Dissecting the heterogeneity of DENV vaccine-elicited cellular immunity using single-cell RNA sequencing and metabolic profiling

Adam T. Waickman1, Kaitlin Victor1, Tao Li1, Kristin Hatch1, Wiriya Rutvisuttinunt1, Carey Medin2, Benjamin Gabriel2, Richard G. Jarman1, Heather Friberg1 & Jeffrey R. Currier1

Generating effective and durable T cell immunity is a critical prerequisite for vaccination against dengue virus (DENV) and other viral diseases. However, understanding the molecular mechanisms of vaccine-elicited T cell immunity remains a critical knowledge gap in vaccinology. In this study, we utilize single-cell RNA sequencing (scRNAseq) and longitudinal TCR clonotype analysis to identify a unique transcriptional signature present in acutely activated and clonally-expanded T cells that become committed to the memory repertoire. This effector/memory-associated transcriptional signature is dominated by a robust metabolic transcriptional program. Based on this transcriptional signature, we are able to define a set of markers that identify the most durable vaccine-reactive memory-precursor CD8+ T cells. This study illustrates the power of scRNAseq as an analytical tool to assess the molecular mechanisms of host control and vaccine modality in determining the magnitude, diversity and persistence of vaccine-elicited cell-mediated immunity.
A fundamental goal of dengue virus (DENV) vaccine design is to generate an immune response that encompasses both humoral and cellular immunity. Consisting of four immunologically and genetically distinct serotypes, DENV-1 to DENV-4, DENV infects up to 280–500 million individuals yearly worldwide\(^1\)-\(^3\). While the majority of DENV-infected individuals recover quickly, nearly 500,000 individuals a year develop severe dengue disease, classified as either Dengue Hemorrhagic Fever (DHF) or Dengue Shock Syndrome (DSS)\(^4\)-\(^6\). Characterized by increased vascular permeability, hypovolemia, and dysregulated blood clotting, DHF/DSS has a 2.5% mortality rate\(^1\)-\(^3\). While the environmental and genetic factors responsible for the development of DHF/DSS are complex and incompletely understood\(^7\)-\(^9\), prior infection with one serotype of DENV has been shown to significantly increase the likelihood of developing DHF/DSS upon heterotypic re-infection\(^10\)-\(^12\). This phenomenon is thought to be facilitated at least in part by poorly-neutralizing, serotype cross-reactive antibodies which enable the opsonization of viral particles but not functional neutralization\(^2\),\(^4\),\(^6\),\(^9\). Hence, the development of vaccine platforms (e.g., vaccinia, yellow fever) and non-replicating viral vectors (rAD/MVA)\(^13\) that can predict the fate of an individual cell.

In this study, we demonstrate that TAK-003 elicits a potent cellular immune response which persists for at least 120 days post-vaccination in human subjects. The antigen specificity of the cellular immune response generated by TAK-003 spans the DENV proteome and demonstrates significant cross-reactivity against all four DENV serotypes. Single-cell RNA sequencing analysis of CD8\(^+\) T cells activated in response to TAK-003 exposure revealed a highly polyclonal CD8\(^+\) T cell repertoire, which had significant clonal overlap between DENV-2 non-structural protein (NS1)- and NS3-reactive CD8\(^+\) T cells identified and isolated 120 days post-vaccination. Transcriptional analysis of CD8\(^+\) T cells acutely activated in response to TAK-003 exposure also revealed a highly diverse transcriptional profile, with NS1- and NS3-reactive memory-precursor CD8\(^+\) T cells at day 14 post-immunization displaying a distinct transcriptional signature dominated by metabolic pathways. Based on these observations, we identified a panel of metabolic markers, which could be used to faithfully identify CD8\(^+\) T cells activated in vitro in response to antigenic stimulation, or activated in vivo in response to TAK-003. In particular, expression of the transferrin receptor (TIR1/CD71)—critical for efficient iron uptake—exclusively marks CD8\(^+\) T cells with high proliferative and effector/memory potential. Therefore, analysis of the metabolic profile in vaccine-responsive CD8\(^+\) T cells can aid in the identification and characterization of the most effective and durable vaccine-elicited clonotypes.

### Results

**TAK-003 generates potent and durable cellular immunity.** T cell activation in response to TAK-003 administration was assessed by flow cytometry in 55 individuals on days 0, 14, 28, and 120 post immunization. Consistent with other live-attenuated vaccine platforms, TAK-003 administration resulted in significant CD8\(^+\) T cell activation on days 14 and 28 post-vaccination (Fig. 1a, b, Supplementary Fig. 1A). CD8\(^+\) T cell activation, as assessed by CD38/HLA-DR upregulation, peaked on day 28 post-vaccination and returned to baseline levels by day 120. Moderate CD4\(^+\) T cell activation was also observed in response to TAK-003 administration (Fig. 1c, d, Supplementary Fig. 1), with the peak of activation observed on day 14 post-vaccination. However, despite significant activation at this time point, CD8\(^+\) T cells do not appear to be functionally licensed to produce IFN-\(\gamma\) in response to DENV peptide stimulation until later post-vaccination. (Fig. 1e, f, Supplementary Fig. 1B).

To determine if the extensive T cell activation observed in response to TAK-003 administration translated to durable cellular immunity, we stimulated PBMCs isolated from study participants on days 0 and 120 of the study with peptide pools corresponding to the NS1, NS3, NS5, and CprM/E proteins of DENV-1 to -4 and quantified the number of vaccine-reactive, IFN-\(\gamma\) producing cells by ELISPOT assay. All subjects receiving TAK-003 displayed a significant increase in the number of circulating peptide-pool-reactive T cells on day 120 relative to baseline (Supplementary Fig. 1C), with the specificity of this reaction spread across the DENV proteome (Supplementary Fig. 1D).

Tetravalent T cell reactivity patterns observed in TAK-003 recipients were detected in both structural (CprM/E) and non-structural (NS1, NS3, NS5) regions of the proteome. Structural region responses could have been generated by any, or all, of the four components of the vaccine, however, non-structural responses can be interpreted as truly cross-reactive since DENV-2 is the common non-structural element of the four vaccine components.

### Identification of antigen-specific CD8\(^+\) memory T cells

To further assess the diversity and persistence of TAK-003-elicited CD8\(^+\) T cell immunity, we utilized single-cell RNA sequencing to track the clonal expansion/contraction of TAK-003-reactive CD8\(^+\) T cells from acute activation time points and memory time points from the same individual. For this analysis, we utilized samples from a subject who demonstrated strong NS1 and NS3 biased memory T cell responses following TAK-003 administration, as quantified by IFN-\(\gamma\) ELISPOT analysis of day 120 post vaccination PBMC samples (Fig. 2a). Matrixed-based ELISPOT analysis utilizing overlapping peptide pools spanning the entirety of DENV2 NS1 and NS3 revealed that the observed NS1 and NS3
reactivity was directed against two distinct epitopes in each peptide pool (Supplementary Table 1).

