layer have stem cell-like properties and divide constantly by mitosis, which leads to a movement of the new daughter cells towards the skin surface[5].

b. The **stratum spinosum** contains several layers of keratinocytes with a limited capacity for cell division[5]. Also sentinels of the immune system, called LCs are found beside T cells in the stratum spinosum[7].

c. The **stratum granulosum** consists of non-dividing keratinocytes which contain granules that are filled with keratohyalin. The nuclei and organelles of these cells break down, also they flatten due to successive push to the skin surface[6].

d. The **stratum corneum** is the outermost layer of the epidermis that contains many sheets of flattened, scalelike corneocytes. This layer is cornified and acts as the actual protection of the skin[6].

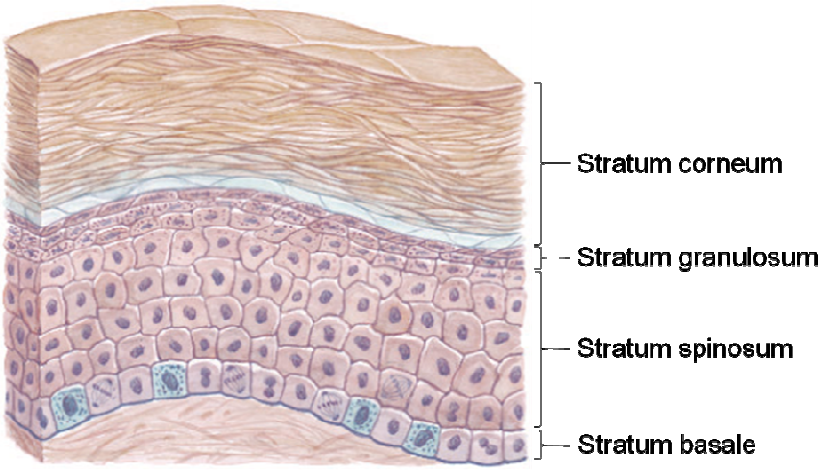

Figure 2. A diagram of epidermal layers[5]

2.1.2 Dermis

The dermis is deeper and much thicker than the epidermis and can be divided into an upper papillary dermis and a lower reticular dermis. Its thickness varies, depending on the anatomic location (e.g. the back and the palms have a much thicker dermis than the eyelids)[4]. The dermis is made up of an extracellular matrix composed of fibrous and non-fibrous proteins, a wide network of blood vessels providing nutrient supply for the stratum basale of the epidermis; lymphatic vessels, nerves, muscles, sweat glands, sebaceous (oil-secreting) glands, hair follicles, resident and trafficking cells[4;5]. The main cell type of the dermis is the fibroblast. Besides it also harbours other cell types such as dermal dendritic cells, plasmacytoid dendritic cells, T cells ($CD8^+$ T cells, T helper 1 [T_H1] cells, T_H2 cells, T_H17 cells, regulatory T cells [T_{reg} cells], natural killer T cells, $\gamma\delta^+$ T cells), macrophages, natural killer cells, and mast cells[8;9].

2.1.3 Hypodermis

The hypodermis is a subcutaneous tissue that is in fact not part of the skin. It binds the dermis to the underlying bones or muscles, stores lipids, regulates the body temperature, and insulates and cushions the body (the hypodermis contains 50% of body fat)[5]. Adipocytes, macrophages and fibroblasts are also present[4].

2.2 Cell types of the skin

2.2.1 Keratinocytes

Keratinocytes are the prevalent cell type in the epidermis and account for more than 90% of all cells. Keratinocytes of the stratum basale are anchored by hemidesmosomes to the basement membrane that separates the epidermis from the dermis[10]. Adjacent keratinocytes of all layers are generally interconnected by desmosomes[11], tight junctions, and adherens junctions[12]. Keratinocytes contain melanosomes, to store the skin color pigment melanin, which is synthesised by melanocytes[13].

Keratinocytes, produce the protein keratin (K) as part of their cytoskeleton. Keratins are one of the most potent epithelial markers and play an important role for the mechanical stability and integrity of epithelial cells and tissues. Two types of keratins can be distinguished, based on their pH-value. Type 1 keratins are acidic (K9-10,

K12-28, and K31-K40), whereas type 2 keratins are neutral/basic (K1-K8 and K71-K86)[14]. Keratinocytes express keratin polypeptides in pairs, composed of a type 1 and a type 2 keratin, but the constitution pattern of keratins depends on the type of keratinocytes. K5 and K14 are expressed in basal keratinocytes, suprabasal ones express K1 and K10. The keratinocytes in the stratum granulosum express K2 and K11, whereas those in the plantar region express K9[4].

Humans have 54 keratin genes and at least 26 are specifically expressed in the hair follicle[14]. The keratin genes are clustered at two chromosomal sites. Type 1 keratins (except K18) are located on chromosome 17q21.2, and type 2 keratins (including K18) are found on chromosome 12q13.13[15].

Keratinocytes are not only bricks of the epidermis, they also function as immune sentinels, because of their ability to express toll-like receptors (TLR) and nucleotide-binding domain, leucine-rich repeat containing proteins (NLRs), which recognise pathogen-associated molecular patterns and danger-associated molecular patterns[8]. The activation of extracellular (TLR1, 2, 4, 5 and 6) or intracellular (TLR3 and 9)[16] TLRs in keratinocytes, results in the activation of the nuclear transcription factor-κB (NF-κB), leading to the production of antimicrobial and antiviral peptides, chemokines and cytokines[17]. If NLRs get activated by irritants or toxins, pro-interleukin (IL)-1β and pro-IL-18 will get cleaved by caspase 1 to generate the active pro-inflammatory cytokines IL-1β and IL-18[18].

The constitutive expression of numerous cytokines like IL-1, IL-6, IL-10, IL-18 and tumour necrosis factor (TNF)[19] by keratinocytes, makes them an important cytokine secreting source.

During inflammation, keratinocytes are also capable to attract different cell types into the skin by secreting a wide range of chemokines. Activated keratinocytes are able to recruit effector T cells to the skin by expressing CC-chemokine ligand (CCL)20, CXC-chemokine ligand (CXCL)9, CXCL10 and CXCL11[18], whereas neutrophils are attracted through CXCL1 and CXCL8[18]. Furthermore, LC precursors are decoyed to the epidermis caused by the release of CCL20/MIP3α[20].

2.2.2 Melanocytes

Melanocytes represent 2-5% of all epidermal cells[21], descend from neural crest and migrate into the basal cell layer of the epidermis[4]. They have a dendritic morphology and express constitutively S100[22], bcl-2[23;24], c-kit/CD117[25] and vimentin[26]. As

melanocytes are the only cell population expressing CD117, this marker can be used to identify these cells in the epidermis[27].

Melanocytes synthesize the skin color pigment melanin within melanosomes by a biochemical process named melanogenesis[28]. During this process, melanin is produced from the substrate tyrosine by the enzymatic activity of tyrosinase and stored in melanosomes[4]. All humans have about the same quantity of melanocytes and various skin colors result from different amounts of produced and stored melanin[5].

Melanocytes are also able to constitutively produce IL-1α, IL-1β, IL-3, IL-6, TNFα and transforming growth factor (TGF) β[21;29]. These cytokines contribute to the regulation of an inflammation within the skin.

2.2.3 Merkel cells

Since their discovery in 1875, the role and origin of Merkel cells has been researched extensively[30]. These cells are located in the epithelial sheath of hair follicles and in the basal layer of the epidermis[4], and represent between 0.2 and 5% of epidermal cells[31;32]. In hair follicles Merkel cells are rarely associated with nerve endings, whereas those in the epidermis are in close contact with terminal nerves[33] and form the Merkel cell-neurite complex. However there is no evidence of a synaptic transmission[34]. Merkel cells contain melanosomes like keratinocytes, and are attached to them by desmosomes[13].

The cytokeratins 18[35] and 20[36;37] give Merkel cells epidermal characteristic features. Antibodies against these cytokeratins can be used to identify Merkel cells in the skin. Furthermore, neuroendocrine markers including chromogranin A[38-40] and the protein gene product 9.5[41;42] are used to detect Merkel cells.

The origin of Merkel cells is not evidenced yet, but two hypotheses are circulating among experts. On the one hand, the neural crest hypothesis exists which proposes, that Merkel cells are derived from the neural crest[43]. On the other hand the epidermal origin hypothesis, based on the presence of cytokeratin 20 and desmosomes, assumes that Merkel cells have an epidermal origin[44-47]. Recent findings showed that during embryonic development, Merkel cells arise by the differentiation of epidermal progenitors[48]. In adults, Merkel cells are not replaced by proliferation of differentiated Merkel cells; a cell type originating from epidermal stem cells, undergoes a slow turnover and replaces the old ones[48].

2.2.4 T cells

T cells, derive from a common lymphoid progenitor, immigrate to the thymus, where they undergo positive and negative selection[49]. The outcome is a small fraction of immature $CD4^+$ and $CD8^+$ thymocytes which show low affinity for self-peptide/major histocompatibility complex (MHC)[50]. These naive T cells express high levels of CCR7 and CD62L, enabling these cells to home to the T cell zones in secondary lymphoid organs[51]. Notably, in the extra-thymic environment, the positive selection continues through dendritic cells (DC) by constant T cell receptor (TCR) interaction with self-peptide/MHC ligands[49] as described later. To keep the post-thymic naive T cells in interphase and alive[52;53], an interaction with IL-7 and self-peptide/MHC molecules is necessary[54]. Interestingly, a small population of cells, termed fibroblastic reticular cells, which is found in secondary lymphoid organs, is secreting CCL21 and CCL19 (the ligands for CCR7) and IL-7[51;55].

If an antigen enters the body, antigen-presenting cells uptake, process and present it on MHC I or II to T cells in the secondary lymphoid organs. Signalling by the TCR alone is not enough to activate T cells; in addition, co-stimulation via CD28 with CD80/CD86, IL-2 release from T cells, as well as the release of antigen presenting cell-derived cytokines like IL-12, IL-15 and interferon α (IFNα) are necessary to convert naive T cells to effector T cells[56-59]. Upon activation, some T cells ($CD4^+$) might either migrate to the B cell follicles in the lymph node or the spleen, where they stimulate B cells; or $CD4^+$ and $CD8^+$ T cells enter the circulation where they respond to an inflammation in the periphery[60].

2.2.4.1 T cells possess different homing phenotypes for the skin and the gut

Two subsets of effector T cells can be distinguished, dependent on their preferential homing capacity for cutaneous or intestinal tissues[61]. One subset expresses the α4β7-integrin, the ligand of which is the mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1)[62] and is expressed by endothelial cells of the intestinal tract and attendant lymphoid tissues under steady state conditions[63]. Most of the skin resident T cells, representing the second subset, express cutaneous lymphocyte-associated protein (CLA)[64], which is the ligand for E- and P-selectin. To support T cell rolling under non-inflamed conditions, dermal microvessels constitutively express E- and P-selectin in a sufficient way[65]. The levels of E- and P-selectin expression

increase during an inflammation in the tissue, which causes an enhanced T cell recruitment to the inflamed region[66-70]. For a long time, it was thought that the identity and localisation of the lymph nodes determine the tissue-homing-receptor profile on T cells[71;72], but recent data indicate that the site from where DCs acquire an antigen constitutes the phenotype[73].

To regulate the trafficking of effector T cells to intestinal or cutaneous sites, chemokine receptors play an important role[74]. In the blood circulating α4β7-integrin$^+$ T cells express CCR9[75-78] and its ligand CCL25 is selectively and constitutively expressed by human intestinal epithelial cells[75;76;79]. Most of the CLA$^+$ T cells in peripheral blood express CCR4 and CCR10[80-84]. CCL17 (the ligand of CCR4) is low expressed by skin venules, but its expression increases during an inflammation[80], whereas CCL27 (the ligand for CCR10) is constitutively expressed by skin keratinocytes[81-84].

