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Supplemental Information

CD49a Expression Defines Tissue-Resident CD8$^+$ T Cells Poised for Cytotoxic Function in Human Skin

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SUPPLEMENTAL FIGURES

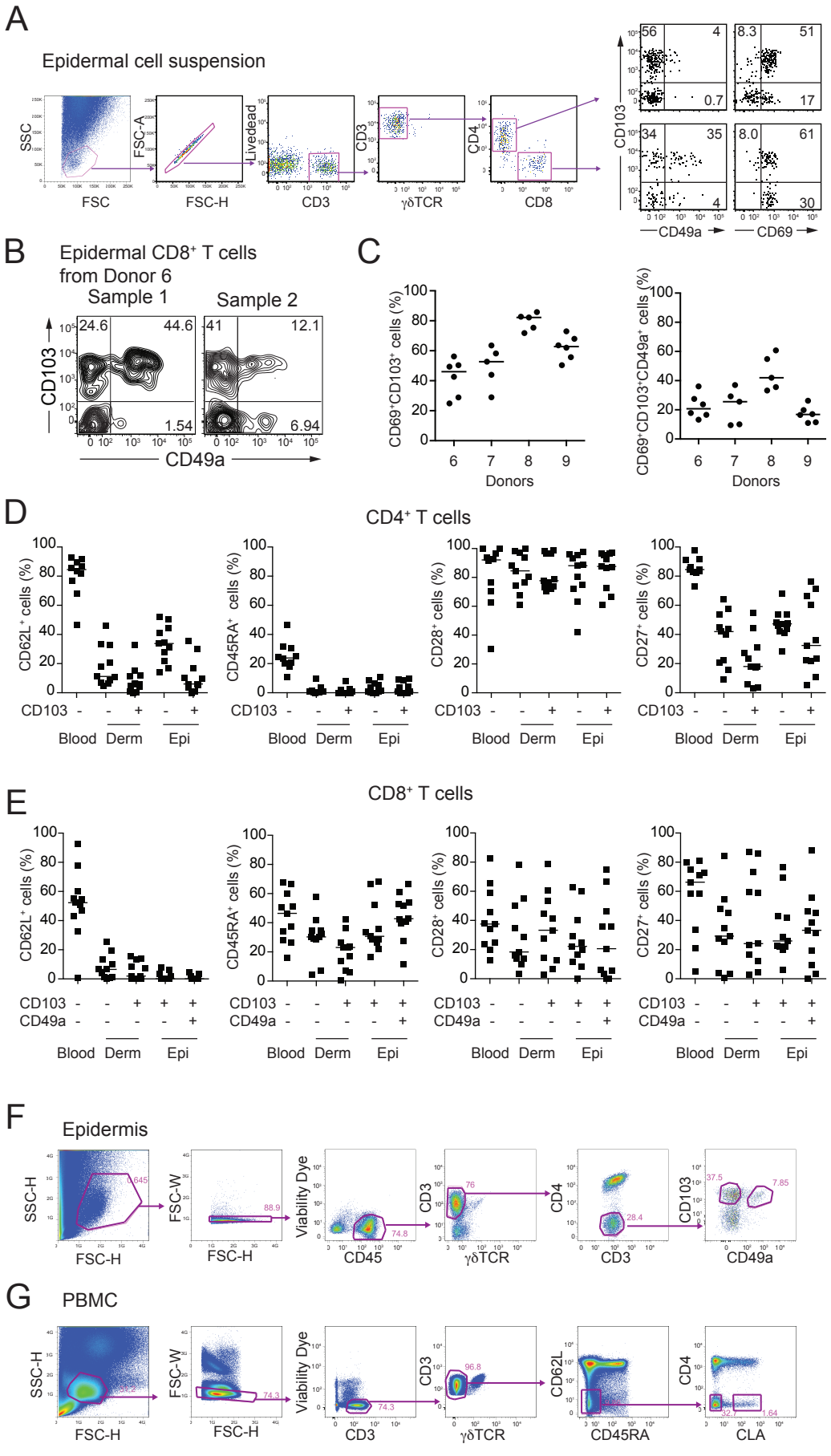

Figure S1. Cheuk *et al*.

Figure S1, related to Figure 1. Phenotypic profile and differentiation of epidermal T cells in healthy skin.

(A) Gating strategy for epidermal cell suspension for flow cytometry analysis. T cells were gated on live, singlet, $CD3^+$ $\gamma\delta TCR^-$ T cells and further subgated according to CD4 and CD8 expression. CD103, CD69 and CD49a expression was assessed within the $CD4^+$ or $CD8^+$ T cell gates. (B) Two representative FACS plots show CD103 and CD49a expression on epidermal $CD8^+$ T cells from two skin samples from different sites of the same individual. (C) Graphs depict the proportion of (right) $CD103^+CD69^+$ and (left) $CD103^+CD69^+CD49a^+$ among epidermal $CD8^+$ T cells from 4 healthy individuals. Five or six samplings at least 20cm apart were analyzed. (D, E) Graphs depict the frequency of CD62L-, CD45RA-, CD28- and CD27-expressing cells among (D) $CD4^+$ or (E) $CD8^+$ T cell subpopulations from blood, dermis and epidermis (n=11), as indicated. (F, G) Gating strategy for cell sorting for TCR and RNA sequencing. (F) In epidermis, $CD8^+CD103^+CD49^-$ and $CD8^+CD103^+CD49^+$ Trm cells were gated as live, singlet $CD45^+CD3^+\gamma\delta TCR^-CD4^-CD103^+$ cells and subsequently sorted according to CD49a expression. (G) In PBMC, two memory T cell subpopulations were gated as live, singlet, $CD3^+\gamma\delta TCR^-CD62L^-CD45RA^-CD4^-$ cells and further sorted according CLA expression.

