Somatic reversion of pathogenic *DOCK8* variants alters lymphocyte differentiation and function, resulting in clinical improvement and effective cure of DOCK8 deficiency

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Conflict of Interest

The authors have declared that no conflict of interest exists

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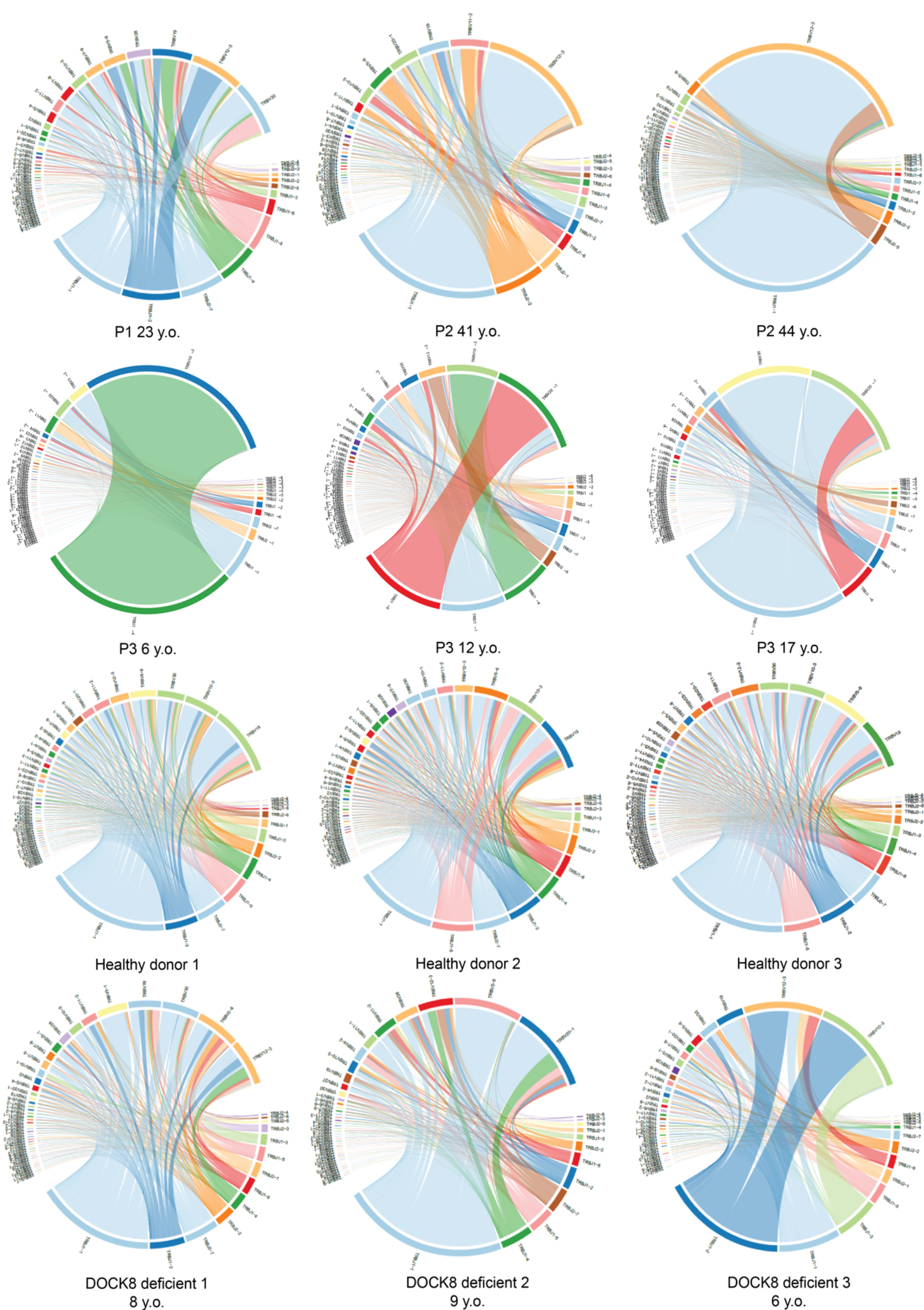
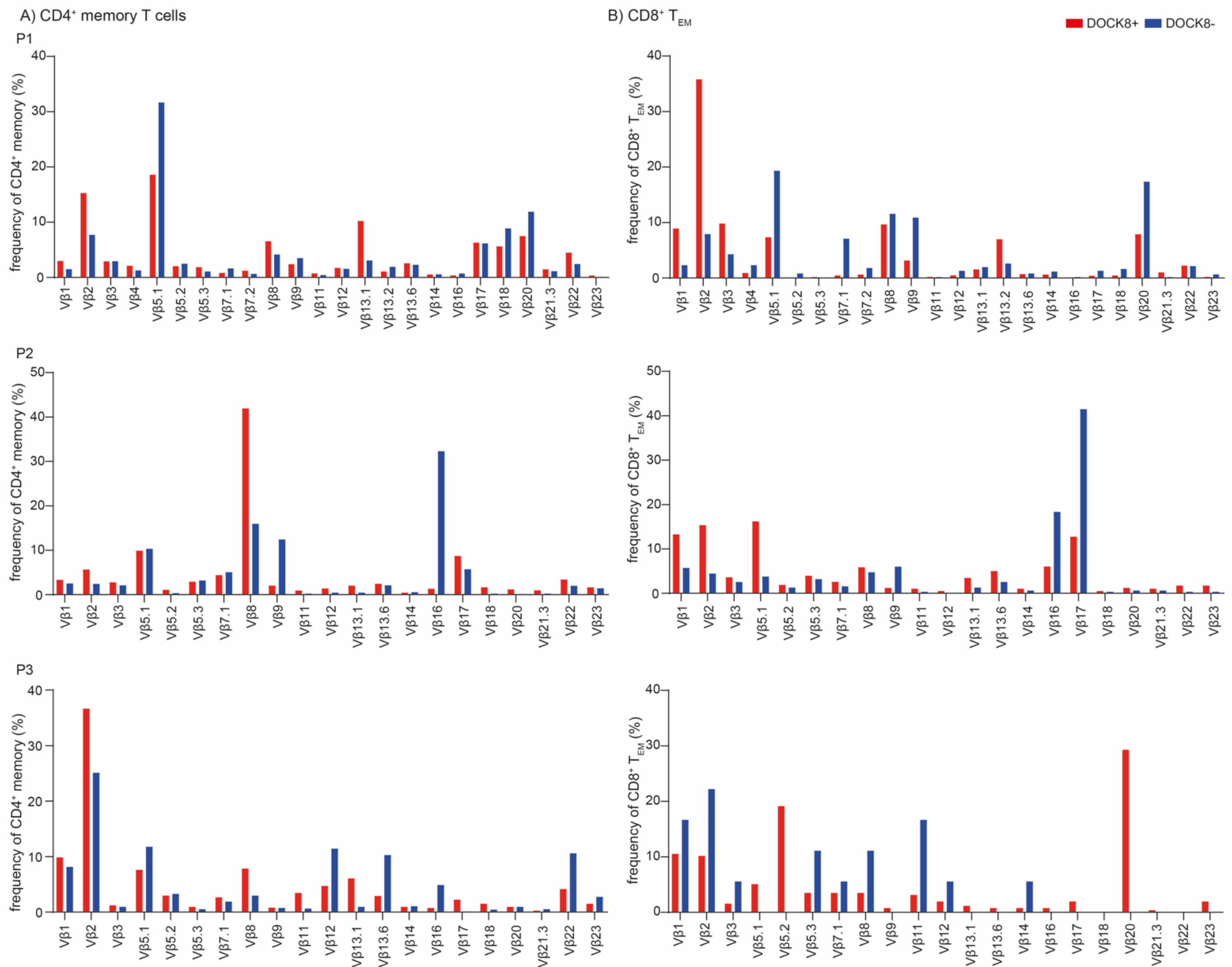
Running title: *DOCK8 reversion can lead to clinical improvement and effective disease cure*



