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Molecular Signatures Distinguish Human Central Memory from Effector Memory CD8 T Cell Subsets

Tim Willinger, Tom Freeman, Hitoshi Hasegawa, Andrew J. McMichael, and Margaret F. C. Callan

Memory T cells are heterogeneous in terms of their phenotype and functional properties. We investigated the molecular profiles of human CD8 naive central memory (T_{CM}), effector memory (T_{EM}), and effector memory RA (T_{EMRA}) T cells using gene expression microarrays and phospho-protein-specific intracellular flow cytometry. We demonstrate that T_{CM} have a gene expression and cytokine signaling signature that lies between that of naive and T_{EM} or T_{EMRA} cells, whereas T_{EM} and T_{EMRA} are closely related. Our data define the molecular basis for the different functional properties of central and effector memory subsets. We show that T_{EM} and T_{EMRA} cells strongly express genes with known importance in CD8 T cell effector function. In contrast, T_{CM} are characterized by high basal and cytokine-induced STAT5 phosphorylation, reflecting their capacity for self-renewal. Altogether, our results distinguish T_{CM} and T_{EM}/T_{EMRA} at the molecular level and are consistent with the concept that T_{CM} represent memory stem cells.

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gain insight into their relationship at the molecular level. We elected to define memory subsets according to the Lanzavecchia model in view of the capacity of the model to distinguish between two apparently functionally distinct subpopulations of CD45RA⁺ primed T cells (T CM and T EM) and the extensive debate in the literature concerning the lineage relationship between T EM and T EMRA cells. Our results define a molecular basis for this model in view of the capacity of the model to distinguish between two apparently functionally distinct subpopulations of CD45RA⁺ primed T cells (T CM and T EM) and the extensive debate in the literature concerning the lineage relationship between T EM and T EMRA cells. Our results define a molecular basis for the different functional properties of human CD8 T cell memory subsets and place T EM cells between T N and T EM/TEMRA cells in terms of their molecular signatures.

Materials and Methods

Isolation of CD8 T cell subsets

PBMC were obtained from buffy coat preparations from four healthy donors by density gradient centrifugation using lymphoprep (Nycomed) in accordance with institutional ethics approval. We isolated CD8⁺ T cells by positive immunomagnetic selection using Dynabeads (Dynal) with detachment of the anti-CD8 mAb. Purity of the selected CD8⁺ T cells was >98% as assessed by flow cytometry. The CD8⁺ T cells were then stained with primary Abs specific for CCR7 (R&D Systems) and CD45RA (Biolegend) and sorted into T N (CCR7⁺ CD45RA⁺), T CM (CCR7⁻ CD45RA⁺), T EM (CCR7⁻ CD45RA⁻), and T EMRA (CCR7⁻ CD45RA⁻) populations on a MoFlow Cytometer (DakoCytomation). Purity of isolated subpopulations was 93–98%. Cell purification procedures were conducted at 4°C to minimize in vitro-induced changes in gene expression.

Preparation of cRNA and array processing

Total RNA was extracted from purified CD8 T cell populations using TRI reagent (Sigma-Aldrich) followed by RNA cleanup with the RNeasy kit (Qigen). We confirmed integrity of the total RNA by Lab-on-a-Chip 2100 Bioanalyzer (Agilent) quality control. Double-stranded cDNA was synthesized using a modification of the SMART-PCR protocol described by Peятalidis et al. (20). This protocol has been validated regarding the fidelity of amplification and compares favorably with the direct labeling approach in terms of sensitivity, speed and cost-effectiveness. Briefly, 300–600 ng of total RNA was reverse transcribed using a modified SMART CDS Primer IIA containing a 5’ T7 promoter sequence. After double-stranded DNA synthesis, the cDNA was subjected to 15 rounds of PCR according to the manufacturer’s recommendations. Biotin-labeled cRNA was generated from 2 μg of double-stranded cDNA by one round of in vitro transcription with the BioArray High Yield RNA Transcript Labeling kit (Enzo). cRNA yields were >50 μg for CCR7 (R&D Systems) and 1.9–2.1. After fractionation, labeled RNA was hybridized to Affymetrix HG-U133 plus 2.0 arrays (containing 54,675 probe identifiers (IDs)) according to the manufacturer’s instructions. Arrays were scaled to a target intensity of 100 using GCOS software (Affymetrix). Scaling factors for all arrays were within 2 SDs of the mean (range, 0.7–1.2). Percentage of genes as scored present on arrays by GCOS software was 32.8 ± 2.5%. 3’ to 5’ GAPDH ratios ranged from 0.83 to 1.4. We performed replicate microexperiments with RNA from four independent donors. R² values derived from scatter plots of signal intensity values were 0.98 or greater for individual replicates of CD8 T cell populations. All data have been deposited in the European Bioinformatics Institute ArrayExpress public database. Accession number: E-TABM-40.