2.2.4.2 Memory T cells

Upon elimination of the pathogen, most of the antigen-specific effector T cells die because of a lack of IL-7 and/or TCR signaling[52;54]. Additionally, T_{reg} cells are able to regulate T cells negatively, by reducing proliferation and facilitated apoptosis[49]. This widespread death of T cells is necessary to restore the steady state in the periphery, but a small fraction of these antigen-specific T cells (about 5%) survive to become long-lived memory T cells[52], which can respond quickly to a secondary pathogen challenge[85-87]. Two types of memory T cells are described: (a) “central” memory cells, which return from the periphery to the lymph nodes due to the expression of the homing receptors CCR7 and CD62L, and (b) “effector” memory cells, which stay in the periphery, and have down regulated CCR7 and CD62L[88-90]. Effector memory T cells are able to enter the blood and migrate at another site of the periphery, to sustain a memory effect in the whole periphery of the body[88-90]. When the pathogen is cleared, antigen-specific memory T cells become effector memory T cells, which gradually switch to central memory T cells over time[91]. The mechanism behind this process is largely unknown.

2.2.4.3 Proliferation capacity of T cells

The number of naive T cells remains relatively stable, because they do not undergo cell division, whereas central memory T cells proliferate every 2-3 weeks[53]. Central memory T cells express high levels of CD127 and CD122 on their surface, which allows them to interact with IL-7 and IL-15[54]. IL-7 is important for their survival as it is for naive T cells, while IL-15 is necessary for their intermittent homeostatic proliferation[54].

Effector memory T cells show also homeostatic proliferation, which is largely driven by IL-15[92], but it is very slow compared to central memory T cells[92;93] leading to a shift towards central memory T cells[92;94]. According to a recent study, memory T cells in the periphery show the ability to resist apoptosis longer than their counterparts in secondary lymphoid organs[95], which is in contrast to the early mentioned view.

Although memory T cells in lymphoid and peripheral sites are able to proliferate, the number of antigen-specific T cells steadily decreases within time after the pathogen is eliminated[91;96;97].

2.2.4.4 Activation of memory T cells

In case of a recurrent infection, memory T cells in the periphery can function as sentinels, which alert the immune system by producing chemokines and pro-inflammatory cytokines, or by limiting the early pathogen replication until the arrival of secondary antigen-specific effector T cells from the lymph nodes[91]. The activation of memory T cells in the periphery may be explained by two mechanisms. Cells of the innate immune system can indirectly act on memory T cells in the periphery by releasing pro-inflammatory cytokines, like type 1 interferons, IL-12 and IL-18, to induce memory T cell effector functions[98-100]. Also it has been proven that DCs in the periphery can directly interact with memory T cells and activate them[101]. Thus, memory T cells can be activated in an antigen-dependent and antigen-independent way[91].

2.2.4.5 T cells in the skin

In normal human skin, over 90% of T cells are found in the dermis[102], whereas only a small fraction of T cells reside in the basal and suprabasal keratinocyte layer of the epidermis[102-106]. Approximately $1x10^6$ T cells are found in 1 cm^2 normal human adult skin[107]. Most of the T cells in human skin express TCR αβ heterodimers[103;108-110], and

only a minor population of T cells belong to the TCR γδ lineage[103;103;108-112]. The functional role of skin-resident γδ T cells is not understood, but data in a recent publication showed that they are able to secrete insulin-like growth factor 1 upon activation and seem to be involved in wound healing[113].

Multiple isoforms of CD45 can be generated by alternative splicing of the extracellular domain of the molecule, and can be used to distinguish between naive and memory T cells.[114]. In adult human skin, naive T cells comprise 5-19% of all $CD3^+$ cells and express CD45RA[106]. Memory T cells constitute about 90% of the T cells in the skin and are all $CD45RO^+$, whereas only about 80% of all $CD3^+$ cells are CLA^+, 90% of the memory T cells express CLA[106]. While the distribution of naive ($CD3^+CD45RA^+$) and memory T cells ($CD3^+CD45RO^+$) in adult blood is quite similar to the skin[115], only 10-25% of the $CD3^+$ blood cells are positive for CLA[116]. Most recent data provide evidence that under resting conditions, 98% of all CLA^+CD3^+ cells are found in the skin[107].

While many reports exist about T cells in adult human skin, less is known about their appearance in the developing skin (prenatal – adult). Recent findings indicate, that within human fetal skin $CD3^+CD45RA^+$, $CD3^+CLA^+$ and $CD3^+CD45RO^+$ cells are already found in low numbers in the 18^{th} week of estimated gestational age (EGA)[117]. The numbers of $CD3^+CLA^+$ and $CD3^+CD45RO^+$ cells do not appreciably rise until the 30^{th} week of EGA, whereas, the density of $CD3^+CD45RA^+$ cells multiplies, leading to a distribution which shows three times more naive than memory T cells within the skin of a fetus[117]. A similar distribution of naive and memory T cells was described for the cord blood of newborn children[115].

2.2.5 Dendritic cells

DCs are important initiators of the innate and adaptive immune response. They are the most potent antigen-presenting cells and are found in peripheral tissues. In the skin, DCs can be divided according to their anatomical localisation. In the epidermis one can detect LCs, and in the dermis interstitial DCs and plasmacytoid DCs are found[8]. DCs reside in an immature state in the periphery and are able to identify foreign antigens by random sampling their environment by phagocytosis[118-120], macropinocytosis[121] and receptor-mediated endocytosis[122;123]. The lack of T cell activating receptors, and the low expression of MHC I and MHC II in the immature state, makes them unable to activate T cells[124]. The recognition of a foreign peptide

results in the activation of the NF-κB pathway and finally in the maturation of the DC, which is accompanied by the expression of co-stimulatory molecules and the enhanced expression of antigen-presenting MHC molecules[125]. This is followed by migration into the T cell areas of lymphoid tissues such as the lymph nodes or spleen, which is guided by a chemotactic cytokine gradient. During this migration process, DCs mature and loose their ability to capture antigens. Antigens are processed, and displayed on either MHC I or MHC II molecules, to be presented to naive T cells[126]. The interaction with receptors on T cells, is accomplished by molecules such as CD40, CD54, CD58, CD80 and CD86[127;128]. On which MHC molecule the foreign antigen is displayed, depends on the kind of antigen. $CD8^+$ T cells interact with MHC I, whereas $CD4^+$ T cells are activated through MHC II[124]. To avoid an excessive immune response, activated T cells induce apoptosis in DCs by expressing Fas Ligand, TNF-α and TNF-related apoptosis-inducing ligand (TRAIL)[129]. DCs also have the unique ability among antigen-presenting cells, to cross-present antigens to naive T cells[125;130]. Additionally, in case of an adaptive mediated immune response, DCs are able to direct it towards a type-1 and/or type-2 reaction[131].

Beyond these skills, DCs are involved in the induction of tolerance in the thymus (central tolerance) and in lymphoid organs (peripheral tolerance). A small number of DCs move from the periphery to the lymph nodes, even in the absence of invading pathogens in the steady state[125]. These tolerogenic DCs do not activate T cells or lead to a clonal expansion, because of the release of IL-10 and catabolizing enzyme ideolamine 2,3-dioxygenase (IDO) which enable T cell anergy, T cell death and T_{reg} cell proliferation[132]. The role of LCs in mediating immune tolerance is not fully delineated, whereas evidence for the tolerogenic function of dermal DCs exist[133].

2.2.5.1 Langerhans cells

Paul Langerhans, a medical student in Berlin, first identified dendritic cells in the epidermis in 1868, which he supposed to be nerve cells of the skin[134;135]. One century later it was appreciated, that he discovered a new population of leukocytes, which was named after him[130]. LCs represent the first line of immunological defence to the external environment. They are strategically positioned in the epidermis and form a network with their external dendrites[4]. LCs account for 3-5% of epidermal cells in humans[136] and derive from the bone marrow[137]. Since the discovery of Birbeck granules in 1961[138], it has been possible to distinguish between LCs and other cells,

because this cytoplasmic organelle is found only in LCs. This was the only method to identify LCs for many years, until it was shown that Langerin (CD207), a mannose-specific C-type lectin receptor, which is localised to Birbeck granules[139] and highly expressed by human and mouse LCs[140], can be used as a marker to identify LCs within the epidermis, too. Until recently, it was thought that Langerin is only expressed by LCs, but newest studies showed that a murine population of dermal DCs is also able to express this receptor[141-144], whereas a human counterpart is not found yet.

LCs are present in an immature state and in the human epidermis express CD1a, CD39, CD45, CD207, Birbeck granules, chemokine receptor (CCR) 6, CLA, E-cadherin, MHC I and MHC II[143;145;146]. Upon activation, LCs mature and upregulate the expression of CD40, CD45, CD80, CD83, CD86, CCR7, very late antigen (VLA) -4, intercellular adhesion molecule (ICAM) -1, MHC I and MHC II[124;125;147-154].

While it is a matter of fact that LCs are derived from bone marrow-derived progenitors[155], it is unsolved whether and if so, how LCs migrate into the epidermis under non-inflamatory conditions. Three different viewpoints exist: first, in mouse experiments it was shown that LCs can divide in the epidermis[156]. Second, it was described that dividing LC-precursors ($CD207^+CD14^+$) reside in the dermis and that the final migrating step is perhaps controlled by the release of TGFβ and CXCL14[157;158] from keratinocytes. Third, it was demonstrated that LCs derive from CLA-expressing LCs precursors[159], which were generated in the bone marrow, released to the blood, through which they reach the skin and populate the epidermis.

Only during an inflammation process in the skin, a recruitment of LC-precursors from the blood is described[156]. To enter the epidermis, LC-precursors depend on the release of the keratinocyte-derived cytokine TGFβ[160] and the chemokine CCL20[161]. This cytokine is necessary for the formation of Birbeck granules[162], whereas CCL20 attracts LC-precursors[20;163].

2.2.5.2 Dermal dendritic cells

Three different subsets of antigen-presenting cells are found in the human dermis. $HLA\text{-}DR^{hi}CD14^-CD1a^+CD207^-$ DCs; $HLA\text{-}DR^{hi}CD14^+CD1a^-$ DCs and $HLA\text{-}DR^{lo}CD14^+CD1a^-FXIIIa^+CD163^+$ dermal macrophages[164-169]. $CD14^-CD1a^+$ DCs are able to induce a less efficient proliferation of allogeneic T cells compared to LCs[167;170]

and are distinct from LCs[166;169]. $CD14^+$ cells include both migratory DCs and nonmigratory macrophages.

It is known that LCs can cross-present antigens to $CD8^+$ T cells and are potent inducer of a T_H1 cell response, but it seems that the role of dermal DCs is another one. They facilitate B cell immunoglobulin class switching and initiating follicular T_H cells[170;171].

Plasmacytoid DCs are also localised in the dermis, but they are rarely found in healthy skin[167;172]. They express CD123, blood dendritic cell antigen (BDCA) -4 and, the only exclusive marker for plasmacytoid DCs, BDCA-2[173]. A special feature of plasmacytoid DCs is that they express a wide range of TLRs which makes them very competent to recognise microbial pathogens[174]. Furthermore, they are the most potent producers of Type I IFNs (IFN-α, β, ω) in case of a viral infection[175;176].

3 The role of RANK and RANKL in the immune system

RANK and its ligand (RANKL) are both members of the TNF receptor superfamily[129]. RANK is constitutively expressed on DCs and osteoclasts whereas RANKL is not found on resting T cells, osteoblasts or keratinocytes, but is expressed upon activation of these cells[129]. The RANK-RANKL-system is associated with numerous physiological processes including bone remodelling, lymph node formation, interactions between DCs and T cells, DC survival, the regulation of DC functions and the formation of lactating mammary glands in pregnancy[177-184]. RANK can be detected on some cancer cells, including prostate and breast cancers and may play a role in the induction of tumor cell proliferation[185;186]. RANK and RANKL are also expressed in astrocytes of the brain and seem to be important for the control of female body temperature and the central fever response in inflammation[187].

