Circulating CD4$^+$ memory T cells give rise to a CD69$^+$ resident memory T cell population in non-inflamed human skin.

Maria M. Klicznik[1], Ariane Benedetti[1] Angelika Stoecklinger[1], Daniel J. Campbell[2,3], Iris K. Gratz[1,2,4]

[1]Department of Biosciences, University of Salzburg, Salzburg, Austria

[2]Benaroya Research Institute, 1201 9th AVE, Seattle, WA 98101 USA

[3]Department of Immunology, University of Washington School of Medicine, Seattle WA 98109, USA.

[4]Division of Molecular Dermatology and EB House Austria, Department of Dermatology, Paracelsus Medical University Salzburg, Austria

Corresponding author: iris.gratz@sbg.ac.at

Abbreviations: T_{RM}, tissue resident memory T cells; PBMC, peripheral blood mononuclear cells; NSG, NOD-*scid IL2rγ*^{null} mice; ES, engineered human skin; huPBMC-ES-NSG, NSG mice with grafted human PBMC and ES

Abstract

The blood of human adults contains a pool of circulating $CD4^+$ memory T cells and normal human skin contains a $CD4^+CD69^+$ memory T cell population that produce IL17 in response to *Candida albicans*. Here we studied the generation of $CD4^+CD69^+$ memory T cells in human skin from a pool of circulating $CD4^+$ memory T cells.

Using adoptive transfer of human PBMC into a skin-humanized mouse model we discovered the generation of $CD4^+CD69^+$ resident memory T cells in human skin in absence of infection or inflammation. These $CD4^+CD69^+$ resident memory T cells were activated and displayed heightened effector function in response to *Candida albicans*. These studies demonstrate that a $CD4^+CD69^+$ T cell population can be established in human skin from a pool of circulating $CD4^+$ memory T cells in absence of infection/inflammation. The described process might be a novel way to spread antigen-specific immunity at large barrier sites even in absence of infection or inflammation.

Healthy human skin is protected by at least four phenotypically and functionally distinct subsets of memory T cells that are either recirculating or tissue-resident (Watanabe et al. 2015). Tissue resident memory T cells (T_{RM}) are maintained within the tissue and show superior effector function over circulating memory T cells. T_{RM} are defined by the expression of CD69 and/or CD103, both of which contribute to tissue retention (Mackay et al. 2015; Mackay et al. 2013). $CD4^+$ $CD69^+$ T_{RM} are generated in response to microbes such as *C.albicans* and provide protective immunity upon secondary infection in mouse skin, and a similar $CD4^+CD69^+$ T cell population that produced IL17 in response to heat killed *C.albicans ex vivo* was found in human skin (Park et al. 2018). By contrast, a population of fast migrating $CD69^-$ $CD4^+$ memory T cells entered murine dermis even 45 days after *C.albcians* infection when the infection was already resolved, hence they were likely not recruited in response to antigen. These data suggest that in absence of infection/inflammation, T cell memory in human skin is heterogenous and composed of resident and migratory memory T cell populations. In mouse parabiosis experiments $CD4^+$ memory T cells were able to modulate CD69 and/or CD103 expression, exit the skin and were found in equilibrium with the circulation. However, it is unclear if these cells could re-migrate to the skin and re-express CD69 upon entry into the tissue (Collins et al. 2016). Re-entry into the tissue to regain residency at distant skin sites would facilitate dispersion of protective immunity across large barrier organs such as the skin. The presence of a migratory memory T cell population in previously infected skin (Park et al. 2018) implies the existence of circulating memory T cells that can enter non-infected skin in steady state. So far it remains to be elucidated if human circulating $CD4^+$ memory T cells can give rise to resident memory T cells in human skin in absence of acute infection.

We therefore set out to test whether human peripheral blood mononuclear cells (PBMC) contain a circulating population of memory $CD4^+$ T cells with the potential to enter non-inflamed/non-infected skin sites distinct from the site of initial antigen-encounter, and assume

