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Skin resident memory CD8$^+$ T cells are phenotypically and functionally distinct from circulating populations and lack immediate cytotoxic function

Short title: Phenotype and function of cutaneous T cells

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Abbreviations:

T_{RM} = Tissue resident T cells

T_{CM} = Central memory T cells

T_{EM} = Effector memory T cells

T_{EMRA} = Effector memory T cells re-expressing CD45RA

Abstract

The in depth understanding of skin resident memory $CD8^+$ T lymphocytes (T_{RM}) may help uncover strategies for their manipulation during disease. We investigated isolated T_{RM} from healthy human skin, which expressed the residence marker CD69, and compared them to circulating $CD8^+$ T cell populations from the same donors. There were significantly increased proportions of $CD8^+CD45RA^-CD27^-$ T cells in the skin that expressed low levels KLRG1, CD57, perforin and granzyme B. The $CD8^+$ T_{RM} in skin were therefore phenotypically distinct from circulating $CD8^+CD45RA^-CD27^-$ T cells that expressed high levels of all these molecules. Nevertheless the activation of $CD8^+$ T_{RM} with TCR/CD28 or IL-2 or IL-15 *in vitro* induced the expression of granzyme B. Blocking signaling through the inhibitory receptor PD-1 further boosted granzyme B expression. A unique feature of some $CD8^+$ T_{RM} cells was their ability to secrete high levels of TNF-α and IL-2, a cytokine combination that was not frequently seen in circulating $CD8^+$ T cells. The cutaneous $CD8^+$ T_{RM} are therefore diverse and appear to be phenotypically and functionally distinct from circulating cells. Indeed, the surface receptors used to distinguish differentiation stages of blood T cells cannot be applied to T cells in the skin. Furthermore the function of cutaneous T_{RM} appears to be stringently controlled by environmental signals *in situ*.

Introduction

T cells are abundant in healthy human adult skin and are an important component of the local immune system (1,2). T_{RM} arise from naïve precursors that are first activated in secondary lymphoid organs and then migrate to the peripheral tissues, where they are retained through expression of CD69 and may act as sentinels for future re-infection (3–5). Current literature suggests that T_{RM} may have constitutive functional activity and are poised to respond rapidly to antigenic challenge (1,2). However the mechanisms controlling the effector functions of these cells, particularly their cytotoxic function, are not known.

There has been extensive characterization of surface markers that can be used to identify circulating T cells at different stages of differentiation (6,7). The correlation between phenotype and function has enabled the discrimination between naïve (T_N; $CD45RA^+$ $CD27^+$), central memory ($T_{CM;}$ $CD45RA^+$ $CD27^+$), effector memory ($T_{EM;}$ $CD45RA^+$ $CD27^+$) and effector memory T cells that re-express CD45RA ($T_{EMRA;}$ $CD45RA^+$ $CD27^+$) within both $CD4^+$ and $CD8^+$ T cell subsets (7,8). In peripheral blood, highly differentiated T_{EM} and T_{EMRA} cells display high constitutive levels of preloaded cytotoxic granules

containing perforin and granzyme B and can secrete the effector cytokines TNFα and IFNγ after activation *in vitro* (7). In addition these cells have short telomeres (9–11), lack the costimulatory receptors CD27 and CD28, cannot produce the cytokine IL-2 but express high levels of CD57 and the inhibitory receptor KLRG1 (7,11,12). These circulating cells also express high levels of the transcription factors Tbet and Eomes that are important for cytotoxic differentiation (13). Overall, T_{EM} and T_{EMRA} are thought to have gained increased effector capabilities at the expense of proliferative activity compared to T_N and T_{CM} cells (7,11,12). Unlike T_N and T_{CM} cells, the T_{EM} and T_{EMRA} populations do not home to lymphoid tissues, but may migrate instead to peripheral organs, including the skin (14,15). Because most T_{RM} in the skin lack the lymph node homing receptor CCR7, they were initially thought to belong to the same lineage as T_{EM} in the blood (1,16). However the mechanisms that control the activity of skin T_{RM} *in situ* and their phenotype are poorly defined.

The aim of this study was to investigate the cytotoxic potential of $CD8^+$ T_{RM} in the skin of healthy donors and to identify the factors that influence the function of these cells. We found that $CD8^+$ T_{RM} are not poised for immediate cytotoxic activity since they contain very low levels of perforin and granzyme B relative to effector $CD8^+$ T cells in the blood. $CD8^+$ T_{RM} cells require cytokine- or TCR-mediated activation in order to adopt a cytotoxic effector phenotype, whilst inhibitory signalling though PD-1 may prevent this. In addition phenotypic markers that identify highly differentiated cells in the blood (KLRG1, CD57, $CD28^-$) are not characteristics of EM ($CD27^-CD45RA^-$) or EMRA ($CD27^-CD45RA^+$) T_{RM} cells in the skin. Therefore the correlation of differentiation stage of $CD8^+$ T cell subsets with surface phenotype, which has been widely used to characterize circulating cells, cannot be extrapolated to the study of T_{RM} populations in the skin. Instead, the skin appears to be populated with phenotypically distinct and diverse quiescent T cells, including a novel $CD8^+$ T cell subset capable of producing both IL-2 and TNFα upon stimulation.

-Material and Methods

Blood and skin samples

Blood, skin and blister samples were obtained from healthy donors or individuals undergoing plastic surgery. Individuals suffering from co-morbidities or on medication were excluded from the study. We investigated 33 skin donors in total (average age: 46, age range 19-82, 4 males 29 females). Written and informed consent was obtained from all participants in agreement with the declaration of Helsinki protocols and the study was approved by the Ethics Committee of the Royal Free Hospital and Guy's Hospital, London.