In the periphery, interactions between RANK on DCs and RANKL on activated T cells, lead to a prolonged survival of DCs[188;189], an increased production and release of proinflammatory cytokines like IL-15, IL-12, IL-6 and IL-1[190;191] through DCs, and it seems that several DC functions, such as the activation of T cells are enhanced[192].

The RANK-RANKL-system is very well studied on osteoclasts and osteoblasts in bones, but less is known about this system in other tissues. UV irradiation in the skin leads to an upregulation of RANKL on keratinocytes which interact with LCs through

the RANK-RANKL-system and activate them[178]. These activated LCs respond by upregulation of certain cytokines such as IL-6, IL-10 and TNF-α and molecules such as CD86 and CD205 inducing preferentially the expansion of $CD4^+CD25^+$ T_{reg} cells thus, suppressing an immune response in the skin[129]. This interplay of keratinocytes with LCs and LCs with T_{reg} cells might be one reason for the immunosuppressive effect of UV irradiation[129]. It is also reported that approximately 95% of skin resident LCs are $RANK^+$ and that RANKL knockout mice show LC numbers in the skin that are reduced to 50% as a consequence of an inferior DC survival[180].

CD40 and CD40L are members of the TNF family[129]. RANKL and CD40L are both expressed on activated T cells and show functional similarities such as the enhanced survival and activation of DCs[188;192]. The interaction of CD40 and CD40L leads to an altered expression of MHC II, CD54, CD80 and CD86 on DCs. Such an effect is not described for the interaction of RANK and RANKL[129]. Furthermore, CD40L is only expressed on activated $CD4^+$ T cells, whereas RANKL is found on activated $CD4^+$ and $CD8^+$ T cells[190;192]. In kinetic experiments CD40L showed highest expression levels between 6 and 8 hours; a downregulation to resting levels was observed between 24 and 48 hours which indicates that the CD40-CD40L-system controls the initial priming stage[188;190]. In contrast, highest RANKL levels were measured after 48 hours, leading to the conclusion that the RANK-RANKL-system is active at later time points[188;190]. Differences have also been described in the control of an immune response. While DCs can interact with T and B cells via the CD40-CD40L-system, it is only possible for them, to interact with T cells by the RANK-RANKL-system, since B cells do not express RANK[129].

4 Aim of the study

Every year dozens of new publications about the skin immune system are published, but most of these studies were performed with mice or adult human skin. Knowledge about the phenotype of immune cells, such as epidermal LCs and T cells in the developing human skin after birth is scarce.

In our lab we were able to show that the morphology and the distribution of LCs is similar in human skin from infants and adults. However, the numbers of LCs in infant epidermis are reduced compared to adults[193]. As RANK is an important molecule for LC survival, we tested whether LCs and other skin cells already express RANK around birth.

As the predominant T cells in human fetal skin are naive T cells, while those in adult skin are memory T cells we wanted to explore, when the switch from naive to memory T cells occurs in the skin.

5 Results and discussion

5.1 Analysis of RANK expression in the developing human epidermis after birth

Barbaroux et al. have recently published that 95% of all CD1a$^+$ cells in adult human epidermis express RANK[180]. In their study they have isolated epidermal cells from normal human skin biopsies, enriched LCs via ficoll flotation and analyzed them by flow cytometry for CD1a and RANK expression[180]. They described two RANK$^+$ cell populations, LCs and an as yet undefined epidermal population[180]. They speculated that the latter cells may be keratinocytes, but did not further investigate this[180]. We wanted to address this question and analyzed single cell suspensions prepared from normal human breast and foreskin. Unlike Barbaroux et al., we have not enriched for LCs because our intention was to characterize all RANK$^+$ epidermal cells. To exclude the possibility that our samples may be inflamed, mRNA levels of random samples were tested for E-selectin[67] and ICAM-1[131] expression by quantitative real-time PCR (performed by Nousheen Iram, Elbe-Bürger group; Laboratory of Cellular and Molecular Immunobiology of the skin, Department of Dermatology, Medical University of Vienna). Only donors with negative to low gene expression compared with the positive control were selected for further analysis (data not shown).

Epidermal cells were stained with anti-CD1a and anti-pancytokeratin monoclonal antibodies (mAbs) to distinguish LCs and keratinocytes, respectively. T cells and melanocytes were labeled with anti-CD3 and anti-CD117 mAbs, respectively. We found that LCs and keratinocytes express RANK, while T cells and melanocytes are definitely not RANK$^+$ (**Figure 3A**). To confirm that LCs and keratinocytes are the only two cell populations within the epidermis which are RANK$^+$, triple staining with RANK, CD1a and pancytokeratin was performed. Indeed, we found that epidermal cells gated for RANK expression mainly contained LCs and keratinocytes (**Figure 3B**).

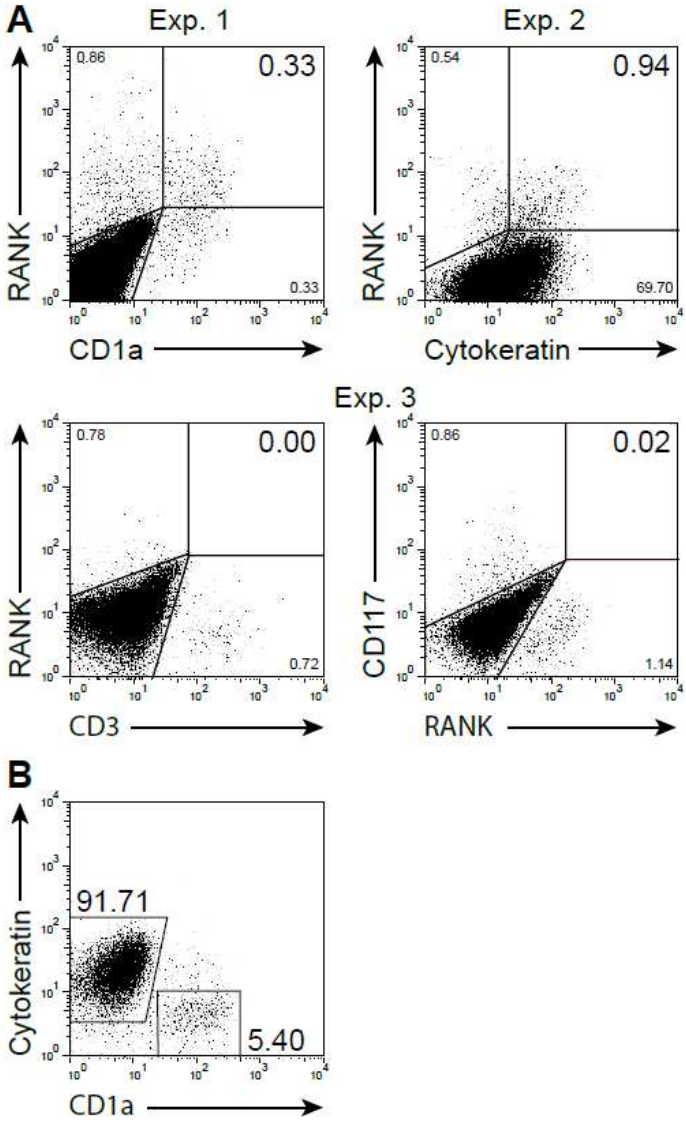

Figure 3. LCs and keratinocytes but not T cells and melanocytes express RANK. (**A**) Epidermal single cell suspensions were prepared from normal human foreskin [31 years (Exp. 1), 1 year (Exp. 2) and 11 years (Exp. 3)], and double stained with the indicated markers. Dead cells were excluded by 7-amino-actinomycin D (7-AAD) uptake. Dot plots show 100,000 cells and are representative of 3-21 experiments. (**B**) To analyze all RANK$^+$ epidermal cells, a triple staining was performed (36 year-old foreskin). Viable cells were gated for RANK expression.

Using flow cytometry, freshly prepared epidermal single cell suspensions from human donors of selected age groups were labeled with anti-RANK and anti-CD1a mAbs to analyze the frequency of RANK$^+$ LCs within human epidermis. When analyzing the percentage of RANK$^+$CD1a$^+$ cells, we identified a significant increase of these cells with age. In foreskin from infants (11.16%±8.67) and old children (12.87%±9.29) we found a significant lower percentage of RANK$^+$ LCs when compared with adult foreskin (32.99%±11.5) (**Figure 4A, red gates, B**). Also adolescent foreskin contained fewer RANK$^+$ LCs when compared with adult foreskin, however, this was not significant. Additionally, normal human breast skin was analyzed for the presence

of RANK$^+$CD1a$^+$ cells in the epidermis to include the percentage of RANK$^+$ LCs from another part of the body. Surprisingly, only 12.49% of all LCs were RANK$^+$ (**Figure 4B**), which is in contrast to the results of Barbaroux et al[180].

Furthermore, the frequency of RANK$^+$CD1a$^-$ cells was determined (**Figure 4A, yellow gates**). Foreskin from infants (96.28%±4.68), old children (93.98%±4.68) and adolescents (86.87%±7.07), and normal human breast skin (91.59%±5.3) showed a higher percentage of RANK$^+$CD1a$^-$ cells in the epidermis compared to adult (64.62%±23.95) foreskin (**Figure 4C**), but only data obtained from infants and old children were significant when compared with adult foreskin.

