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Identification of an Evolutionarily Conserved Transcriptional Signature of CD8 Memory Differentiation That Is Shared by T and B Cells¹

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After Ag encounter, naive lymphocytes differentiate into populations of memory cells that share a common set of functions including faster response to Ag re-exposure and the ability to self-renew. However, memory lymphocytes in different lymphocyte lineages are functionally and phenotypically diverse. It is not known whether discrete populations of T and B cells use similar transcriptional programs during differentiation into the memory state. We used cross-species genomic analysis to examine the pattern of genes up-regulated during the differentiation of naive lymphocytes into memory cells in multiple populations of human CD4, CD8, and B cell lymphocytes as well as two mouse models of memory development. We identified and validated a signature of genes that was up-regulated in memory cells compared with naive cells in both human and mouse CD8 memory differentiation, suggesting marked evolutionary conservation of this transcriptional program. Surprisingly, this conserved CD8 differentiation signature was also up-regulated during memory differentiation in CD4 and B cell lineages. To validate the biologic significance of this signature, we showed that alterations in this signature of genes could distinguish between functional and exhausted CD8 T cells from a mouse model of chronic viral infection. Finally, we generated genome-wide microarray data from tetramer-sorted human T cells and showed profound differences in this differentiation signature between T cells specific for HIV and those specific for influenza. Thus, our data suggest that in addition to lineage-specific differentiation programs, T and B lymphocytes use a common transcriptional program during memory development that is disrupted in chronic viral infection. *The Journal of Immunology*, 2008, 181: 1859–1868.

When populations of naive lymphocytes differentiate through an effector state into memory cells, they acquire a set of functions that confer protective immunity to the host, including a more rapid proliferative response to Ag re-exposure and the ability to self-renew (1). Defining the molecular basis for the acquisition and maintenance of these functions in humans is central to the development of vaccines and therapies for chronic viral infections such as HIV and hepatitis B and C, diseases in which effective T cell immunity fails to develop (2, 3).

A molecular definition of lymphocyte memory differentiation is complicated by the extraordinary heterogeneity within and between memory T and B cell lineages (4–6). For instance, in humans the heterogeneity of CCR7, CD62L, CD27, and CD28 expression can divide the CD8 compartment into multiple populations that differ in proliferative response, cytokine secretion, and effector potential (7, 8). In the CD4 lineage, naive T cells can differentiate along several different pathways to give rise to cell types as diverse as Th1, Th2, and Th17 cells (9). Nevertheless, for any of these populations to confer life-long immunity, they must acquire the fundamental characteristics of memory cells. How these heterogeneous populations of lymphocytes develop and maintain a similar set of memory functions remains a central question in immunology.

At least two explanations are formally possible: 1) divergent molecular processes in each lymphocyte lineage give rise to analogous memory functions; or 2) common transcriptional programs underlie memory differentiation in multiple lineages. Current concepts of mature lymphocyte differentiation suggest that discrete subsets of Ag-experienced lymphocytes develop under the guidance of lineage-specific transcription factors that confer “specialist” functions (10–14), supporting the first possibility. In contrast, fewer data support the existence of differentiation programs shared by discrete populations of memory cells, still less by different lymphocyte lineages (15, 16).

Memory CD8 T cell differentiation has been extensively studied in TCR transgenic T cell models in the mouse because they permit the measurement of “gold-standard” properties of CD8 memory T cells, including the ability of memory cells to persist and confer

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protection following transfer. As a result, genome-wide transcriptional profiling of mouse naive, effector, and memory cells have revealed genes and processes that are critical to memory differentiation in the CD8 lineage (15, 17, 18). In humans, however, it has not been possible to identify the transcriptional signature of memory differentiation because the functional characteristics of the memory cells that are used to define the differentiation state in mouse models cannot easily be measured in discrete populations of human T cells. For instance, testing the ability of a population of cells to persist and confer immunity following transfer to a different host is not feasible in humans. Comparisons of transcriptional profiles in human CD8 T cells have therefore necessarily involved populations defined by phenotypic markers of the memory state rather than those known to have true memory function.

We hypothesized that the common characteristics of memory cells would be reflected in a transcriptional profile that would be shared by diverse populations of memory cells. We therefore performed cross-species comparison of genome-wide expression profiles from multiple populations of human memory-phenotype and naive cells in CD8, CD4, and B cell lineages with functionally defined memory CD8 T cells from two mouse models to identify transcriptional patterns of memory differentiation shared between species and lineage.

Materials and Methods

Subjects and samples

Blood samples were collected from a group of 29 healthy volunteers (median age 31 years, range 22–67 years). HLA-A*0201 donors were identified by staining PBMC with BB7.2 mAb and verified by high-resolution DNA testing. Chronic HIV-infected individuals met one of the following criteria: a viral load of $>45,000$ copies/ml (Roche Amplicor Assay, version 1.0), or a CD4 count that was $<350/\mu\text{l}$. The upper count for viral load testing was 750,000 copies/ml. All subjects had been without antiretroviral treatment for at least 3 mo at the time of the blood draw. All human samples were collected after informed consent was given according to a protocol approved by the Dana-Farber Cancer Institute's Institutional Review Board (Boston, MA).