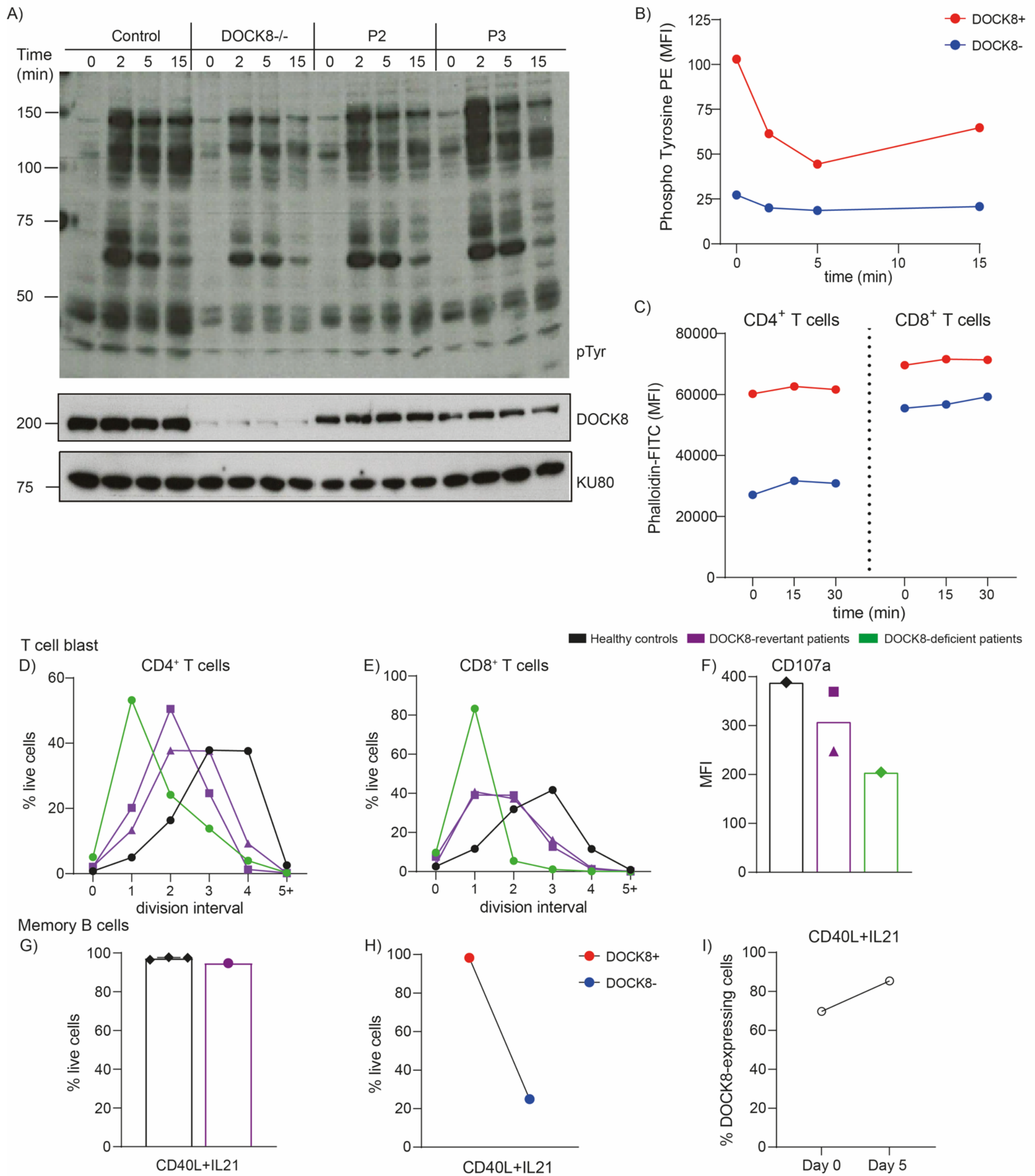
Supplemental Figure 1: TCR β repertoire evaluation