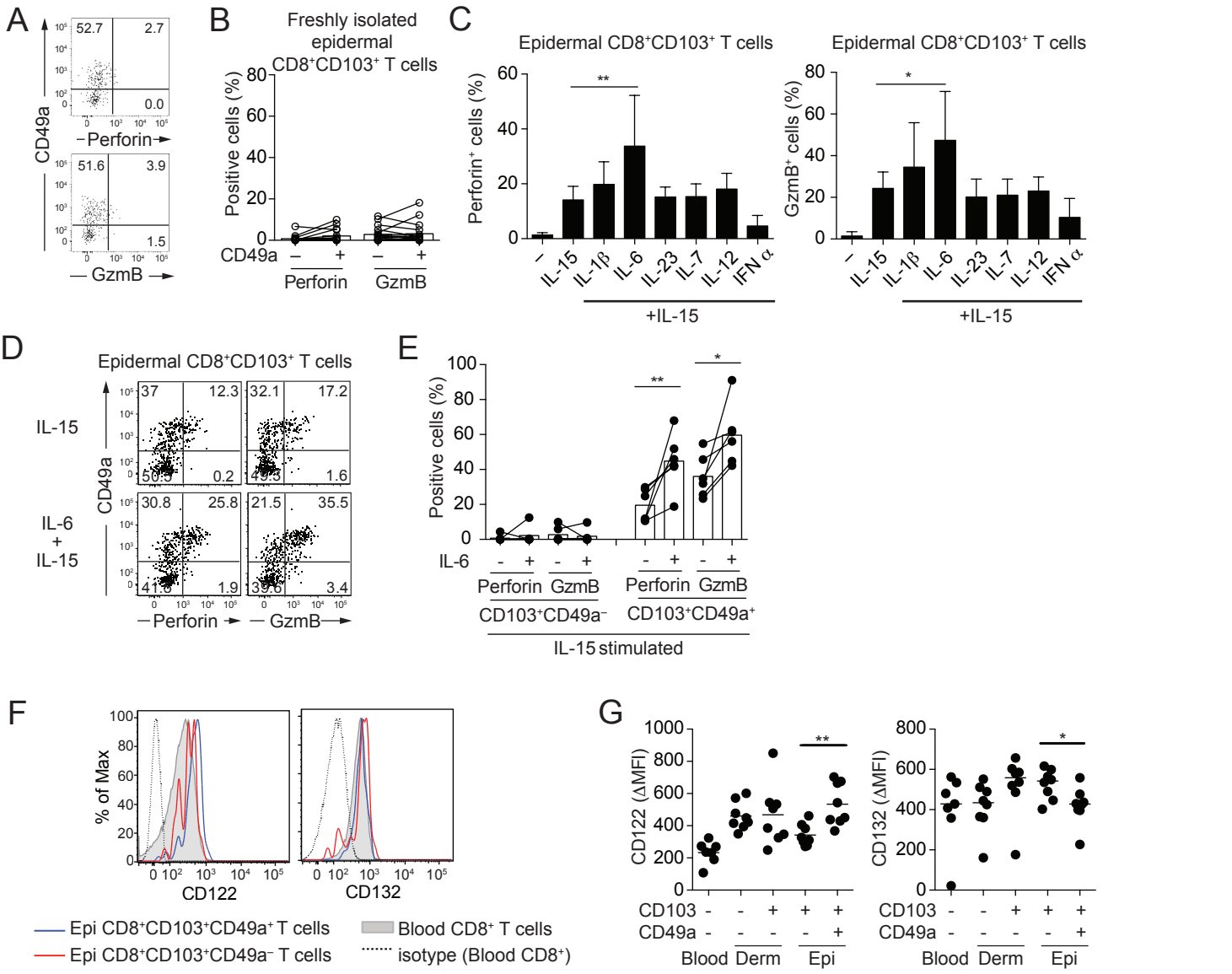

Figure S2, related to Figure 3. Expression of cytotoxic granule constituents upon cytokine stimulation and expression of IL-2 receptor subunit in epidermal Trm cells.