Following overnight stimulation with either the complete DENV2 NS1 or DENV2 NS3 peptide pool, DENV-reactive memory CD8+ T cells (day 120) were identified by their upregulation of the early activation markers CD25 and CD69. CD8+CD25+CD69+ T cells were isolated by flow cytometric cell sorting (Fig. 2b, Supplementary Fig. 2) and subjected to single-cell RNA sequencing analysis. In addition, vaccine-reactive CD8+ T cells from an early post-vaccination time point (day 14) were identified by expression of the activation markers CD38 and HLA-DR and isolated by flow sorting (Fig. 2c). A similar number

**Fig. 1** TAK-003 elicits a potent and durable DENV-specific cellular immune response following vaccination. PBMCs from individuals immunized with TAK-003 were assessed for markers of T cell activation by flow cytometry on days 0, 14, 28, and 120 post-vaccination. **a** Representative plots demonstrating CD8+ T cell activation as assessed by CD38 and HLA-DR upregulation on days 0, 14, 28, and 120 post-vaccination. **b** Aggregate analysis from 55 TAK-003 recipients, demonstrating maximal CD8+ T cell activation on day 28 post-vaccination, returning to baseline by day 120. **c** Representative plots demonstrating CD4+ T cell activation as assessed by CD38 and HLA-DR upregulation on days 0, 14, 28, and 120 post-vaccination. **d** Aggregate analysis from 55 TAK-003 recipients, demonstrating maximal CD4+ T cell activation on day 14 post-vaccination, returning to baseline by day 120. **e** Representative plots demonstrating DENV-specific IFN-γ production and CD8 T cell degranulation (as assessed by surface CD107a expression) days 0, 14, 28 and 120 post-vaccination. **f** Aggregate analysis from 12 TAK-003 recipients. Cytokine production was assessed following stimulation with peptide pools demonstrated by ELISPOT analysis as being immunogenic at day 120 post vaccination in each individual. *P < 0.05, **P < 0.01, ****P < 0.0001 (Paired two-tailed t-test); ns, not significant. Source data are provided as a Source Data file.
CD8$^+$ CD38$^+$ HLA-DR$^+$ T cells are highly polyclonal and persist as long-lived memory cells. TCR clonotype diversity was assessed within acutely-activated and DENV-reactive memory CD8$^+$ T cells using single-cell RNA sequencing. a The antigenic specificity of DENV-reactive memory CD8$^+$ T cells from a TAK-003 recipient was assessed by IFN-γ ELISPOT 120 days post immunization. The number of spots is presented relative to 1 million PBMCs. b NS1-reactive and NS3-reactive memory CD8$^+$ T cells from 120 post-vaccination were isolated by flow cytometry based on upregulation of CD25 and CD69 expression following in vitro stimulation for 18 h with 1 μg ml$^{-1}$ of the indicated peptide pools. c Activated (CD38$^+$ HLA-DR$^+$) CD8$^+$ T cells and non-activated (CD38$^-$ HLA-DR$^-$) CD8$^+$ T cells were additionally isolated by flow cytometry from the same TAK-003 recipient 14 day post-vaccination. d TCR clonotype diversity from sorted NS1-reactive and NS3-reactive CD8$^+$ T cells isolated 120 days post-vaccination. The top 10 most abundant NS1- and NS3-reactive clones are demarcated in color. e TCR clonotype diversity in sorted non-activated (CD38$^-$ HLA-DR$^-$) and activated (CD38$^+$ HLA-DR$^+$) CD8$^+$ T cells using scRNAseq from 14 days post-TAK-003 administration. TCR clones that overlap with the dominant NS1- or NS3-reactive memory CD8$^+$ T cell clones observed at day 120 are indicated with the appropriate color designation. f Assessment of the relative contribution and stability of the dominant NS1- and NS3-reactive memory precursors to the overall CD38$^+$HLA-DR$^+$ CD8$^+$ T cell pool at day 14 post-TAK-003 immunization.
memory CD8$^+$ T cells isolated on day 120 post-vaccination exhibited a significant degree of clonal diversity, with an average (mean) clonal abundance of 1.71 cells/clone for NS1-reactive cells, and 2.49 cells/clone for NS3-reactive cells. However, these statistics are significantly skewed by a large number of clones with a single representative in the dataset. In contrast, the top 10 most abundant clones found in both the NS1- and NS3-reactive memory T cell pool account for 34.4 and 44.4% of recovered cells in the dataset, respectively (Fig. 2d, f). Furthermore, 80% of these memory T cell pool account for 34.4 and 44.4% of recovered cells in the dataset. In contrast, the top 10 most abundant clones found in both the NS1- and NS3-reactive memory T cell pool account for 34.4 and 44.4% of recovered cells in the dataset.

Characterization of TAK-003-stimulated CD8$^+$ T cells. In light of the significant clonal diversity observed in the activated CD8$^+$ HLA-DR$^+$CD8$^+$ T cell compartment 14 days after TAK-003 administration, we decided to determine if the heterogeneity of this population extended to the functional transcriptional profile of these cells. To this end, we assessed the functional gene expression profile of the sorted CD8$^+$ CD38$^+$HLA-DR$^+$ T cells isolated 14 days post-TAK-003 inoculation using single-cell RNAseq. Of the 1019 cells contained within the TCR analysis, this population extended to the functional transcriptional profile of 1003 cells (98.4%) that met the quality control threshold of our analysis pipeline.

Unsupervised tSNE clustering of sorted CD8$^+$ CD38$^+$HLA-DR$^+$CD8$^+$ T cells based on differential gene expression profiles revealed four statistically-distinct populations (Fig. 3a, b, Table 2). Expression of genes associated with an effector CD8$^+$ T cell program such as GZMA, GZMB, and PRF1 were significantly enriched in cluster 1 (Fig. 3b, c, Table 2). Transcripts associated with a more resting/naïve T cell phenotype such as CCR7, MAL, and TCF7 were significantly enriched in clusters 2 and 3, and showed minimal overlap with cells expressing effector gene products (Fig. 3b, c, Table 2). In addition, gene expression associated with cellular metabolism, proliferation and cell-cycle progression, such as TYMS, IDH2, and GAPDH were significantly enriched in cluster 1 relative to all other groups.