Sequencing of TCR beta genes was performed using genomic DNA extracted from blood samples from healthy donors (HD#1, #2, #3), DOCK8-deficient patients (n=3), P1, P2 (at 2 time points) and P3 (at 3 time points) using multiplex PCR and Euro-Clonality primers. Primary analysis was carried on with IMGT-HighVquest analysis tool. Figures were generated using VDJtools software. Evolution of TRB repertoire in P2 across time was visualized using chord diagrams showing VJ usage.



Supplementary Figure 2: DOCK8⁻ vs DOCK8⁺ TCR repertoire

DOCK8-revertant patient PBMCs were stained for surface expression of CD4, CD8, CCR7 and CD45RA as well as Vβ diversity using the Vβ repertoire kit and then intracellularly stained for DOCK8 to identify DOCK8⁻ and DOCK8⁺ cells. The frequency of cells expressing different Vβ chains in (A) CD4⁺ memory (CD4⁺ CD45RA⁻) and (B) CD8⁺ T_{EM} T cells was determined by flow cytometry.



Supplementary Figure 3: Further functional assessment of DOCK8-revertant patients.

(A) T cells blasts from P2 and P3 were activated by anti-CD3 mAb plus crosslinking Ab; activation was stopped with lysis buffer at the indicated times. Global phosphorylation was evaluated by immunoblotting with anti-phosphotyrosine antibody (upper blot). Expression of DOCK8 and KU80 (as loading control) are also shown. Size markers on the left. (B) PHA-blasts from P1 were stimulated with anti-CD3 mAb, followed by crosslinking, and fixed at the indicated times. Global tyrosine phosphorylation in DOCK8^{-} and DOCK8^{+} cells was evaluated by permeabilisation and staining with anti-phosphotyrosine mAb and

DOCK8 followed by flow cytometric analysis. (C) PBMCs from P1 were treated with IL-2 for the indicated time. F-actin content in $DOCK8^-$ and $DOCK8^+$ cells was assessed by surface staining for CD3, CD4 and CD8 and intracellular staining for Phalloidin-FITC and DOCK8 followed by flow cytometry. (D-F) T cell blasts from a healthy donor, P2, P3 and a DOCK8-deficient patient were labelled with cell trace violet and cultured for 7 days with anti-CD3 mAb and then stained for CD4 and CD8 to determine proliferation of (D) $CD4^+$ T cells and (E) $CD8^+$ T cells or (F) stimulated for 3hr with anti-CD3 and stained for CD107a and CD8. (G-I) Sorted memory B cells from healthy donors (n=3) and P1 were stained with a live/dead cell marker and intracellularly stained to detect DOCK8 after 5 days of in vitro culture with CD40L+IL21 to determine survival of (G) total cells and of (H) DOCK8- and DOCK8+ cells and (I) proportion of DOCK8-expressing cells. (P1●, P2 ■, P3▲).

Supplementary methods

Cell culture

Memory B cells were isolated as $CD20^{+}$ $CD27^{+}$ cells and cultured with CD40L (200ng/ml) and IL21 (50ng/ml). T cell blasts were generated either by culturing PBMCs for 72h with 2.5ug/ml PHA or with the additional steps of dead cells removal by Ficoll-Paque density-gradient centrifugation and subsequent cultur with 100IU/ml human IL2. To determine proliferation, T cell blasts were synchronised, labelled with CellTrace violet dye and cultured with anti-CD3 before being stained for CD4 and CD8 and assessed by dilution of CellTrace violet. CD107a staining was carried out on T cell blasts stimulated for 3h with immobilised anti-CD3 and stained for CD8.