Microarray data analysis

We used the comprehensive software package BRB-ArrayTools (21, 22) for data analysis. First, the robust multiarray average algorithm was applied for data normalization, background correction, and to calculate log intensity values were 0.98 or greater for individual replicates of CD8 T cell populations. All data have been deposited in the European Bioinformatics Institute ArrayExpress public database. Accession number: E-TABM-40.

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gene expression profiling using Affymetrix oligonucleotide microarrays. Unsupervised data analysis methods were used to explore the relationship between CD8 T cell subsets. First, we subjected the 16 samples (four replicates per subset) to hierarchical clustering using a filtered set of 10,854 probe IDs. We found that for both T_N and T_CM all four replicates clustered together (Fig. 1A). In contrast, T_EM and T_EMRA samples from the same donors formed individual clusters. Thus, two main clusters could be distinguished: T_N/T_CM and T_EM/T_EMRA. We confirmed the robustness of these two main clusters by obtaining a high overall cluster reproducibility measure (R index = 0.949, dendrogram cut at level of four clusters). Second, we performed MDS analysis to assess the distances between the expression signatures of the CD8 T cell subsets. MDS could separate T_EM and T_EMRA, but these two populations were still in close proximity (Fig. 1B). In contrast, their transcriptional profiles placed T_CM replicates between T_N and T_EM/T_EMRA. We also calculated average linkage distances between T_N and the different memory subsets as a measure for their relatedness. This showed that the gene expression profile of T_CM was closer to T_N than were the profiles of T_EM or T_EMRA (Fig. 1C).

Furthermore, we determined the number of differentially expressed genes between the CD8 T cell subsets by individual pairwise comparisons: It was highest for T_N vs T_EMRA and T_CM vs T_EMRA and lowest for T_EM vs T_EMRA (data not shown), again confirming the dichotomy T_N/T_CM vs T_EM/T_EMRA. Finally, we conducted a global permutation test to assess whether gene expression profiles between the CD8 subsets differed. The significance values (T_EM vs T_EMRA, p = 0.05714; all other pairwise comparisons p = 0.02857) again suggest that T_EM and T_EMRA have the most similar gene expression profiles among all CD8 T cell subsets. In summary, analysis of our results, using several different approaches, indicate that the T_EM and T_EMRA subsets are closely related, whereas T_CM have an expression signature that is distinct from that of the other primed T cells and is more closely related to the T_N population.

Genes differentially expressed between CD8 T cell memory subsets

Next, we identified genes that showed significant differential expression among the four CD8 T cell subsets. We used stringent statistical criteria, including a permutation test to minimize the FDR (see Materials and Methods). A total of 2092 probe IDs corresponding to 940 named genes met our criteria for differential expression: p < 0.001, FDR < 1% with 95% confidence. Hierarchical clustering of the differentially expressed genes revealed six main clusters (Fig. 2). Number of probe IDs in each cluster was as follows: 103 (cluster 1), 1369 (cluster 2), 92 (cluster 3), 16 (cluster 4), 129 (cluster 5), and 383 (cluster 6). Two major patterns could be identified among the clusters: First, genes with low expression in T_N with increasing expression from T_CM to T_EM to T_EMRA (clusters 2 and 3), termed “effector memory signature.” Second, genes with high expression in naive cells with decreasing expression from T_CM to T_EM to T_EMRA (clusters 5 and 6), termed “naive signature.” Cluster 1 consisted of genes with higher expression in T_CM and T_EM. Cluster 4 comprised genes with a “T_CM-specific” expression pattern. Surprisingly, very few genes fell within this category and probe IDs for CD28 accounted for 3 of the 16 included in this cluster (Fig. 2). Overall, >70% of all differentially expressed genes had expression levels in T_CM that were intermediate between their expression levels in the T_N population and the T_EM and T_EMRA subsets. These analyses again suggest that, at the

*The online version of this article contains supplemental material.*
level of gene expression, T_CM represent a differentiation state that is intermediate between T_N cells and the T_EM and T_EMRA subsets.