(A, B) Perforin and granzyme B expression on freshly isolated epidermal CD8⁺CD103⁺ T cells. (A) Representative FACS plots show the expression of perforin and granzyme B against CD49a on freshly isolated epidermal CD8⁺CD103⁺ T cells. (B) Graphs depicts the proportion of perforin- and granzyme B-expressing cells among CD8⁺CD103⁺CD49a⁻ and CD8⁺CD103⁺CD49a⁺ Trm cells from freshly prepared epidermal cell suspension (n=17). (C-E) IL-6 further enhances perforin and granzyme B expression in IL-15 stimulated epidermal T cells (n=5). (C) Bar chart depicts the proportion of (left) perforin- and(right) granzyme B-expressing cells left unstimulated or incubated with IL-7 (20 ng/ml), IL-1β (20 ng/ml), IL-6 (20 ng/ml), IL-23 (20 ng/ml), IL-12, (50 ng/ml), IFN-α (2000 U/ml) in the presence of IL-15 (20 ng/mL) for 48 hours. Mean ± SD is depicted. Dunn's multiple comparison tests of each condition against IL-15 stimulated was performed. (D) Representative FACS plot showing the expression of perforin and Granzyme B in relation to CD49a in IL-15 treated epidermal CD8⁺CD103⁺ Trm cells with or without IL-6 stimulation. (E) Graph depicts the proportion of perforin- and granzyme B-expressing cells among CD8⁺CD103⁺CD49a⁻ and CD8⁺CD103⁺CD49a⁺ Trm cell from epidermal cell suspension incubated in IL-6 (20 ng/ml) or IL-6 (20 ng/ml) + IL-15 (20 ng/ml). Wilcoxon test. (F-G) Expression of IL-2Rβ (CD122) and IL-2Rγ (CD132, common γ-chain) in blood, dermal and epidermal CD8⁺ T cells subpopulations from healthy donors. (F) Representative histograms of CD122 and CD132 expression of peripheral blood CD8⁺ T cells, epidermal CD8⁺CD103⁺CD49⁺ and CD8⁺CD103⁺CD49a⁻ Trm cells. (G) Plot showing the expression level (ΔMFI= MFI-MFI of isotype) of CD122 and CD132 in the indicated CD8 T cells subpopulations (n=8). Wilcoxon test. *p<0.05 and ** p<0.01.

Figure S2. Cheuk *et al*.