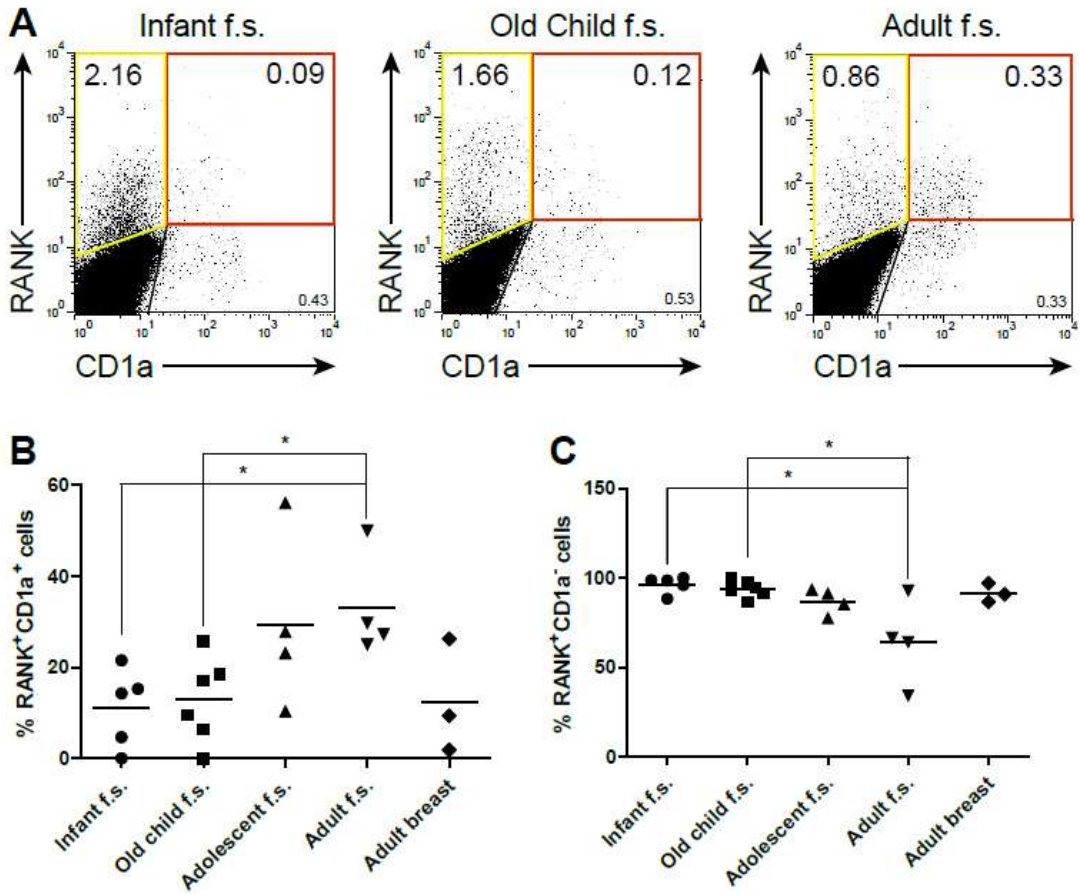

Figure 4. Analysis of RANK$^+$CD1a$^+$ and RANK$^+$CD1a$^-$ cells in developing human epidermis. (**A**) Single cell suspensions from foreskin of selected age groups [infant (1 year), old child (10 years), adult (31 years)] were labeled with anti-RANK and anti-CD1a mAbs. Dot plots show RANK$^+$CD1a$^+$ (red gates) and RANK$^+$CD1a$^-$ (yellow gates) cell populations. Dead cells were excluded by 7-AAD uptake. Dot plots show 100,000 cells. (**B**) The diagram displays the percentage of RANK$^+$CD1a$^+$ epidermal cells of the indicated age groups. Significant differences between infant and adult (* P<0.05) and old child and adult (* P<0.05) RANK$^+$CD1a$^+$ LCs were observed. (**C**) The diagram shows the percentage of RANK$^+$CD1a$^-$ epidermal cells of different age groups. A significant difference between infant and adult (* P<0.05) and old child and adult (* P<0.05) RANK$^+$CD1a$^-$ cells was observed. f.s. = foreskin.

5.2 Not all LCs in adult human epidermis express RANK

To test the validity of our flow cytometry data in adult skin, epidermal sheets were prepared and labeled with anti-RANK and anti-CD1a mAbs (**Figure 5A, B**). We found that many LCs do not express RANK, what is again in contrast to observations from Barbaroux et al.[180]. The discrepancy of these results cannot be explained at the moment.

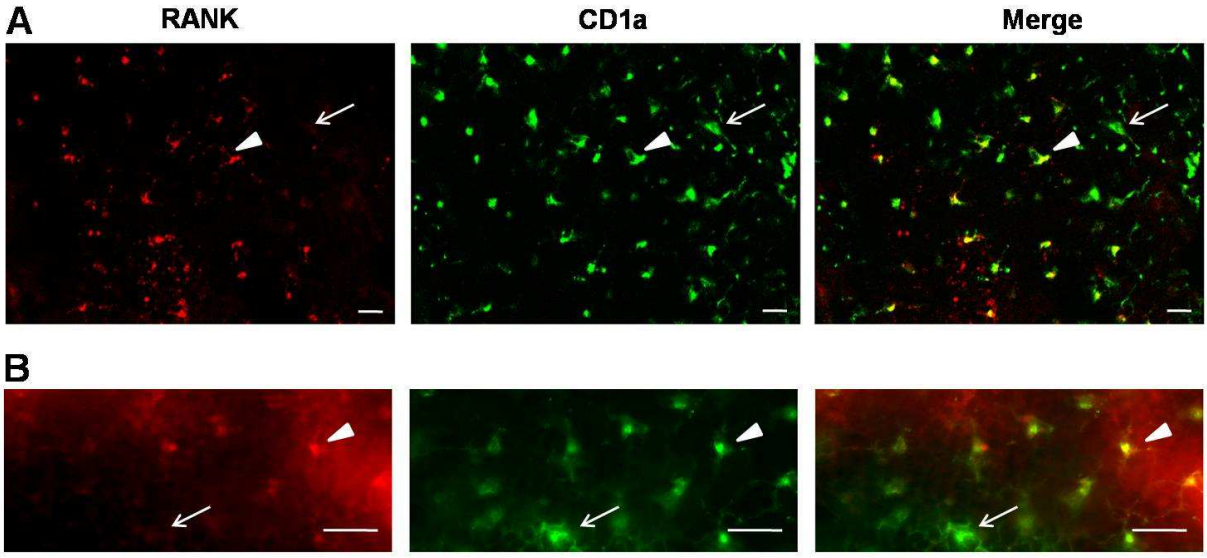

Figure 5. Not all CD1a$^+$ LCs in adult human epidermis express RANK. Epidermal sheets from (**A**) belly skin (41 years) and (**B**) gluteal skin (55 years) were stained with an anti-RANK mAb, visualized with an AlexaFluor-546 goat anti-mouse IgG and counterstained with an anti-CD1a-FITC mAb. Arrows indicate RANK$^-$CD1a$^+$ cells. Arrowheads indicate RANK$^+$CD1a$^+$ cells. Scale bars: 20 µm.

5.3 Only a minor percentage of in vitro generated LCs express RANK

We next determined the percentage of LCs expressing RANK, when these were differentiated in vitro from CD34$^+$ cells isolated from cord blood. Upon culture of CD34$^+$ cells with GM-CSF, Flt3L, SCF, TNF-α and TGF-β1[194] for 9 days, cells were labeled with anti-RANK and anti-CD1a mAbs or with anti-Langerin and anti-CD1a mAbs for flow cytometric analysis. Langerin/CD1a staining was performed to confirm that the generated cells are indeed LCs. Unfortunately, it was not possible to accomplish a Langerin/CD1a/RANK staining. Therefore, it was necessary to correlate the percentage of CD1a$^+$ cells in both stainings. The generation, staining and recording of the results were performed by Thomas Bauer (Stroble group; Institute of Immunology, Medical University of Vienna).

The mean of RANK$^+$CD1a$^+$ cells is about 12.12%±7.45 (**Figure 6**), which corresponds with the results obtained for RANK$^+$CD1a$^+$ cells in infant or old child skin in the in vivo experiments.

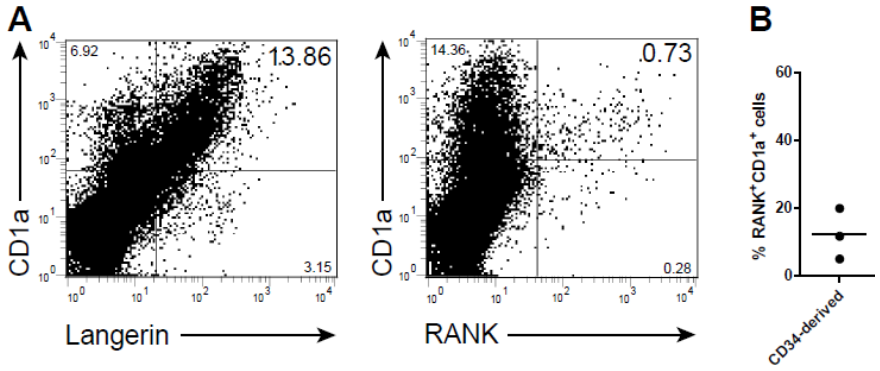

Figure 6. In vitro generated LCs. (**A**) LCs were in vitro generated from CD34$^+$ cells and labeled with the indicated mAbs. Only a small fraction of CD1a$^+$ cells express RANK. (**B**) The diagram displays the percentage of in vitro generated RANK$^+$CD1a$^+$ cells.

Thus, similar to our observation with freshly isolated epidermal cells, only a minor population of CD1a$^+$ cells express RANK. Double labeling experiments need to be performed to test how many Langerin$^+$ cells express RANK. All together our results are in direct contrast to those of Barbaroux et al.[180] The use of a different isolation procedure is one explanation. However, it does not explain the results in epidermal sheets.

The validity of our flow cytometry data is supported by our observations in epidermal sheets and in vitro differentiation data of LCs from their precursors . Experiments are planned to investigate whether RANK$^+$ and RANK$^-$ LCs represent different LC subtypes and also have a different functional capacity. It will be also interesting to investigate which factors in the developing skin are responsible for the upregulation of RANK on LCs during development. Furthermore, we plan to test when during prenatal development RANK expression can first be detected on LCs.

5.4 Keratinocytes express RANK

Upon identification of RANK$^+$Cytokeratin$^+$ keratinocytes (**Figure 3**), freshly prepared epidermal single cell suspensions from selected age groups were labeled with an anti-RANK and an anti-pancytokeratin mAb to determine the frequency of RANK$^+$ keratinocytes (**Figure 7A**). A decrease of RANK$^+$ keratinocytes from infants compared to adults was found. The percentage of keratinocytes expressing RANK in infant epidermis (0.78%±0.51) is higher compared with those of old children

(0.34%±0.14) or adults (0.17%±0.14). In normal human breast skin, the ratio of RANK$^+$ keratinocytes is about 0.5%±0.39.

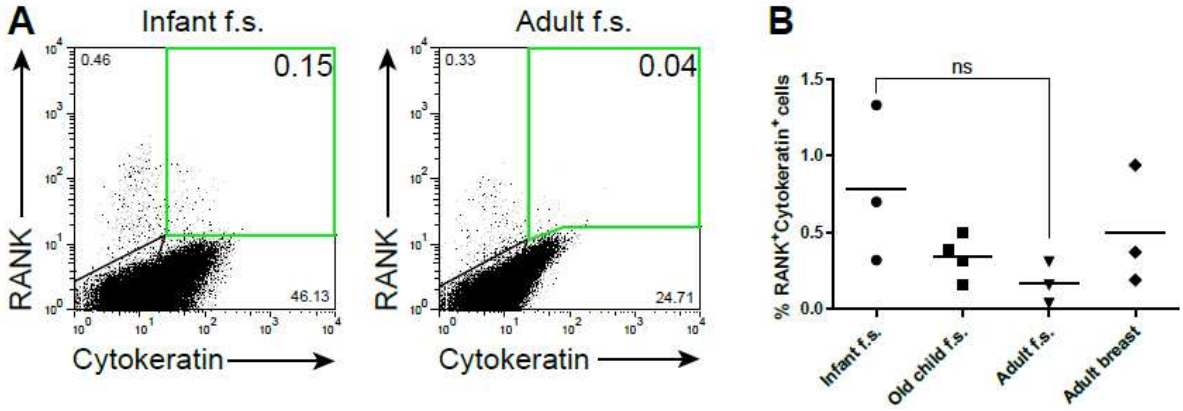

Figure 7. RANK expression on keratinocytes. (**A**) Epidermal single cell suspensions from infant (1.5 years) and adult (20 years) foreskin were labeled with anti-RANK and anti-pancytokeratin mAbs. Green gates show the percentage of RANK$^+$ keratinocytes. Dead cells were excluded by 7-AAD uptake. Dot plots display 100,000 cells. (**B**) The diagram displays the percentage of RANK$^+$Cytokeratin$^+$ cells. f.s. = foreskin; ns = not significant.

The percentage of RANK$^+$ keratinocytes in all age groups is less then 1%. However, it is important to put these results in context to the total cell numbers which are present in the epidermis. Keratinocytes represent approximately 90% and LCs only 3-5% of all epidermal cells. If we consider a population of 1000 epidermal cells, then only approximately 40 LCs and 900 keratinocytes would be identified. In infants, 11.16% of all LCs and 0.78% of all keratinocytes are RANK$^+$. Only 4.46 LCs and 7 keratinocytes would express RANK in our theoretical epidermis, leading to a ratio of 1:1.51. The total number of RANK$^+$ keratinocytes in infant skin is higher than those of RANK$^+$ LCs. In the epidermis of old children we have a ratio of 1:0.59 and in adult foreskin 1:0.12, showing that there seems to be a shift from higher RANK$^+$ keratinocyte numbers to more RANK$^+$CD1a$^+$ cells. In normal breast skin the ratio is 1:0.9, which leads to the conclusion that in normal human breast skin, the distribution of total RANK$^+$ LCs and keratinocytes is almost equal.

