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Distinct transcriptomic and epigenomic modalities underpin human memory T cell subsets and their activation potential

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Human memory T cells (MTC) are poised to rapidly respond to antigen re-exposure. Here, we derived the transcriptional and epigenetic programs of resting and ex vivo activated, circulating CD4⁺ and CD8⁺ MTC subsets. A progressive gradient of gene expression from naïve to T̄CM to T̄EM is observed, which is accompanied by corresponding changes in chromatin accessibility. Transcriptional changes suggest adaptations of metabolism that are reflected in altered metabolic capacity. Other differences involve regulatory modalities comprised of discrete accessible chromatin patterns, transcription factor binding motif enrichment, and evidence of epigenetic priming. Basic-helix-loop-helix factor motifs for AHR and HIF1α distinguish subsets and predict transcription networks to sense environmental changes. Following stimulation, primed accessible chromatin correlate with an augmentation of MTC gene expression as well as effector transcription factor gene expression. These results identify coordinated epigenetic remodeling, metabolic, and transcriptional changes that enable MTC subsets to ultimately respond to antigen re-encounters more efficiently.
Memory T cells (MTC) arise after a naïve T cell responds to an initial interaction with antigen and play an essential role in mounting a robust secondary response upon reinfection. These cells, composed of both CD4 and CD8 expressing T cells, exhibit enhanced effector functions and a heightened response to subsequent activations with antigen. Human MTC are extremely long-lived, with one study finding antigen-specific T cells still detectable in individuals 75 years after vaccination, suggesting a capacity for self-renewal not seen in shorter-lived effector cells. Naïve and memory CD4+ and CD8+ T-cell subsets are commonly subdivided based on cell surface expression of CCR7, CD45RA, and CD62L into naïve (Nav, CCR7+CD45RA+), central memory (TCM, CCR7-CD45RA-), effector memory (TEM, CCR7-CD45RA-), and terminally differentiated effector memory (TEMRA, CCR7-CD45RA+). CCR7 and CD62L are highly expressed in TCM and naïve T cells and enable cellular homing to secondary lymphoid organs. TEM exhibit lower expression of both CCR7 and CD62L and are thought to instead localize to inflamed tissues where they exhibit a high degree of effector function. Additionally, naïve T cells can be separated from TCM and TEM cells by higher expression of the CD45RA isof orm, which is downregulated in TCM and TEM cells. However, a subset of effector-memory-like human CD8+ T cells in the blood express high levels of CD45RA (CCR7-CD45RA+) while simultaneously expressing effector function genes such as GZMB and PRF1 (encoding granzyme B and perforin, respectively). These cells (TEMRA) are believed to be highly differentiated effector cells or potentially even senescent versions of effector MTC, which arise after chronic infections with virus. Despite their importance in immunological memory, the full spectrum of transcriptional and epigenetic characteristics of these main memory subsets, the transcription factor programs associated with each, and how they play a role in memory responses remains to be fully understood.

Previous studies have profiled various epigenetic and transcriptional aspects of CD8+ MTC; however, the full phenotypic and epigenetic characteristics of the subsets found within the entire circulating human MTC compartment remains largely under-defined. At the same time, changes to the transcriptomic and epigenetic landscape of T cells after initial activation of naïve cells have been shown to be important in both establishing the differentiated memory subsets, and in rapid recall response upon re-stimulation. Understanding how these memory-subset-specific epigenetic changes affect their formation and function upon re-encounter with antigen will ultimately inform more effective therapeutic design.

To better understand the epigenomic parameters of human MTC subsets and how such parameters dictate gene expression, we investigated transcriptional and epigenetic differences between CD4+ and CD8+ memory cell subtypes (TCM, TEM, and TEMRA) from human blood, as well as in response to ex vivo stimulation. Analysis of changes in mRNA transcripts and chromatin accessibility revealed that MTC share a substantial set of genes expressed at similar levels irrespective of lineage and cell subset. Moreover, we observed evidence of a progressive increase in the amount of differentiation from naïve T cells, to TCM, and finally to TEM and TEMRA memory populations. One set of genes that were upregulated, included those that reflected changes in metabolic capacity of the MTC subsets. Biochemical analyses confirmed differences in the metabolic capacity of various MTC subsets. Combining gene expression and chromatin accessibility analyses before and after stimulation identified a series of patterned regulatory modalities that may define memory-subset differentiation, as well as increased reactivity in response to secondary activations. Such dynamic regions of chromatin were enriched for motifs known to bind a small group of transcription factors (TFs) from the basic leucine zipper (bZIP), high mobility group (HMG), T-box, and basic helix-loop-helix (BHLH) families. Altogether, these data lay out the full spectrum of transcriptional and epigenetic differences of the primary memory-subset categories found in human blood, while also identifying the unique transcription factor networks associated with memory-subset differentiation and highlighting loci that may be important in establishing a memory cell’s response to activation upon secondary antigen exposure.

Results

The shared transcriptional programs of human CD4+ and CD8+ MTC. To define the transcriptional profiles of the major subsets of MTC, naïve, TCM, and TEM cells from CD8+ and CD4+ lineages were FACS separated from the blood of four human donors using CCR7 and CD45RA as distinguishing cell surface markers (Fig. 1a, b, and Supplemental Fig. 1a). Naïve T cells were defined as CCR7+CD45RA+; TCM as CCR7+CD45RA-; and TEM as CCR7-CD45RA-. CD8+ TEMRA T cells (CD45RA+CCR7-), a terminal effector subset was also included for comparison (Supplemental Fig. 1b-c). To elucidate transcriptional differences between naïve and memory subsets RNA-sequencing (RNA-seq) was conducted on sorted populations. The mRNA content of each sorted subset group was assessed for shared and unique transcripts defining MTC from naïve T cells. Relative to their naïve counterparts, TCM and TEM cells exhibited 805 and 1,486 differentially expressed genes (DEG), respectively (Fig. 1c, Supplemental Data 1). Comparing subsets between lineages (CD4+ vs. CD8+), showed that TEM and TCM cells each shared approximately 30% of their DEG, highlighting conserved relationships between the CD4+ and CD8+ lineages. Gene ontology analysis of the lineage specific and shared DEG showed that genes shared between lineages were highly enriched for pathways related to T-cell activation, co-stimulation, lymphocyte homeostasis, and cytokine response/production in both TCM (Supplemental Fig. 2a) and TEM subsets (Supplemental Fig. 2b). TEM DEG shared between CD4+ and CD8+ T cells were also more highly enriched for antigen processing and presentation, as well as GTPase signaling pathways.

Examples of genes expressed in both lineages included the transcription factors LEFI and TCF7, which were more highly expressed in both CD4+ and CD8+ TCM and naïve T cells. PDCDI1, encoding PD-1, and MHC class II genes encoding HLA-DR were expressed higher in TEM cells of both lineages (Fig. 1d). Other genes exhibited lineage-specific expression patterns, including integrins (e.g., ITGAL/CD11a, ITGA4/VLA4), which were expressed more highly in CD8+ MTC; whereas the chemokine receptors CCR4 and CCR6, which have been implicated in homing to specific peripheral tissues such as the skin, were more expressed in CD4+ MTC (Fig. 1e). Lineage-specific DEG encoding components of the interleukin-2 receptor (IL2R) were also observed, with CD4+ MTC expressing higher levels of the α chain (IL2RA, Fig. 1f), while CD8+ MTC expressed considerably higher levels of IL2RB (Fig. 1f). These differences emphasize the fact that while all MTC share important gene pathways, which make them unique from naïve cells, transcriptional differences can also distinguish each lineage and/or subset underlying their distinct immunological functions.