To compare how the transcriptional profile of phenotypically activated CD8$^+$ HLA-DR$^+$CD8$^+$ T cells compared to that of phenotypically non-activated CD8$^+$ HLA-DR$^+$ CD8$^+$ T cells, we performed additional single cell gene expression analysis on the sorted CD8$^+$ HLA-DR$^+$CD8$^+$ T cells shown Fig. 2c. The analysis resulted in the identification of 1391 cells with a complete gene expression profile along with a full-length TCR. This

| Table 1 Dominant TCR clonotypes from acutely activated and memory CD8$^+$ T cells |

| Population | Day | Frequency | TCRα CDR3aa | TRAV | TRAJ | TCRβ CDR3aa | TRBV | TRBD | TRB J |
|------------|-----|-----------|-------------|------|------|-------------|------|------|------|
| CD8$^+$CD38$^+$HLA-DR$^+$ (resting) | 14 | 0.01847 | CAVMDSNYQILW | TRAV1-2 | TRAJ33 | CASSEGAQNYQF | TRBV6-4 | TRBD2-4 | TRB J |
| CD8$^+$CD38$^+$HLA-DR$^+$ (activated) | 120 | 0.0127157 | CAVQAQGYSTLF | TRAV14 | TRAJ11 | CASAEADNEQFF | TRBV7-9 | TRBD2-4 | TRB J |
| NS1 reactive memory CD8$^+$ | 120 | 0.017517 | CAVRRGDYKLSF | TRAV12 | TRAJ11 | CASSEAGNTGELFF | TRBV7-9 | TRBD2-4 | TRB J |
| NS3 reactive memory CD8$^+$ | 120 | 0.017517 | CAVRRGDYKLSF | TRAV12 | TRAJ11 | CASSEAGNTGELFF | TRBV7-9 | TRBD2-4 | TRB J |
scRNAseq gene expression data was merged with the activated CD38+HLA-DR+ CD8+ T cell gene expression data, and differential gene expression analysis performed (Supplementary Fig. 4A, Supplementary Table 2). Notably, there was minimal transcriptional overlap between the sorted CD38+HLA-DR+ and CD38−HLA-DR− CD8+ T cells, with each statistically defined cluster in the merged dataset exhibiting significant bias towards one-or-the-other parental population (Supplementary Fig. 4A, Supplementary Fig. 4B). Canonical T cell activation markers (HLA-DR, CD27, GZMH) and genes associated with cellular proliferation/migration (STMN1, HMGB2, TUBB) are preferentially expressed in phenotypically activated CD8+ T cells (Supplementary Fig. 4A, C, Supplementary Table 2). Interestingly for this subject, a statistically unique transcriptional cluster can be defined within the merged dataset (cluster 1) that is significantly enriched in CD38+HLA-DR− CD8+ T cells and characterized by expression of the canonical MAIT-associated TRAV1-2 TCR receptor gene segment28,30, along with the key lineage-defining genes KLRB1, KLRG1 and IL-7R (Supplementary Fig. 4C, Supplementary Table 2)31,32. The prevalence of CD8+ MAIT T cells within the sorted CD38−HLA-DR− CD8+ T cell population is further validated by the corresponding TCR clonotype information derived from these cells (Table 1), which shows a significant enrichment in cells expressing the canonical TRAV1-2 TRAJ33 semi-invariant TCR alpha chain28,30. These data demonstrate that although there is significant transcriptional heterogeneity within the sorted CD38+HLA-DR+ CD8+ T cells circulating after TAK-003 administration, this population is transcriptionally distinct from phenotypically non-activated CD38−HLA-DR− CD8+ T cells.

Having demonstrated that CD38+HLA-DR+ CD8+ T cells are a distinct yet transcriptionally heterogeneous population 14 days post TAK-003 administration, we asked if DENV-reactive CD8+ T cells that are destined to form long-lived memory T cells can be identified within the population, and if they exhibit a unique transcriptional profile relative to all other activated CD8+ T cells. To this end, we asked whether any of the TCR clonotypes defined in the NS1− or NS3- reactive memory CD8+ T cell population 120 days post TAK-003 administration could be found amongst CD38+HLA-DR+CD8+ T cells on day 14 post vaccination.

Of the 1003 CD38+HLA-DR+ CD8+ T cells recovered on day 14 post-vaccination, 145 cells (14.5%) expressed TCRs found in NS1- and NS3-reactive memory cells on day 120 post-vaccination (Fig. 3d). The transcriptional profile of these memory precursors positioned them predominantly within the previously defined phenotypic cluster 1 (Supplementary Fig. 5), suggesting that this distinct subset of CD38+HLA-DR−CD8+ T cells found 14 days post-vaccination is uniquely primed to develop into long-lived memory cells. In addition, those cells expressing a clonally expanded TCR (defined as a TCR expressed in >2 cells) were preferentially enriched in cluster 1 (Fig. 3e).

To further define the transcriptional and phenotypic signatures of vaccine-reactive CD8+ T cells within our dataset, we utilized the Ingenuity Pathway Analysis (IPA) software package33 to identify gene pathways selectively expressed in putative DENV-reactive effector/memory-precursor CD8+ T cells (Table 3). We assessed the gene pathways preferentially expressed within cluster 1, which contained the majority of identified memory-precursor cells, relative to all other cells in the dataset. Interestingly, there was some preferential expression of gene pathways associated with effector function and cellular migration in cluster 1. However, the dominant cellular transcriptional signatures that distinguished cluster 1 from the rest of the dataset were associated with cellular metabolism and proliferation, such as oxidative phosphorylation, mTOR signaling, and eIF4/p70S6K signaling (Table 3). These data suggest that the assessment of cellular metabolism pathways may provide a robust and unbiased indication of cellular memory-precursor potential, as well as effector status and antigen reactivity.

Polyclonal T cell activation modulates cellular metabolism. To explore the broader implications of the observed relationship between metabolic gene activity and effector/memory potential in vaccine-reactive T cells, we aimed to establish a set of markers to quantify the metabolic potential of human T cells following TCR engagement. As the metabolically-associated gene products identified by scRNAseq analysis and preferentially expressed in memory-precursor CD8+ T cells were intracellular in origin, we selected a panel of cell surface markers and traceable metabolites that we hypothesized would be preferentially expressed in, or taken up by, memory-precursor CD8+ T cells. The criteria used to select these markers were that they (1) were directly regulated by the gene pathways differentially expressed in our populations of interest and/or (2) the associated metabolites were utilized by metabolic pathways differentially expressed in our populations of interest.

To this end, we selected the transferrin receptor complex (TRIR1/CD71) and the Large-neutral Amino Acid Transporter 1 (LAT1/CD98) for additional analysis. The expression and surface-localization of both of these transporters is regulated by mTOR/p70S6K signaling (pathways preferentially expressed in our memory-precursor cells)34-36. Furthermore, the metabolites they import—iron and amino acids, respectively—are critical co-factors for mitochondrial oxidative phosphorylation or direct catabolic and anabolic substrates required in proliferating T cells, which can both directly impact mTOR/p70S6K signaling37-39. In addition, we assessed the ability of T cells to uptake glucose (measured by uptake of 2-NBDG, a fluorescent analog of glucose)40,41 or fatty acids (assessed by BODIPY FL-C16 uptake, a florescent palmate derivative)42,43 by flow cytometry.