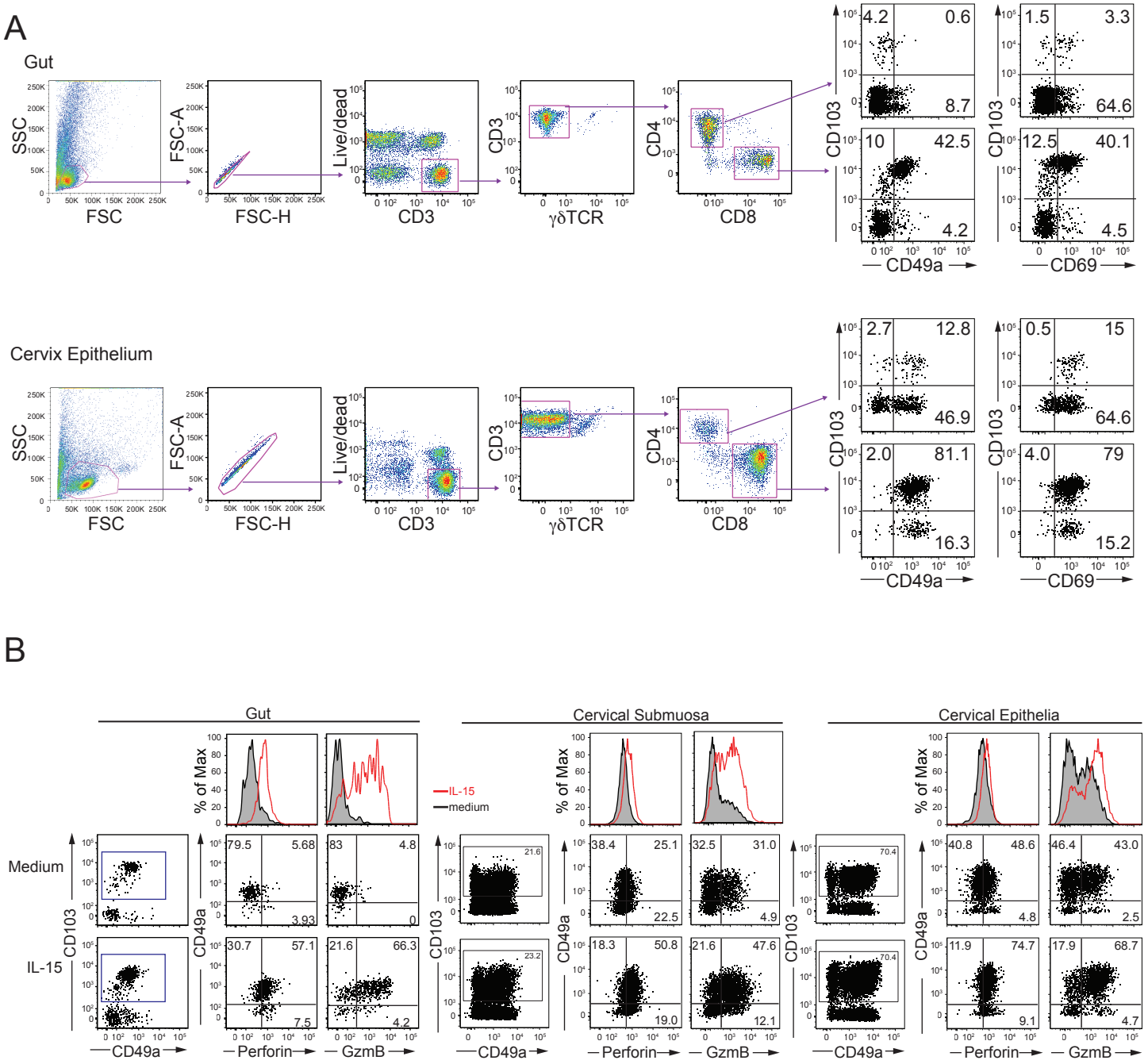

Figure S3, related to Figure 4. Profiling of Trm cells in the gut and cervix.

(A) Gating strategy for intestinal and cervical cell suspension for flow cytometry analysis. T cells were gated on live, singlet $CD3^{+}\gamma\delta TCR^{-}$ cells and further subgated on $CD4^{+}$ or $CD8^{+}$ cells. CD103, CD69 and CD49a expression were assessed within the $CD4^{+}$ or $CD8^{+}$ T cell gates. (B) Gating strategy of $CD8^{+}CD103^{+}$ T cells and representative histograms and FACS plots on perforin and granzyme B expression in $CD8^{+}$ T cells from gut, cervical submucosa and epithelia after 24 hours incubation with or without IL-15 (20ng/mL) as indicated.

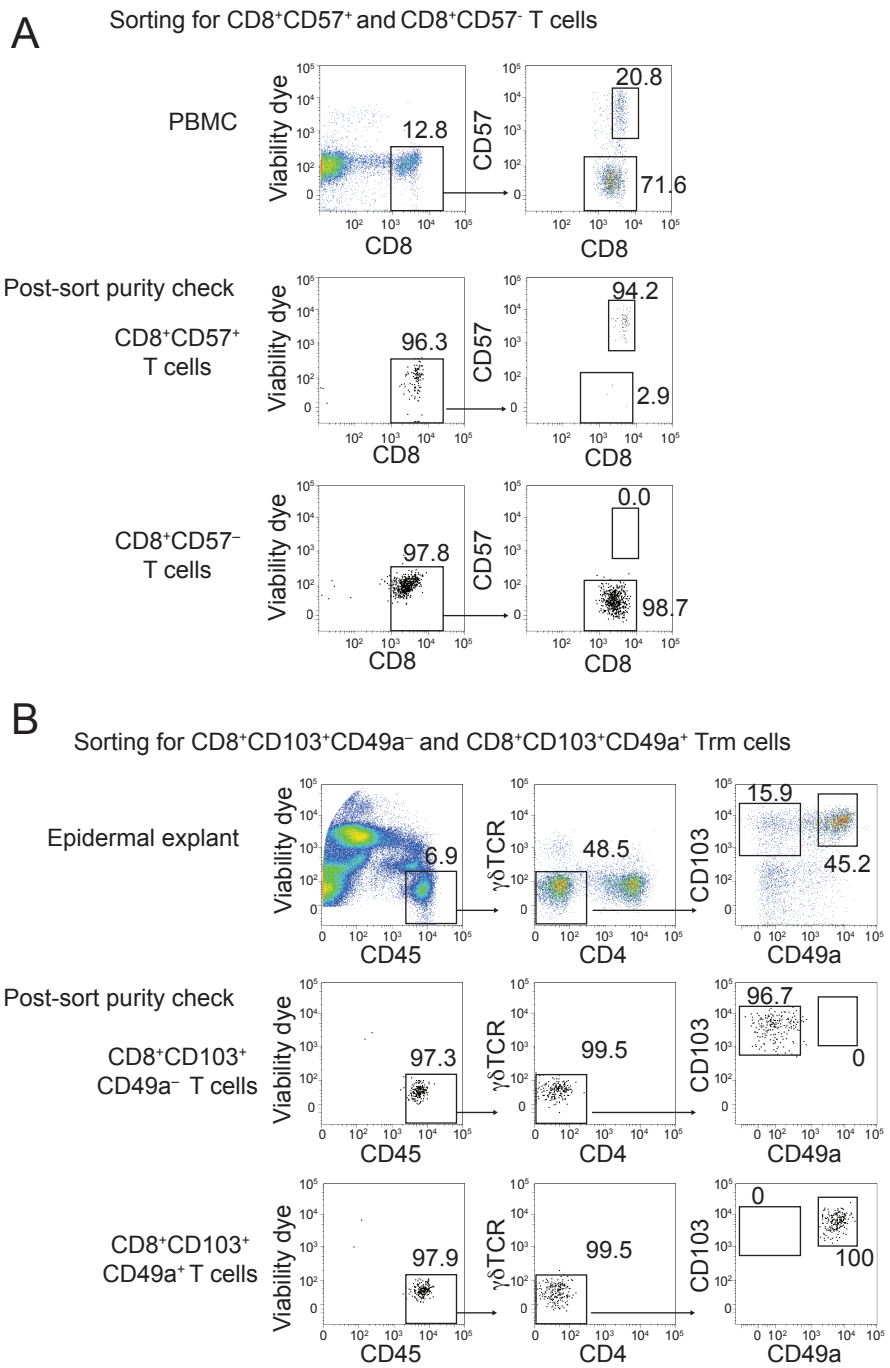

Figure S4, related to Figure 5. Cell sorting for cytotoxicity assays.