the phenotype and function of $CD69^+CD4^+$ resident memory T cells upon tissue entry. For this, we utilized a novel xenografting mouse model (detailed in Klicznik et al. linked submission) in which we adoptively transferred human PBMC into immunodeficient NOD-*scid IL2rγ*^{null} (NSG) mice (King et al. 2008) mice that carried engineered human skin (ES), which was devoid of any resident leukocytes. Within this model, designated huPBMC-ES-NSG, we found that transferred PBMC preferentially infiltrated the human but not murine skin, and that the human skin tissue promotes T cell maintenance and function in absence of inflammation or infection (Klicznik et al. linked submission). In line with previous reports (Clark et al. 2006), we found that human PBMC contained a proportion of skin homing $CLA^+CD45A^-CD4^+$ memory T cells and, upon adoptive transfer skin-tropic CLA^+CD4^+ T cells were significantly enriched in the ES compared to the spleen (Fig. 1a). Interestingly among these CLA^+ cells, the majority expressed the T_{RM} marker CD69 in the ES (Fig.1b). Importantly, the increased proportion of $CD4^+$ T cells expressing CD69 in the ES was likely not due to tissue-derived inflammatory cues (Mackay et al. 2012) such as IL1α, IL1β, IL18, IL23, IFNγ, TNFα and TNFβ, which were found at equal or lower levels as in healthy human skin (Supplement Fig.1). Nor was CD69 expression due to local activation of T cells by antigen-presenting cells (APC), which are virtually absent in the ES of this PBMC-ES-NSG model (Klicznik et al., linked submission). Some of the $CD69^+$ cells in the ES expressed CD103, a marker of human $CD4^+CLA^+$ T_{RM} (Klicznik et al. 2018) (Fig. 1c). Additionally, $CD69^-$ but not $CD69^+$ cells within the ES and the spleen expressed CD62L, a marker of circulating memory T cells (Watanabe et al. 2015) (Fig. 1d). These two distinct memory subsets, a resident memory-like $CD4^+$ $CD69^+$ and a $CD69^-CD62L^+$ migratory population, replicate two major sets of memory T cells in healthy human skin (Park et al. 2018). Taken together, these data show that circulating $CD4^+$ memory T cells have the ability to up-regulate markers of tissue-residency upon entry into non-inflamed/non-infected human skin.

$CD69^+$ T_{RM} represent a transcriptionally, phenotypically, and functionally distinct T cell subset at multiple barrier sites with enhanced capacity for the production of effector cytokines compared to circulating cells (Kumar et al. 2017). A proportion of skin-tropic human $CD4^+$ T cells is specific for *C.albicans* (Acosta-Rodriguez et al. 2007), and to compare the function of $CD69^+$ and $CD69^-$ $CD4^+$ T cells infiltrating the ES, we injected the ES after adoptive transfer of human PBMC with autologous monocyte derived DC that were pulsed with heat killed *C.albicans.* (Fig. 2a). Upon antigen-challenge $CD69^+$ memory T cells produced higher levels of effector cytokines such as IL17, IFNγ, TNFα and IL2 (Fig. 2b-e), which is in line with a rapid memory response upon secondary infections.

Here we show for the first time that circulating human memory $CD4^+$ T cells can enter skin in the absence of infection or inflammation and give rise to a $CD69^+CD4^+$ memory T cell population (Fig.1b). Importantly these recently immigrated resident memory T cells produce substantially higher levels of effector cytokines (Fig.2b-e) in response to *C.albicans,* indicating that they represent the main responding memory population, which is in line with recent findings in murine skin (Park et al. 2018). It remains to be elucidated whether $CD69^+CD4^+$ memory T cells reside within the ES long-term or if they can modulate CD69 expression and leave the tissue again. The ability of circulating $CD4^+$ memory T cells to enter non-inflamed skin sites, where they assume the phenotypical and functional profile of resident memory T cells reveals a novel mechanism to generate and disperse protective memory in large barrier organs, such as the skin.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions:

IGK, DJC and MMK conceptualized the study, MMK designed and performed the experiments, MMK and AB acquired the data, GS acquired human samples, MMK performed data analysis, MMK and IGK interpreted data and wrote the manuscript. All authors reviewed the final version of the manuscript.

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Figure legends:

Figure 1: CD4$^+$ memory T cells derived from the circulation up-regulate markers of residency and skin-tropism in engineered human skin. Engineered skin (ES) was generated on immunodeficient NSG mice. After complete wound healing (> 30 days) 3x10^6 autologous human PBMC were adoptively transferred. Single cell suspensions of spleen and ES of huPBMC-ES-NSG were prepared 21 days after adoptive transfer and analyzed by flow cytometry together with ingoing PBMC (**a-b**) Representative flow cytometry analysis and graphical summary of expression of indicated markers by CLA$^+$CD45RA$^-$CD4$^+$CD3$^+$ live leukocytes. Mean +/- SD, statistical significance determined by paired t-test; (**c-d**) Representative flow cytometry analysis and graphical summary of the expression of indicated markers by CD69$^+$ or CD69$^-$ cells in ES and spleen (S) gated on CLA$^+$CD45RA$^-$CD4$^+$CD3$^+$ live leukocytes. Mean +/- SD; one-way RM ANOVA with Dunett's test for multiple comparison to CD69$^+$ cells in ES.