Our observation that some keratinocytes are able to express RANK, leads to many questions. It remains to be determined which keratinocytes express RANK. If expressed only on some basal keratinocytes, we plan to analyze whether its expression may correlate with proliferating (=Ki67$^+$) keratinocytes. Are RANK$^+$ keratinocytes able to interact with RANKL-expressing T cells. If yes, why? It is currently unclear why the percentage of RANK$^+$ keratinocytes in infants even though not significant, is much higher than in any other age group and what leads to the reduction of RANK$^+$ keratinocytes with time?

5.5 Staining of naive and memory T cells in human dermal single cell suspensions

To investigate the expression of specific markers on skin T cells, it was necessary to first evaluate whether they are sensitive to enzymes used to prepare skin single cell suspensions. Therefore, dermal single cell suspensions were prepared and double staining were performed with the T cell specific marker CD3 and markers for naive (CD45RA) and memory T cells (CD45RO). Unfortunately, neither CD45RA nor CD45RO expression was observed (**Figure 8**), implying that both receptors are sensitive to either dispase or liberase or even both enzymes used to separate the epidermis from the dermis.

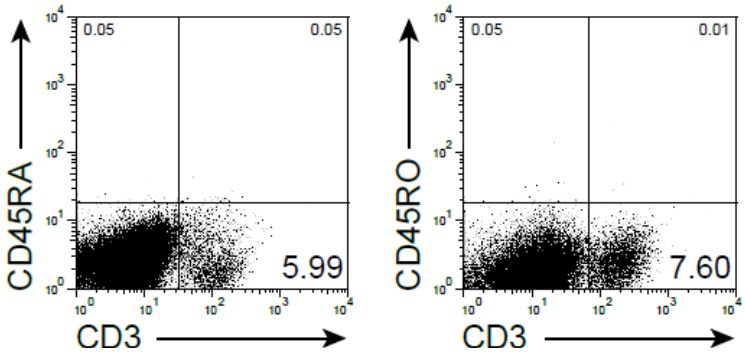

Figure 8. Staining of naive and memory T cells in a dermal single cell suspension. A dermal single cell suspension was prepared from human adult foreskin (31 years) and labeled with anti-CD3 and anti-CD45RA or anti-CD45RO mAbs to distinguish naive and memory T cells. Dead cells were excluded by 7-AAD uptake. Dot plots display 100,000 cells and are representative of 3 experiments.

5.6 Staining of naive and memory T cells in human peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from peripheral blood of healthy adult volunteers. One half of the cells was stained with anti-CD45RA, anti-CD45RO and anti-CLA mAbs and analyzed by flow cytometry (**Figure 9A**). The other half of the cells was incubated in dispase II (1 hour, 37℃), washed and digested in liberase (2 hours, 37℃) to imit ate the preparation of a dermal single cell suspension and to test the dispase and liberase sensitivity of the mentioned markers. Afterwards the cells were analyzed by flow cytometry. Surprisingly, no significant difference was observed, upon dispase and liberase treatment leading to the conclusion that the three tested markers are not sensitive for the treatment with dispase and/or liberase (**Figure 9B**).

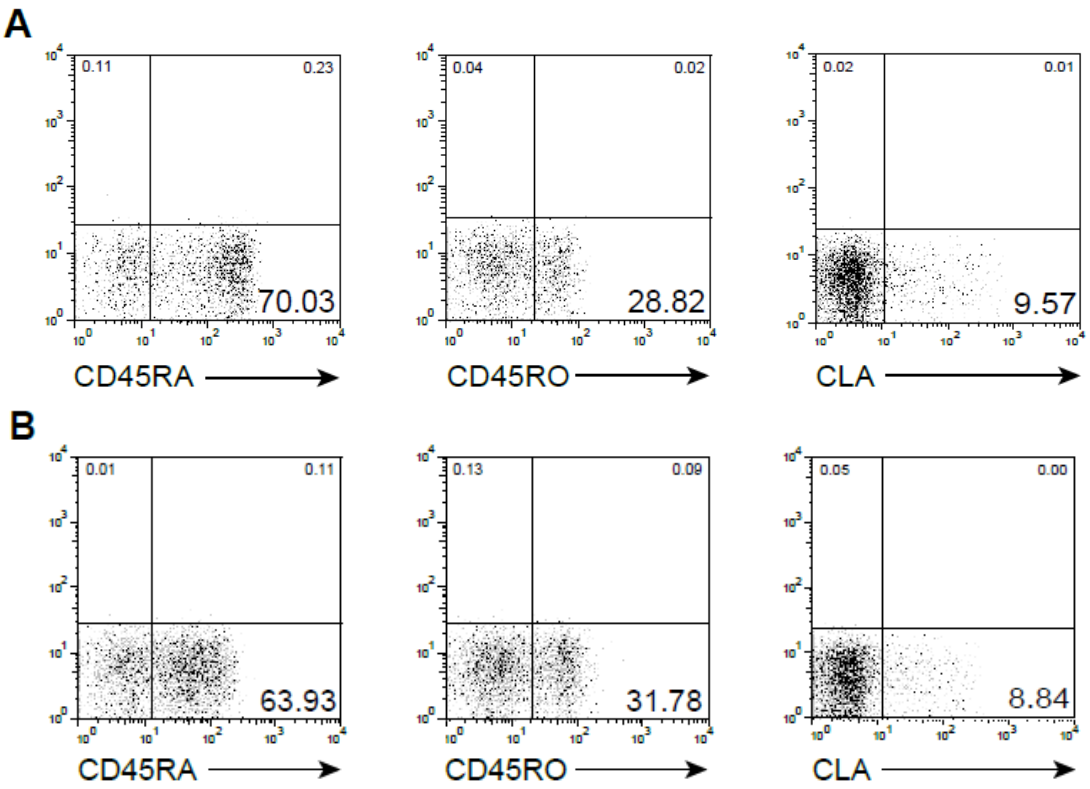

Figure 9. Staining of naive and memory T cells in PBMCs. (**A**) PMBCs were isolated from the blood of healthy adult donors and stained directly after isolation or (**B**) incubated with dispase and afterwards with liberase and stained with the indicated markers. Dead cells were excluded by 7-AAD uptake. Dot plots display 10,000 cells and are representative of 3 experiments.

5.7 Staining of naive and memory T cells in skin single cell suspensions

As we found no difference in the expression levels of naive and memory T cell markers in T cells upon liberase treatment compared with the non-treated group, single cell suspensions from whole skin were prepared using liberase for digestion of the dermis and were stained with anti-CD45RA and anti-CD45RO mAbs. As a second marker for memory T cells we have used an anti-CLA mAb. Approximately 50% of the T cells either expressed CD45RA or CLA, implying that these markers are partially sensitive to liberase. CD45RA and CLA showed unequivocal populations and no reactivity with an anti-CD45RO mAb was observed (**Figure 10**).

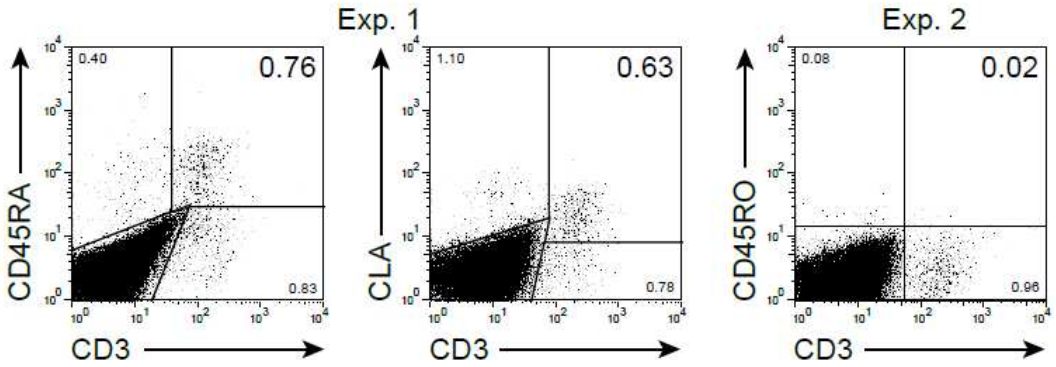

Figure 10. CD45RO molecules are liberase sensitive. Skin single cell suspensions were prepared from human foreskin [16 years (Exp. 1), 3 years (Exp. 2)] and labeled with anti-CD3, anti-CD45RA, anti-CD45RO or anti-CLA mAbs to distinguish between naive and memory T cells. Dead cells were excluded by 7-AAD uptake. Dot plots display 100,000 cells and are representative of 3-11 experiments.

5.8 Determination of the percentage of naive and memory T cells in developing human skin

Human breast and foreskin samples from selected age groups were triple stained for CD3, CD45RA and CLA to determine the percentage of naive and memory T cells. Analyzed cells were gated for CD3, and CD45RA is displayed against CLA (**Figure 11A**). Sensitivity of CD45RA and CLA molecules for diverse enzymes has already been demonstrated, but no report about liberase sensitivity was familiar for us. Unfortunately, most of the cells displayed only CD3, implying a loss of CD45RA or CLA, which makes it impossible to differentiate between naive or memory T cells in skin single cell suspensions. Only a small fraction of naive and memory T cells kept their markers, and a third fraction with a similar percentage showed a triple positive phenotype. This $CD3^+CD45RA^+CLA^+$ population has a receptor expression profile previously described for regulatory T cells[195-198]. The obtained data were evaluated and displayed in a diagram to show the relation of naive and memory T cells even though we are aware that they do not represent the “true” picture for reasons described above (**Figure 11B**).

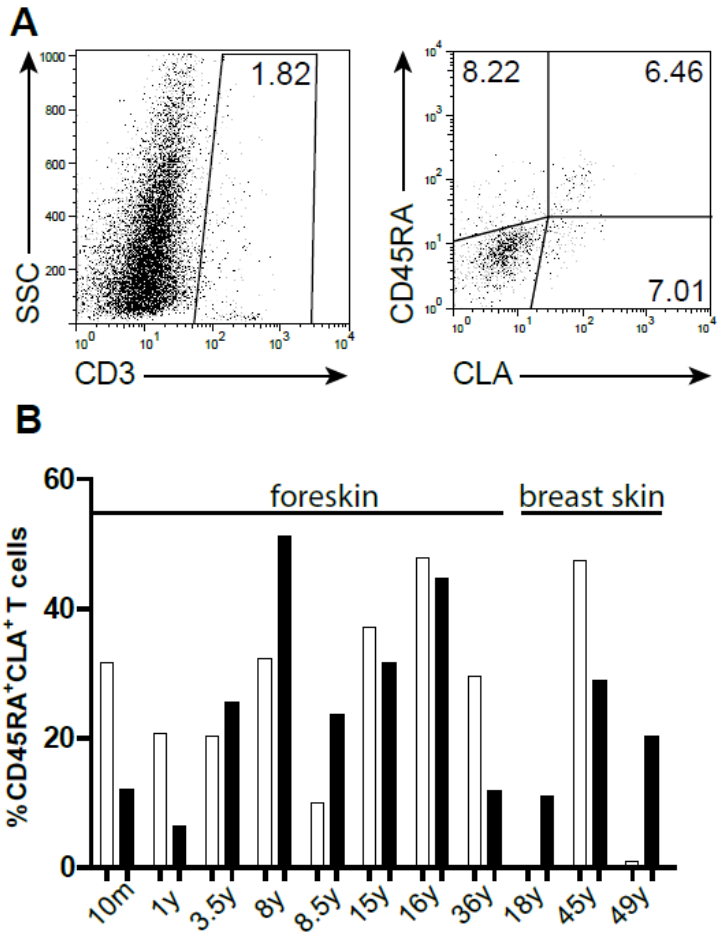

Figure 11. Distribution of naive and memory T cells in skin from selected age groups. (**A**) A single cell suspension was prepared from human foreskin (3.5 years). Left dot plot shows a gate for $CD3^+$ T cells. Right dot plot displays the expression of CD45RA and CLA on $CD3^+$ cells. Dead cells were excluded by 7-AAD uptake. Dot plots display 100,000 cells and are representative of 3-11 experiments. (**B**) The diagram shows the relation of naive ($CD3^+CD45RA^+$, white bars) and memory ($CD3^+CLA^+$, black bars) T cells in human skin. Infant (10 months and 1 year), child (3.5 years, 8 years and 8.5 years), adolescent (15 years and 16 years), adult (36 years) foreskin and breast skin (18 years, 45 years and 49 years) were analyzed.