(A) Gating strategy and representative post-sort purity check for blood-derived $CD8^+CD57^-$ and $CD8^+CD57^+$ T cells. Live, singlet, $CD8^{hi}$, $CD57^+$ or $CD57^-$ cells were sorted. (B) Gating strategy and representative post-sort purity check for epidermal $CD8^+CD49a^-$ and $CD8^+CD49a^+$ Trm cells. Live, singlet, $CD45^{hi}\gamma\delta TCR^-CD4^-CD103^+$, $CD49a^-$ or $CD49a^+$ cells were sorted.

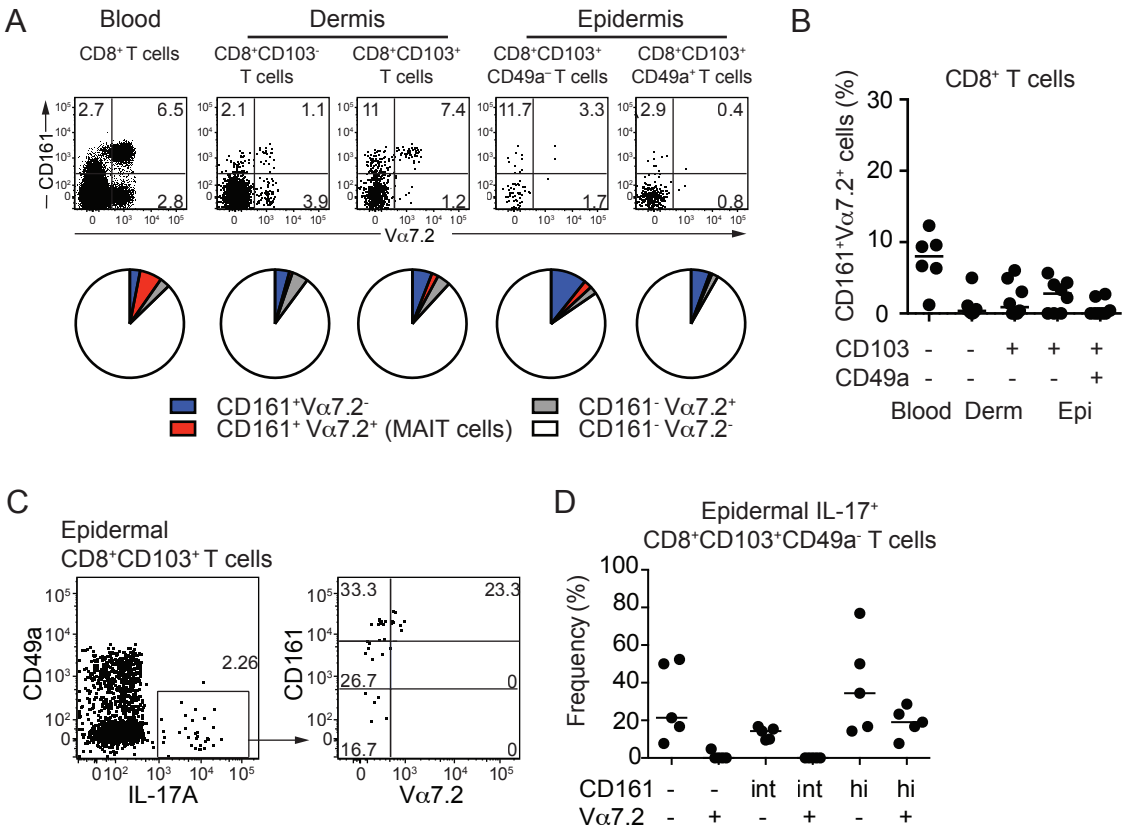

Figure S5, related to Figure 6. MAIT cells do not constitute a large population of CD8⁺ T cells in healthy skin.
(A) Representative FACS plots (upper) and pie charts (lower) illustrating expression CD161 and Vα7.2 in the corresponding CD8⁺ T cell subpopulation from blood, dermis and epidermis from healthy donors (n=6). (B) Plot depicting the proportion of CD161⁺Vα7.2⁺ MAIT cells among the indicated CD8⁺ T cells subpopulation. (C) Representative FACS plots show the gating strategy for evaluating CD161 and Vα7.2 expression on IL-17A-producing CD8⁺CD103⁺CD49a⁻ T cells from PMA and ionomycin stimulated epidermal cell suspension from healthy skin. (D) Graph depicts the relative proportion of IL-17A-producing CD8⁺CD103⁺CD49a⁻ T cells subpopulations according to expression of CD161 and Vα7.2 expression from PMA and ionomycin stimulated epidermal cell suspension from healthy skin (n=5).