Figure 2: CD69$^+$CD4$^+$ cutaneous memory T cells show a distinct cytokine and activation profile in response to *C.albicans*. ES of huPBMC-ES-NSG mice was intradermally injected with heat killed *C.albicans* loaded onto autologous moDCs. Single cell suspensions of the ES were prepared and analyzed for intracellular cytokine secretion upon PMA/ionomycin stimulation (**a**) Experimental set-up; (**b-e**) Graphical summary of the proportion of positive cells of the indicated markers among CD69$^+$ or CD69$^-$ cells gated on CLA$^+$CD45RA$^-$CD4$^+$CD3$^+$ live leukocytes. Mean +/- SD; statistical significance determined by paired Student's t-test

Supplementary Figure 1: Analysis of pro-inflammatory cytokine levels in ES of huPBMC-ES-NSG mice in comparison to healthy human skin. ES and skin were

weighed and analyzed for indicated cytokine levels using a bead-based multicomponent assay. Graphical summary shows concentration of indicated cytokine per mg skin; Mean +/- SD; statistical significance determined by paired Student's t-test

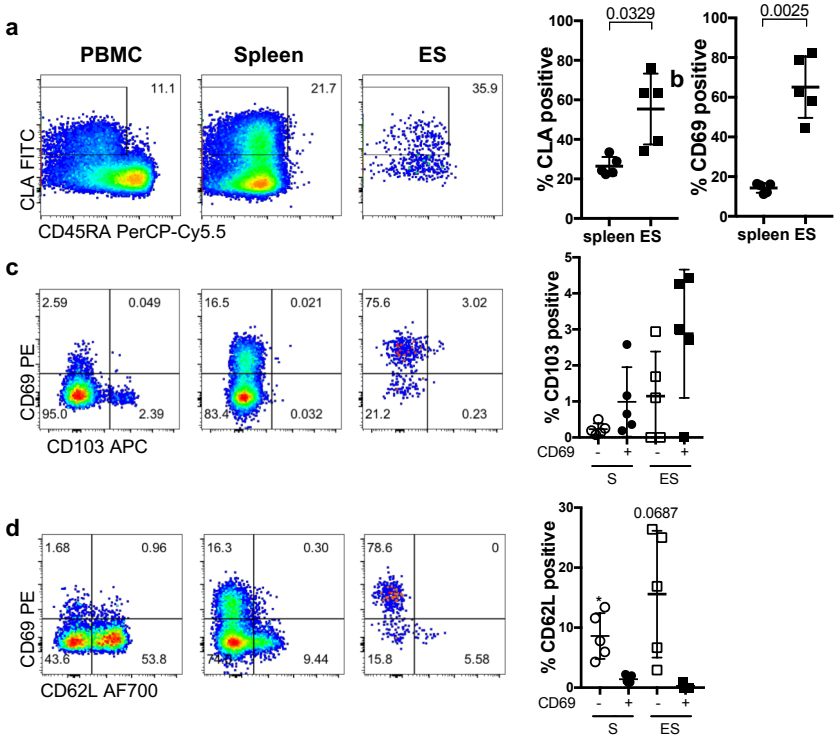


Figure 1: CD4⁺ memory T cells derived from the circulation upregulate markers of residency and skin-tropism in engineered human skin.