5.9 Naive T cells are present in infant and adult but not in newborn epidermis

Due to the sensitivity of certain T cell markers to skin cell separation enzymes, scientists have developed new methods to isolate T cells from the skin. T cell chemoattracting factors and skin explants were used to allow migration of T cells out of the skin[199]. It is a procedure, which takes some days and might lead to a modification of the T cell phenotype. Therefore, we decided to use immunofluorescence staining of T cells in cryostat sections. Skin sections were labeled with anti-CD3, -CD45RA and -CD45RO mAbs. Inflamed samples were excluded, based on T cell cluster formations in the epidermis (**Figure 12A**) and on high T cell numbers in the dermis (**Figure 12B**).

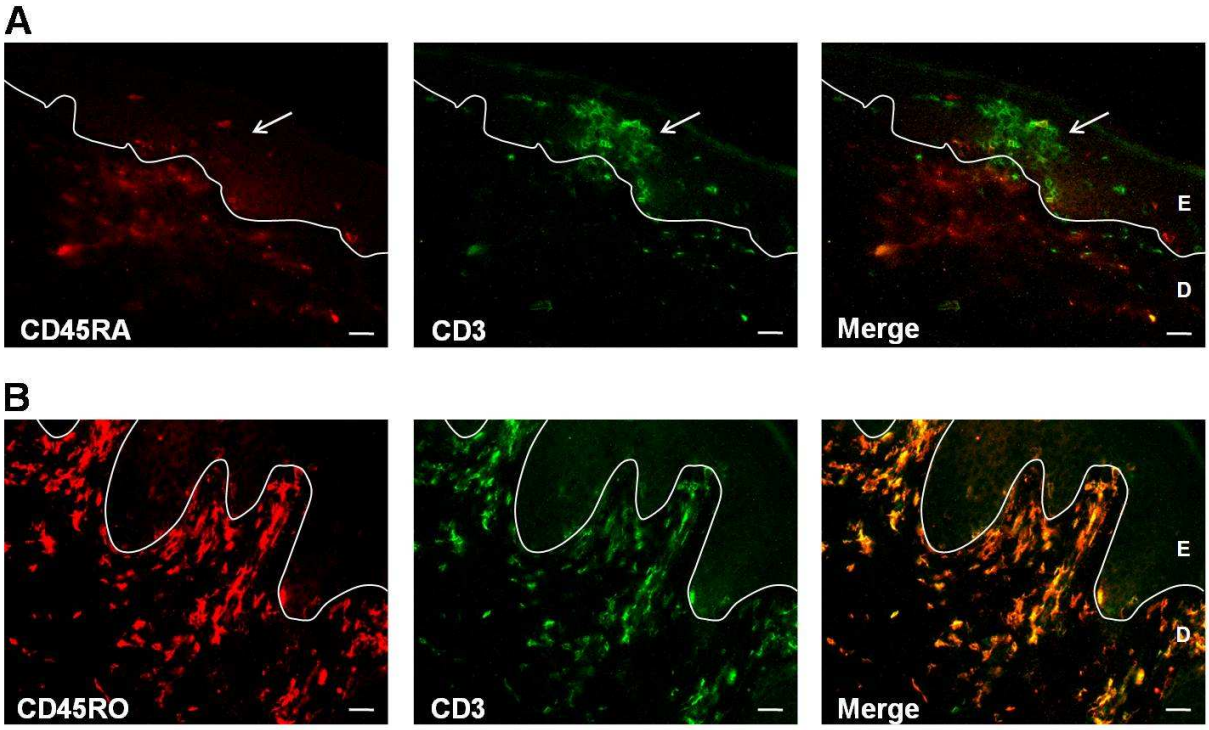

Figure 12. Identification of inflamed skin. (**A**) Cryosections from human foreskin (10 months) were labeled with anti-CD3-FITC and anti-CD45RA-PE mAbs. The epidermis shows a cluster formation of T cells (arrow), indicating an inflammation. (**B**) Human foreskin (2 months) was stained with an anti-CD45RO mAb, visualized with an AlexaFluor-546 goat anti-mouse IgG and counterstained with an anti-CD3-FITC mAb. High T cell numbers were detected within the dermis, indicating an inflammation. Scale bars: 20 µm. E = Epidermis, D = Dermis.

Upon exclusion of inflamed samples, naive and memory T cell staining was performed with newborn, infant, and adult foreskin samples. From each donor and staining, at least 10 pictures were taken and analyzed. Unfortunately, it was impossible to analyze the dermis from adult foreskin samples due to high levels of collagen. Therefore, we have included normal human male breast skin which showed lower levels of collagen (**Figures 13-15**).

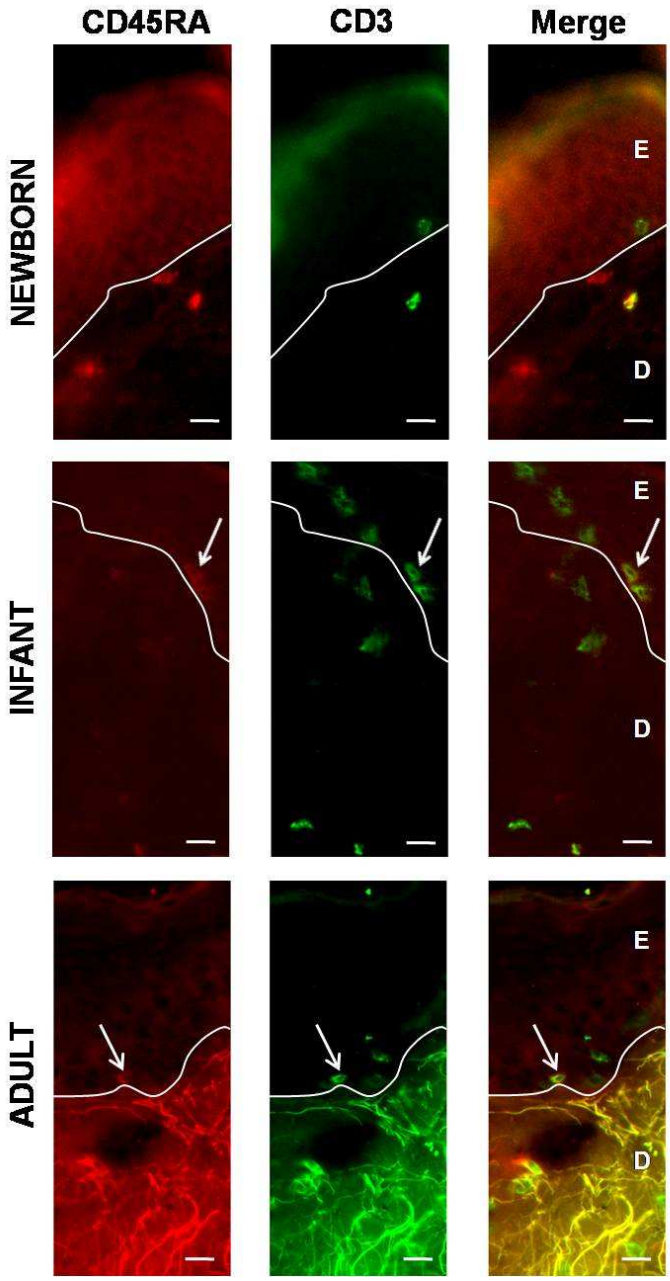

Figure 13. Naive T cells are present in infant and adult, but not in newborn epidermis. Cryosections from newborn (3 days), infant (5 months) and adult (31 years) foreskin were labeled with anti-CD3-FITC and anti-CD45RA-PE mAbs. No naive T cells were found in the newborn epidermis, whereas infant and adult epidermis harbors $CD45RA^{+}CD3^{+}$ cells. Arrows indicate $CD45RA^{+}CD3^{+}$ cells in the epidermis. Scale bars: 5 µm. E = Epidermis, D = Dermis.

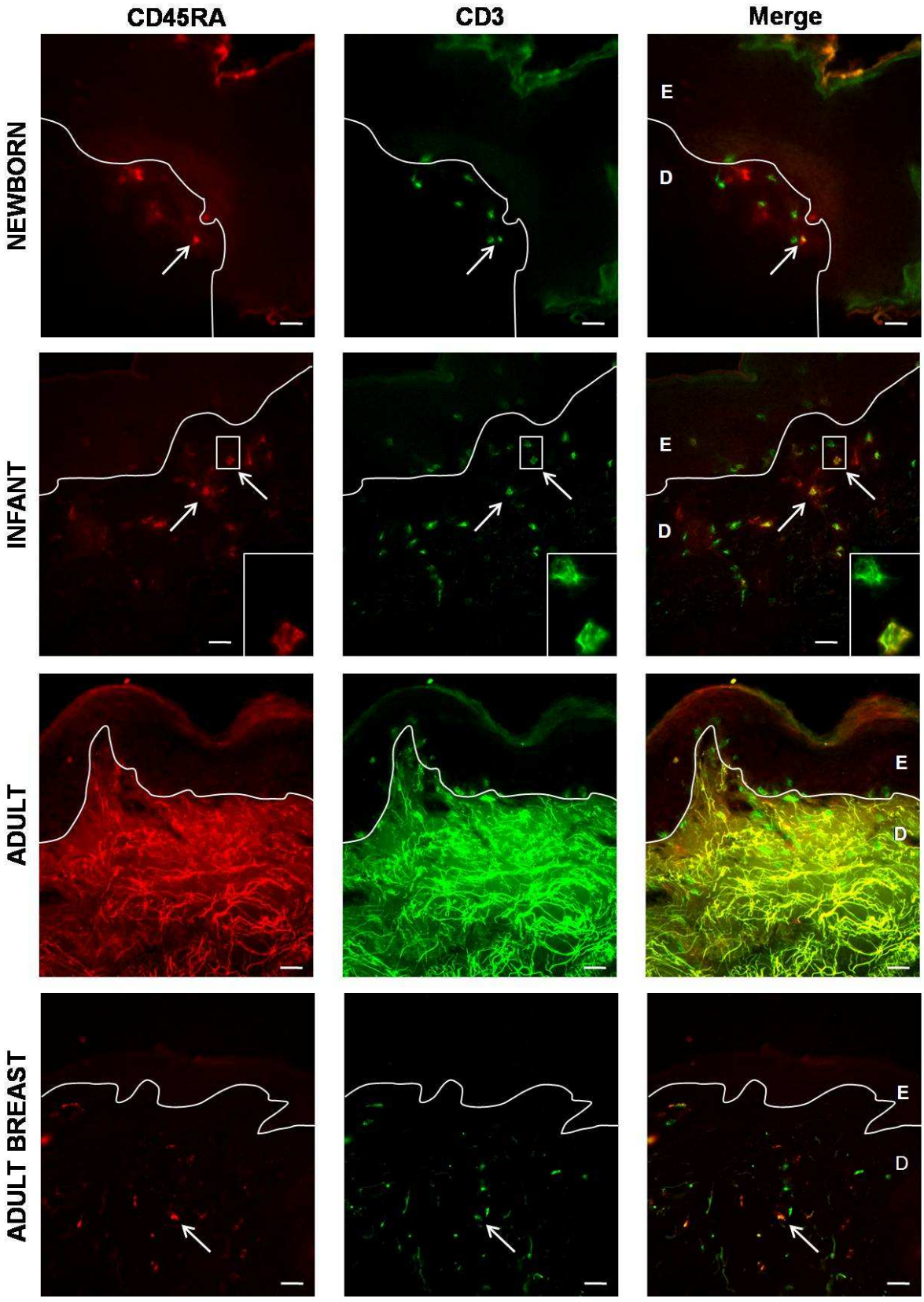

Figure 14. Naive T cells are present in newborn, infant and adult dermis. Cryosections from newborn (5 days), infant (5 months) and adult (31 years) foreskin were labeled with anti-CD3-FITC and anti-CD45RA-PE mAbs. The dermis from adult foreskin was difficult to analyze due to high collagen expression. Therefore, normal human adult male breast skin (19 years) was included. $CD45RA^{+}CD3^{+}$ cells (white arrows) were found in the dermis of the indicated age groups. Scale bars: 20 µm. E = Epidermis, D = Dermis.