Resting TEM/TEMRA cells exhibit progressively greater transcriptional differentiation from naïve progenitors than TCM. A total of 4,943 DEG distinguished naïve cells from CD4+ and CD8+ MTC subsets. Principal component analysis (PCA) of these DEG indicated that the bulk of this variation separated naïve T cells from TCM, TEM, and TEMRA subsets (Fig. 2a).
Fig. 1 MTC display distinct patterns of shared and differentially expressed genes that define their subsets and lineage. a Experimental workflow for isolation, ex vivo stimulation, and sequencing analysis of MTC from human blood samples. b Color code of naïve T cell and MTC lineages and subsets used throughout are shown. c Venn diagrams representing total number of DEG for T_CM and T_EM compared to naïve T cells. d Heatmap and hierarchal clustering of gene expression for select genes commonly expressed in either T_CM or T_EM regardless of lineage. e Heatmap of lineage-specific gene expression in MTC. f Gene expression bar plots (reads per kilobase million, rpkm) for indicated IL-2 receptor genes. Data are plotted as mean ± SD (standard deviation); asterisks indicate DEG (FDR ≤ 0.05) as detected by DESeq2 algorithm. Cell type color codes are shown in (b).

Differentiation from naïve cells showed progressive increases in both up and down DEG from T_CM to T_EM in both CD4+ and CD8+ T cells and finally to T_EMRA in CD8+ T cell subsets (Fig. 2b). Fold-change levels of DEG in CD8+ T-cell memory subsets were compared using hexagonal “tri-wise” visualization (Fig. 2c)20. These plots contain three axes corresponding to each subset and each radiating axis represents fold-change levels. DEG that fall directly on each axis represent those expressed exclusively by that subset, while those sharing high fold-change levels across two groups relative to the third subset fall midway between spokes. This analysis revealed that many of the DEG with highest fold change were shared between T_EM and T_EMRA subsets but absent in T_CM cells. Overlaid genes from several gene ontology gene sets relevant to MTC showed that genes involved in pathways related to cell cytotoxicity as well as NK-related genes involved in cell killing were exclusively upregulated in T_EM and T_EMRA subsets (Fig. 2d). Alternatively, a custom gene set representing genes expressed by stem-like T cells derived from Hudson et al. 21, as well as gene ontology gene sets representing the WNT-beta catenin pathway were highly upregulated in the T_CM subset relative to either effector subset (Fig. 2d).

Fuzzy c-means clustering of the DEG was used to identify gene modules across the CD8+ and CD4+ MTC subsets. Fuzzy c-means clustering allows genes to be assigned membership to multiple overlapping clusters before an ultimate single cluster classification is determined by ranking the magnitude of membership score for that gene. This analysis identified five distinct modules of gene expression programs within the DEG of CD8+ T cells across all memory subsets (Fig. 2e, Supplemental Data 2). Modules 1 and 3 corresponded to genes that were upregulated in CD8+ T_EM and T_EMRA cells compared to naïve T cells. Gene ontology analysis showed that genes in module 1 were most highly expressed in T_EMRA cells and enriched for pathways related to T-cell killing, cytokosis, and innate immune response (Supplemental Fig. 3a). Module 2 contained fewer genes expressed in CD8+ T_EM and T_EMRA cells but more highly expressed in naïve or T_CM cells (Fig. 2f) with individual genes plotted and colored according to cluster membership (Fig. 2g). These were enriched for DNA methyltransferases such as DNMT3A or pathways related to T-cell differentiation, respectively (Supplemental Fig. 3b). Modules 4 and 5 represented genes repressed in CD8+ T_EM and T_EMRA subsets but more highly expressed in naïve or T_CM cells (Fig. 2f) with individual genes plotted and colored according to cluster membership (Fig. 2g). Application of the same clustering method to CD4+ T subsets showed a similar pattern of clustering with five total gene modules (Fig. 2h, i, Supplemental Data 3) with individual genes plotted (Fig. 2j). Modules 1, 3, and 4 were most highly expressed in CD4+ T_EM cells (Fig. 2i) but showed differences in gene ontology
pathway enrichment (Supplemental Fig. 3f-h). These included secretory granules and carbohydrate metabolism (module 1), cytokine secretion, and T-cell activation (module 3), and MAP Kinase activity or cytolysis (module 4). The expression of genes in module 5 was highest in CD4+ naive T cells and contained similar gene set enrichments to module 5 in CD8+ MTC lineage. Pathway enrichment for this module showed enrichment for several metabolism-related pathways including cofactor and lipid biosynthesis (Supplemental Fig. 3i), including self-renewal related transcription factors, such as \(\text{LEF1}\. Module 2 was expressed in both CD4+ TCM and T EM subsets as in the CD8+ lineage. Pathway enrichment for this module showed enrichment for several metabolism-related pathways including cofactor and lipid biosynthesis (Supplemental Fig. 3i), as with CD8+ MTC,
MTC subsets exhibit distinct migration and metabolism characteristics. Contained within the above modules were genes representing the potential for important functional differences across MTC subsets, including genes involved in T-cell migration and metabolism. For example, as expected,$^{3}$ CD4$^+$ and CD8$^+$ naïve and TCM cells expressed high levels of both CCR7 and SELL (L-selectin). Whereas CD4$^+$ TCM and TEM exclusively expressed CCR4 and CCR2 (Fig. 2k). CD8$^+$ TEM/TEMRA cells had the highest expression of SIPR5, which has been shown to be associated with promoting egress of lymphocytes from secondary lymphoid organs or bone marrow (Fig. 2i)$^{22}$. MTC in general were found to have upregulated a greater number of metabolism-related genes compared to resting naïve T cells (Fig. 3a). Subset-specific differences were observed in expression of genes responsible for fatty acid metabolism, glycolysis, and oxidative phosphorylation (Fig. 3a). Many of these genes relate to the regulation of acetyl CoA or lactate metabolism as exemplified by expression differences of the genes PDK1, PDP1, and LDHB. For example, in CD8$^+$ TEM and TEMRA cells (Fig. 2j), LDHB, which encodes lactate dehydrogenase enzyme subunit B has been previously associated with aerobic glycolytic metabolism in effector T cells, as well as in cancer.$^{23,24}$

Several assays to measure and compare the metabolic states of naïve and MTC were performed. Using the Seahorse-based Glycolysis Stress Test assay on unstimulated CD4$^+$ T-cell subsets, an extracellular acidification rate (ECAR) was determined (Fig. 3b). In response to glucose, CD4$^+$ MTC did not exhibit a significant difference in glycolysis; however, TCM and TEM populations showed significantly higher glycolytic capacity (Fig. 3c) and glycolytic reserve (Fig. 3d) compared to naïve CD4$^+$ T cells, suggesting that they have higher potential to increase ATP production via glycolysis under stress or other physiologically energy-demanding conditions. Anti-CD3/CD28 bead stimulated CD4$^+$ T-cell subsets showed an elevated level of glycolysis compared to unstimulated cells, which was consistent with metabolic reprogramming phenomena in response to stimulation (Fig. 3b). No significant difference between the stimulated MTC subsets regarding glycolysis, glycolytic capacity, or glycolytic reserve was observed, suggesting that regardless of subset, CD4$^+$ MTCs can achieve similar glycolytic metabolic rates.