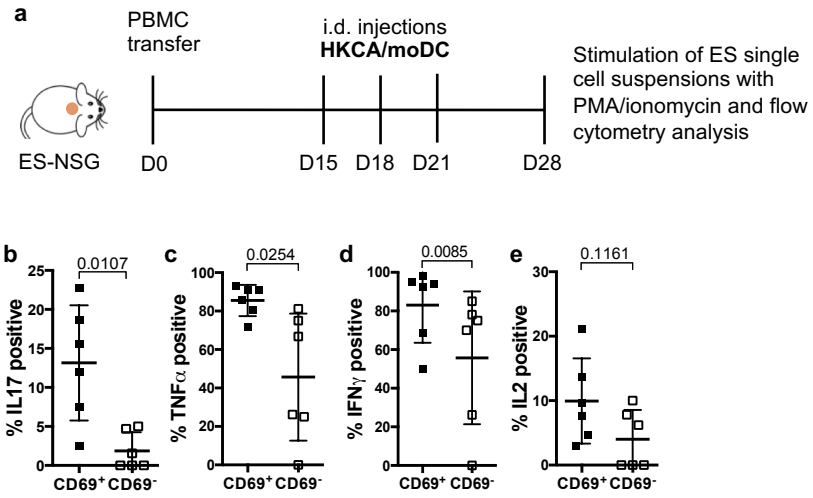
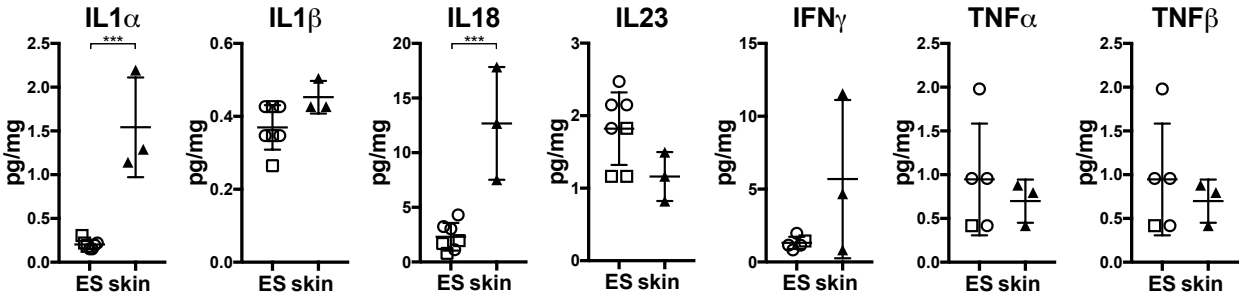


Figure 2: CD69⁺ CD4⁺ memory T cells show a distinct cytokine and activation profile in response to *C.albicans*.



Supp. Figure 1: Inflammatory cytokines are secreted at lower or equal levels in ES compared to healthy human skin

Material and Methods

Mice. Animal studies were approved by the Austrian Federal Ministry of Science, Research and Economy. NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were obtained from The Jackson Laboratory and bred and maintained in a specific pathogen-free facility in accordance with the guidelines of the Central Animal Facility of the University of Salzburg.

Human specimens. Normal human skin was obtained from patients undergoing elective surgery, in which skin was discarded as a routine procedure. Blood and/or discarded healthy skin was donated upon written informed consent at the University Hospital Salzburg, Austria.

PBMC isolation for adoptive transfer into NSG recipients and flow cytometry. Human PBMC were isolated from full blood using Ficoll-Hypaque (GE-Healthcare; GE17-1440-02) gradient separation. PBMC were frozen in FBS with 10% DMSO (Sigma-Aldrich; D2650), and before adoptive transfer thawed and rested overnight at 37°C and 5% CO_2 in RPMIc (RPMI 1640 (Gibco; 31870074) with 5% human serum (Sigma-Aldrich; H5667 or H4522), 1% penicillin/streptomycin (Sigma-Aldrich; P0781), 1% L-Glutamine (Gibco; A2916801), 1% NEAA (Gibco; 11140035), 1% Sodium-Pyruvate (Sigma-Aldrich; S8636) and 0.1% β-Mercaptoethanol (Gibco; 31350-010). Cells were washed with PBS and $3x10^6$ PBMC/mouse intravenously injected. Murine neutrophils were depleted with mLy6G (Gr-1) antibody (BioXcell; BE0075) as described before (Racki et al. 2010).

Generation of engineered skin (ES). Human keratinocytes and fibroblasts were isolated from human skin and immortalized using human papilloma viral oncogenes E6/E7 HPV as previously described (Merkley et al. 2009). Cells were cultured in Epilife (Gibco, MEPICF500) or DMEM (Gibco; 11960-044) containing 2% L-Glutamine, 1% Pen/Strep,

10% FBS, respectively. Per mouse, 1-2x10^6 keratinocytes were mixed 1:1 with autologous fibroblasts in 400µl MEM (Gibco; 11380037) containing 1% FBS, 1% L-Glutamine and 1% NEAA for *in vivo* generation of engineered skin as described (Wang et al. 2000).