To assess the suitability of these markers to quantify the metabolic potential of human T cells following TCR engagement, we stimulated PBMCs from normal healthy donors in vitro with aCD3/CD28 to induce polyclonal T cell activation. After in vitro stimulation, CD4+ and CD8+ T cells showed a well-characterized pattern of activation marker upregulation (Fig. 4a, b, Supplementary Fig. 6). Furthermore, we observed a sustained increase in expression of TRIR1 and CD98 following TCR stimulation (Fig. 4c, d, Supplementary Fig. 6), as well as 2-NBDG and BODIPY FL-C16 uptake (Fig. 4e, f, Supplementary Fig. 6). Uptake of 2-NBDG and BODIPY FL-C16 by activated T cells could be reduced upon addition of the corresponding unlabeled metabolites (Supplementary Fig. 7A), although the inhibition of 2-NBDG uptake by unlabeled glucose was modest. Of particular note, the expression of TRIR1 and CD98 and uptake of BODIPY FL-C16 showed exceptional promise as markers of T cell activation and metabolic potential due to their large dynamic range and persistence relative to other markers of T cell activation. In particular, the expression of TRIR1 on in vitro stimulated CD8+ T cells increased ~600 fold 48 h after polyclonal stimulation. The increase in TRIR1 expression upon in vitro stimulation corresponded to a significant increase in the ability of T cells to uptake transferrin (Supplementary Fig. 7B).

Clonal T cell activation modulates cellular metabolism. In light of the observation that the expression of TRIR1 and CD98 and the uptake of BODIPY FL-C16 are quantifiable metabolic indicators of polyclonal T cell activation following in vitro activation, we endeavored to determine if these markers could be utilized to identify and characterize T cells activated in an antigen-specific
**Fig. 3** TAK-003-elicited CD8\(^+\) memory precursors exhibit a unique transcriptional profile. **a** Transcriptional heterogeneity of sorted CD38\(^+\)HLA-DR\(^+\)CD8\(^+\) T cells isolated 14 days post-TAK-003 administration as assessed by single-cell RNA sequencing. Unsupervised cell clustering and data visualization were performed using sparse nearest-neighbour graphing, followed by Louvain Modularity Optimization. **b** Hierarchical heat map of differentially expressed genes within the sorted CD38\(^+\)HLA-DR\(^+\)CD8\(^+\) T cell. **c** Expression of select transcripts within the sorted CD38\(^+\)HLA-DR\(^+\)CD8\(^+\) T cells. **d** Distribution of CD38\(^+\)HLA-DR\(^+\)CD8\(^+\) T cells at day 14 with TCR clonotypes overlapping with NS1- and NS3-reactive memory CD8\(^+\) T cells at day 120 (memory precursors). **e** Distribution of clonally expanded CD38\(^+\)HLA-DR\(^+\)CD8\(^+\) T cells at day 14.
CD8+ T cell scRNAseq analysis

Table 2 Cluster defining gene lists from day 14 sorted CD38+ HLA-DR+ CD8+ T cell scRNAseq analysis

| Cluster 1 | Cluster 2 | Cluster 3 | Cluster 4 |
|-----------|-----------|-----------|-----------|
| Ensembl ID | Gene Name | P-Value  | Log2 FC  | Ensembl ID | Gene Name | P-Value  | Log2 FC  | Ensembl ID | Gene Name | P-Value  | Log2 FC  | Ensembl ID | Gene Name | P-Value  | Log2 FC  |
| ENSG00000081059 | TCF7 | 3.80E-02 | 1.73 | ENSG00000227507 | LTB | 2.98E-02 | 0.98 |
| ENSG00000120129 | DUSP1 | 2.11E-03 | 1.30 |
| P | Value | Log2 FC | Ensembl ID | Gene Name | P-Value  | Log2 FC  | Ensembl ID | Gene Name | P-Value  | Log2 FC  | Ensembl ID | Gene Name | P-Value  | Log2 FC  |
| ENSG00000196126 | HLA-DRB1 | 7.29E-21 | 3.18 |
| ENSG00000179144 | GIMAP7 | 6.65E-07 | 1.69 |
| ENSG00000130844 | ZNF331 | 3.44E-02 | 0.98 |
| ENSG00000177606 | JUN | 3.66E-03 | 1.30 |
| ENSG00000222041 | LINC00152 | 2.24E-19 | 2.78 |
| ENSG00000114861 | FOXP1 | 2.21E-04 | 1.34 |
| ENSG00000100100 | PIK3IP1 | 3.90E-02 | 0.96 |
| ENSG00000100906 | NFKBIA | 1.23E-02 | 1.13 |
| ENSG00000196352 | CD55 | 7.50E-02 | 0.88 |
| ENSG00000128016 | ZFP36 | 1.31E-02 | 1.15 |
| ENSG00000100453 | GZMB | 9.00E-18 | 3.16 |
| ENSG00000108848 | LUC7L3 | 1.74E-03 | 1.21 |
| ENSG00000171522 | PTGER4 | 7.68E-02 | 0.87 |
| ENSG00000153563 | CD8A | 1.35E-16 | 2.34 |
| ENSG00000245164 | LINC00861 | 5.02E-03 | 1.15 |
| ENSG00000119801 | YPEL5 | 9.27E-02 | 0.81 |
| ENSG00000081059 | TCF7 | 7.89E-02 | 0.88 |
| ENSG00000144655 | CSRNP1 | 1.45E-02 | 1.17 |
| ENSG00000152518 | ZFP36L2 | 6.28E-02 | 0.96 |
| ENSG00000271503 | CCL5 | 4.11E-14 | 2.73 |
| ENSG00000126353 | CCR7 | 1.27E-02 | 1.06 |
| ENSG00000168421 | RHOH | 1.29E-01 | 0.79 |
| ENSG00000105374 | NKG7 | 4.11E-14 | 2.71 |
| ENSG00000127528 | KLF2 | 1.88E-02 | 0.94 |
| ENSG00000059804 | SLC2A3 | 1.48E-01 | 0.75 |
| ENSG00000168209 | DDIT4 | 7.39E-02 | 0.99 |
| ENSG00000227815 | ACTG1 | 4.11E-14 | 2.28 |
| ENSG00000188404 | SELL | 1.42E-02 | 0.98 |
| ENSG00000111678 | C12orf57 | 1.42E-01 | 0.75 |
| ENSG00000143384 | MCL1 | 7.30E-02 | 0.98 |
| ENSG00000184009 | ACTG1 | 4.11E-14 | 2.71 |
| ENSG00000126353 | CCR7 | 1.27E-02 | 1.06 |
| ENSG00000168421 | RHOH | 1.29E-01 | 0.79 |
| ENSG00000105374 | NKG7 | 4.11E-14 | 2.71 |
| ENSG00000127528 | KLF2 | 1.88E-02 | 0.94 |
| ENSG00000059804 | SLC2A3 | 1.48E-01 | 0.75 |
| ENSG00000168209 | DDIT4 | 7.39E-02 | 0.99 |
*not significant* (CD69, CD25), metabolite transporters (TfR1, CD98), as well as assessed the upregulation of conventional activation markers cytometry.

BODIPY FL-C16 following hCMV (Fig. 5f), adenovirus (Fig. 5g), and the increased expression of TfR1, CD98, or the uptake of activation markers CD25/CD69 following in vitro stimulation correlation between the upregulation of the conventional pattern of CD4+ T cells, we aimed to determine whether these same markers can be used to dissect the functional heterogeneity of in vivo activated T cells. To this end, we compared the expression of the conventional activation markers CD38/HLA-DR on T cells isolated from individuals inoculated with TAK-003 on days 0, 14, 28 and 120 post-immunization, to the ability of cells to uptake 2-NBDG and BODIPY FL-C16 and the upregulation of the metabolic transporters TfR1 and CD98.