Due to the limitations in obtaining purified subset cell numbers, we could not perform a similar set of assays on CD8$^+$ T cells. Instead, two flow cytometry-based assays were used to assess oxidative phosphorylation (OXPHOS) and fatty acid metabolic states of both the CD4$^+$ and CD8$^+$ T cells (Fig. 3e–h). In the first assay, cells were stained with MitoTracker Green (MTG), a mitochondrial specific dye that provides a relative assessment of mitochondrial mass, and tetramethylrhodamine methyl (TMRM), an OXPHOS marker that accumulates in functional mitochondria caused by differential membrane potential.$^{25,26}$ Unstimulated TCM and TEM populations had higher frequencies of cells with highly functional mitochondria (MTG$^{Hi}$ and TMRM$^{Hi}$) compared to naïve populations in both CD4$^+$ and CD8$^+$ T cells (Fig. 3f). Stimulated CD4$^+$ and CD8$^+$ TCM and CD8$^+$ TEM subsets also had higher frequency of cells with functional mitochondria than naïve populations (Fig. 3h). In contrast, a lower percentage of CD8$^+$ TEMRA had functional mitochondria and this number decreased when the cells were stimulated, which is consistent with previous studies showing mitochondrial impairment of this population.$^{27}$

In a second assay, we performed the single cell energetic metabolism by profiling translation inhibition (SCENITH) assay that measures metabolism of individual cells based on relative translation rates and the incorporation of the puromycin into elongating ribosomes in the presence and absence of metabolic pathway inhibitors to assess glycolysis, OXPHOS, or fatty acid metabolism.$^{28}$ (Fig. 3i–p). The SCENITH data confirmed the Seahorse results showing that unstimulated CD4$^+$ TEM had higher glycolytic capacity than the naïve CD4$^+$ T cells (Fig. 3i). In addition, all CD8$^+$ MTC showed higher glycolytic capacity than the naïve CD8$^+$ T cells. Glucose dependence; however, was significantly lower in only CD8$^+$ TEMRA (Fig. 3j). Overall, unstimulated naïve T cells exhibited more mitochondrial dependence than the MTC (Fig. 3k). Together with the mitochondria staining data, these results suggest that the unstimulated MTC subsets have elevated OXPHOS metabolism compared to naïve populations, a finding that is also consistent with the RNA-seq data; however, resting naïve T cells depended on mitochondria more than the MTC subsets. CD8$^+$ TEMRA cells exhibited an elevated fatty acid and amino acid oxidation capacity (FAO and AAO) compared to naïve CD8$^+$ T cells (Fig. 3i). In general, when these cells were stimulated they reprogrammed their metabolism to be less dependent on mitochondria and instead increased glycolytic capacity to its maximum (Fig. 3m, n). We did not detect any significant difference in the metabolisms of stimulated naïve, TCM and TEM populations of CD4$^+$ and CD8$^+$ T cells (Fig. 3m–p). However, CD8$^+$ TEMRA cells showed less glycolytic capacity and glucose dependence, but higher mitochondrial dependence and FAO and AAO capacity than naïve CD8$^+$ T cells. Taken together these data show the distinct transcriptional differences between naïve and MTC, the resulting functional consequences to cell metabolism and phenotype, and that these differences appear to increase in both number and magnitude as cells differentiate towards an effector-memory phenotype.
The chromatin landscape of memory subsets correlates with transcriptional differentiation. To determine the extent to which the differences in gene expression were coordinated with changes to the chromatin organization in MTC subsets, the assay for transposase accessible chromatin-sequencing (ATAC-seq) was performed on the MTC subsets described above. This analysis resulted in the identification of 57,315 differentially accessible regions (DAR) between naïve, TCM, TEM, and TEMRA cells of both CD4+ and CD8+ T-cell lineage groups (Supplemental Data 4). As with the RNA-seq data, principal component (PC) 1 separated effector-memory subsets (TEM, TEMRA) from TCM and naïve T cells in a progressive manner (Fig. 4a). Hierarchical clustering of the DAR also revealed CD8+ effector-memory and naïve T cells as being the most distinct, with central memory and all CD4 MTC subsets sharing more similarity to naïve cells, and CD8 TEM/TEMRA cells clustering closely together (Fig. 4b).
**Fig. 3** MTC subsets exhibit distinct metabolic features. 

a Heatmap of selected genes representing differences in metabolism. Data represent the mean expression of each cell subset.

b Line plot showing Seahorse-derived ECAR data (mP / min / 10^5 cells) over time for unstimulated and stimulated naïve, T_{CM}, and T_{EM} CD4^+ T cells. Shaded regions indicate regions of metabolic relevance as labeled. Arrows indicate time of inhibitor addition (G, glucose; O, oligomycin; and DG: 2-deoxy-glucose).

c, d Bar plots showing glycolytic capacity and glycolytic reserve calculated for unstimulated and stimulated CD4^+ T cells. Error bars indicate ±SD.

e, f Bar plots showing frequency of MTG^+TMRM^+ cells in naïve and MTC subsets. Bar plots showing metabolic attributes calculated from SCENITH data as indicated for unstimulated (e–I) and stimulated (m–p) cell types. Error bars indicate ±SD. For all experiments, 3–6 independent samples were analyzed. One-way ANOVA, with multiple comparisons were used to determine significance. ^*P<0.05, **P<0.01, ***P<0.001.

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**Fig. 4** Effector-memory subsets exhibit a greater number of changes to chromatin accessibility compared to central memory subsets. 

a PCA plot of DAR from ATAC-seq data.

b Heatmap displaying DAR z-score normalized across samples.

c Scatter plots showing log2-fold changes (FC) of MTC subsets vs. naïve T cells of DEG (x-axis) mapped to DAR (y-axis) for CD8^+ T_{CM}, CD8^+ T_{EM}, and CD8^+ T_{EMRA}. Dotted lines represent ±1 log2FC.

d Genome plots showing the average accessibility levels in each of the memory subsets and naïve T cells at the indicated locus. Data represent the mean of each cell type. DAR are highlighted by boxes.

e Volcano plots showing DAR that are more accessible in CD4^+ T_{EM} vs CD4^+ T_{CM}, CD8^+ T_{EM} vs CD8^+ T_{CM}, and CD8^+ T_{EMRA} vs CD8^+ T_{EM}. Number of DAR in each direction are totaled at the top of each plot.
Differential accessibility at regions that mapped to DEG was found to be positively correlated for each of the memory subsets (Fig. 4c, Supplemental Fig. 4a-b). Comparing the magnitude of these differences by fold change of both DEG and nearby DAR showed that gene expression and chromatin accessibility increased in both quantity and intensity in effector-memory cells compared to smaller differences from naïve T cells in central MTC (Fig. 4c). Some examples of DAR near DEG include the PRF1 locus (Fig. 4d), which was found to have gained accessibility only in effector-memory subsets. Alternatively, chromatin at two regions upstream of the LDHB gene, which is downregulated in these subsets (Fig. 2), was found to have decreased in accessibility in the effector-memory T cells (Fig. 4d). In a similar fashion, accessibility around gene loci expressed more highly in naïve or TCM cells, such as genes encoding LEF1 and the G protein coupled receptor GPR15 (involved in T-cell homing), showed lower chromatin accessibility in TEM subsets (Supplemental Fig. 4c and d). Overall CD4+ MTC exhibited relatively fewer DAR between TCM and TEM subsets compared to the large number of accessibility changes between CD8+ MTC subsets (Fig. 4e), and chromatin accessibility differences between CD8+ TEM and terminally differentiated TEM were the fewest in number (Fig. 4e). Collectively these data demonstrate that the transcriptional differentiation found in memory subsets is highly correlated with changes to chromatin accessibility.