The effector memory signature of human CD8 T cells

Genes in clusters 2 and 3 (effector memory signature) appear to represent genes that underpin the known high effector capacity of T_EM and T_EMRA. We identified GO categories that displayed statistical overrepresentation among cluster 2 and 3 genes (hypergeometric statistics, Holm multiple testing correction, α = 0.05): GO Biological Processes such as “immune response,” “cytolysis,” and “protein transport” were overrepresented among the effector memory signature genes (Table I). Thus, T_EM and T_EMRA highly expressed genes encoding lytic granule proteins like granzyme A (GZMA), granzyme B (GZMB), granzyme H (GZMH), and perforin (PRF1) as well as TNFSF10 (TRAIL) and TNFSF6 (FASL) that mediate perforin-independent apoptosis of target cells. Also T_EM and T_EMRA showed strong expression of genes involved in protein sorting to granules and granule transport/exocytosis such as HPS3, MYO5A, RAB27A, and RABGGTA. Humans with genetic defects of any of these genes have impaired T cell cytotoxicity (30). Finally, genes encoding inflammatory cytokines (e.g., IFNG (IFNγ)) and chemokines (e.g., CCL5 (RANTES)) were also present in clusters 2 and 3. Consistent with this, in T_EM and T_EMRA subsets, we found higher expression of transcription factors that control effector function in CD8 T cells (31), i.e., EOMES, TBX21 (T-BET), REL, NFATC2, and NFATC3.4

In the case of some proteins, particularly those expressed at the cell surface, Abs capable of detecting expression are available. Using these we have confirmed higher expression of CCR7, CD62L, CD27, and CD28 in T_N and T_CM subsets at the protein level (data not shown). Apart from effector molecules, differential expression of other genes from clusters 2 and 3 between different CD8 T cell subsets has previously been described at the protein level: ITGAL (CD11a), ITGB2 (CD18), ITGAM (CD11b), ITGA4 (CD49d), KLRB1 (CD161), KLRD1 (CD94), killer Ig-like receptor family members, and TNFRSF6 (CD95). Therefore, the results of the protein expression studies are consistent with the results of our gene expression study. Overall, this further strengthens the validity of our gene expression data.

Cytokine signaling signatures of CD8 T cell memory subsets

We observed differential expression of cytokine receptor mRNA in our microarray analysis (Table II). For example, naïve CD8 T cells were characterized by higher expression of receptors for IL-6 (IL-6ST) and IL-7 (IL-7R). In contrast, T_EM and T_EMRA showed preferential expression of receptors for IL-2 family cytokines (IL-2RB,
IL-2RG) and for inflammatory Th1-type cytokines (TNFR2, IL-12RB1, IL-18R1, IL-18RAP, IFNGR1). This prompted us to investigate cytokine signaling in CD8 T cell subsets at the single-cell level using multiparameter flow cytometry (32, 33). In doing so, we aimed to generate a functional data set that could be subject to clustering and scaling analysis and give further insight into the relationship between the TCM and TEM/TEMRA subsets.

Activated STAT and NF-κB proteins that transduce cytokine signals were detected with phospho-specific Abs by intracellular FACS staining. Methanol permeabilization can compromise detection of some surface Ags (29), and we observed loss of discrimination for CCR7 surface staining (data not shown). Therefore, we substituted CCR7 with CD62L as a surface marker to identify CD8 T cell subsets in conjunction with CD45RA. CCR7 and CD62L expression largely overlap in CD8 T cells (Ref. 10, and own data not shown). In all, for each of the four T cell subsets, we analyzed basal expression levels of intracellular phosphorylated STAT1, STAT3, STAT4, STAT5, STAT6, and NF-κB. We also analyzed expression levels of phosphorylated STAT1 in cells stimulated with IFN-γ or IFN-α, phosphorylated STAT3 in cells stimulated with IL-6 or IL-10, phosphorylated STAT4 in cells stimulated with IL-12 or IFN-γ, phosphorylated STAT5 in cells stimulated with IL-2, IL-7, or IL-15, phosphorylated STAT6 in cells stimulated with IL-4, and phosphorylated NF-κB in cells stimulated with IL-18 or TNF-α. Examples of phospho-specific intracellular staining are shown in Fig. 3. Interestingly, ex vivo CD8 T cells had elevated levels of P-STAT1 and P-STAT5, which could be increased further by cytokine stimulation (Fig. 3 A).