Sample preparation

PBMC were isolated by density centrifugation using Ficoll-Paque PLUS (GE Healthcare). Cutaneous single cell suspensions were obtained by overnight digestion of finely minced skin specimen with 0.8mg/ml collagenase type IV (Life Technologies, Paisley, UK) in RPMI. Addition of 20% FCS to the digestion mix prevented surface receptor degradation (17). The cells from digested skin showed

negligible surface marker degradation and similar phenotypic properties to those analysed in snap-frozen skin sections *in situ* via immunofluorescence, as previously reported (18). Skin cells were obtained as previously described from suction blisters raised on normal skin (19).

Flow cytometry

Antibodies used for flow cytometry are summarized in Supplementary table 1. Single cell suspensions were initially stained for extracellular markers, followed by intracellular staining according to the manufacturers' instructions using either the Fix & Perm Cell Permeabilization Kit (An Der Grub, Buckingham, UK) for cytokines, perforin and granzyme B, or intranuclear FoxP3 Staining Buffer Set (Miltenyi), for T-bet and eomes staining. To assess cytokine production cells were stimulated with 25ng/ml PMA and 500ng/ml ionomycin for 1 hour, followed by addition of 5 µg/ml brefeldin A (Sigma-Aldrich) and incubation for a further 5 hours. Unstimulated cells were used as the negative control. Telomere lengths were measured via flow cytometry using the flow-FISH method as described previously (9). Pooled samples of blood and CFSE stained blister cells were used for the Flow-FISH method in order to ensure equivalent staining between both cell populations. All samples were acquired using LSRII or Fortessa flow cytometers (both from BD Biosciences, San Jose, CA, USA) and analysed using FlowJo (Tree Star Inc., Asland, USA).

Immunofluorescence

Punch biopsies (5 mm diameter) from normal skin were frozen in OCT (optimal cutting temperature compound; Bright Instrument Company Ltd). 6µm sections were cut, left to dry overnight, then fixed in ethanol and acetone and stored at -80ºC. Samples were incubated with optimal dilutions of primary antibodies and followed by an appropriate secondary antibody conjugated to various fluorochromes as described (18). Antibodies used were: CD69 (clone FN50, Biolegend), CD103 (clone 2G5.1, Thermofisher Scientific), CD8 (clone RPTA-T8, BD Bioscience), CD4 (YNB46.1.8, BD Bioscience) and PD1 (clone NAT105, Abcam). Images were acquired on the AxioScan Z1 slide scanner and Imaged on Zen Blue (Zeiss, Cambridge U.K.).

Culture conditions

Cells were maintained in RPMI with 10% FCS, 1% penicillin/streptomycin and 1% L-Glutamine (all by Sigma-Aldrich). PBMCs or cells derived from collagenase digested skin were incubated for four days in the presence of no stimulant, 10ng/ml TNFα, 5ng/ml IL-2 (both PeproTech, London, UK), 10ng/ml IL-15 (R&D systems) or anti-CD3/CD28 coated beads (Dynabeads; Life Technologies), followed by flow cytometric staining. For PD-1 signalling blockade, cells were incubated with 10mg/ml of anti-PDL-1 and anti-PDL-2 antibodies (MIH1 and MIH18) or 20mg/ml isotype control (all eBioscience) for two hours prior to stimulation with 0.05 µg/ml immobilized anti-CD3 (OKT3) for 3 days.

Statistical Analysis

Graphs were drawn and statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, USA). Non-parametric data were identified using the D'Agostino and Pearson omnibus normality test. Statistical tests used included the Student's T-test or paired T-test when data followed a Gaussian distribution and the Whitney-Mann test or Wilcoxon test for non-parametric data. Correlations were calculated using Pearson correlation coefficient or Spearman correlation for non-parametric data. Lines of best fit were generated using linear regression. If more than two groups were compared simultaneously, one-way ANOVA (for parametric data) or the Friedman test (for non-parametric data) were applied, followed by Holm-Sidak or Dunn's multiple comparison tests respectively for paired comparisons. Differences were considered significant when $p<0.05$.

Results

Skin derived $CD8^+$ T_{RM} cells express CD69 and are enriched for $CD27^-$ $CD45RA^-$ (effector memory) populations

Single cell suspensions were generated through overnight collagenase digestion of fresh healthy human skin and $CD8^+$ T cell phenotype was analysed at the same time as PBMCs from the same healthy donors (representative gating strategy in Fig.1A). The majority of skin but not blood-derived $CD8^+$ T cells expressed CD69 that identifies them as tissue resident T cells (20), Fig.1B,C,D). In the blood, combined use of the surface markers CD27 and CD45RA allows the identification of $CD8^+$ T cells that have functional characteristics of naïve ($CD45RA^+CD27^+$), central memory ($CD45RA^-CD27^+$), effector memory ($CD45RA^-CD27^-$) and late stage/senescent effector memory ($CD45RA^+CD27^-$) cells (7,21). Among skin $CD8^+$ T cells, the relative expression of CD45RA and CD27 identified 4 subsets as found in blood (Fig. 1E,F). However, skin $CD8^+$ T cells were significantly enriched for the $CD45RA^-$ $CD27^-$ T subset while $CD45RA^+CD27^+$ subset was significantly reduced in this tissue (Fig.1E,F). Among the skin $CD8^+$ T cells CD69 expression was equally high in all the four subsets (Fig.1G). Similar changes were also observed in $CD4^+$ T cells, where skin cells were mainly $CD69^+$ and were enriched for $CD45RA^-CD27^-$ T cells compared to the blood of the same donors (Supplementary Figure 1A,B).