Differentiated chromatin between MTC subsets is enriched for bZIP, HMG, T-box, and bHLH transcription factor motifs. Transcription factors play key roles in orchestrating the global gene expression changes involved in establishing and maintaining cell type differentiation and establishing distinct gene regulatory states. To identify potential transcription factors regulating differentiation of MTC subsets, we analyzed the variation of chromatin accessibility between subsets at sites with known binding motifs for transcription factors in the genome using ChromVAR. ChromVAR identifies motifs around which chromatin accessibility varies the most in a given set of samples (in this case cell subsets). We found that the chromatin accessibility around binding motifs for AP-1 (Jun, FOS, BATF), T-box (T-BET, EOMES), and HMG family transcription factors (LEF1) was the most highly variable across all MTC and naïve T cells (Fig. 5a). Visualization of the ChromVAR analysis data using tSNE projection distinguished samples by MTC subtype (Fig. 5b). Overlapping the ChromVar deviation score for individual transcription factor motifs onto the tSNE projection showed that TEM cells had higher scores for T-box factors such as T-BET, encoded by TBX21 (Fig. 5c), while naïve T cells and TCM samples showed higher scores for LEF1 binding sites (Fig. 5d). Higher ChromVar scores were also found at AP-1 and IRF family binding motifs in the effector-memory samples (Supplemental Figs. 5a-f).

To further interrogate the various transcription factor regulatory networks involved in differentiating the MTC subsets the PageRank algorithm was used to correlate the presence of transcription factor binding motifs in accessible regions with changes in target gene expression, ultimately calculating a rank of transcription factor importance to the network. PageRank identified several transcription factors that increased in network rank in at least one MTC subset relative to naïve T cells and recapitulated naïve-associated transcription factors such as LEF1 and TCF7 (Fig. 5e). To compare subset differences, the fold change in PageRank scores from naïve cells was plotted against RNA expression for each factor in CD8+ TCM and TEM cells (Fig. 5f-g, Supplemental Fig. 5g-i). These data also corroborated the ChromVAR analysis by indicating both higher relative expression and PageRank scores for AP-1 and T-box factors, such as FOSL2, T-BET, and EOMES in the effector-memory subsets.

One transcription factor, MSC (encoding for musculin or ABF-1), was observed to have the highest rank score in all MTC subsets while being absent in naïve T cells. MSC has been previously identified as a transcriptional repressor capable of binding to E-box elements and implicated in the activation pathways of B cells, as well as the differentiation of peripheral CD4+ Treg cells. Intracellular staining of MSC showed expression in all subsets with significant increases in stimulated T-cell subsets (Supplemental Fig. 6a), suggesting a potential role in T-cell activation. Analysis of the PageRank-generated regulatory network for MSC identified its potential for regulating many important genes in MTC subsets, including repression of LEF1 and TCF7 (Fig. 5b). The MSC target gene network was enriched for several key pathways such as cell activation, adhesion, cytokine production, and differentiation (Fig. 5i).

bHLH Family Factors AHR and HIF1A potentially regulate different environmental responses in CD8+ TCM and TEM MTC subsets. Reconstructing transcription factor regulatory networks via PageRank-predicted interactions emphasized the centrality of key transcription factors LEF1, TCF7, T-BET, and EOMES, as well as differences in TF importance by subset. MSC was predicted by PageRank to regulate LEF1 and TCF7, potentially leading to higher repression of these factors in CD4+ TEM and other effector-memory subsets (Fig. 6a). The bHLH transcription factor AHR was highly induced as seen by intracellular protein staining in all T-cell subsets (Supplemental Fig. 6a).

Intriguingly, AHR also showed both high relative expression and PageRank score exclusively in the CD8+ TEM subset, while bHLH transcription factor, HIF1A (hypoxia-inducible factor 1), was more highly ranked by PageRank in CD8+ TEM cells (Fig. 6b, Supplemental Fig. 5k). AHR protein levels were increased in all subsets upon stimulation. HIF1A was increased after stimulation in all T-cell subsets except for TEM, which maintained more constant concentrations of this protein (Supplemental Fig. 6a). AHR has been implicated in sensing xenobiotics and T-cell homing to tissues, while HIF1A has previously been associated with sensing hypoxic conditions and modulating metabolism in these circumstances. HIF1A and AHR are known to have an antagonistic relationships with respect to target genes and their own expression. GSEA using the list of genes putatively regulated by AHR showed significant enrichment of these genes in those upregulated in CD8+ TCM vs CD8+ TEM (Fig. 6c). Expression of several of these genes in the leading edge of GSEA enrichment were positively correlated with AHR expression in MTC and were exclusively expressed in TEM (Fig. 6d). Two of these genes, VBP1 and PVT1, along with HIF1A are part of the hypoxia response. Two DAR with greater accessibility in TCM, were found near the promoter region of the VBP1 gene, one of which contains a binding motif known to bind AHR (Fig. 6e). VBP1 encodes for the protein VHL which is the substrate recognition subunit of an E3 ligase known to target HIF1A for degradation. Another DAR with higher accessibility in TCM and naïve T cells was found at the promoter of the INPP4B gene, encoding inositol polyphosphate-4-phosphatase type II B (Fig. 6e). This region contains three AHR binding motifs as well as a motif specific for HIF1A binding, suggesting potentially competitive regulation between these two factors. In total, these data suggest that bZIP, HMG, and T-box family transcription factors are important for memory-subset differentiation, and additionally bHLH family factors, such as MSC, AHR, and HIF1A may play key roles in regulating distinct subsets and their transcriptional programs before and following stimulation.
Genes that are uniquely upregulated in stimulated memory-subset cells include Induced and Augmented transcripts. To better understand the transcriptional and epigenetic properties that allow memory T-cell subsets to rapidly respond to stimulation, all five MTC subsets, as well as naïve T cells were stimulated using anti-CD3/CD28 beads ex vivo for 42–48 h. RNA-seq analysis of the stimulated T cells showed a profound change in transcriptional programs after stimulation and much of this response was homogeneous across the different MTC subsets. This is highlighted by PCA wherein PC1 (52.59% variation) separated resting cells from all those that have been activated (Fig. 7a). GSEA showed that activated naïve cells more strongly separated resting cells from all those that have been activated. This is highlighted by PCA wherein PC1 (52.59% variation) separated resting cells from all those that have been activated.
upregulated genes associated with IL-2, STAT5 signaling, and other immune response signaling pathways (Fig. 7b). Despite a large degree of consistency in response to stimulation in the different memory subsets, gene sets related to fatty acid metabolism, glycolysis, and MTOR signaling were enriched to varying degrees in GSEA between TCM and TEM cell subsets after stimulation (Fig. 7c).