In vivo T cell activation modulates cellular metabolism. Having established the utility of TfR1/CD98 expression, as well as BODIPY FL-C16 and 2-NBDG uptake, for the identification and functional characterization of antigen-specific in vitro activated T cells, we aimed to determine whether these same markers can be used to dissect the functional heterogeneity of in vivo activated, vaccine-reactive T cells. To this end, we compared the expression of the conventional activation markers CD38/HLA-DR on T cells responding to virus-antigen stimulation (Fig. 5a, b) and CD4+ T cell expansion observed on day 28 post-inoculation (Fig. 5a, Supplementary Fig. 8). A similar, albeit reduced, pattern of CD4+ T cell activation can be concurrently observed in vaccinated individuals, with the peak of CD4+ T cell expansion occurring 14 days post-inoculation (Supplementary Fig. 9).

Consistent with the single-cell RNA sequencing phenotype observed in TAK-003-reactive T cells and our in vitro T cell stimulation data, a significant increase in both 2-NBDG and BODIPY FL-C16 uptake can be observed in HLA-DR positive CD8+ T cells on day 14 and 28 post-vaccination. Additionally, a subset of HLA-DR positive CD8+ and CD4+ T cells upregulate TfR1 (Fig. 6d, Supplementary Fig. 9) on days 14 and 28 post-TAK-003 administration. Notably, while the peak expression of CD38/HLA-DR on CD8+ T cells occurs on day 28 post-vaccination, the expression of both TfR1 and CD98 peaks on day 14, suggesting that these markers may provide an earlier indicator of vaccine T cell immunogenicity than conventional surface markers. The expression of the conventional activation markers CD38 and HLA-DR and the uptake of BODIPY FL-C16 and 2-NBDG appear to occur concomitantly and uniformly in vivo activated T cells whereas
the expression of TfR1 (transferrin receptor) shows a significant amount of variability within the HLA-DR+ T cell compartment (Fig. 6d). As quantification of CD38, TfR1, and CD98 was all performed using antibodies conjugated to the same fluorophore (PE), the observed heterogeneity in TfR1 expression within the HLA-DR+ T cell compartment is unlikely to be attributable to a technical aberration. This suggests the possibility that heterogeneity of TfR1 expression may reflect and capture the functional diversity previously observed in the scRNAseq-derived transcriptional profiles of vaccine-reactive CD8+ T cells, and can be utilized to more stringently identify effector/memory-precursor CD8+ T cells.

**TfR1 expression correlates with effector/memory potential.** To investigate whether differences in the surface expression of TfR1

### Table 3 Enriched gene pathways in memory-precursor CD8+ T cells

| Pathway                              | P-value     | z score | Ratio | Major contributing genes                                      |
|--------------------------------------|-------------|---------|-------|---------------------------------------------------------------|
| Oxidative phosphorylation            | 1.00E-36    | 4.667   | 0.346 | NDUFB9, COX7B, MT-ND5, UQCR10, ATP5PB                         |
| EIF2 signaling                       | 1.00E-32    | -4.146  | 0.202 | RPS26, RPL32, RPS27, EIF4A2, RPL35A, RPS5                    |
| Mitochondrial dysfunction            | 3.16E-31    | -0.816  | 0.23  | NDUFB9, COX7B, MT-ND5, UQCR10, ATP5PB                       |
| mTOR signaling                       | 2.51E-16    | 0.654   | 0.106 | JUN, NDUFB9, MT-ND5, ATP5PB, PGAM1                         |
| Regulation of elf4 and p70S6K signaling | 6.31E-15  | -0.149  | 0.137 | RPS29, RPS5, RPS26, RPS27, RPS18, PABPC1                    |
| Sirtuin signaling pathway            | 6.31E-15    | 2.325   | 0.137 | RPS29, RPS5, RPS26, RPS27, RPS18, PABPC1                    |
| Regulation of Actin-based motility by Rho | 2.00E-13 | 3.349   | 0.119 | ARPC3, MYL12B, WIPF1, ARPC5, RAC2, ACTR3                    |
| Signaling by Rho Family GTPases      | 5.01E-12    | 2.836   | 0.099 | JUN, WIPF1, ARPC5, RAC1B, FOS, ARPC5                       |
| Mitochondrial dysfunction            | 3.16E-31    | -0.23   | 0.119 | ARPC3, MYL12B, RPS26, RPS27, RPS18, PABPC1                  |
| EIF2 signaling                       | 1.00E-32    | -4.146  | 0.202 | RPS26, RPL32, RPS27, EIF4A2, RPL35A, RPS5                    |
| mTOR signaling                       | 2.51E-16    | 0.816   | 0.137 | RPS26, RPS27, FKBP1A, EIF4B, EIF4A2, RPL35A                  |
| Regulation of elf4 and p70S6K signaling | 6.31E-15 | -0.149  | 0.137 | RPS29, RPS5, RPS26, RPS27, RPS18, PABPC1                    |
| Sirtuin signaling pathway            | 6.31E-15    | 2.325   | 0.137 | RPS29, RPS5, RPS26, RPS27, RPS18, PABPC1                    |
| Regulation of Actin-based motility by Rho | 2.00E-13 | 3.349   | 0.119 | ARPC3, MYL12B, WIPF1, ARPC5, RAC2, ACTR3                    |
| Signaling by Rho Family GTPases      | 5.01E-12    | 2.836   | 0.099 | JUN, WIPF1, ARPC5, RAC1B, FOS, ARPC5                       |
| Mitochondrial dysfunction            | 3.16E-31    | -0.23   | 0.119 | ARPC3, MYL12B, RPS26, RPS27, RPS18, PABPC1                  |
| EIF2 signaling                       | 1.00E-32    | -4.146  | 0.202 | RPS26, RPL32, RPS27, EIF4A2, RPL35A, RPS5                    |
| mTOR signaling                       | 2.51E-16    | 0.816   | 0.137 | RPS26, RPS27, FKBP1A, EIF4B, EIF4A2, RPL35A                  |
| Regulation of elf4 and p70S6K signaling | 6.31E-15 | -0.149  | 0.137 | RPS29, RPS5, RPS26, RPS27, RPS18, PABPC1                    |
| Sirtuin signaling pathway            | 6.31E-15    | 2.325   | 0.137 | RPS29, RPS5, RPS26, RPS27, RPS18, PABPC1                    |
| Regulation of Actin-based motility by Rho | 2.00E-13 | 3.349   | 0.119 | ARPC3, MYL12B, WIPF1, ARPC5, RAC2, ACTR3                    |
| Signaling by Rho Family GTPases      | 5.01E-12    | 2.836   | 0.099 | JUN, WIPF1, ARPC5, RAC1B, FOS, ARPC5                       |
| Mitochondrial dysfunction            | 3.16E-31    | -0.23   | 0.119 | ARPC3, MYL12B, RPS26, RPS27, RPS18, PABPC1                  |
| EIF2 signaling                       | 1.00E-32    | -4.146  | 0.202 | RPS26, RPL32, RPS27, EIF4A2, RPL35A, RPS5                    |
| mTOR signaling                       | 2.51E-16    | 0.816   | 0.137 | RPS26, RPS27, FKBP1A, EIF4B, EIF4A2, RPL35A                  |
| Regulation of elf4 and p70S6K signaling | 6.31E-15 | -0.149  | 0.137 | RPS29, RPS5, RPS26, RPS27, RPS18, PABPC1                    |
| Sirtuin signaling pathway            | 6.31E-15    | 2.325   | 0.137 | RPS29, RPS5, RPS26, RPS27, RPS18, PABPC1                    |
| Regulation of Actin-based motility by Rho | 2.00E-13 | 3.349   | 0.119 | ARPC3, MYL12B, WIPF1, ARPC5, RAC2, ACTR3                    |
| Signaling by Rho Family GTPases      | 5.01E-12    | 2.836   | 0.099 | JUN, WIPF1, ARPC5, RAC1B, FOS, ARPC5                       |
| Mitochondrial dysfunction            | 3.16E-31    | -0.23   | 0.119 | ARPC3, MYL12B, RPS26, RPS27, RPS18, PABPC1                  |
| EIF2 signaling                       | 1.00E-32    | -4.146  | 0.202 | RPS26, RPL32, RPS27, EIF4A2, RPL35A, RPS5                    |
| mTOR signaling                       | 2.51E-16    | 0.816   | 0.137 | RPS26, RPS27, FKBP1A, EIF4B, EIF4A2, RPL35A                  |
| Regulation of elf4 and p70S6K signaling | 6.31E-15 | -0.149  | 0.137 | RPS29, RPS5, RPS26, RPS27, RPS18, PABPC1                    |
| Sirtuin signaling pathway            | 6.31E-15    | 2.325   | 0.137 | RPS29, RPS5, RPS26, RPS27, RPS18, PABPC1                    |
| Regulation of Actin-based motility by Rho | 2.00E-13 | 3.349   | 0.119 | ARPC3, MYL12B, WIPF1, ARPC5, RAC2, ACTR3                    |
| Signaling by Rho Family GTPases      | 5.01E-12    | 2.836   | 0.099 | JUN, WIPF1, ARPC5, RAC1B, FOS, ARPC5                       |
| Mitochondrial dysfunction            | 3.16E-31    | -0.23   | 0.119 | ARPC3, MYL12B, RPS26, RPS27, RPS18, PABPC1                  |