Figure S5. Cheuk *et al*.

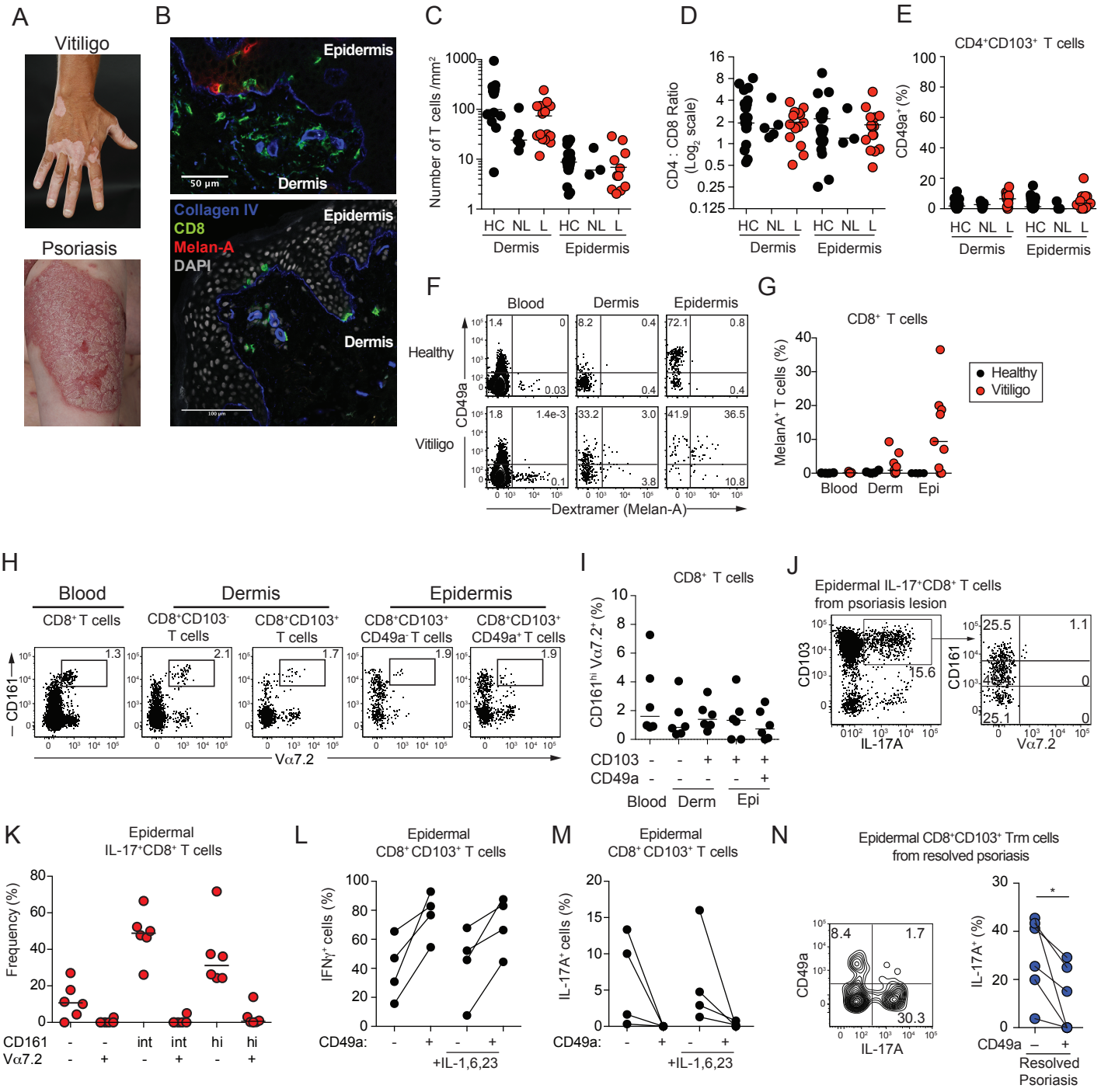

Figure S6. Cheuk *et al*.

Figure S6, related to Figure 7. Enumberation of T cells in vitiligo lesions and IL-17A production in CD8 T cells in psoriasis.