Table I. GO categories overrepresented in effector memory signature (clusters 2 and 3)

<table>
<thead>
<tr>
<th>GO Biological Process</th>
<th>No. of Genes</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune response</td>
<td>47</td>
<td>0</td>
</tr>
<tr>
<td>Cellular defense response</td>
<td>15</td>
<td>0.0000000002</td>
</tr>
<tr>
<td>Intracellular signaling cascade</td>
<td>35</td>
<td>0.000000304</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>66</td>
<td>0.000000563</td>
</tr>
<tr>
<td>Cell growth and/or maintenance</td>
<td>29</td>
<td>0.000028800</td>
</tr>
<tr>
<td>Cell motility</td>
<td>13</td>
<td>0.000143000</td>
</tr>
<tr>
<td>Response to stress</td>
<td>10</td>
<td>0.010673760</td>
</tr>
<tr>
<td>Cytolysis</td>
<td>3</td>
<td>0.000517991</td>
</tr>
<tr>
<td>Nucleobase, nucleoside, nucleotide, and nucleic acid metabolism</td>
<td>6</td>
<td>0.010724858</td>
</tr>
<tr>
<td>Cell surface receptor-linked signal transduction</td>
<td>12</td>
<td>0.011990255</td>
</tr>
<tr>
<td>Protein transport</td>
<td>15</td>
<td>0.012280396</td>
</tr>
<tr>
<td>Cell matrix adhesion</td>
<td>8</td>
<td>0.015343897</td>
</tr>
<tr>
<td>Lipid transport</td>
<td>6</td>
<td>0.015350140</td>
</tr>
<tr>
<td>Response to oxidative stress</td>
<td>5</td>
<td>0.002518426</td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>16</td>
<td>0.034058555</td>
</tr>
<tr>
<td>DNA metabolism</td>
<td>3</td>
<td>0.040130732</td>
</tr>
</tbody>
</table>

This table lists differentially expressed genes with the biological processes as an analysis (Onto-Express). Biological processes (levels 4 and 5) with a total number of three or more genes and p < 0.05 are displayed.

Table II. Expression of cytokine receptor mRNA in CD8 T cell subsets

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>p</th>
<th>TM</th>
<th>TCx</th>
<th>TCM</th>
<th>TEM</th>
<th>TEMRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6ST</td>
<td>&lt;0.000000</td>
<td>596.9</td>
<td>235.2</td>
<td>164.9</td>
<td>171.3</td>
<td></td>
</tr>
<tr>
<td>TGFBR1</td>
<td>&lt;0.000000</td>
<td>349</td>
<td>197.5</td>
<td>465.1</td>
<td>853.6</td>
<td></td>
</tr>
<tr>
<td>TGFBR3</td>
<td>0.00000003</td>
<td>302.1</td>
<td>1065.7</td>
<td>1541.1</td>
<td>2021.2</td>
<td></td>
</tr>
<tr>
<td>IL10RA</td>
<td>0.00000016</td>
<td>571.6</td>
<td>1034.8</td>
<td>1393.5</td>
<td>1299.4</td>
<td></td>
</tr>
<tr>
<td>TGFBR2</td>
<td>0.00000022</td>
<td>233.6</td>
<td>141.5</td>
<td>123.5</td>
<td>126.8</td>
<td></td>
</tr>
<tr>
<td>TGFBR2</td>
<td>0.00000022</td>
<td>233.6</td>
<td>141.5</td>
<td>123.5</td>
<td>126.8</td>
<td></td>
</tr>
<tr>
<td>IL2RB</td>
<td>0.00000024</td>
<td>933.5</td>
<td>2021.4</td>
<td>2767.1</td>
<td>2859</td>
<td></td>
</tr>
<tr>
<td>TNFRSF1B</td>
<td>0.00000034</td>
<td>181.6</td>
<td>480.3</td>
<td>763.9</td>
<td>775.6</td>
<td></td>
</tr>
<tr>
<td>IL18RAP</td>
<td>0.00000074</td>
<td>136.3</td>
<td>366.3</td>
<td>966.5</td>
<td>902.4</td>
<td></td>
</tr>
<tr>
<td>ACVR1C</td>
<td>0.00000168</td>
<td>201.5</td>
<td>124.9</td>
<td>97.1</td>
<td>84.5</td>
<td></td>
</tr>
<tr>
<td>CRLF3</td>
<td>0.0000191</td>
<td>1655.5</td>
<td>851</td>
<td>1077.4</td>
<td>1299.3</td>
<td></td>
</tr>
<tr>
<td>IL18R1</td>
<td>0.0000288</td>
<td>39.1</td>
<td>73.9</td>
<td>108.4</td>
<td>110.7</td>
<td></td>
</tr>
<tr>
<td>IL12RB1</td>
<td>0.00001641</td>
<td>496.3</td>
<td>577.6</td>
<td>906.6</td>
<td>982.4</td>
<td></td>
</tr>
<tr>
<td>IL27RA</td>
<td>0.00001734</td>
<td>220.8</td>
<td>115</td>
<td>151.3</td>
<td>215.5</td>
<td></td>
</tr>
<tr>
<td>IL18R1</td>
<td>0.00002810</td>
<td>1743.4</td>
<td>1458.7</td>
<td>1978.9</td>
<td>2416</td>
<td></td>
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<tr>
<td>IFNGR1</td>
<td>0.0003961</td>
<td>689.7</td>
<td>1031.9</td>
<td>1461.6</td>
<td>1241.3</td>
<td></td>
</tr>
<tr>
<td>ACVR2</td>
<td>0.00004012</td>
<td>431</td>
<td>646.3</td>
<td>345.8</td>
<td>343.8</td>
<td></td>
</tr>
<tr>
<td>IL7R</td>
<td>NS</td>
<td>3699.1</td>
<td>2732.9</td>
<td>2189.8</td>
<td>1348.6</td>
<td></td>
</tr>
<tr>
<td>IL4R</td>
<td>NS</td>
<td>734.8</td>
<td>858.5</td>
<td>709.9</td>
<td>539.7</td>
<td></td>
</tr>
</tbody>
</table>