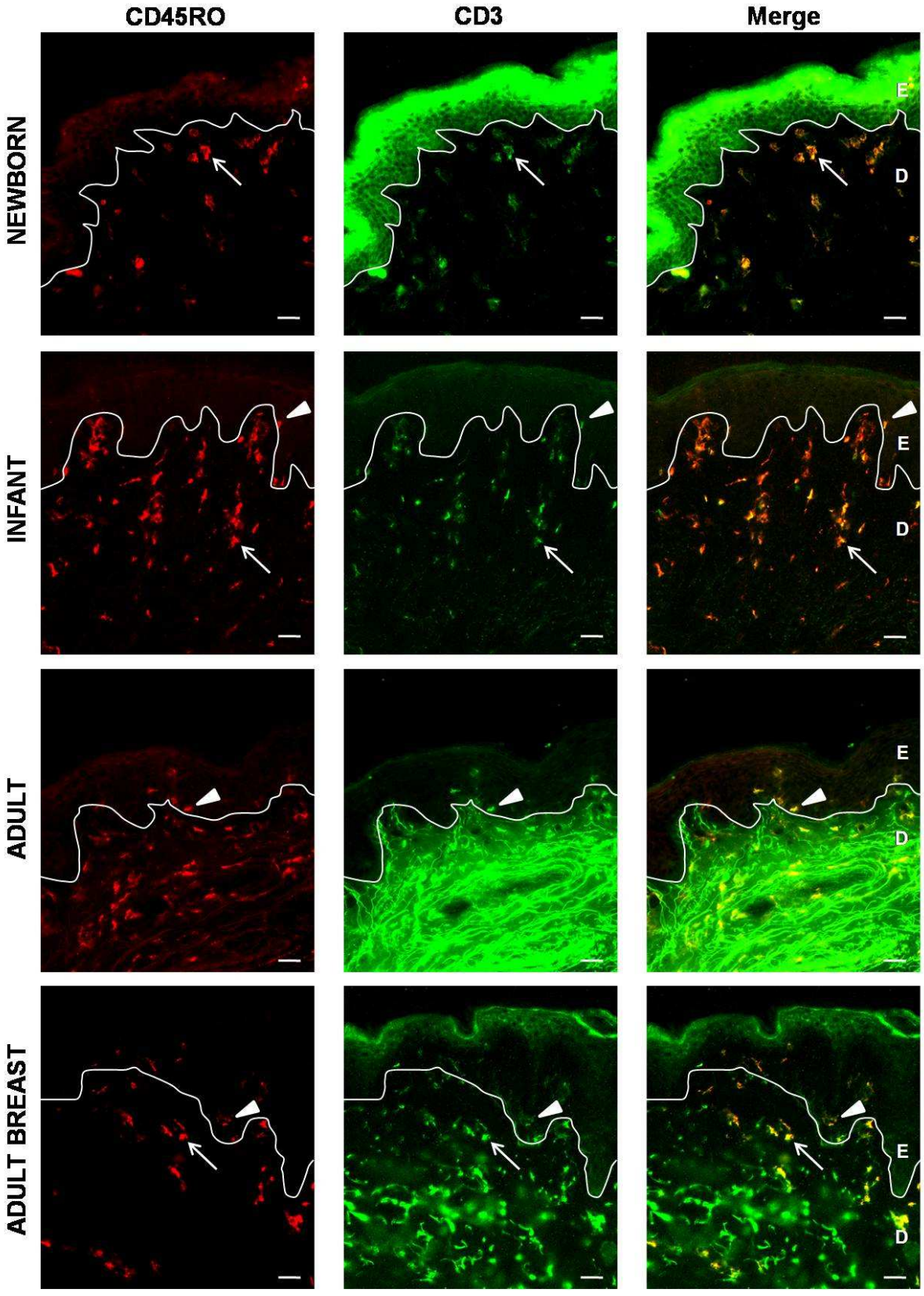

Figure 15. Memory T cells are present in newborn, infant and adult epidermis and dermis. Frozen human foreskin samples from newborn (3 days), infant (6 months) and adult (31 years) individuals were labeled with an anti-CD45RO mAb, visualized with an AlexaFluor-546 goat anti-mouse IgG and counterstained with an anti-CD3-FITC mAb. We used a normal human adult male breast skin (19 years) as a control. $CD45RO^{+}CD3^{+}$ cells were extremely rare in newborn epidermis, but their numbers increased with age. $CD45RO^{+}CD3^{+}$ cells are indicated with arrows (dermis) and arrowheads (epidermis). Scale bars: 20 µm. E = Epidermis, D = Dermis.

As naive and memory T cells were found in the epidermis and dermis of almost all age groups, we decided to enumerate these populations (**Figure 16**).

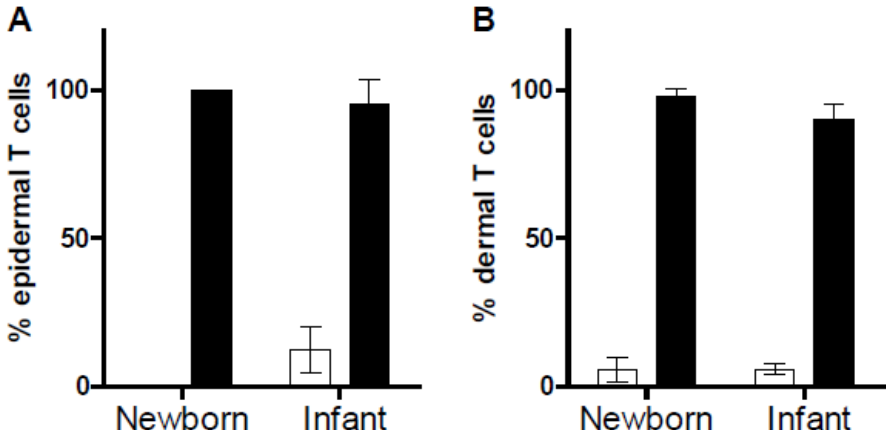

Figure 16. Frequency of naive and memory T cells in newborn and infant skin. Frozen human foreskin samples from newborn (3 to 5 days) and infant (5 months, 6 months and 14 months) individuals were analyzed. Diagrams show the relation of naive ($CD45RA^+CD3^+$, white bars) and memory (CLA^+CD3^+, black bars) T cells in human skin. T cells numbers in the (**A**) epidermis and (**B**) dermis were evaluated, by counting naive and memory T cells in different donors on cryosections. At least three different, non-inflamed donors from each age group were analyzed. In the epidermis from newborns, no $CD45RA^+CD3^+$ cells were found, whereas 12.26%±7.67 of all T cells found in the epidermis of infants were naive T cells. Most of the epidermal $CD3^+$ cells are memory T cells (100% in newborns, 95.3%±8.14 in infants). The distribution of naive and memory T cells is similar in newborn (5.77%±4.2 naive T cells, 97.67%±2,72 memory T cells) and infant (5.95%±1.69 naive T cells, 90.22%±4.89 memory T cells) dermis.

Immunofluorescence staining showed that epidermal T cells are very rare, but already present in newborn skin. No naive T cells were found in the newborn epidermis. Infants show a small population of $CD45RA^+CD3^+$ cells with an average of 12.26%±7.67 of all T cells found in the epidermis. All epidermal T cells in newborns show a memory T cell expression profile, whereas 95.3%±8.14 of infant epidermal T cells are $CD45RO^+CD3^+$.

Naive T cells are also present in the newborn dermis. Our results show that 5.77%±4.2 from newborn and 5.95%±1.69 from infant dermal T cells are $CD45RA^+CD3^+$ and that 97.67%±2.72 from newborn and 90.22%±4.89 from infant dermal T cells have a $CD45RO^+CD3^+$ expression profile. These values are already comparable to those found in the literature when adult skin was analyzed[106].

We show for the first time that already the newborn skin contains more memory then naive T cells. We are aware that more skin samples need to be analyzed to come to a definitive conclusion.

6 Materials and methods

6.1 Apparatuses and instruments

Binocular (Leitz-Austria, Vienna, Austria)

Buffy Coats (Rotes Kreuz, Vienna, Austria)

Capillary gab microscope slides, 100 µm (Dako, Glostrup, Denmark)

Carbon steel surgical blades (Heintel, Vienna, Austria)

Centrifuge (Heraeus, Vienna, Austria)

Centrifuge, megafuge 2.0 (Kendro Laboratory Products, Vienna, Austria)

CO_2 incubator (Heraeus, Vienna, Austria)

Confocal laser scanning microscope, CLSM 510 (Zeiss, Jena, Germany)

Cryostat (Leica, Wetzlar, Germany)

FloJo software (Tree Star Inc., Ashland, OR, USA)

Fluorescence-activated cell sorter (FACSCalibur, BD, Franklyn Lakes, NJ, USa)

Freezers (-20℃, -80℃)

Fridges

Forceps

GraphPad Prism 5 Software (GraphPad, San Diego, CA, USA)

ImmEdge, H-4000 (Vector Laboratories Inc., Burlingame, CA, USA)

Laminar flow (Holten, Allerod, Denmark)

LSM image browser software (Zeiss, Jena, Germany)

Lucia general software

Neubauer counting champer

Optical microscope, Nikon eclipse 80i (Nikon Corporation, Tokyo, Japan)

Pipetman, 1-50 ml (Hirschmann, Eberstadt, Germany)

Pipette (Gilson, Middelton, WI, USA)

Scalpel

Vacutainer CPT 8ml (BD, Franklin Lakes, NJ, USA)

Vortex Genie 2 (Lactan, Graz, Austria)

Water bath

6.2 Plastic material

Cell strainer 70 µm (Falcon, Lincoln Park, New Jersey, USA)

Gloves

Microscope glass cover slips, 24x60 mm (Menzel-Gläser, Braunschweig, Germany)
Microscope slides, 76x26 mm (Menzel-Gläser, Braunschweig, Germany)
Microtubes for flow cytometry (Micronic, Lelystad, The Netherlands)
Petri dishes for tissue culture, 100x20 mm (Costar, Lowel, MA, USA)
Pipettes, 5, 10 ml (Costar, Lowel, MA, USA)
Polypropylene tubes, 14 and 50 ml (Falcon, Ulm, Germany)
Sterile tips, 0.5-10, 1-100 and 200-1000 µl (Costar, Lowel, MA, USA)
Tissue culture plate, 96-well, flat bottom without lid (BD, Franklin Lakes, NJ, USA)
Tissue-tek cryomold standard (Salcuna Finetek, Zoeterwoude, The Netherlands)