Skin $CD8^+$ T_{RM} cells exhibit distinct expression of differentiation-associated surface markers compared to circulating memory T cells

We next investigated whether skin $CD8^+$ T_{RM} cells had the same phenotypic properties as their circulating counterparts. Circulating $CD8^+CD45RA^-CD27^-$ and $CD8^+CD45RA^+CD27^-$ T cells have been shown to express low levels of CD28 but high levels of the NK-cell associated receptors KLRG1 and CD57 compared to less differentiated populations (11,22,23). Total skin $CD8^+$ T_{RM} expressed similar levels of CD28 and CD57 but significantly lower levels of KLRG1 compared to the total circulating $CD8^+$ T cell population (Fig. 2A, B). However, when comparing the two tissues' $CD8^+CD45RA^+CD27^-$ subsets, CD28 expression was significantly higher while CD57 and KLRG1 expression were

significantly lower in the skin (Fig. 2C). In addition, the $CD8^+CD45RA^+CD27^+$ T_{RM} cells in the skin expressed significantly lower levels of CD28 and higher levels of CD57 than this subset in the blood. Similar patterns were observed in skin $CD4^+$ T cells, where the dominant $CD45RA^-CD27^+$ population did not express KLRG1 (Supplementary Fig. 1C,D). These results suggest that patterns of differentiation differ between blood and skin T cells.

To test the possibility that the $CD8^+$ T cells in the skin and the circulation have disparate expression of differentiation-related markers because of differences in their replicative history, we compared the telomere lengths of these cells. In peripheral blood, $CD8^+CD45RA^+CD27^+$ T cells have the longest and $CD8^+CD45RA^-CD27^-$ T cells the shortest telomeres among T memory cell subsets (24). Since debris present in digested skin samples interfered with telomere detection, we isolated $CD8^+$ T_{RM} cells by suction blister technology (25) and compared them to circulating populations from the same donors (Representative gating strategy Supplementary Fig. 2A). Naïve T cells had longer telomeres than $CD45RA^-CD27^-$ (T_{EM}) and $CD45RA^+$ $CD27^-$ T (T_{EMRA}) subsets in both skin and blood $CD8^+$ T cells (Fig. 2D). Furthermore telomere length of blood and skin total $CD8^+$ T cells and $CD8^+$ T cell subsets were similar (8.9±1.8kbp in the skin and 9.7±2kbp in blood $CD8^+$ T cells; Fig.2D). This indicated that the altered expression of differentiation-related markers between $CD8^+$ T_{RM} and their blood counterparts was not due to differences in replicative history. However, telomere length was significantly reduced in skin compared to blood in total $CD4^+$ T cells (8.0±1.0kbp in the skin vs. 10.3±1.8kbp in the blood, Supplementary Fig 2B) indicating that skin $CD4^+$ T_{RM} cells have experienced more proliferation *in vivo*.

A unique population of skin $CD8^+$ T_{RM} secretes IL-2 and TNFα after activation *in vitro*

Cutaneous $CD8^+$ T_{RM} are considered a first line of defence against reinfection by pathogens (2,26). We investigated differences in cytokine production between isolated $CD8^+$ T_{RM} and circulating $CD8^+$ T cells. The production of 3 cytokines IL-2, TNFα and IFNγ individually or in combination was measured after stimulation with PMA and ionomycin *in vitro* (Fig.3A,B). Among peripheral blood T cells different subsets secrete either IL-2 alone, or TNFα together with IFNγ (23). In the skin we observed a reduction in cells producing IL-2 alone and an increase in novel populations of $CD8^+$ T cells, which lacked IFNγ but secreted either TNFα alone or in combination with IL-2 (Fig. 3B). $CD8^+$ T_{RM} that secreted both IL-2 and TNFα were not confined to one particular CD45RA/CD27 population (Fig. 3C).

Average abundance of $CD8^+$ T cells that secreted all 3 cytokines together after activation was not significantly different between blood and skin samples (Fig.3B). However, slight but not significant differences were noted in the distribution of these multiple cytokine producers among the $CD45RA^+CD27^-$ subsets (Fig.3D). These data indicate that the functional profiles of $CD8^+$ T cells and their CD45RA/CD27 subsets are distinct in the blood and skin and that the skin may contain unique populations of cells with different functional properties.

$CD8^+$ T_{RM} in normal skin have low cytotoxic potential

$CD8^+$ T_{RM} cells are considered to be poised for rapid responses in case of pathogen re-encounter (26). Previous work from our group has shown that the T_{EM} and T_{EMRA} T cell subsets in the blood contain preloaded cytotoxic granules containing perforin and granzyme B (11,23). Here, we show that the levels of both proteins were consistently lower in skin $CD8^+$ T cells compared to the same subsets in blood, with perforin being almost undetectable in most individuals (Fig. 4A,B). Furthermore perforin and granzyme B levels were low in the $CD8^+CD45RA^-CD27^-$ and $CD8^+CD45RA^+CD27^-$ T cell subsets in the skin while being particularly high in the equivalent populations in the blood (Fig.4C).

We next investigated whether the low cytotoxic mediator expression was induced locally in the skin or whether this was predetermined in T cells with skin homing potential. To do this we examined perforin and granzyme B expression in circulating T cells expressing the cutaneous lymphocyte antigen (CLA; Fig. 4D). Blood-derived $CD8^+CLA^+$ T cells expressed significantly less perforin and granzyme B than the CLA^- population (Fig.4D), suggesting that cutaneous T cells lack cytotoxic effector functions prior to populating the skin.