This table lists differentially expressed genes encoding cytokine receptors. Gene expression values (RMA expression measures), fold change values relative to TCM (in bold), and associated p are shown.
that, as known from the literature, some cytokines did not induce phosphorylation of certain STAT proteins, e.g., STAT1 and STAT3 in response to IFN-β, IL-10, and IL-15, respectively (thick line histograms). Thin line histograms indicate basal phosphorylation and dashed line histograms indicate isotype control staining. Histograms are gated on CD8+ lymphocytes. B, Phospho-STAT7/IFN-κB proteins were detected in unstimulated or cytokine-stimulated PBMC after staining with Abs for CD8, CD62L, CD45RA, and P-STAT/P-IFN-κB. Gating of CD8+ cells based on CD62L/CD45RA expression was used to define Tn, TCM, TEM, and TEMRA subsets. A representative example from one donor is shown for STAT3, STAT5, and IFN-κB phosphorylation in response to IL-6, IL-7, and TNF-α, respectively (thick line histograms). Thin line histograms indicate basal phosphorylation.

A. Phospho-STAT proteins were detected in unstimulated or cytokine-stimulated PBMC after staining with Abs for CD8 and P-STAT. A representative example from one donor is shown for STAT1, STAT3, and STAT5 phosphorylation in response to IFN-β, IL-10, and IL-15, respectively (thick line histograms). Thin line histograms indicate basal phosphorylation and dashed line histograms indicate isotype control staining. Histograms are gated on CD8+ lymphocytes. B. Phospho-STAT7/IFN-κB proteins were detected in unstimulated or cytokine-stimulated PBMC after staining with Abs for CD8, CD62L, CD45RA, and P-STAT/P-IFN-κB. Gating of CD8+ cells based on CD62L/CD45RA expression was used to define Tn, TCM, TEM, and TEMRA subsets. A representative example from one donor is shown for STAT3, STAT5, and IFN-κB phosphorylation in response to IL-6, IL-7, and TNF-α, respectively (thick line histograms). Thin line histograms indicate basal phosphorylation.

In all we performed experiments on samples of blood taken from 10 different donors and found differences in levels of basal STAT/IFN-κB phosphorylation between the four different T cell subsets. Interestingly, TCM were characterized by significantly higher cumulative basal STAT/IFN-κB phosphorylation compared with TEM and TEMRA subsets (TCM vs TEM, p = 0.006, TCM vs TEMRA, p = 0.001, one-way ANOVA/Tukey’s post hoc test). We also found higher basal P-STAT1 (p = 0.037) and P-STAT5 levels (p = 0.013) in TCM compared with TEMRA.