**Fig. 4** In vitro activated T cells can be identified by changes in metabolite transporter expression and metabolite utilization. CD8+ T cells from healthy donors were analyzed by flow cytometry at the indicated time points after in vitro stimulation with 0.1 μg ml−1 αCD3 and 1 μg ml−1 αCD28. T cell activation was assessed based on expression of **a**, **b** CD69, CD25, **c**, **d** TfR1, CD98, and **e**, **f** 2-NBDG BODIPY FL-C16 uptake. Error bars show mean and SEM. Results are representative of two independent experiments with a total of four individual donors. Source data are provided as a Source Data file.
on in vivo activated CD8+ T cells can be used to determine their effector/memory potential, we utilized scRNAseq to assess the relative abundance of memory precursor CD8+ T cell clonotypes within either the TIR1+HLA-DR+ or CD38+HLA-DR+ CD8+ T cell compartment 14 days post-TAK-003 administration. To this end, TIR1+HLA-DR+ CD8+ T cells from the same individual used in the previous scRNAseq analysis were sorted 14 days post-TAK-003 inoculation and subjected to scRNAseq analysis (Fig. 7a). This analysis resulted in the capture of 117 individual TAK-003-reactive cells with 80 unique TCR clonotypes (Fig. 7b, Supplementary Table 3). NS1- and NS3-reactive memory precursors within the sorted TIR1+HLA-DR+ CD8+ T cell pool were identified by the presence of TCR clonotypes found in NS1- and NS3-stimulated memory CD8+ T cells 120 days post-vaccination (Fig. 2b, d, Table 1). Of the 117 captured TIR1+HLA-DR+ CD8+ cells within the scRNAseq dataset, 31 and 24 were NS1- or NS3-reactive memory precursors, respectively (Fig. 7b). These numbers represent 47% of all cells within the sorted population, ~four-fold higher than the frequency of the same TCR clonotypes within the sorted CD38+HLA-DR+ CD8+ T cell pool previously analyzed (Fig. 2d, Fig. 7b).

To determine if the expression of TIR1 accurately delineates DENV-reactive memory precursors within the larger CD38+HLA-DR+ CD8+ T cell pool (Fig. 3, Cluster 1), we
performed scRNAseq gene expression analysis on the sorted TIR1-HLA-DR+ CD8+ T cells analyzed above. The resultant single cell gene expression dataset was merged with the previously generated gene expression analysis of sorted CD38+HLA-DR+ CD8+ T cells (Fig. 3), and the relationship and overlap between the two populations assessed (Fig. 7c). As predicted by the previous flow cytometry analysis, TIR1-HLA-DR+ CD8+ T cells form a distinct transcriptional cluster, overlapping with a subset of the larger CD38+HLA-DR+ CD8+ T cell pool (Fig. 7c, Supplementary Table 4). TIR1-HLA-DR+ CD8+ T cells transcriptionally co-localize with (and are enriched in) cells expressing either NS1- or NS3-reactive T cell receptors. Furthermore, TIR1-HLA-DR+ CD8+ T cells co-localize exclusively with those CD38+HLA-DR+ CD8+ T cells formerly falling into the previously defined Cluster 1 identified in Fig. 3a. These data demonstrate that the expression of TIR1 is a robust marker for the identification and isolation of vaccine-reactive CD8+ T cells enriched for effector/memory potential.

To extend the observation that TIR1 expression may better define vaccine-reactive CD8+ T cells with effector/memory potential, we further analyzed CD8+ T cells from an additional 12 individuals 14 days after immunization with TAK-003 by flow cytometry with the addition of intracellular markers of CD8+ T cell effector function, proliferation, and effector/memory potential. As expected, only a subset of CD38+HLA-DR+ CD8+ T cells express TIR1 14 days post-TAK-003 administration (Fig. 7d, Supplementary Fig. 10). Additionally, we observed that TIR1 expression within the CD38+HLA-DR+ CD8+ T cell compartment positivity correlates with the presence of markers of cellular proliferation (Ki67) (Fig. 7e, f), cytolytic function (Granzyme B) (Fig. 7e, g), and effector/memory lineage commitment (EOMES) (Fig. 7e, h). Expression of these markers were significantly enriched in TIR1+CD38+HLA-DR+ CD8+ T cells relative to TIR1-CD38+HLA-DR+ CD8+ T cells, or CD38−HLA-DR− CD8+ T cells. These data demonstrate that the surface expression of TIR1 is a marker of effector/memory potential, and may aid in the identification and characterization of vaccine-reactive T cells within the total activated CD8+ T cell pool (Supplementary Fig. 11).