F-actin measurement

PBMC were incubated with 125U/ml IL2 for various times before being washed with PBS and stained for surface markers and live/dead cell marker. Cells were then fixed and permeabilised with saponin and stained with Phalloidin-FITC and anti-DOCK8 Ab/ anti-rabbit IgG Alexa-647 secondary Ab.

Phospho-tyrosine assessment

Stimulation of cells, protein extraction, and immunoblotting protocols have been described previously (57, 58). Briefly, cells (5×10^{6} cells per ml) were stimulated by anti-CD3 antibody and cross-linked with a rabbit anti-mouse IgG for the designated time periods. Cells were washed in PBS, and proteins were extracted with cell lysis buffer (1% NP-40 [NP-40 alternative; Calbiochem], 50 mM Tris, pH 8, 150 mM NaCl, 20 mM EDTA, 1 mM Na3VO4, 1 mM NaF, complete protease inhibitor cocktail [Roche], and phosphatase inhibitor cocktails 2 and 3 [Sigma]). Proteins were denatured by boiling for 10 min with sample buffer (125 mM Tris, pH 6.8, 3% SDS, 10% glycerol, 5% 2β-mercaptoethanol, and 0.01% bromophenol blue), separated by SDS-PAGE, and transferred on polyvinylidene fluoride membrane (Millipore). Membranes were blocked with milk or BSA-based buffer before incubation with antibodies. The following antibodies were used for immunoblotting: anti-phosphorylated tyrosine (P-Tyr-100, #9411S, Cell Signaling Technology), anti-Ku80 (C48E7, #2180, Cell Signaling Technology), anti-DOCK8 (G-2, sc-376911, Santa Cruz). Membranes were then washed and incubated with secondary anti-mouse (GE-Healthcare) or anti-rabbit (Cell Signaling Technology) HRP-linked antibodies. Pierce ECL WB substrate and Bio-Rad Clarity ECL substrate were used for revelation. For reprobing, membranes were incubated with Restore WB stripping buffer (Thermo Scientific) before blocking.

For flow cytometric evaluation of phosphotyrosine, PHA blasts were stimulated with 1µg/ml anti-CD3 mAb for 30s before crosslinking with 2µg/ml goat anti-mouse IgG for various time at 37°C. Cells were washed in and fixed with 1.5% formaldehyde for 20min at 4°C. Cells were permeabilised with methanol for 10min at 4°C and stained with PE-conjugated anti-phosphotyrosine and anti-DOCK8 Abs before additional staining with anti-rabbit Alexa-647 secondary antibody.

Supplementary References

1. Martin, E., Palmic, N., Sanquer, S., Lenoir, C., Hauck, F., Mongellaz, C., Fabrega, S., Nitschké, P., Esposti, M.D., Schwartzentruber, J., et al. 2014. CTP synthase 1 deficiency in humans reveals its central role in lymphocyte proliferation. *Nature* 510:288-292.
2. Winter, S., Martin, E., Boutboul, D., Lenoir, C., Boudjemaa, S., Petit, A., Picard, C., Fischer, A., Leverger, G., and Latour, S. 2018. Loss of RASGRP1 in humans impairs T-cell expansion leading to Epstein-Barr virus susceptibility. *EMBO Mol Med* 10:188-199.