To identify genes that were differentially expressed upon stimulation in MTC subsets, expression patterns were examined that were specific to MTC. Overall, the data revealed the presence of three main groups of genes that change in expression compared to naïve T cells before and after stimulation as hypothetically illustrated in Fig. 7d. Each group has two expression states: (a) its constitutive expression in unstimulated MTC vs. naïve; and (b) its change in expression following stimulation. Expressed genes had high fold-change differential expression between naïve and MTC, which naïve T cells induced only after stimulation. Induced genes were similarly expressed in both resting naïve and MTC and are induced in only the MTC group following stimulation. Augmented genes showed expression differences between MTC and naïve cells in both the resting and stimulated states (Fig. 7d). Overlaying these categories on gene expression differences of naïve versus memory resting and stimulated T cells showed that the majority of such DEG were positively upregulated in MTC (Fig. 7e). The memory-induced gene category was highly enriched for cytokine genes including IL4 (Fig. 7h), IL1A, and IL22. These genes were uniquely expressed by MTC subsets to varying degrees after stimulation but repressed in activated naïve T cells at 48 h after the same stimulation. To confirm these results, we measured a panel of common cytokines using intracellular staining coupled with flow cytometry (Supplemental Fig. 7). Some cytokines, such as IL-2 and TNFα were expressed in a significant number of cells by all activated T cells studied. In contrast, the production of effector
Fig. 7 MTC induce or augment unique gene expression programs after stimulation. a PC analysis plot of DEG for resting (circle) and stimulated (triangle) memory and naïve T-cell samples. b GSEA plots for indicated gene sets showing the enrichment score for each stimulated MTC subset as compared to stimulated naïve T cells. c Heatmap of normalized enrichment scores (NES) from GSEA of both stimulated and unstimulated MTC compared to respective naïve T cells. d Schematic describing examples of DEG categorization; before and after stimulation (Stim). e Scatter plot showing log2FC of DEG in unstimulated vs. stimulated MTC. Dots are colored according to DEG category as indicated. f Heatmap showing log2 transformation of the percent of cells positive for respective cytokine protein expression. g Bar plot showing number of DEG compared to naïve T cells belonging to Expressed, Induced, or Augmented categories for each memory subset. h Gene expression bar plots showing rpkm for indicated genes. Error bars represent ±1 SD from mean in each group. Asterisks indicate relevant significant differences as detected by DESeq2 algorithm.
T-cell-associated cytokines (IFNγ, IL-1α, IL-4, IL-17, and IL-5) was differentially increased in various MTC after stimulation compared to stimulated naïve T cells (Fig. 7f). II-22 protein was significantly induced in naive and TEM CD4+ MTC. The T_EM MTC subsets of both CD4+ and CD8+ lineage produced the highest level of cytokines after stimulation in comparison to naïve or T_CM cells (Supplemental Fig. 7). The stimulation-independent highest level of cytokines after stimulation in comparison to naïve TEM cells included important effector T-cell molecules, such as granzyme H, encoded by GZMH; T-BET; and CXCR3, a chemokine receptor associated with T_H CD4+ T cells and effector CD8+ T cells41 (Fig. 7h). Both CD8+ and CD4+ T_EM cells showed higher numbers of Induced and Augmented genes than T_CM. Intriguingly, MSC was also found to exhibit an Augmented expression pattern in these cells after stimulation, while remaining repressed in stimulated naïve T cells (Fig. 7h).

Thus, despite both MTC and naïve T cells activating several similar gene programs upon TCR stimulation, MTC subsets have unique inducible expression profiles upon stimulation, a property that might provide them with a greater degree of efficiency and capacity upon rechallenge.

Augmented gene expression is correlated with epigenetic changes introduced by earlier activation of naïve cells. To determine the role chromatin accessibility in the unique response to stimulation described above, ATAC-seq was performed on the stimulated memory and naïve T cells. Similar to the global transcriptional response to stimulation, PCA of the ATAC-seq datasets showed that the greatest amount of variation separated stimulated from unstimulated cells, and that differences between memory subsets were diminished in the stimulated samples (Fig. 8a). Individual peaks of accessibility in the ATAC-seq analysis followed a set of a patterns that we have termed patterned accessibility regions (PAR) (Supplemental Data 5). PAR are defined by chromatin state before and after stimulation. Five categories of PAR emerged from the analysis: conserved, stimulated, primed, memory, and naïve (Fig. 8b). Conserved-PAR were unchanged across all subsets and stimulation states, whereas stimulation-PAR were present only after stimulation. Each of these two PAR groups occurred equally in both naïve and MTC and made up the majority of the accessible chromatin regions found in the ATAC-seq data in all memory subsets (Fig. 8c, Supplemental Fig. 8a). Conserved-PAR were also found to be more highly enriched in promoter regions than other PAR categories (Supplemental Fig. 8b). PAR specific to MTC occurred in one of three ways: memory-PAR were specific to MTC and were unchanged by stimulation; naïve-PAR were present in resting naïve T cells, but neither their stimulated counterparts nor in MTC; and finally primed-PAR were accessible in resting and stimulated MTC and became accessible upon activation in naïve T cells. Primed-PAR were more than twice as abundant as memory- or naïve-PAR.

In examining the relationships between PAR types and gene expression, we observed that the presence of at least one primed-PAR was strongly correlated with upregulation of DEG in MTC for both resting and stimulated states (Fig. 8d). A similar influence of stimulated-PAR was found in the stimulated but not the resting MTC transcriptional data (Fig. 8e). The presence of memory-PAR was found to be correlated with both resting and stimulated expression in MTC, while the presence of naïve-PAR biased gene expression in most nearby genes towards higher expression in naïve T cells (Supplemental Figs. 9a, b). Comparing these nearby DEG to the MTC-specific gene programs as defined by unique expression after stimulation (Expressed, Augmented, or Induced) revealed that nearly 80% of the upregulated genes in the augmented category had at least one primed peak in cis (Fig. 8f). Additionally, compared to the genome-wide prevalence, primed- and memory-PAR made up a significantly higher proportion of all the accessible regions surrounding DEG that were augmented in MTC (Fig. 8g). This is exemplified by the PDCD1 locus, a gene showing augmented expression in MTC, which is also composed primarily with primed-PAR (Fig. 8h).

These data support an epigenetic mechanism of enhanced recall in which primed-PAR are inscribed in the epigenome upon previous activation of naïve T cells42 and occur within regions of the genome that are associated with higher expression levels of important genes in MTC. PAR contain distinct sets of transcription factor motifs that segregate T cells by lineage and memory subtype. To identify transcription factors associated with each of the identified PAR categories, motif discovery, and enrichment analysis was performed using HOMER43. To control for uneven power, driven by unequal numbers of individual peaks in each category (Fig. 8c), enriched transcription factor binding motifs in DAR were compared across all five peak categories by relative ranking of enrichment p-values (Fig. 9a). Enrichments of HMG family transcription factors (LEF1 and TCF7) were more highly ranked within naïve-PAR, and CTCF-motif enrichment was ranked highest in the constitutively accessible conserved-PAR (Fig. 9b). Interestingly, several transcription factor motifs were enriched in both primed- and stimulation-PAR, including the AP-1 family factors (Fig. 9b). Motifs specific for other transcription factors were enriched in both primed-and memory-PAR, including the T-box factors EOMES and T-BET.

Enrichment rank scores of transcription factor motifs associated with primed-PAR across the different memory subsets in CD8+ and CD4+ T cells clustered according to cell lineage (Fig. 9c); whereas transcription factor motifs enriched in memory-PAR clustered according to memory cell subtype (Fig. 9d). Differences between memory subsets were also observed, with CD4+ T cells showing slightly higher percentages of primed-PAR containing AP-1 factor motifs (Fig. 9e). The percentage of memory-PAR containing T-BET motifs was highest in CD8+ T_EM cells and lowest in CD4+ T_CM, a finding that is consistent with the role of T-box factors in effector T-cell programming (Fig. 9f)44. The PageRank algorithm was used to compare the relative importance of different transcription factors in terms of their effect on target DEG after stimulation. EOMES was found to have the highest PageRank score in stimulated CD8+ MTC (compared to stimulated naïve), as well as the greatest fold change in augmented gene expression (Fig. 9g, i), a finding consistent with its role in maintaining CD8+ T-cell memory44,45. PageRank scores for EOMES and T-BET were similar. T-BET expression was lowest in naïve cells but varied across MTC subsets and was induced to similar levels irrespective of subset (Fig. 9h). Protein measurements of both intracellular EOMES and T-BET via flow cytometry showed that each were increased after stimulation (Supplemental Fig. 6a); however, EOMES levels were already significantly high in resting CD8 T_EM and remained unchanged at these high levels after stimulation. T-BET levels were induced to the highest degree in naïve T cells in both CD4+ and CD8+ lineage groups. Likewise, the PageRank score and expression of IRF8 were highest in stimulated naïve T cells (Fig. 9g). Expression data showed that stimulation induced high levels of IRF8 in naïve cells, but all memory subsets induced significantly lower levels of IRF8 after the 48 h of stimulation (Fig. 9h) compared to naïve T cells. Collectively these data show
that both distinct and overlapping sets of transcription factor binding motifs are enriched in each of the identified PAR categories. Moreover, the expression of many of the transcription factors known to bind such motifs (IRF8, EOMES, T-BET, and LEF1) are coordinated to regulate and enable unique aspects of MTC differentiation following stimulation.