Furthermore, the four different T cell subsets responded differently, in terms of changes in STAT/IFN-κB phosphorylation, to stimulation with cytokines (Fig. 4). Despite their already elevated P-STAT5 levels, overall TCM had higher induction of STAT5 phosphorylation in response to IL-2 family cytokines (IL-2: TCM vs TN, p < 0.001; TCM vs TEM, p = 0.044, TCM vs TEMRA, p < 0.001; IL-7: TCM vs TEMRA, p < 0.001; IL-15: TCM vs TN, p = 0.002, TCM vs TEMRA, p = 0.011). In Tn we detected strong phosphorylation of STAT1 in response to IFN, STAT3 in response to IL-6, STAT5 in response to IL-7, and STAT6 in response to IL-4. Overall, Tn and TCM displayed the highest cumulative cytokine-stimulated phosphorylation response (ranging from TN to TCM, p = 0.01, TCM vs TEMRA, p = 0.02). Finally, we found preferential phosphorylation of NF-κB in response to IL-18 and TNF-α in the TEM and TEMRA subsets (IL-18: TEM vs TN, p < 0.001, TEM vs TN, p < 0.001, TEM vs TEMRA, p = 0.008; TNF-α: TEM vs TN, TEM vs TEMRA, TEM vs TEMRA vs TN, and TEMRA vs TCM, all p < 0.001). Thus, human CD8 T cell subsets are characterized by the differential activation of cytokine signaling pathways.

We clustered CD8 T cell subsets based on their cytokine signaling profiles. The 18-parameter phospho-signature, based on the 6 basal and 12 stimulated levels of phosphorylated STAT and NF-κB molecules, again placed TCM between TN and TEM and TEMRA subsets (Fig. 5A). Interestingly, the responses to the proinflammatory cytokines IL-12, IL-18, and TNF-α clustered together and formed a bigger cluster with IL-10 and IL-15. In contrast, IFNs were found in a cluster with IL-4, IL-6, and IL-7. We also performed MDS analysis based on our FACS phospho-proteome data. Similar to the gene expression data, TCM took up an intermediate place between TN and TEM/TEMRA (Fig. 5B). Consistent with this, the average linkage distance between TCM and TN was smaller than that between TEM or TEMRA and TN (Fig. 5C). Thus, the cytokine signaling signatures support the observations from transcriptional profiling in terms of the molecular relationship of CD8 memory cells.

Discussion

In this study, we identified both the gene expression and cytokine signaling profiles of CD8 Tn, TCM, TEM, and TEMRA cells. Our results provide a molecular basis for the different functional properties of different memory subsets, especially in terms of their self-renewal and effector capacity. They also shed light on the relationship between the primed T cell subsets.

In contrast with a recent study (34) that used the van Lier classification of CD8 T cells, we used the Lanzavecchia model as a basis for our experiments. This allowed us to compare the properties of the TCM subset with those of other T cell populations. Interestingly, we found a clear dichotomy between TCM and the two effector memory (TEM and TEMRA) CD8 T cell subsets at the molecular level. Analysis of both gene expression and cytokine signaling showed that TCM cells were significantly more closely related to TN cells than TEM or TEMRA cells were. Furthermore, the results of the gene expression analysis imply that TCM cells form a population that is broadly intermediate between Tn and TEM or TEMRA cells. The observation that there are very few truly “TCM specific” genes is most consistent with the idea that these cells form part of a continuum of differentiation or functional states (35–37). However, it is possible that some of the defining features of TCM are epigenetically imprinted, but not yet actively transcribed, and are therefore not identified by transcriptional profiling.

In many respects, the genetic profile of CD8 TCM cells suggests they are equipped with features of both naive and effector memory cells. Thus, TCM possess some stem cell-like qualities, such as a high proliferative capacity, that are also found in the TN cell population. In this regard, TCM displayed high basal and IL-2 family-induced STAT5 phosphorylation. Cytokines of the IL-2 family, which mainly signal through STAT5, are known to play an important role in the differentiation and homeostasis of CD8 T cells (38, 39). Thus, IL-15 can induce the homeostatic proliferation of CD8 memory T cells, whereas IL-7 has recently been implicated in the survival of murine CD8 memory precursors, which can be
identified by selective expression of the IL-7Rα (40). Furthermore, a recent study found that STAT5 regulates the self-renewal and differentiation of human memory B cells (41). Therefore our results might explain why TCM have a greater expansion potential and self-renewal capacity than effector memory cells, TEMRA in particular. In contrast, we found that both effector memory subsets strongly express genes involved in CD8 T cell effector function. However, our data also show that the gene expression profile of TCM is biased to some extent toward effector differentiation, characteristic of effector memory cells. Thus, mRNAs encoding effector molecules like perforin, granzyme A, IFN-γ, and RANTES were up-regulated in TCM compared with TN.