6.3 Reagents

7-amino-actinomycin D (Sigma-Aldrich, St. Louis, MO, USA)
Acetone (Merck, Darmstadt, Germany)
Ammoniumchloride
Ammoniumthiocyonate (Merck, Darmstadt, Germany)
Antibodies: summarized in **Table 1**
Betaisodona (Mundipharma, Vienna, Austria)
Bovine serum albumin, BSA (Sigma-Aldrich, St. Louis, MO, USA)
Dispase II (Roche Applied Science, Basel, Switzerland)
DNAse (Invitrogen, Lofer, Austria)
Ethylenediaminetetraacetic acid, EDTA (Merck, Darmstadt, Germany)
Ethanol (Merck, Darmstadt, Germany)
Fetal calf serum, FCS (PromoCell, Heidelberg, Germany)
Fixation medium, Solution A (ADG, Kaumberg, Austria)
Liberase Blendzyme 3 (Roche Applied Science, Basel, Switzerland)
Lymphoprep (Axis Shield, Rodelokka, Norway)
Mounting medium for cryostat sections (Vector Laboratories Inc., Burlingame, CA, USA)
Mounting medium for sheets (Difco Bacto, BD, Franklin Lakes, NJ, USA)
Optimum cutting temperature formulation (O.C.T., Tissue-Tek, Sakura Finetek, Zoeterwoude, The Netherlands)
Phosphate-buffered saline, PBS (Invitrogen, Lofer, Austria)
Saponin (Sigma-Aldrich, St. Louis, MO, USA)
Trypan blue, 0.4% (Sigma-Aldrich, St. Louis, MO, USA)

Trypsin cryst. lyophalizied (Merck, Darmstadt, Germany)

6.4 Buffers and media

6.4.1 10x DNAse

RPMI 1640 (Gibco) supplemented with:	10% FCS (PromoCell)
	1x penicillin/streptomycin (Gibco)
	100 mg DNAse (Invitrogen)

6.4.2 Wash medium

RPMI 1640 (Gibco) supplemented with:	10% FCS (PromoCell)
	1x penicillin/streptomycin (Gibco)

6.4.3 0.8% Trypsin

1x PBS (Invitrogen) supplemented with:	4 g Trypsin cryst. lyophalizied (Merck)

6.4.4 FACS Buffer

1x PBS (Invitrogen) supplemented with:	1% FCS (PromoCell)
	0.5 mM EDTA (Merck)

6.4.5 MACS Buffer

1x PBS (Invitrogen) supplemented with:	0.5% BSA (Sigma-Aldrich)
	2 mM EDTA (Merck)

6.5 Skin preparation and cell isolation

6.5.1 Preperation of epidermal single cell suspensions

Skin samples were obtained from infants (28 days to 24 months), young children (3 to 5 years), old children (6 to 11 years), adolescents (12 to 17 years) and adults (18 to 49 years). Human foreskin (routine circumcisions), normal human breast skin (mammary reduction), normal gluteal and belly skin were obtained as discarded material after the ethical committee approval of the Medical University of Vienna. Skin samples were stored at 4℃ in PBS until use and pro cessed no longer than 6

hours after surgery. For the preparation of single epidermal cell suspensions, subcutaneous tissue was removed and remaining skin was disinfected by incubation in Betaisodona for approximately 10 min, followed by a short plunge in 70% ethanol and another incubation in PBS for 1-2 min. Then skin was cut into small stripes and placed epidermal side up on 25% dispase (over night, 4°C). On the next day, the epidermis was separated from the dermis with forceps and digested in 5 ml PBS, 2 ml 0.8% trypsin and 1 ml 10x DNAse (12 min, 37°C). The digested epidermis was sifted through a 70 µm cell strainer and the enzymatic reaction of trypsin was stopped by the addition of 30 ml wash medium. The obtained cells were washed and resuspended in 500 µl MACS buffer. An aliquot was stained with trypan blue and the numbers of viable cells were determined with a Neubauer counting chamber.

6.5.2 Preperation of dermal single cell suspensions

The preparation process is identical with that described for the preparation of an epidermal single cells suspension until the separation step of the epidermis from the dermis. Dermis was digested in 15 ml PBS and Liberase Blendzyme 3 (10.7 Units; 2 hours, 37°C). Subsequently, the digested dermis was sifted through a 70 µm cell strainer and washed with 40 ml wash medium and 1 ml 10xDNAse solution. Isolated cells were resuspended in 500 µl MACS buffer, and viability of the cells was determined as described in 6.5.1.

6.5.3 Preperation of skin single cell suspensions

The preparation process was identical with that described above. Upon incubation in PBS (1-2 min), skin was cut as small as possible and digested in 15 ml PBS and Liberase Blendzyme 3 (10.7 Units; 2 hours, 37°C). Subsequently, the digested skin was sifted through a 70 µm cell strainer and washed with 40 ml wash medium and 1 ml 10xDNAse solution. Isolated cells were resuspended in 500 µl MACS buffer, and cell viability was dertermined as described in 6.5.1.

6.5.4 Preparation of human epidermal sheets

The preparation process was identical with that for the preparation of an epidermal single cell suspension until the removal of subcutaneous tissue from the samples. Skin was cut into small squares (approximately 1 cm^2) and placed on a 3.8% ammonium thiocyanate solution with the epidermal side up (30 min, 37°C). Then the

epidermis was separated from the dermis and washed in PBS (10 min, room temperature). Thereafter, the epidermis was fixed in acetone (10 min, room temperature), washed in PBS (10 min, room temperature), and incubated in 50 µl of a purified anti-RANK antibody (Ab) solution in a 96-well flat bottom cell culture plate (over night, 4℃). After two washing steps in PBS (5 m in, room temperature), 50 µl of an AlexaFluor-546 $F(ab')_2$ goat anti-mouse IgG dilution were added per well to the sheets (2 hours, room temperature, dark). After two washing steps in PBD (5 min, room temperature), the sheets were incubated in 50 µl of an anti-CD1a-FITC Ab solution (over nights, 4℃, dark). Finally, the sheets were washed again (twice, 5 min, room temperature, dark), placed on microscope slides, mounted with mounting media and covered with microscope cover glasses. Control samples were stained with appropriate isotype Ab. Images were recorded using a CLSM 510 Confocal laser scanning microscope.

6.5.5 Isolation of PBMCs

Buffy coats from peripheral blood of healthy adult volunteers were purchased from the local transfusion service (Rotes Kreuz, Vienna, Austria). PBMCs were isolated as interface cells after density gradient centrifugation and erythrocytes were removed with ammonium chloride (0.8% NH_4Cl/0.1 mM EDTA). Isolated cells were resuspended in 500 µl MACS buffer, an aliquot of the cells was stained with trypan blue and their viability and numbers were determined with a Neubauer counting chamber.

6.6 Flow Cytometry

Skin or epidermal single cell suspensions were incubated in microtubes (100 µl/sample) with APC-, FITC-, PE-conjugated or purified Ab (30 min, 4℃, dark). Then, cells were washed with FACS buffer and when a purified Ab was used, a PE-conjugate was added to this staining. To analyze intracellular antigens, cells were permebailized and fixed with Solution A (1 ml/ml; room temperature, 15 min). Afterwards, the cells were washed with 0.1% saponin/PBS and stained with an appropriate conjugated Ab. Dead cells were excluded by 7-AAD (1 µg/ml) and cells were analyzed with a FACS Calibur cytometer. Data were analyzed with FlowJo software. Control samples were stained with appropriate isotype-matched Ab. To determine the percentage of different subsets of cells (e.g. $RANK^+CD1a^+$ cells), gates

were set (**Figure 3A**) based on isotype controls. The percentage of the upper right gate was divided through the summed up percentages of the lower and upper right gates, and multiplied with 100.

6.7 Immunofluorescence

Skin specimens from selected age groups were embedded in O.C.T., snap-frozen in liquid nitrogen and stored at –80℃ until further processing. Five µm sections were cut, transferred onto capillary gap microscope slides, fixed in ice-cold acetone for 10 min and air-dried. Sections were incubated with 10% normal mouse serum and 1% purified mouse-IgG1 Ab (20 min, room temperature). To stain memory T cells, sections were incubated (over night, 4℃) with a purified anti-CD45RO mAb dilution. Skin sections were washed in PBS (3x10 min) and incubated with an AlexaFluor-546 $F(ab')_2$ goat anti-mouse IgG dilution (2 hours, room temperature). Afterwards, the samples were washed again in PBS (3x10 min), blocked with 10% normal mouse serum and 1% purified mouse IgG1 Ab (20 min, room temperature), incubated with an anti-CD3-FITC-conjugated mAb (over night, 4℃), and mounted with fluorescence mounting medium.

To stain naive T cells, sections were simultaneously incubated with anti-CD45RA-PE and anti-CD3-FITC mAbs (over night, 4℃), washed in PBS (3x10 min) and mounted with fluorescence mounting medium. Control samples were stained with appropriate isotype-matched Ab and images were taken with a Nikon eclipse 80i optical microscope. For the enumeration of naive and memory T cells, at least 10 images per donor were taken. Then epidermis and dermis were measured and the numbers of single and double positive cells were determined and placed in relation. Measurement and counting was made with Lucia general software.

6.8 Statistical analysis

The gained values were compared in a statistic diagram designed with the GraphPad Prism 5 software. P-values were calculated with the Mann-Whitney–U test. Significance results at p-values smaller than 5%.

Table 1. Antibodies & second-step reagents

AB specifity	Clone	Ig Class	Working dilution	Source*	Conjugate
CD1a	HI149	Mouse IgG_1, κ	1:100	Biolegend	AlexaFluor-488
CD1a	HI149	Mouse IgG_1, κ	1:100	BD Pharmingen	APC, PE, FITC
CD3	SK7	Mouse IgG_1, κ	1:100	BD Pharmingen	APC
CD3	UCHT1	Mouse IgG_1, κ	1:50	ADG	FITC
CD45RA	T6D11	Mouse IgG_{2b}, κ	1:50	Miltenyi	PE
CD45RO	UCHL1	Mouse IgG_{2a}, κ	1:50	BD Pharmingen	PE
CD45RO	UCHL1	Mouse IgG_{2a}, κ	1:50	AbD	Purified
CD117	YB5.B8	Mouse IgG_1, κ	1:100	BD Pharmingen	PE
CLA	HECA-452	Rat IgM, κ	1:50	BD Pharmingen	FITC
Pancytokeratin	C-11	Mouse IgG_1, κ	1:500	Abcam	FITC
RANK	80704	Mouse IgG_1, κ	1:100	R&D Systems	purified
Second-step reagents					
Goat anti-mouse	F(ab')2 IgG (H+L)		1:250	Invitrogen	AlexaFluor-546
Goat anti-mouse	Goat F(ab')2 IgG + IgM (H+L)		1:250	Dako	PE

*Abcam, Cambridge, United Kingdom; AbD Serotec, Kidlington, United Kingdom; ADG, Kaumberg, Austria; BD Pharmingen, San Diego, CA, USA; Biolegend, San Diego, CA, USA; Dako, Glostrup, Denmark; Miltenyi Biotech, Bergisch Gladbach, Germany; R&D Systems, Minneapolis, MN, USA.

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8 Curriculum vitae

Personal information

Name	AKGÜN Johnnie
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Education

01/2009 – 12/2009	Diploma Thesis at the Department of Dermatology, Medical University of Vienna; Thesis Title: Differential expression of RANK on Langerhans cells and CD45RA and CD45RO/CLA on T cells in developing human skin after birth Supervisor: Ao. Prof. Dr. Adelheid Elbe-Bürger
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1994 – 2003	Grammar school “Realgymnasium Erlgasse”, 1120 Vienna, Austria
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