**Discussion**

In this study, we demonstrate that the live-attenuated tetravalent DENV vaccine TAK-003 is capable of eliciting potent and durable cellular immunity. CD8+ T cell activation in response to TAK-003 administration peaked 28 days post-vaccination, while maximal CD4+ T cell expansion occurred on day 14. DENV-specific cellular immunity persisted for at least 120 days following immunization as assessed by IFN-γ ELISPOT. The antigenic specificity of the cellular memory immune response elicited by TAK-003 spanned the entire DENV proteome and exhibited significant cross-reactivity against all four DENV serotypes. Analysis of the clonotypic and functional diversity of TAK-003-stimulated CD8+ T cells 14 days after vaccination utilizing scRNAseq revealed a significant amount of transcriptional heterogeneity within a phenotypically homogenous population. Isolation and scRNAseq-based analysis demonstrated that the dominant TCR clones within the NS1- and NS3-reactive memory CD8+ T cell populations assessed 120 days post-vaccination can also be observed within the activated CD38+HLA-DR+CD8+ T cell compartment 14 days post-vaccination. scRNAseq-based analysis of these memory precursor cells present at day 14 post-vaccination revealed a unique transcriptional signature, dominated by the gene expression pathways associated with cellular metabolism and proliferation.

Based on these observations, we were able to develop a panel of markers to assess the metabolic potential of both CD8+ and CD4+ T cells following in vitro or in vivo activation. We were able to demonstrate that surface expression of TIR1 (transferrin receptor) marks cells with the highest functional and proliferative capacity, as assessed by Ki67, Granzyme B, and EOMES expression, providing a robust marker for identifying the CD8+ T cells shortly after vaccination with the greatest effector/memory potential. These data not only provide insight into molecular mechanisms responsible for regulating memory T cell development, but also suggest possible therapeutic targets for enhancing vaccine efficacy by selectively priming the metabolism of effector/memory precursor CD8+ T cells during the critical para-vaccine T cell expansion phase. We believe these data demonstrate that highly activated effector cells and memory precursor cells are not categorically incompatible descriptors, as a significant fraction of the memory CD8+ T cells generated in response to TAK-003 vaccination clonally overlap with a population of cells with a proliferative and highly activated transcriptional profile present shortly after vaccination.

The regulation of memory T cell development and homeostasis is a complex and incompletely understood process, involving the integration of a constellation of immunological cues such as antigen density, TCR/peptide/MHC affinity, duration of antigen exposure, and cytokine availability. However, it is becoming increasingly clear that the development of a stable memory T cell population is dependent on the availability of a handful of key metabolites and the expression of a corresponding metabolic cellular program. In particular, the availability of glucose, long-chain fatty acids, amino acids, and micronutrients such as iron can profoundly impact CD8+ T cell effector and/or memory potential. Depriving T cells of any of these key metabolites either through pharmacological or genetic means has significant implications for T cell development, effector function, and long-term persistence.

Directly manipulating the metabolism of T cells in vitro or in vivo to influence effector function or persistence has primarily focused on restricting or enhancing access to the metabolite glucose. Due to the unique metabolic requirements of nascently activated T cells, which overwhelmingly eschew conventional mitochondrial oxidative phosphorylation in favor of oxidative glycolysis, glucose metabolism is a convenient therapeutic target. The proliferation and terminal effector function of both CD4+ and CD8+ T cells can be significantly enhanced by increasing glucose bioavailability, whereas restricting glucose metabolism can facilitate the development of long-lived and/or suppressive T cell lineages. However, the utility of manipulating systemic glucose metabolism for therapeutic immunoregulatory effect in vivo is questionable. Systemic glucose metabolism is tightly regulated, and even modest perturbations in systemic glucose availability can have profound negative consequences for the well-being of patients. However, the selective upregulation of TIR1 on vaccine-reactive CD8+ T cells suggests that manipulating iron availability following vaccination may selectively enhance the expansion of the most functional vaccine-reactive CD8+ T cells. Indeed, loss-of-function mutations of TIR1 have been observed and are associated with severe defects in adaptive immune function. Furthermore, expression of the gene products highlighted in this study—such as TIR1—is not restricted to vaccine-elicted T cell activation, as natural infection with dengue is also associated with increased TIR1 expression on activated T cells.
These findings suggest the preferential survival of T cells undergoing clonal expansion in vivo is dependent on metabolite availability and the initiation of a transcriptional program permissive to nutrient uptake. Cumulatively, these data highlight the utility of high-content single-cell transcriptomic analysis coupled with more traditional cellular immune monitoring in assessing vaccine-elicited T cell immunity. The ability to accurately and longitudinally track T cell clones from acute infection to stable memory provides a unique opportunity to identify correlates of T cell-mediated immunity with single-cell resolution. Future work is required to verify the distinctiveness of the metabolic and transcriptional programming that determines long-term T cell

**Fig. 6** Vaccine-reactive T cells can be identified by changes in metabolite transporter expression and metabolite utilization. CD8+ T cells from TAK-003 recipients were analyzed by flow cytometry at days 0, 14, 28 and 120 post-vaccination. Vaccine-reactive CD8+ T cells were quantified based on expression of a CD38/HLA-DR, b CD38/2-NBDG, c CD38/BODIPY FL-C16, d TfR1/HLA-DR, and e CD98/HLA-DR. Error bars show mean and SEM. n = 10 individuals. *P < 0.05, ***P < 0.001, ****P < 0.0001 (Paired two-tailed t-test). Source data are provided as a Source Data file.
Fig. 7 TIR1 expression correlates with CD8\(^+\) T cell effector/memory potential in vaccine-reactive CD8\(^+\) T cells. CD8\(^+\) T cells from TAK-003-inoculated individuals were analyzed 14 days after immunization. a TIR1\(^+\)HLA-DR\(^+\) CD8\(^+\) T cells were sorted from a TAK-003 inoculated individual 14 days post immunization and subjected to single-cell RNAseq analysis. b The abundance of memory-precursor clonotypes was assessed at day 14 post-vaccination within the TIR1\(^+\)HLA-DR\(^+\) or CD38\(^+\)HLA-DR\(^+\) CD8\(^+\) T cell compartments. Memory-precursor clonotypes were defined as TCR clones found at both day 14 within the pools of phenotypically activated CD8\(^+\) T cells, and at day 120 within either NS1 or NS3 reactive memory CD8\(^+\) T cell populations. c scRNAseq gene expression analysis of sorted CD38\(^+\)HLA-DR\(^+\) CD8\(^+\) T cells and TIR1\(^+\)HLA-DR\(^+\) CD8\(^+\) T cells 14 days post TAK-003 administration. Merged datasets showing cellular library origin, memory precursor specificity, and Cluster 1 origin cells. d Flow cytometric analysis of TAK-003-reactive CD8\(^+\) T cells 14 days post-vaccination. Cells were subdivided into CD38\(^-\)HLA-DR\(^-\), and CD38\(^+\)HLA-DR\(^+\) TIR1\(^-\), and CD38\(^+\)HLA-DR\(^+\) TIR1\(^+\) populations, then assessed for intracellular expression of Ki67, GzmB, and EOMES. Error bars show mean and SEM. n = 12 individuals. *P < 0.05, ***P < 0.001, ****P < 0.0001 (Paired two-tailed t-test). Source data are provided as a Source Data file.
memory and whether these markers define the protective capacity of T cell immunity.