(A) Representative clinical pictures of macroscopic vitiligo and psoriasis lesions. (B) Confocal microscopy depicting CD8 (green), Melan A (red) and collagen IV (blue) in lesional vitiligo at the border of pathology (upper picture) and in depigmented skin (lower picture). (C) Plot depicting the density of dermal and epidermal T cells per surface area (mm^2) of healthy skin or vitiligo lesions determined by flow cytometry. (D) Plot showing the CD4 to CD8 ratio of dermal and epidermal T cells from healthy skin or vitiligo lesions. (E) Graph depicting the proportion of $CD49a^+$ cells among dermal and epidermal $CD4^+CD103^+$ T cells in skin of healthy donors (HC) or non-lesional (NL) and lesional (L) skin of vitiligo patients. (F) Representative FACS plot of dextramer (Melan-A) staining and CD49a expression in CD8 T cells from peripheral blood, dermis and epidermis of HLA-A2 positive healthy donors or vitiligo patients. (G) Graph depicting the proportion of dextramer positive cells among $CD8^+$ T cells in peripheral blood and $CD8^+CD103^+CD49a^+$ T cells from dermis or epidermis of healthy donors (n=5) or vitiligo patients (n=9). (H, I) Representative FACS plots showing expression of CD161 and Vα7.2 and the proportion of $CD161^{hi}$ $Vα7.2^+$ MAIT cells among the indicated $CD8^+$ T cells subpopulations from blood, lesional dermis or epidermis from psoriasis patients. (J) Representative FACS plots showing expression of CD161 and Vα7.2 for IL-17A-expressing $CD8^+CD103^+$ T cells from PMA and ionomycin stimulated epidermal cell suspension of psoriasis lesions. (K) Plot showing the proportion of $CD8^+$ T cells subpopulations according to their CD161 and Vα7.2 expression among IL-17 producing $CD8^+CD103^+$ cells from PMA and ionomycin stimulated epidermal cell suspension from psoriasis lesion. (L, M) Epidermal cell suspensions were incubated in medium or stimulated by a cocktail of IL-1β (20ng/mL), IL-6 (20ng/mL) and IL-23 (20ng/mL) for three days and cytokine expression were assessed upon short-term PMA + ionomycin stimulation for 4.5 hours (n=4). Plots depicting the proportion of (L) IFN-γ- and (M) IL-17A-expressing in $CD8^+CD103^+CD49a^{+/-}$ T_{RM} cells were depicted. (N) Representative contour plots and dot plots showing IL-17A expression in epidermal $CD8^+CD103^+CD49a^-$ and $CD8^+CD103^+CD49a^+$ T_{RM} cells from resolved psoriasis (n=6). Wilcoxon test, * $p<0.05$. (O) Schematic of Trm cell composition in healthy skin, vitiligo and psoriasis. In healthy human skin, $CD49a^+$ Trm cells are spatially restricted to epidermis and respond to IL-15 or TCR stimulation with induction of cytotoxic proteins and IFN-γ. Additionally, healthy epidermis harbors $CD49a^-$ Trm cells that respond to activation with IL-17 production. In vitiligo, activated $CD49a^+$ Trm cells expressing perforin and granzyme B accumulate and translocate to dermis. In psoriasis, both $CD49a^-$ and $CD49a^+$ Trm cells infiltrate hypertrophic epidermis and predominately produce IL-17 and IFN-γ, respectively.

Table S1, related to Figure 2. List of upregulated genes ($CD49a^+$ > $CD49a^-$).

A List of upregulated genes in epidermal CD49a+ Trm ($CD49a^+$ > $CD49a^-$), with unadjusted P-vlaue < 0.05 from using likelihood ratio test in the EdgeR. Genes with adjusted p-value < 0.05 by FDR method were marked by *

Table S1 is attached as a separated file: Table S1.xlsx

Table S2, related to Figure 2. List of downregulated genes ($CD49a^+$ < $CD49a^-$).

A List of downregulated genes in epidermal CD49a+ Trm ($CD49a^+$ < $CD49a^-$) with unadjusted P-vlaue < 0.05 from using likelihood ratio test in the EdgeR. Genes with adjusted p-value < 0.05 by FDR method were marked by *

Table S2 is attached as a separated file: Table S2.xlsx

Table S3, related to Figure 1-7. Clinical data for vitiligo, psoriasis and healthy donors.

Clinical data of patients and healthy donors included in this study.

	Number of donors	Area affected	PASI	PsA	Years of Disease	Age	Family history
Vitiligo	21	26.3% (s.d. 27.3)	-	-	25.1 yrs (s.d. 14.9)	51.9 yrs (s.d. 14.4)	12 (57.1%)
Psoriasis	28		7.7 (s.d. 5.2)	7 (26.9%)	22.2 yrs (s.d. 13.3)	53.1 yrs (s.d. 14.2)	17 (68.6%)
Healthy controls	91	-	-	-	-	43.5 yrs (s.d. 12.8)	-

Table S4, related to Figure 1, 3-7. Antibodies used in this study.

Information of antibodies used in this study

Marker	Clone	Company
CD45RA	H100	BD Bioscience
CD49a	SR84	BD Bioscience
CD62L	Dreg-56	BD Bioscience
CD69	FN50	BD Bioscience
CD8	RPA-T8	BD Bioscience
IFNγ	B27	BD Bioscience
TNF	MAb11	BD Bioscience
IL-2	5344.111	BD Bioscience
CCR6	11A9	BD Bioscience
CD27	M-T271	BD Bioscience
CD45	J33	Beckman Coulter
TCRγδ	IMMU510	Beckman Coulter
CD103	Ber-ACT8	Biolegend
CD3	OKT3	Biolegend
CD4	OKT4	Biolegend
CLA	HECA-452	Biolegend
Granzyme A	CB9	Biolegend
Perforin	dG9	Biolegend
CXCR3	G025H7	Biolegend
CD161	HP-3G10	Biolegend
Vα7.2	3C10	Biolegend
CD132	TUGh4	Biolegend
CD122	TU27	Biolegend
CD28	CD28.2	Biolegend
CD8	SK1	Biolegend
IL-17A	eBio64DEC17	eBioscience
TCRγδ	B1.1	eBioscience
CD3	S4.1	Invitrogen
CD8	3B5	Invitrogen
Granzyme B	GB11	Invitrogen
CD4	S3.5	Invitrogen
IL23R	BAF1400	R&D system