**Discussion**

In this study we used an integrated transcriptomic and epigenetic sequencing approach to understand the cellular programming of the largest memory-subset groups found in human blood and how these programs change in response to ex vivo stimulation. In addition to specific and differential relationships between subsets and lineages, a series of regulatory modalities, pathways, and transcription factors were found to be associated with specific MTC genes. States of chromatin accessibility in key genes within MTC revealed a robust mechanism to control gene expression in response to secondary activation that allow MTC to respond more efficiently and with fewer epigenetic reprogramming steps. Regardless of cell lineage, MTC subsets shared nearly one third of the DEGs that distinguish them from naive T cells, suggesting a common memory signature. However, expression differences between each MTC subset and naive T cells were also observed in gene pathways related to important functions such as cytotoxicity, metabolism, and self-renewal.

It is known that MTC exploit distinct metabolic pathways based on their differentiation and memory status\(^{46-48}\). Here, in addition to the transcriptional evidence, we provided additional characterization of the metabolic states of naive and MTC subsets. Consistent with previous studies\(^{49}\), we showed that resting MTC populations predominantly utilize OXPHOS and fatty acid oxidation for their main or primary metabolic needs and switch.
their metabolism to aerobic glycolysis when they are stimulated in vivo. In addition, some of the MTC subsets use the other pathways to different degrees as a secondary means of metabolism. Previous studies revealed that although both resting naïve and MTC rely on OXPHOS, naïve T cells harbor less mitochondrial mass. In addition, mitochondria of MTC display more elongated structures with tight cristae compared to naïve counterparts, indicating that OXPHOS is highly efficient in these cells. Supporting these studies, our mitochondria analysis of T-cell subsets demonstrates distinct distribution of populations among the subsets according to their activation state. In particular, T_EM and T_CM subsets exhibit...
significantly higher frequency of cells with greater mitochondria mass and potential than naïve T cells. By contrast, CD8+ TEMRA cells did not show a significant difference compared to their naïve counterpart, and stimulation of these cells further decreased the frequency of MTC52 and TRM53, suggesting that these cells are metabolically distinct than the other MTC subsets. CD4+ MTC subsets have a similar fatty acid oxidation capacity; however, CD8+ TEM and TEMRA subsets showed increased capacity for this metabolic pathway compared to naïve counterparts. Consistent with previous studies53, all activated T cells decreased fatty acid utilization.

Recent studies showed that MTC shift towards aerobic glycolysis more rapidly than naïve T cells to facilitate a rapid secondary response54. We showed that TEM and TCM subsets differ in expression of genes related to the regulation of metabolism and in particular to acetyl CoA production with differential expression of glycolysis-promoting enzymes such as LDH. Our metabolic data confirm that all resting MTC subsets have higher glycolytic capacity than their naïve counterparts, suggesting a potential contribution to more efficient secondary responses. Moreover, the glycolytic capacity of the effector MTC is significantly higher than central memory and naïve subsets that might be required for their rapid effector function.

Overall, our data show evidence of a gradient of differentiation from naïve T-cell progenitors in MTC subsets which is highest in TEM. We show that this subset exhibits the greatest numbers of changes from the naïve T-cell transcriptome as well as higher numbers of changes to chromatin accessibility. TCM on the other hand, maintained the highest expression levels of genes that were also expressed in naïve T cells, a result consistent with other recent findings55. In addition shared expression of genes in naïve T cells and TCM MTC includes the transcription factors downstream of the WNT-beta catenin signaling pathway (LEFI and TCF1), which have been previously associated with self-renewal and stem-like properties in mature CD8+ T cells56. It is important to note that the naïve CCR7 + CD45RA + T cells isolated in this study may also contain a small percent of stem-cell MTC: however, this population has been shown to make up less than 10% of total CD8+ T cells and so likely had a small effect on our data57.

One mechanism by which MTC may be able to alter the way in which they respond to antigen stimulation compared to naïve T cells is through modification of chromatin structure. Large numbers of chromatin accessibility and epigenetic modifications such as DNA methylation changes have been observed previously in mouse CD8+ MTC after acute or chronic viral infections33,58. Here, we found that all human CD8+ and CD4+ MTC contain large numbers of DAR compared to naïve T cells. These DAR fell within loci that were correlated with differential gene expression, and which progressively increased in both number and magnitude from central memory to effector MTC subsets. It remains unclear whether this variation in epigenetic changes between central and effector MTC reflects a one-way linear differentiation path in CD8+ T cells as previously suggested by some models59, or results from a more complicated plastic development trajectory58,60. However, fewer differences were found when comparing the chromatin of CD8+ TEM to TEMRA, suggesting a closely shared differentiation pathway for these cells. Interestingly, accessibility differences between circulating CD4+ TCM and TEM were subtle, reflecting their close relationship. This may reflect a heterogeneous population not as well captured by traditional TCM and TEM definitions, as the diverse roles CD4+ effector T cells play in the immune response is likely to lead to a memory population that is equally as diverse. Upon activation, however, CD4+ TEM show a greater degree of transcription and chromatin accessibility changes (greater than CD4+ TCM), which may be related to the activation requirement of effector functions of different CD4+ T-cell effector subsets.

Transcription factor motifs associated with DAR and DEG identified sets of potential factors that could be responsible for defining MTC transcriptional networks. T-box, AP-1, and HMG family transcription factor binding motifs were enriched within DAR that separate memory from naïve T cells, particularly those that distinguish effector memory. Supporting these results, the T-box factors T-BET and EOMES are known to be important in the formation of CD8+ effector T-cell programming and memory cell populations respectively60,61. The HMG factor LEFI has also been implicated in early T-cell differentiation52. Members of the bHLH family of transcription factors, such as AHR, HIF1A, and MSC were found to be important for distinguishing between the memory subsets. AHR and HIF1A are capable of sensing changes in environmental conditions38,63. An AHR binding motif found within a DAR near the promoter of VPB1 (encoding VHL) suggests one potential mechanism by which AHR modulates metabolic differences of CD8+ TCM and TEM, as conditional deletion of this gene has been shown to promote constitutive glycolysis in CD8+ T cells reminiscent of that exhibited by TEM64. Our data suggests that AHR plays a greater role in the CD4+ MTC and CD8+ TCM subsets, including the highest protein expression of AHR in TCM after stimulation. The integrated assay data also suggested that HIF1A might be more important in the CD8+ TEM population. However, interestingly, protein data for this factor showed that TEM populations were the only subset studied for which HIF1A concentration did not increase after stimulation. Ultimately other mechanisms (such as dynamic degradation by AHR-regulated genes like VBP1 (VHL)) could be a part of differential environmental sensing mechanisms between MTC subsets65. The evidence for unique regulation of HIF1A and its targets in TEM shown here suggests a potentially important role in this MTC subset which will require further experimentation to elucidate.

MSC was the most highly ranked transcription factor in all MTC by the PageRank algorithm, but its expression was absent in naïve T cells. MSC is a repressor capable of binding E-box elements as either a homodimer or heterodimer with E2A (TCF3) and was initially found to be highly expressed in activated B cells.
where it has been shown to play a role in promoting memory B cell differentiation35,66. We showed that it is also highly expressed in activated T cells as well. The potential interactions of MSC with E2A is particularly interesting in the context of memory T cells as motifs specific for this factor were found to surround several genes repressed in activated CD8+ T cells in mice13. MSC is expressed by several CD4+ effector T-cell subsets, including Th1, Th17, and Treg cells in mice34,67. In human Th17 cells, MSC transcripts translates directly to higher levels of cytokine protein expression and chromatin accessibility. Various cytokines genes are upregulated in activated naïve T cells to peripheral sites of inflammation41. Several MTC-specific genes were augmented in their expression, having high levels in the resting state that were increased further following stimulation. These genes were correlated with accessible chromatin loci that were present only in MTC prior to stimulation, suggesting that these genes were poised for expression upon rechallenge. The transcription factor EOMES exemplifies this set, with higher expression in MTC potentially facilitating its central role in the maintenance/programming of these cells44,70.