**Methods**

**Cells/samples.** The samples used in this study were collected during a Phase 1 trial of a novel dengue vaccine. Live attenuated dengue, TAK-003 (NCT01728792; WRAIR #1987), was used as a proof-of-concept vaccine. The vaccine was given to healthy volunteer recruits, and the samples were collected 140 days post-vaccination. Cryopreserved PBMC samples were thawed and resuspended in complete cell culture media at a concentration of 5 × 10^6 cells/mL and incubated for 30 min at 37°C. For assessment of glucose uptake, cells were subsequently washed 2X with glucose-free RPMI (ThermoFisher, 11879200), resuspended at a concentration of 5 × 10^6 cells/mL in glucose-free RPMI, and rested for an additional 10 min at 37°C. 2-NBDG (ThermoFisher, N13195) was added to the final concentration of 1 μM, and cells were incubated for 30 min at 37°C. Cells were washed 2X with PBS + 5% FBS, then surface stained for flow cytometry analysis as described above. For assessment of fatty-acid uptake, BODIPY FL-C16 (ThermoFisher, D3821) was added to cells in complete cell culture media at a final concentration of 1 μM. Cells were incubated for 30 min at 37°C, then washed 2X with PBS + 5% FBS and surface stained for flow cytometry analysis as described above. For assessment of transferrin uptake, transferrin-AF488 (ThermoFisher, T13342) was added to cells in serum-free RPMI at a final concentration of 50 μg/mL. Cells were incubated for 30 min at 37°C, then washed twice with PBS + 5% FBS and surface stained for flow cytometry analysis as described above. Binding specificity of 2-NBDG and BODIPY FL-C16 on activated T cells was assessed by flow cytometry analysis as described above.

**Sequencing.** scRNAseq 5′ gene expression libraries were sequenced on an Illumina NextSeq platform with a 500/500 High Output Kit v2 (150 cycles) to a read depth of ~30,000 reads/cell. Sequencing parameters were set for Read1 (26 cycles), Index1 (eight cycles), and Read2 (98 cycles). scRNAseq TCR V(D)J enriched library sequencing was performed on an Illumina MiSeq platform with a v3 Reagent Kit (600 cycles) to a read depth of ~10,000 reads/cell. Sequencing cycles were set at 150 for Read1, 8 for Index1, and 150 for Read2. Prior to sequencing, library quality and concentration were assessed using an Agilent 4200 TapeStation with High Sensitivity D5000 ScreenTape Assay and Qubit Fluorometer (Thermo Fisher Scientific) with dsDNA BR assay kit according to the manufacturer’s recommendation.

**TCR sequence analysis.** Sorted CD8+ T cell TCR clonotype identification, alignment, and annotation was performed using the 10x Genomics Cell Ranger pipeline. Sample demultiplexing and clonotype alignment was performed using the Cell Ranger software package (10x Genomics, CA, v2.1.0) according to the manufacturer’s recommendations, with the default settings, and nkfast/vdj consensus mode was selected. TCR clonotype annotation was performed using a filtered human V(D)J reference library generated using the Cell Ranger mkvdj command and the Ensemble GRCh38 v87 top-level genome FASTA and the corresponding Ensemble v87 gene GTF. TCR clonotype visualization, diversity assessment, and analysis were performed using the Loupe VDJ Browser (10x Genomics, CA, v2.0.0). TCR gene or enriched V(D)J library was set to VDJtools. TCR clonal overlap between NS1- and NS3-specific CD8 T cells and putative memory precursors was assessed by using the CDR3nt sequence of defined memory cells and the corresponding full-length clonotype as the search seed. T cells isolated at day 14 which shared the same CDR3nt sequence and corresponding clonotype as a defined memory cell were defined as memory precursors. Cells with only a full-length alpha-or-beta chain were matched based on only the overlap of the single chain.

**10x Genomics 5′ gene-expression data analysis.** 5′ gene expression analysis from sorted CD8+ T cells was performed using the 10x Genomics Cell Ranger pipeline. In short, sample demultiplexing and analysis was performed using the Cell Ranger software package (10x Genomics, CA, v2.1.0) according to the manufacturer’s recommendations, with the default settings and nkfast/count command, respectively. Transcript alignment was performed against a human reference library generated using the Cell Ranger mkvdf command and the Ensemble GRCh38 v87 top-level genome FASTA and the corresponding Ensemble v87 gene GTF. Data visualization and differential gene expression analysis were performed using the Loupe Cell Browser (10x Genomics, CA, v2.0.0). t-SNE plot visualization of gene expression data was based on the coordinate cells calculated by the Cell Ranger count command. Cell Ranger count outputs were subsequently filtered to only contain cells with a recovered TCR sequence as identified above, and to contain cells not expressing CD14 (monocytes) or CD19 (B cells) using the Cell Ranger reanalyze command. Aggregation and re-analysis of multiple gene expression datasets was performed using the Cell Ranger aggr command. Read-
depth normalization was performed on all merged datasets by subsampling mapped reads to achieve an equal number of confidently mapped reads per cell. Differential gene expression in Cell Ranger was calculated using a negative binomial exact test in sqeeq, paired with a false asymptotic beta test in edgeR for samples with a large number of counts. For each unique cluster, the algorithm was run on that cluster relative to all other clusters, generating a list of genes that were differentially expressed in the cluster of interest relative to all other cells in the sample. The top 20 differentially expressed genes in each cluster are listed in the relevant tables, first ranked by p-value (using the Benhamini-Hochberg procedure to control for FDR), then ranked by fold-change. Differentially expressed genes that failed to reach a Benhamini-Hochberg adjusted p-value of 0.05 were still listed, but marked as non-significant in the relevant tables.

Statistical analysis. All statistical analysis was performed using GraphPad Prism 6 Software (GraphPad Software, La Jolla, CA). A p-value < 0.05 was considered significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability The authors declare that all data supporting the findings of this study are available within this article and its Supplementary Information files, or from the corresponding author upon reasonable request. Single-cell RNAseq gene expression data have been deposited in the Gene Expression Omnibus database under the accession code GSE132950. The nature data underlying Figs. 1b, d, f, 4, 5 b, c, d, e, f, g, h, 6a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, are provided as a Source Data File.

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Author contributions
A.T.W. conceived of the project, designed, and executed experiments, analyzed data, and wrote the paper. K.V., T.L., and K.H. generated data. W.R. and H.F. designed and executed experiments and analyzed data. C.M. and B.G. analyzed data. R.G.J. and J.R.C. provided project oversight and secured funding.

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