Among skin derived T cells, true resident cells express both CD103 and CD69 whilst cells transitioning in the tissue do not express either surface receptor (20). We found that among skin $CD8^+$ T cells granzyme B expression was highest in the minor "transitioning" $CD69^-CD103^-$ population and lowest in the "true resident" $CD69^+CD103^+$ population and that perforin was low in all these subsets (Fig.4E). Furthermore the transcription factors Tbet and Eomes, which are associated with effector memory T cell populations (13), were both absent among $CD8^+T_{RM}$ cells, even among those that were granzyme B positive (Fig.4F). Therefore unlike circulating $CD8^+$ T cells, $CD8^+$ T_{RM} in healthy skin do not have a conventional effector phenotype and these cells lack immediate cytotoxic potential.

Surface markers that correlate with cytotoxic potential in circulating $CD8^+$ T cells are not applicable in the skin

In published literature blood effector memory T cells are identified by their effector functions together with the increased expression of surface markers such as KLRG1, and absence of other receptors such as CD27 or CD28 (7). We confirmed a strong positive correlation between KLRG1 expression and granzyme B expression (Fig. 5A) and a negative correlation between both CD27 (Fig. 5B) and CD28 (Fig. 5C) with granzyme B expression in blood $CD8^+$ T cells. This was not observed in cutaneous $CD8^+T_{RM}$ cells. There is therefore a dissociation between the expression of this cluster of phenotypic markers and effector function of skin $CD8^+T_{RM}$ cells, further indicating that memory $CD8^+$ T cells from the circulation and the skin are controlled differently.

Cutaneous CD8$^+$ T_{RM} skin T cells can be induced to express perforin and granzyme *in vitro*

Circulating CD8$^+$ T cells can upregulate perforin and granzyme B after stimulation (27,28). In order to investigate whether skin derived CD8$^+$ T cells also have the ability to acquire cytotoxic potential, we activated total T_{RM} and PBMCs *in vitro* with or without either TNFα, IL-2, IL-15 or anti-CD3/CD28 antibody coated beads. After 4 days, perforin and granzyme B expression were measured via intracellular flow cytometry (Fig.6A). All these stimulants apart from TNFα induced a significant increase in granzyme B in both blood and skin CD8$^+$ T cell populations (Fig. 6A). Perforin levels were significantly increased in both blood and skin CD8$^+$ T cells by IL-15 or CD3/CD28 stimulation (Fig.6A). Skin CD8$^+$ T_{RM} cells are therefore capable of upregulating their cytotoxic machinery to the same extent as blood populations, suggesting that these cells are not inherently unresponsive.

PD-1 signalling may be involved in low cytotoxic potential of skin T cells

PD-1 is a regulatory surface receptor often implicated in peripheral tolerance and we hypothesized that PD-1 signalling may control granzyme B and/or perforin expression in skin T_{RM} cells. PD-1 levels were low among circulating CD8$^+$ T cells but significantly higher in skin CD8$^+$ T_{RM} cells (Fig.6B,C). PD-1 staining of T_{RM} was confirmed by immunohistology (Fig. 6D). To test whether PD-1 signalling affects perforin and granzyme B expression, healthy skin derived cells were stimulated with suboptimal levels of plate-bound anti-CD3 antibody. This was performed in the presence of PDL-1 and PDL-2 blocking antibodies or isotype controls. In the presence of PD-1 ligand blockade, intracellular perforin was only significantly increased in blood derived CD8$^+$ T cells but not in skin derived cells while granzyme B was significantly increased compared to the isotype control in CD8$^+$ T cells from both tissues (Fig.6E). Therefore while PD-1 signalling may contribute partially to the inhibition of cytotoxic potential of CD8$^+$ T_{RM} in the skin, other mechanisms are likely to be also involved.

Discussion

A major advance in the understanding of immune memory was the recognition that memory T cells in tissues are an important barrier to re-infection and reactivation of pathogens (26). Circulating T cells and T_{RM} cells have different transcriptional profiles suggesting that their activity is differentially controlled (4,5). However the mechanisms involved have not been defined. The aim of the present study was to compare the functional activity and extent of phenotypic differentiation of skin CD8$^+$ T cells to those in the blood in healthy humans.

Blood-derived T cell subsets with distinct differentiation and functional properties are often identified through well-defined surface markers such as CD27 and CD45RA (7). We found that almost all skin derived CD8+ T cells expressed the tissue retention marker CD69, identifying them as T_{RM} (29). Among them, those that were CD45RA$^-$CD27$^-$ and CD45RA$^+$CD27$^-$ displayed low levels of perforin and granzyme B, substantially reduced expression of KLRG1 and CD57 and lacked of the transcription factors T-bet and Eomes, but secreted both IL-2 and TNFα after activation. This is clearly distinct from equivalent phenotypically defined CD8$^+$ T cells in the circulation that have high

perforin, granzyme, KLRG1, CD57, Eomes and T-bet and secrete TNFα and IFNγ but no IL-2. Other studies using mRNA as a readout also report low levels of perforin, granzyme B or Eomes mRNA in healthy skin of humans or mice (5,30). This indicates that the correlation between effector phenotype and function that is observed in blood $CD8^+$ T cells cannot be extrapolated to $CD8^+$ T_{RM} populations and a similar situation exists for $CD4^+$ T_{RM} populations. Stated simply, cutaneous $CD45RA^+CD27^+$ $CD8^+$ T_{RM} are not naïve and $CD8^+CD45RA^-CD27^-$ T cells are not immediately operational effector-like cells, unlike their blood counterparts.