Binding motifs specific for known activation-induced transcription factors such as AP-1 family members (e.g., BATF) and their binding partners NFAT and IRF were highly enriched in both stimulation- and primed-PAR, suggesting that these regions may also be maintained in an open state in MTC in order to more rapidly respond to TCR stimulation. Binding site motifs for transcription factors at primed-PAR separated the samples by lineage (CD4+ vs CD8+) suggesting distinct epigenetic control of activation for each lineage. Interestingly, binding motifs within memory-PAR separated the samples by subset (TCM vs TEM) irrespective of lineage. The exact cues leading to specific MTC subset differentiation remain elusive; however, it is likely that epigenetic mechanisms targeting these memory-subset-specific PAR play a role when paired with simultaneous expression of master regulator transcription factors.

Transcription factor motifs may play multiple roles depending on whether they are maintained in an accessible state within naïve or MTC, and when matched with expression of their corresponding TF. For example, in all MTC, IRF8 motifs are enriched in primed- and stimulation-PAR, but network analysis suggests that this factor plays a greater role in expression changes of activated naïve T cells than in activated MTC, due perhaps to its lowered expression in MTC. Coupled with IRF8’s known role in driving effector T-cell generation in mice21,72, this may suggest repression of IRF8 as an important aspect of T-cell memory. Conversely, as noted above, the expression of EOMES is augmented in CD8+ MTC before and after stimulation while expression of the competing factor T-BET is similar between naïve and MTC after stimulation. Expression of EOMES after stimulation in CD8+ MTC is slightly decreased after stimulation, but still expressed to a greater extent than in stimulated naïve cells, perhaps leading to a higher overall ratio of EOMES/T-BET expression in stimulated MTC. This along with the presence of EOMES/T-BET binding motifs within both memory- and primed-PAR highlights a potential mechanism by which EOMES helps drive memory-specific gene expression, particularly in CD8+TEM cells, which express high levels of this factor in a resting state. In its absence, T-BET controls the effector cell transcriptional response after activation44. Accordingly, higher resting expression of T-BET and greater accessibility of its target sites in TEM may drive the effector-like phenotype of this subset.

In summary, MTC display lineage and subset-specific gene expression and chromatin accessibility patterns. These provide MTC with a unique epigenetic text dependent on their previous activation during encounters with antigen. In total, these memory-specific features enable MTC to adopt expression profiles of effector T cells more rapidly during secondary immune challenge.

Methods

Human subjects. Whole blood samples from four deidentified individuals were obtained with informed consent in accordance with Emory University School of Medicine Institutional Review Board protocols, IRB00045821. PBMCs were separated by density gradient centrifugation (Ficoll-Paque, GE Healthcare), treated with ACK lysing buffer to remove red blood cells, and washed in PBS.

MACS isolation and ex vivo stimulation. CD4+ or CD8+ T cells were isolated from PBMC samples using MACS microbead (Milteny Biotec) isolation kits (CD4: #130-096-533, CD8: #130-096-495) via negative selection of non-target cells. Briefly, 1 × 10^6 cells were resuspended in 40 μL MACS buffer and incubated with CD4+ or CD8+ biotin-antibody cocktail and then incubated with 20 μL of T-cell MicroBead cocktail for 10 min. The flow-through was collected from a MACS separator column and washed with 3 mL MACS buffer. Collected cells were separated for either immediate flow sorting (resting cells) or incubated for 42–48 h in complete Roswell Park Memorial Institute (RPMI) media with anti-CD3/CD28 beads (Gibco: #11131D) for ex vivo stimulation. 10^6 cells were added to cell suspensions in a 2:1 bead-to-cell ratio before being removed prior to flow staining and sorting.

Flow cytometry isolation of human memory T-cell subsets. Cells were resuspended at 1 × 10^6/100 μL in FACS buffer (PBS, 1% BSA, and 2 mM EDTA), stained with CCR7-BSB515 (BD biosciences: 565870) for 30 min at 37 °C, and then a cocktail of the following: CD3-V450 (Tonbo Biosciences: 75-0038), CD4-PE/Cy7 (Biolegend; 300511), CD8-ITC (Life technologies: MHCD08014), CD45RA/PE/TxRed (Biolegend: 304145), Zombie Yellow Fixable Viability Kit (Biolegend; 423104)) for 30 min at 4 °C and then washed with 1 mL of FACS buffer. The following gating strategy was used to define memory subsets: lymphocytes were gated based on SSC-A/FSC-A, single cells by FSC-H/FSC-A, and live cells were based on exclusion of Zombie Yellow Fixable Viability Kit. T cells of the appropriate lineage were selected using the markers CD3, CD4, and CD8. Memory and naive T-cell subsets were isolated using the markers CCR7 and CD45RA. Cell sorting was performed at the Emory Flow Cytometry Core using a FACSaria II (BD Biosciences) and BD FACSDiva software (BD Biosciences). Data were analyzed and figures generated using Flowjo v10.6.2. Supplementary Table 1 contains a list of all antibodies and the concentrations used.

RNA sequencing. One thousand cells were sorted directly into RLT buffer (79216; Qiagen) containing 1% 2-mercaptoethanol. RNA was isolated using the QuickRNA MicroPrep kit (Zymo Research; R1050). The SMART-Seq v4 Ultra Low Input RNA Kit (634894; Takara Bio) was used for cDNA synthesis, and 400 pg of cDNA was used as input for the NexteraXT kit (Illumina) to create sequence libraries. DNA libraries were sequenced at the University of Alabama at Birmingham’s Heffin center for genomics using a NextSeq500.

RNA-sequencing data analysis. Raw sequencing data were mapped to hg38 using STAR v2.5.3j. Duplicate reads were identified and removed using PICARD.
formed by computing a Euclidean distance matrix between enrichment rank values. Integrated analysis and statistics of enriched motifs found in that peak set. Resulting values were then z-score scaled enrichment p-values for individual motifs in each set of peaks by the total number.

ATAC-seq. For each sample, 1000–2000 cells were sorted into FACS buffer and Tn5 transposition was performed. Briefly, cells were resuspended in 12.5 μl 2× tagmentation DNA Buffer, 2.5 μl Tn5, 2.5 μl 1% Tween-20, 2.5 μl 0.2% Digintron, and 5 μl H2O and incubated at 37 °C for 1 h. Cells were then lysed with the addition of 2 μl 10 mg/ml Proteinase-K, 23 μl Tagmentation Clean-up buffer (326 mM NaCl, 109 mM EDTA, 0.63% SDS), and incubated at 40 °C for 30 min. Tagmented DNA was purified and size selected for small fragments using AMPure XP beads (Beckman Coulter, A63881) and PCR amplified (Roche, KK6202) with dual indexing primers (Illunina, FC-131-2004) to generate a sequencing library. Final libraries were again purified, and size selected using AMPureXP beads, quantitated by Qubit (Life Technologies, Q32331), size distributions determined by bioanalyzer (Agilent 2100), pooled at equimolar ratios, and sequenced at the Emory Non-human Primate Genomics Core on a NovaSeq6000 using a PE100 run.