T cell isolation from skin tissues for flow cytometry. Healthy human skin and ES were digested as previously described (Sanchez Rodriguez et al. 2014). Approximately 1cm^2 of skin was digested overnight in 5%CO_2 at 37°C with 3ml of digestion mix containing 0.8mg/ml Collagenase Type 4 (Worthington; #LS004186) and 0.02mg/ml DNase (Sigma-Aldrich; DN25) in RPMIc. ES were digested in 1ml of digestion mix. Samples were filtered, washed and stained for flow cytometry or stimulated for intracellular cytokine staining.

Flow cytometry. Cells were stained in PBS for surface markers. For detection of intracellular cytokine production, spleen and skin single cell suspensions and PBMC were stimulated with 50 ng/ml PMA (Sigma-Aldrich; P8139) and 1 µg/ml Ionomycin (Sigma-Aldrich; I06434) with 10 µg/ml Brefeldin A (Sigma-Aldrich; B6542) for 3.5 hrs. For permeabilization and fixation Foxp3 staining kit (Invitrogen; 00-5523-00) were used. Data were acquired on BD FACS Canto II (BD Biosciences) or Cytoflex LS (Beckman Coulter) flow cytometers and analyzed using FlowJo software (Tree Star, Inc.) A detailed list of the used antibodies can be found in the Supplements.

Statistical analysis. Statistical significance was calculated with Prism 7.0 software (GraphPad) by RM ANOVA with Dunett's multiple comparisons test, or by un-paired student's t-test as indicated. Error bars indicate mean $^+$/- standard deviation.

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Table S1: Detailed list of antibodies and reagents

Tissue preparation

Reagent	Company	Catalog number
Collagenase Type 4	Worthington	LS004186
DNAse	Sigma-Aldrich	DN25
RPMI 1640	Gibco	31870074
human serum	Sigma-Aldrich	H5667/H4522
Penicillin/streptavidin	Sigma-Aldrich	P0781
L-Glutamine	Gibco	A2916801
NEAA	Gibco	11140035
Sodium-Pyruvat	Sigma-Aldrich	S8636
β-Mercaptoethanol	Gibco	31350-010
PBS	Gibco	14190169
Ficoll Paque Plus	GE-Healthcare	GE17-1440-02

Cellular activation

Reagent	Company	Catalog number
Brefeldin A	Sigma-Aldrich	B6542
Foxp3 / Transcription Factor Staining Buffer Set	Invitrogen	00-5523-00
Ionomycin	Sigma-Aldrich	I06434
PMA	Sigma-Aldrich	P8139
Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex™	Invitrogen	EPX-450-12171-901

Skin cell culture and transplantation

Reagent	Company	Catalog number
Epilife	Gibco	MEPICF500
DMEM	Gibco	11960-044
MEM	Gibco	11380037
TrypLE express	Gibco	12604021

Tissue preparation from mice

Reagent	Company	Catalog number
BD Pharm Lyse	BD	555899

Antibodies

Reagent	Company	Catalog number
CLA FITC (HECA-452)	Biolegend	321306
CD1a FITC (HI149)	BioLegend	300104
CD3 BV421 (UCHT1)	BioLegend	300434
CD3 bv605 (SK7)	BioLegend	344835
CD4 PE-594 (RPA-T4)	BioLegend	300548
CD4 PE-Cy5.5 (RPA-T4)	BioLegend	300510
CD8 PE (OKT8)	eBioscience	12-0086-42
CD14 bv421 (M5E2)	BioLegend	301830
CD45 BV785 (HI30)	BioLegend	304048
CD45RAPerCP-Cy5.5 (HI100)	BioLegend	304122
CD62L AF700 (DREG-56)	Biolegend	304820
CD69 PE (FN50)	BioLegend	310906
CD69 PerCP/eFluor710 (FN50)	eBioscience	46-0699-42
CD86 PE (B7-2)	eBioscience	12-0869-41
CD103 APC (Ber-ACT8)	BioLegend	350216
CCR7 bv605 (G043H7)	BioLegend	353224
IFNg PE-Cy7 (B27)	BioLegend	506518
IL-2 PE (MQ1-17H12)	eBioscience	12-7029-42
IL17A APC (BL168)	BioLegend	512334
TNFa FITC (Mab11)	BioLegend	502915
Fixable Viability Dye eFluor™ 780	eBioscience	65-0865-14
mLy6G (Gr-1) InVivoMab RB6-8C5	BioXcell	BE0075