Circulating cells expressing the skin homing marker CLA also lacked perforin and granzyme B suggesting that the low cytotoxic potential of T_{RM} is pre-programmed in $CD8^+$ T cells with propensity for homing to the skin. Instead we found that some cutaneous $CD8^+$ T_{RM} cells can upregulate their cytotoxic machinery after activation. Furthermore, cytotoxic T cells can be found in inflamed skin in patients with atopic dermatitis, psoriasis, herpes simplex-2 infection and vitiligo (2,31,32). This suggests that some T cells can become cytotoxic within the skin under appropriate stimulatory conditions *in vivo* and is in line with a recent report showing that among stimulated epidermal T_{RM}, one specific subset of CD49a+ cells is able to upregulate cytotoxic effector molecules (32). This, together with our observation that T_{RM} share few phenotypic characteristics with highly differentiated cells in the blood, also supports previous murine work showing that skin T_{RM} can extensively proliferate locally and are derived from less differentiated circulating KLRG1- memory precursor T cells (5,33).

We observed that the inhibitory receptor PD-1 was significantly higher on skin T_{RM} cells compared to $CD8^+$ T cells in the circulation. Subsequent blockade of PD-1 signalling during TCR stimulation *in vitro* resulted in increased granzyme B but not perforin expression in the skin $CD8^+$ T_{RM} cells, suggesting that other mechanisms may be involved in the repression of cytotoxic granule expression in skin CD8 $T_{RM.}$ Granzyme B can cause extracellular matrix degradation (34) and PD-1 signalling may therefore be involved in limiting $CD8^+$ T cell mediated tissue damage. PD-1 is reportedly elevated on brain, gastric mucosa and liver T cells (35–37), suggesting that PD-1 signalling may also be important in limiting the activity of resident $CD8^+$ T cells in other tissues.

Cytokines produced by activated skin T_{RM} may also influence local immune responses. Large clusters of infiltrating T cells associated with a cytotoxic gene signature rapidly appear in human skin during challenge with VZV antigens after vaccination against herpes zoster (38,39). We suggest that after activation a subset of $CD8^+$ T_{RM}, with their unique cytokine secreting profile ($IL\text{-}2^+TNF\alpha^+$), may activate endothelial cells through TNFα (40), thereby promoting the recruitment of cells from the circulation. Further IL-2 secretion by these cells would promote the expansion of the recruited cells at the site of challenge in the skin. Given that T cells expressing the skin homing receptor CLA did not readily express perforin or granzyme B either, it is likely that recruited cells too may require further local stimulation to convert to a cytotoxic phenotype. Indeed, the importance of local effector T cell licensing in the skin was demonstrated in a murine graft-versus-host model, where T cell infiltration into the skin graft was not sufficient for rejection but required local Langerhans-mediated help for T cell cytotoxicity towards the mismatched graft (41).

Although skin resident $CD8^+$ T_{RM} uniformly expressed CD69 and lacked perforin, our data also indicate heterogeneity among these cells in terms of cytokine production and expression of surface receptors such as CD27, CD28 CD45RA, KLRG1 and CD57. This suggests that similarly to blood cells, skin $CD8^+$ T cells are composed of phenotypically distinct subsets. Indeed, differences between dermal and epidermal skin T cells have been reported previously (20). Epidermal $CD8^+$ T cells in turn could be further subdivided into CD49a+ and CD49- cells, which had the ability to produce cytotoxic effector molecules or IL-17 respectively (32). We show that skin resident T cells cannot simply be grouped into subsets using surface marker expression patterns that have been established using blood cells. Full identification, characterization and compartmentalization of all phenotypic and functional subsets of the skin T cells therefore remains outstanding.

In summary, skin T_{RM} $CD8^+$ T cells have low cytotoxic potential unless they are stimulated either by cytokines such as IL-15 or through the TCR. This, together with the negative regulation through PD-1, may be a mechanism that limits non-specific tissue damage in the steady state. Importantly, skin $CD8^+$ T cells are phenotypically and functionally distinct from circulating T cells and must not be categorized based on blood-based surface markers, such as CD27 and CD45RA, alone. The unique T_{RM} subset diversity, including IL-2/TNFα-producers, will require further exploration with this in mind.

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JAS, SMH and ANA designed the study. JAS, MVS and ANA wrote the paper. BMD performed the telomere measurements, NP the histological stains and JAS performed the remaining experiments. JFD provided technical expertise for histological analysis of skin samples. JGK advised on the analysis of skin samples and the interpretation of the results. MHAR advised on recruitment of subjects and edited the manuscript. FON provided samples for analysis and advised on the initial outline of the project. KEL provided access to the samples and edited the manuscript.

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Figure Legends

Fig. 1: *Ex vivo* CD69 expression and CD27/CD45RA differentiation subset distribution in $CD8^+$ T cells derived from the blood and skin.

Collagenase digested skin and PBMCs were analyzed via flow cytometry. (A) Representative gating strategy used to identify $CD8^+$ T cells in both tissues derived from the same donor. (B) Representative histograms and (C) cumulative data showing expression of the tissue residence marker CD69 and CD103 (n=8) (D) Immunofluorescence staining for CD8, CD69 and CD103 in a human skin section. (E) Representative flow cytometry staining for CD27 and CD45RA and (F) cumulative CD27/CD45RA subset distribution in blood and skin derived cells (n=12). (G) CD69 expression by CD27/CD45RA subset in blood and skin cells. T_{CM}= Central memory, T_{EM}= Effector Memory and T_{EMRA}= Effector memory re-expressing CD45RA.