ATAC-seq data analysis. Raw sequencing data was mapped to the hg38 genome using Bowtie v1.1.1.9. Peaks of accessibility enrichment were called using MACS2 v2.1.0.40 and annotated to the nearest gene using HOMER44. Differential testing of accessible peak regions was performed using DESeq2 using the cutoffs of FDR ≤ 0.05 and >1.5 log2 fold-change in peaks for either resting or stimulated conditions. For example, primed-PAR were defined as significantly differential between stimulated and unstimulated naïve cells with a non-zero logFC as well as significant in differential comparison between unstimulated naive and MTC. A list of all PAR assignments for each locus can be found in Supplemental Data 5. The HOMER 'findMotifsGenome.pl' function was used for de novo motif enrichment analysis or known motif enrichment from this database. Relative enrichment rank change values were calculated using HOMER by normalizing the enrichment p-values for individual motifs in each set of peaks by the total number of enriched motifs found in that peak set. Resulting values were then z-score scaled for relative comparison across groups.

Integrated analysis and statistics. For principal component analysis, normalized count data was mean scaled by row (across samples) and then analyzed using the 'princomp' function from the stats package in R. Phylogenetic analysis was performed by computing a Euclidean distance matrix between enrichment rank values and clustering using the 'hclust' function in R. Trees were plotted with ape v3.45 as unrooted trees. Bootstrapping was used to assess the reproducibility of clustering using the 'boot.phyllo' function with 10,000 permutations. PageRank44 analysis was performed using both normalized differential expression values derived from the RNA-seq data as well as raw ATAC-seq data. All statistical analyses were performed with R using DESeq2 for large-scale statistical analysis of RNA-seq or ATAC-seq data or using Wilcoxon Rank sum tests on individual genes with p ≤ 0.05 considered significant.

For all metabolic assays, a one-way ANOVA with multiple comparisons was used to determine significance. Three to six independent samples were used in these assays as indicated in the legends. Values p ≤ 0.05 were considered significant.

Metabolic flux analysis (Seahorse assay). ECAR was measured with XF96 Extracellular Flux Analyzer (Seahorse Bioscience). Human PBMCs isolated from three different donors. T cells were enriched by using human Pan T-cell isolation kit (Miltenyi Biotec). Cells were stained with antibodies to CD3 (BV450), CD4 (APC), CD8 (APC-Cy7), CD45RA (BV650), CCR7 (AF488) and ghost viability dye (BV510). Then, CD4+ (naive, Tcm, Tc7) populations were sorted by FACS. The cells were cultured in RPMI media supplemented with IL-7 and IL-15 for 2 days with or without anti-CD3/CD28 beads (1:2 ratio). Cells were harvested, washed, and then resuspended in XF RPMI media. Cells (180,000–250,000) were transferred into a poly-D-lysine coated 96-well plate as three technical repeats for each cell type and centrifuged at 400×g for 5 min to allow the cells to collect into monolayer at the bottom of the plate. The plate was incubated at 37 °C non-CO2 incubator for 1 h and then placed into the XF96 Extracellular Flux Analyzer. Cells were monitored under basal conditions and in response to 10 mM glucose, 1 μM oligomycin, 100 mM 2-Deoxy-Glucose. ECAR values were extracted from Agilent Seahorse Wave Desktop software and normalized according to the initial cell number. Glycolysis, glycolytic capacity and glycolytic reserve values were calculated according to the equations provided at Agilent Report Generator User Guide.

Mitochondrial measurements. T cells were enriched from human PBMCs by using a human Pan T-cell isolation kit (Miltenyi Biotec). The cells were cultured in RPMI media supplemented with IL-7 and IL-15 for 2 days with or without anti-CD3/CD28 beads (1:2 ratio). For assessment of mitochondrial mass, the cells were incubated with 25 nM MitoTracker Green FM (MTG, Invitrogen) for 1 h. To investigate the mitochondrial membrane potential of the cells, TMRRM dye (Invitrogen) was added to a final concentration of 100 nM of the last 30 min of the above MTG incubation. Cells were stained with anti-CD3 (BV450), CD4 (PE-Cy7, APC-Cy7), CD8 (APC-Cy7), CCR7 (APC), and CD145RA (AF700) and treated with 2- deoxy-glucose (250 mM, Sigma–Aldrich), oligomycin (1.5 μM, Sigma–Aldrich), or a combination of both drugs for 20 min at 37 °C. Puromycin (10 μg/ml, Sigma–Aldrich) was added, and the cells were incubated at 37 °C for another 25 min. The cells were immediately washed with FACS buffer, then the surface staining was performed. The cells were incubated with anti-CD3 (V450), CD4 (PE-Cy7), CD8 (APC-Cy7), CCR7 (FITC), CD45RA (AF700) antibodies and ghost viability dye (BV510) in FACS buffer at 4 °C for 20 min. After washing with FACS buffer, the cells were fixed and permeabilized using Foxp3 intracellular staining kit (Invitrogen eBioscience) for 1 h at room temperature. Intracellular staining of puromycin was performed using 1:1000 dilution of anti-PU.1-puromycin (Clone 1D10, Sigma–Aldrich) in kit supplied perm/wash buffer. The cells were incubated at room temperature for one hr, and then washed with perm/wash buffer twice. Finally, the cells were resuspended in FACS buffer and analyzed by flow cytometry. The mitochondrial dependence, glucose dependence, glycolytic capacity, fatty acid, and amino acid oxidation capacity (FAO and AAO) were calculated from the MFI of puromycin in the different treatments with adjustment of range values across samples within a group if a value within the group was negative. Briefly, percent mitochondrial dependence is calculated as the difference between control and oligomycin-treated cells divided by the difference control and 2-deoxy-glucose plus oligomycin-treated cells. Percent glucose dependence was calculated as difference between control and 2-deoxy-glucose-treated cells divided by the difference between control and 2-deoxy-glucose plus oligomycin-treated cells. Glycolytic capacity and FAO and AAO capacity were calculated by subtracting the percent mitochondrial capacity or percent glycolytic capacity from 100, respectively.

Detection of intracellular proteins by flow cytometry. For intracellular cytokine detection experiments, T cells were enriched from PBMC as above and stimulated for 48 h with anti-CD3/CD28 beads (2:1 ratio). The cells were incubated with both Golgi Stop (BD Biosciences) and Brefeldin A (Biolegend) for 5 h at 37 °C. Surface staining was performed with washed cells using anti-CD (BV605), CD4 (BV785), CD8 (APC-Cy7), CCR7 (AF488), CD45RA (AF700) antibodies and ghost viability dye (BV510). After washing with FACS buffer, cells were fixed and permeabilized for 20 min using the Cytofix/Cytoperm kit (BD Bioscience). The cells were again washed with permeabilization buffer. Intracellular staining was performed to detect the cytokines by using IL-1α (PE), IL-2 (APC), IL-5 (AF488), IL-10 (AF647), IL-17 (AF700) and PECy7 antibodies in permeabilization buffer. The isotype controls used were Mouse IgG1, k Isotype (PE), Rat IgG1, K Isotype (APC), Rat IgG2a, k Isotype (PE/Cy7), Rat IgG1
Henson, S. M. et al. p38 signaling inhibits mTORC1-independent autophagy

Hamann, D. et al. Phenotypic and functional separation of memory and

Srivastava, R. et al. Phenotypic and functional signatures of Herpes Simplex

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Code availability

References


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Author contributions
A.R., C.S., B.A., and J.M.B. designed the experiments, and J.R.R. analyzed the omics data. B.A. and A.R. performed the experiments, and analyzed the cellular data, and S.H. created the sequencing libraries. B.A. and M.F. analyzed the flow cytometry data. J.M.B. and C.S. conceived and supervised the study. J.R., J.M.B., and B.A. wrote the manuscript. All authors discussed and commented on the manuscript.

Competing interests
The authors declare no competing interests.

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