Supplementary Table 1: Antibodies and reagents used

Target-Fluorochrome	Supplier	Catalog number
CCR7-PECy7	BioLegend	353226
CD107a-PE	Becton Dickson Biosciences	555801
CD10-APC	Becton Dickson Biosciences	340923
CD10-APCR700	Becton Dickson Biosciences	659120
CD10-BV650	Becton Dickson Biosciences	563734
CD10-BUV737	Becton Dickson Biosciences	564959
CD127-BV421	BioLegend	351310
CD127-BV650	BioLegend	351326
CD161-BV786	Becton Dickson Biosciences	744096
CD161-PerCPCy5.5	eBioscience	45-1619-42
CD20-BUV805	Becton Dickson Biosciences	612905
CD20-PE	Becton Dickson Biosciences	346595
CD25-BUV563	Becton Dickson Biosciences	612918
CD25-FITC	Becton Dickson Biosciences	347643
CD27-BB515	Becton Dickson Biosciences	564642
CD27-BV786	Becton Dickson Biosciences	563327
CD28-PerCPCy5.5	Becton Dickson Biosciences	560685
CD3-BV421	Becton Dickson Biosciences	562426
CD3-BV570	BioLegend	300346
CD3-BV786	Becton Dickson Biosciences	565491
CD45RA-BV605	Becton Dickson Biosciences	562886
CD45RA-BUV395	Becton Dickson Biosciences	740298
CD4-APCCy7	Becton Dickson Biosciences	557871
CD4-BV510	Becton Dickson Biosciences	562970
CD4-BUV737	Becton Dickson Biosciences	564305
CD56-BB790	Becton Dickson Biosciences	624296
CD56-BV605	BioLegend	318334
CD57-FITC	Becton Dickson Biosciences	555619
CD57-PE	BioLegend	322311
CD8-APCCy7	BioLegend	301016
CD8-BUV496	Becton Dickson Biosciences	612942
CD8-PerCPCy5.5	Becton Dickson Biosciences	560662
DOCK8	Abcam	ab175208
Goat anti-rabbit IgG (H&L)-AF647	Abcam	ab150083
GranzymeB-AF700	Becton Dickson Biosciences	560213
IFNγ-BV605	Becton Dickson Biosciences	562974
IL2-BV650	Becton Dickson Biosciences	564166

IL4-PECy7	eBioscience	25-7049-82
IL21-e660	eBioscience	50-7219-42
IL22-PE	eBioscience	12-7229-42
PD1-BV605	Becton Dickson Biosciences	563245
PD1-PECF594	Becton Dickson Biosciences	565024
Perforin-PECy7	BioLegend	353316
Phalloidin-FITC	Sigma Aldrich	P5282
Phosphotyrosine-PE	Becton Dickson Biosciences	558008
Rabbit IgG isotype control	Abcam	ab172730
TCRαβ-PECy7	BioLegend	306720
TCRαβ-BUV737	Becton Dickson Biosciences	613014
TCRγδ-PerCPe710	eBioscience	46-9959-41
TCRγδ-BV711	Becton Dickson Biosciences	745505
TCRVα24-FITC	Beckman Coulter	IM1589
TCRVα24-JaQ BV605	Becton Dickson Biosciences	743999
TCRVα7.2-BV421	BioLegend	351715
TCRVβ11-PE	Beckman Coulter	IM2290
TNFα-BUV395	Becton Dickson Biosciences	563996

Reagent	Supplier
Anti-CD3 (OKT3)	eBioscience
Anti-mouse IgG (rabbit)	Abcam
Anit-mouse IgG (goat)	Jackson ImmunoResearch
Brefeldin A	Sigma-Aldrich
Carboxyfluorescein succinimidyl ester (CFSE)	Invitrogen
CD40L	R&D Systems
Cell trace violet dye	Invitrogen
Ionomycin	Sigma-Aldrich
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich
Phytohemagglutinin (PHA)	Sigma-Aldrich
recombinant human IL-2	R&D Systems
recombinant human IL-21	PeproTech
saponin	Sigma-Aldrich
T-cell activation and expansion (TAE) beads	Miltenyi Biotech
TCR Vβ repertoire kit	Beckman Coulter
Zombie aqua fixable dye	BioLegend

AF, Alexa fluor; APC, allophycocyanin; BB, brilliant blue; BUV, brilliant ultra violet; BV, brilliant violet; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein complex.