Fig. 2: Differentiation-associated phenotypic properties of skin compared to blood CD8+ T cells

(A) Representative and (B) cumulative *ex vivo* measurements of CD28 (n=6), KLRG1 (n=9), and CD57 (n=8), in $CD8^+$ T cells and (C) in CD27/CD45RA subsets, measured via flow cytometry in collagenase digested skin and PBMC samples derived from the same healthy donors. (D) KLRG1 expression among CD69- and CD69+ skin cells (n=19). (E) Cumulative data for total $CD8^+$ T cells and T cell subsets for telomere length, measured via flow cytometry using the Flow-FISH method on PBMCs and skin blister derived cells as described in the material and methods section (n=5).

Fig. 3: IL-2, IFNγ and TNFα cytokine production in blood and skin-derived $CD8^+$ T cells.

Collagenase digested skin cells or PBMCs were stimulated *in vitro* with PMA/Ionomycin before intracellular flow cytometry was performed to detect cytokine production (n=9). (A) Representative dot plots for IFNγ and TNFα staining. Histograms show IL-2 production by different subsets based on IFNγ and TNFα production; (B) Cumulative data for total $CD8^+$ T cells producing IL-2, TNFα or IFNγ alone or in combination. Cytokine production by different CD8 subsets for (C) IL-2/TNFα or (D) IL-2/IFNγ/TNFα cytokine co-production.

Fig. 4: Ex vivo expression of cytotoxic granule components in skin compared to blood $CD8^+$ T cells.

(A) Representative flow cytometry histograms and (B) cumulative data showing granzyme B and perforin expression in collagenase digested skin and blood derived $CD8^+$ T cells from paired samples (n=9). (C) Granzyme B and perforin expression among the $CD8^+$ T cell subsets derived from blood (n=33) and skin samples (n=16) (D) Granzyme B and perforin expression in circulating $CD8^+$ T cells positive or negative for the skin homing receptor CLA (n=12). (E) Granzyme B and Perforin expression in $CD8^+$ CD69/CD103 subsets in the skin of healthy donors (n=3). (F) Expression of the transcription factors tbet or eomes with granzyme B in skin and blood $CD8^+$ T cells.

Fig. 5: Predictive value of CD27, CD28 and KLRG1 surface expression for intracellular granzyme B in skin and blood $CD8^+$ T cells.

Representative dot plots and cumulative data showing (A) KLRG1, (B) CD27 and (C) CD28 expression in relation to granzyme B expression in blood and skin derived $CD8^+$ T cells. Graphs show the correlations between percentage CD27, CD28 or KLRG1 expression and percentage granzyme B expression (Lines of best fit, r values and significance are shown where applicable). Further, cumulative data of granzyme B expression among $CD27^-/CD27^+$, $CD28^-/CD28^+$ and $KLRG1^+/KLRG1^-$ (with averages and standard errors) are shown. KLRG1 was measured in 18 blood and 9 skin donors; CD27 and CD28 were measured in 33 blood and 17 skin donors.

Fig. 6: Factors influencing granzyme B and perforin expression in blood and skin derived $CD8^+$ T cells.

Skin and blood derived cells from healthy donors were cultured for four days in the presence of TNFα, IL-2, IL-15 or anti-CD3/CD28, followed by intracellular flow cytometric staining. (A) Granzyme B and perforin expression (Mean and SE) in blood (n=12) and skin derived cells (n=10). (B) Representative and (C) cumulative data showing PD-1 expression measured by flow cytometry. (D) Immunofluorescent staining of healthy skin for CD8, PD-1 and nuclei (DAPI). (E) Granzyme B and perforin expression following stimulation for 3 days with anti-CD3 in the presence of PDL-1/2 blocking antibodies or isotype controls in blood (n=8) and skin (n=6) specimen (Mean and SE and p values are shown).

Supplementary Figure Legends

Figure S1: Ex vivo CD69 expression and CD27/CD45RA differentiation subset distribution in CD4$^+$ T cells derived from the blood and skin.

Collagenase digested skin and PBMCs were analyzed via flow cytometry. (A) Representative flow cytometry staining and (B) cumulative CD27/CD45RA subset distribution in blood and skin derived cells CD4+ T cells (n=12). Expression of CD28 (n=6), KLRG1 (n=9) and CD57 (n=8) among total blood and skin CD4+ T cells (C) and their subsets (D). (E) Skin cells or PBMCs were stimulated in vitro with PMA/Ionomycin before intracellular flow cytometry was performed to detect cytokine production. Cumulative data for total CD8+ T cells producing IL-2, TNFα or IFNγ alone or in combination.

Figure S2: Telomere length of skin and blood-derived CD4$^+$ T cells

(A) Representative gating strategy for blood and skin CD4$^+$ and CD8$^+$ T cell subset telomere length measurements. (B) Cumulative data for total CD4$^+$ T cells and CD27/CD45RA subsets for telomere length, measured via flow cytometry using the Flow-FISH method on PBMCs and skin blister derived cells as described in the material and methods section (n=5).

References

1. Clark RA, Watanabe R, Teague JE, Schlapbach C, Tawa MC, Adams N, et al. Skin Effector Memory T Cells Do Not Recirculate and Provide Immune Protection in Alemtuzumab-Treated CTCL Patients. Sci Transl Med. 2012 Jan 18;4(117):117ra7-117ra7.