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell sorting

For RNA or DNA extraction, freshly isolated cell suspension, equivalent to 20 – 40 cm^2 surface area, were stained for CD45 CD4, γδTCR, CD3, CD103 and CD49a at 4°C; and were kept on ice before sorting. For DNA extraction, PBMCs were stained for CD3, CD4, γδTCR, CD45RA, CD62L and CLA. Sorting into Qiazol (Qiagen) for later RNA extraction, or Cell lysis Solution from Qiagen Puregene Kit (Qiagen) for DNA extraction, was performed within 2-4 hours using MoFlo XDP (Beckman Coulter) cell sorter. Sorted Qiazol-cell lysate were kept at -80 °C before extraction while lysate for DNA extraction were kept at 4 °C.

For functional experiments investigating cytotoxicity at least $6x10^4$ cells per epidermal T cell subset was sorted using BD FACZJAZZ (BD) from short-term epidermal explants (McGovern et al., 2014). In brief, epidermal sheets, equivalent to 150 – 300 cm^2 skin area were placed in complete RPMI medium with or without IL-15 (20 ng/ml) for 48 hours after which emigrated cells were collected and stained. For time-course experiments, $5x10^3$ – $1x10^4$ sorted $CD8^+CD103^+$ Trm were incubated in 96-well V-bottom plates in a final volume of 100 µl. The purity of sorted cells was at least 90%. Sorted cells were maintained in complete medium overnight before further experiment.

TCR clonality analysis

Shared clonotypes were calculated by the percentage of number of shared clonotypes between two populations over the total number of unique clonotypes of the two populations. The percentage of shared reads was calculated as the average of summation of read frequencies of the shared clonotypes shared in each of the two analyzed populations as previously described (Becattini et al., 2015). Cumulative frequencies of the top 10 clones in epidermal $CD8^+$ $CD103^+CD49a^+$ Trm and $CD8^+$ $CD103^+CD49a^-$ Trm from each donor were tracked in the dermal and peripheral blood populations.

Transcriptome analyses

Differential gene expression analysis was performed using likelihood ratio test and FDR-correction as implemented in the EdgeR package (Robinson et al., 2010) with rounded expected read count data by RSEM (Li and Dewey, 2011). For functional annotation analysis, upregulated or downregulated genes in $CD8^+CD103^+CD49a^+$ as compared to $CD8^+CD103^+CD49a^-$ Trm with p-value < 0.0005 were separately subjected to analysis by the DAVID tool (Huang da et al., 2009a; Huang da et al., 2009b). Principal component analysis was performed on the log2 FPKM gene expression value of the top 200 most varied genes, corrected for batch effects. Principal component analysis was analyzed and visualized by the JMP 13 software.

Flow cytometry for phenotypic and functional analysis of tissue-derived cells

Surface antigen expression was assessed in cell-suspensions following 16 – 20 hours of incubation in complete medium. For dextramer staining, dextramer for HLA-A*0201 (ELAGIGILTV), MART-1, APC (WB2162-APC, Immudex) were added 15min prior the antibody cocktail.

Cytokine expression was assessed in freshly prepared cell suspensions stimulated with PMA (50 ng/ml, Sigma) and ionomycin (1 µg/ml, Sigma) for 4.5 hours in the presence of brefeldin A (Golgiplug, BD Biosciences). Alternatively, sorted cells from epidermal explant with or without IL-15 (20 ng/mL) pre-treatment were co-cultured with P815 cells at 1:1 ratio in the presence of anti-CD3 (1 µg/mL, OKT3, R&D Systems) and brefeldin A (BD Biosciences). For experiments testing functional response to collagen IV engagement, sorted epidermal $CD8^+CD103^+$ T cells were plated in collagen IV coated or uncoated 96-well flat bottom plate in medium or in the presence of IL-15 (20 ng/mL) for 20-24 hours before addition of P815 cells and anti-CD3 for 4.5 hours in the presence of brefeldin A (Golgiplug, BD Biosciences).

Cytotoxic granule constituents was assessed in cells suspensions, from 2 – 3 cm^2 surface skin area, or in sorted cells treated with cytokines as indicated. BD Cytofix/Cytoperm Kit was employed according to manufacturer's protocols and fixed samples were acquired within 48 hours after staining. A fixable yellow viability dye (Life Technologies) was used in all experiments to distinguish the live and dead cell populations. Data were acquired using a LSR-II or LSR Fortessa flow cytometer (BD Bioscience) and were analyzed using FlowJo V9.7.6.

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