The lineage relationship between CD8 T cell subsets remains controversial. Our study is cross-sectional in design and therefore does not directly address questions relating to T cell lineage. It is however interesting to consider the results of our study in the light of recently proposed models. Lanzavecchia and colleagues (6) have suggested a signal strength model of T cell memory differentiation. According to this model, TCR signal strength determines the memory cell fate a naïve T cells acquires: A low Ag dose leads to the differentiation T to noneffector to TCM, whereas a high Ag dose to T to effector to TEM differentiation. Their work also suggests that human TCM further differentiate into TEM following Ag stimulation and into TEMRA in response to homeostatic cytokines (9). The results of our microarray analysis showing that the gene expression profile of TCM cells is largely intermediate between TN and TEM or TEMRA subsets would be consistent with this type of model. In contrast, another model based on the idea that TCM and TEM are independent populations has been proposed by Pannetier and coworkers (19), who found that the TCR repertoires of influenza-specific TCM and TEM are largely distinct. We did not find many truly TCM- and TEM-specific genes and this argues against an independent TCM/TEM differentiation pathway. Finally, Ahmed and coworkers (18) have defined a linear differentiation pathway T to effector to TEM in a murine model of acute LCMV infection. Interestingly, this TEM to TCM conversion does not occur in chronic LCMV infection (42). A second study by Pannetier and colleagues (43) using murine H-Y-specific CD8 T cells confirmed this linear differentiation pathway although there was also evidence for an independent TCM/TEM differentiation pathway. The observation that TCM cells are intermediate between TN and TEM cells does not obviously support this type of linear differentiation model. However, the present study is cross-sectional in nature and therefore we do not exclude the possibility that TEM convert to TCM. Interesting questions about the plasticity of the genetic program of the CD8 memory T cell remain and further work will be needed to address these issues. Finally, it is noteworthy that the studies by Ahmed and Pannetier have not examined the TEMRA subset in relation to TCM and TEM as we did in the present work. Effector memory cells re-expressing CD45RA form a sizeable population in human peripheral blood and are likely to have an important role in human CD8 T cell memory.
In conclusion, our genomic and phospho-proteomic study demonstrates a dichotomy between CD8 T\textsuperscript{CM}, T\textsubscript{EM}, and T\textsubscript{EMRA} cells at the molecular level and suggests that human T\textsubscript{CM} cells represent an intermediate state between the T\textsubscript{N} and the T\textsubscript{EM}, T\textsubscript{EMRA} populations in terms of CD8 memory differentiation and function. By defining molecular signatures for CD8 memory subsets, we provide a framework for the further molecular characterization of human CD8 T cell memory.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


**FIGURE 5.** Cytokine signaling signatures of CD8 memory subsets. A. Hierarchical clustering of cytokine phospho-responses in CD8 T cell subsets. Complete linkage clustering using the Pearson correlation was conducted to define a 18-parameter biosignature for CD8 subsets. The color bar indicates the mean log\textsubscript{2} ratios of cytokine-stimulated vs unstimulated phospho-specific fluorescent intensities from 10 donors. Basal responses are relative to the minimum among samples. B, MDS analysis of CD8 T cell subsets. The three-dimensional plot shows the between sample distances for 10 replicate CD8 T cell subset samples based on the 18-parameter biosignature from A. Pearson correlation was used as the distance metric. Each dot represents a single replicate sample. Color coding: T\textsubscript{N} (red), T\textsubscript{CM} (green), T\textsubscript{EM} (dark blue), and T\textsubscript{EMRA} (light blue). C, Average linkage distances between CD8 T\textsubscript{N} and memory subsets. Average linkage distances between each CD8 memory T cell subset and T\textsubscript{N} were calculated as described in *Materials and Methods* based on the 18-parameter biosignature from A. Error bars represent SEs (SD of pairwise linkages divided by the square root of the number of linkages).