2. Zhu J, Peng T, Johnston C, Phasouk K, Kask AS, Klock A, et al. Immune surveillance by CD8αα+ skin-resident T cells in human herpes virus infection. Nature. 2013 May 23;497(7450):494–7.

3. Davies B, Prier JE, Jones CM, Gebhardt T, Carbone FR, Mackay LK. Cutting Edge: Tissue-Resident Memory T Cells Generated by Multiple Immunizations or Localized Deposition Provide Enhanced Immunity. J Immunol. 2017 Mar 15;198(6):2233–7.

4. Li J, Olshansky M, Carbone FR, Ma JZ. Transcriptional Analysis of T Cells Resident in Human Skin. PLOS ONE. 2016 Jan 29;11(1):e0148351.

5. Mackay LK, Rahimpour A, Ma JZ, Collins N, Stock AT, Hafon M-L, et al. The developmental pathway for CD103(+)CD8(+) tissue-resident memory T cells of skin. Nat Immunol. 2013 Oct 27;

6. Akbar AN, Beverley PCL, Salmon M. Will telomere erosion lead to a loss of T-cell memory? Nat Rev Immunol. 2004;4(9):737–43.

7. Appay V, van Lier RAW, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: Consensus and issues. Cytometry A. 2008;73A(11):975–983.

8. Sallusto F, Geginat J, Lanzavecchia A. Central Memory and Effector Memory T Cell Subsets: Function, Generation, and Maintenance. Annu Rev Immunol. 2004 Apr;22(1):745–63.

9. Riddell NE, Griffiths SJ, Rivino L, King DCB, Teo GH, Henson SM, et al. Multifunctional cytomegalovirus (CMV)-specific CD8+ T cells are not restricted by telomere-related senescence in young or old adults. Immunology. 2015 Apr;144(4):549–60.

10. Di Mitri D, Azevedo RI, Henson SM, Libri V, Riddell NE, Macaulay R, et al. Reversible Senescence in Human CD4+CD45RA+CD27− Memory T Cells. J Immunol [Internet]. 2011 Jul 25 [cited 2012 Jun 26]; Available from: http://www.jimmunol.org/content/early/2011/07/25/jimmunol.1100978

11. Henson SM, Lanna A, Riddell NE, Franzese O, Macaulay R, Griffiths SJ, et al. p38 signaling inhibits mTORC1-independent autophagy in senescent human CD8+ T cells. J Clin Invest. 2014 Sep 2;124(9):4004–16.

12. Libri V, Azevedo RI, Jackson SE, Di Mitri D, Lachmann R, Fuhrmann S, et al. Cytomegalovirus infection induces the accumulation of short- lived, multifunctional CD4+ CD45RA+ CD27− T cells: the potential involvement of interleukin- 7 in this process. Immunology. 2011 Mar 1;132(3):326–39.

13. McLane LM, Banerjee PP, Cosma GL, Makedonas G, Wherry EJ, Orange JS, et al. Differential localization of T-bet and Eomes in CD8 T-cell memory populations. J Immunol Baltim Md 1950. 2013 Apr 1;190(7):3207–15.

14. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999;401:708–12.

15. Faint JM, Annels NE, Curnow SJ, Shields P, Pilling D, Hislop AD, et al. Memory T Cells Constitute a Subset of the Human CD8+CD45RA+ Pool with Distinct Phenotypic and Migratory Characteristics. J Immunol. 2001 Jul 1;167(1):212–20.

16. Thome JJC, Yudanin N, Ohmura Y, Kubota M, Grinshpun B, Sathaliyawala T, et al. Spatial Map of Human T Cell Compartmentalization and Maintenance over Decades of Life. Cell. 2014 Nov 6;159(4):814–28.

17. Mulder WMC, Koenen H, Muysenberg AJC, Bloemena E, Wagsfaff J, Scheper RJ. Reduced expression of distinct T-cell CD molecules by collagenase/DNase treatment. Cancer Immunol Immunother. 1994 Jul;38(4):253–8.

18. Vukmanovic-Stejic M, Sandhu D, Sobande TO, Agius E, Lacy KE, Riddell N, et al. Varicella zoster-specific CD4+Foxp3+ T cells accumulate after cutaneous antigen challenge in humans. J Immunol Baltim Md 1950. 2013 Feb 1;190(3):977–86.

19. Akbar A, Reed J, Lacy K, Jackson S, Vukmanovic-Stejic M, Rustin M. Investigation of the Cutaneous Response to Recall Antigen in Humans in vivo. Clin Exp Immunol. 2013 Mar 27;

20. Watanabe R, Gehad A, Yang C, Scott LL, Teague JE, Schlapbach C, et al. Human skin is protected by four functionally and phenotypically discrete populations of resident and recirculating memory T cells. Sci Transl Med. 2015 Mar 18;7(279):279ra39-279ra39.

21. Pereira BI, Akbar AN. Convergence of Innate and Adaptive Immunity during Human Aging. Front Immunol [Internet]. 2016 [cited 2017 Dec 7];7. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2016.00445/full

22. Effros RB, Boucher N, Porter V, Zhu X, Spaulding C, Walford RL, et al. Decline in CD28+ T cells in centenarians and in long-term T cell cultures: A possible cause for both in vivo and in vitro immunosenescence. Exp Gerontol. 1994 Nov;29(6):601–9.

23. Henson SM, Macaulay R, Riddell NE, Nunn CJ, Akbar AN. Blockade of PD-1 or p38 MAP kinase signaling enhances senescent human CD8+ T-cell proliferation by distinct pathways. Eur J Immunol. 2015 May 1;45(5):1441–51.

24. Plunkett FJ, Franzese O, Belaramani LL, Fletcher JM, Gilmour KC, Sharifi R, et al. The impact of telomere erosion on memory CD8+ T cells in patients with X-linked lymphoproliferative syndrome. Mech Ageing Dev. 2005 Aug;126(8):855–65.

25. Reed JR, Vukmanovic-Stejic M, Fletcher JM, Soares MVD, Cook JE, Orteu CH, et al. Telomere Erosion in Memory T Cells Induced by Telomerase Inhibition at the Site of Antigenic Challenge In Vivo. J Exp Med. 2004 May 17;199(10):1433–43.

26. Ariotti S, Beltman JB, Chodaczek G, Hoekstra ME, van Beek AE, Gomez-Eerland R, et al. Tissue-resident memory CD8+ T cells continuously patrol skin epithelia to quickly recognize local antigen. Proc Natl Acad Sci U S A. 2012 Nov 27;109(48):19739–44.

27. Gamero AM, Ussery D, Reintgen DS, Puleo CA, Djeu JY. Interleukin 15 induction of lymphokine-activated killer cell function against autologous tumor cells in melanoma patient lymphocytes by a CD18-dependent, perforin-related mechanism. Cancer Res. 1995 Nov 1;55(21):4988–94.

28. Janas ML, Groves P, Kienzle N, Kelso A. IL-2 regulates perforin and granzyme gene expression in CD8+ T cells independently of its effects on survival and proliferation. J Immunol Baltim Md 1950. 2005 Dec 15;175(12):8003–10.

29. Kumar BV, Ma W, Miron M, Granot T, Guyer RS, Carpenter DJ, et al. Human Tissue-Resident Memory T Cells Are Defined by Core Transcriptional and Functional Signatures in Lymphoid and Mucosal Sites. Cell Rep. 2017 Sep 19;20(12):2921–34.

30. Hunger RE, Brönnimann M, Kappeler A, Mueller C, Braathen LR, Yawalkar N. Detection of perforin and granzyme B mRNA expressing cells in lichen sclerosus. Exp Dermatol. 2007;16(5):416–420.

31. Yawalkar N, Schmid S, Braathen L r., Pichler W j. Perforin and granzyme B may contribute to skin inflammation in atopic dermatitis and psoriasis. Br J Dermatol. 2001 Jun 1;144(6):1133–9.

32. Cheuk S, Schlums H, Sérézal IG, Martini E, Chiang SC, Marquardt N, et al. CD49a Expression Defines Tissue-Resident CD8+ T Cells Poised for Cytotoxic Function in Human Skin. Immunity [Internet]. 2017 Feb 14 [cited 2017 Feb 22];0(0). Available from: http://www.cell.com/immunity/abstract/S1074-7613(17)30031-6

33. Park SL, Zaid A, Hor JL, Christo SN, Prier JE, Davies B, et al. Local proliferation maintains a stable pool of tissue-resident memory T cells after antiviral recall responses. Nat Immunol. 2018 Feb;19(2):183–91.

34. Parkinson LG, Toro A, Zhao H, Brown K, Tebbutt SJ, Granville DJ. Granzyme B mediates both direct and indirect cleavage of extracellular matrix in skin after chronic low-dose ultraviolet light irradiation. Aging Cell. 2015 Feb 1;14(1):67–77.

35. Sadagopal S, Lorey SL, Barnett L, Sutherland D, Basham R, Erdem H, et al. Enhanced PD-1 Expression by T Cells in Cerebrospinal Fluid Does Not Reflect Functional Exhaustion during Chronic Human Immunodeficiency Virus Type 1 Infection. J Virol. 2010 Jan 1;84(1):131–40.

36. Saito H, Kuroda H, Matsunaga T, Osaki T, Ikeguchi M. Increased PD-1 expression on CD4+ and CD8+ T cells is involved in immune evasion in gastric cancer. J Surg Oncol. 2013 Apr 1;107(5):517–22.

37. Pallett LJ, Davies J, Colbeck EJ, Robertson F, Hansi N, Easom NJW, et al. IL-2high tissue-resident T cells in the human liver: Sentinels for hepatotropic infection. J Exp Med. 2017 May 19;jem.20162115.

38. Patel N, Vukmanovic-Stejic M, Suarez-Farinas M, Chambers ES, Sandhu D, Fuentes-Duculan J, et al. The impact of Zostavax vaccination on T cell accumulation and cutaneous gene expression in the skin of old humans after varicella zoster (VZV) antigen-specific challenge. J Infect Dis. 2018;(In press).

39. Vukmanovic-Stejic M, Chambers ES, Suárez-Fariñas M, Sandhu D, Fuentes-Duculan J, Patel N, et al. Enhancement of cutaneous immunity during aging by blocking p38 mitogen-activated protein (MAP) kinase-induced inflammation. J Allergy Clin Immunol. 2017 Nov 17;

40. Agius E, Lacy KE, Vukmanovic-Stejic M, Jagger AL, Papageorgiou A-P, Hall S, et al. Decreased TNF-{alpha} synthesis by macrophages restricts cutaneous immunosurveillance by memory CD4+ T cells during aging. J Exp Med. 2009 Aug 31;206(9):1929–40.

41. Bennett CL, Fallah-Arani F, Conlan T, Trouillet C, Goold H, Chorro L, et al. Langerhans cells regulate cutaneous injury by licensing CD8 effector cells recruited to the skin. Blood. 2011 Jun 30;117(26):7063–9.