Flow cytometry and sorting

PBMC were obtained by density centrifugation and stained with a mixture of Abs and reagents designed to exclude irrelevant lineages and dead cells. Naive and memory-phenotype CD8, CD4, or B cells were identified using the Abs shown in Figs. 1 and 4 and sorted with a FACSria cell sorter (BD Biosciences). Sorted cells were pelleted in a cold centrifuge and resuspended in TRIzol (Invitrogen) within 4 h of phlebotomy. Analysis and visualization of flow cytometry data were done using FlowJo software (version 6.3.2; Tree Star).

Cell culture

Populations of human peripheral blood CD8 T cells were selected by flow sorting or magnetic selection, CFSE labeled according to manufacturer's instructions, and plated in 96-well plates with combinations of IL-18 (25 ng/ml), IL-12 (25 ng/ml; both from R&D Systems), or suboptimal dilutions of plate-bound CD3 Ab (titrated to give $\sim 40\%$ of maximum proliferation) and soluble CD28 Ab (500 ng/ml). Cells were harvested after 6 days and proliferation was assessed by quantifying the CFSE dim fraction.

Microarray data acquisition

Cells purified by flow sorting were immediately resuspended in TRIzol for RNA purification. The concentration of small quantities of RNA was determined using the Molecular Probes RiboGreen RNA quantitation kit (Turner Biosystems). Linear amplification of 10 ng of total RNA was performed using the Ovation Biotin RNA amplification and labeling system (NuGEN). Fragmented, labeled cDNA was hybridized to Affymetrix HG_U133AAofAv2 microarrays.

Microarray data analysis

Preprocessing and normalization of the data were performed using robust multi-chip averaging (19). Affymetrix MG_U74Av2 from previously published data sets (17, 18, 20) were processed identically using robust mul-

tichip averaging. Genes that are differentially expressed between two classes (such as naive CD8⁺ T cells and memory CD8⁺ T cells) were ranked using the signal to noise metric (21) with the GenePattern software package (22). The statistical significance of differentially expressed genes was determined using the comparative marker selection module in GenePattern (22). The nominal p value was calculated by random permutation of the class labels and the false discovery rate was calculated to adjust for multiple hypothesis testing (22). Hierarchical clustering was performed using GenePattern (22).

To identify an evolutionarily conserved gene expression signature of T cell memory formation, we analyzed microarray experiments performed using human and mouse cells. Cross-species mapping of probe sets was performed as described previously (23). Probe set identifiers were converted to gene symbols using NetAffx (www.affymetrix.com). A total of 6,022 genes were identified that are represented on both the HG_U133AAofAv2 and the MG_U74Av2 Affymetrix microarrays. Gene set enrichment analysis (GSEA)⁴ was performed as described previously (24).

Results

The transcriptional profile of CD8 memory differentiation is highly conserved in mice and humans

To identify transcriptional changes central to memory differentiation, we began by determining whether the transcriptional changes in human and mouse CD8 memory differentiation were similar. We first compared the gene expression profiles of naive and memory CD8 T cells in humans and mice. The population of memory-phenotype CD8 T cells that is likely to contain the human counterparts of the mouse memory CD8 T cells can be phenotypically defined, but within this heterogeneous population the identity of the cells that possess the properties of protective memory cells is not known. Our goal was therefore first to define the general signature of human memory-phenotype CD8 T cells and then to filter that signature using expression data from mouse models of memory differentiation in order to refine the transcriptional signature to the subset of genes that characterize the memory state in both species.

We sorted naive, central memory, and effector memory CD8 T cells from peripheral blood samples of 6–8 healthy donors using markers corresponding to well-recognized memory-phenotype and naive subsets (25) (Fig. 1A). We compared the transcriptional signature of three major populations of human memory-phenotype CD8 T cells with their naive counterparts using oligonucleotide microarrays (Fig. 1B). Genes that were differentially expressed between memory-phenotype and naive T cells were ranked using a signal-to-noise metric, which identifies those genes whose expression pattern best correlated with each differentiation state (21). We identified 220 genes with significantly increased expression in memory-phenotype compared with naive cells ($p < 0.01$), confirming the marked changes in gene expression seen in similar comparisons (26).

To identify genes up-regulated during CD8 memory differentiation in the mouse, we analyzed published data from memory and naive cells in the OTI TCR transgenic mouse model of vaccinia virus infection (17). Using the same analysis as with the human data, we compared the expression of memory OTI transgenic T cells to that of their naive counterparts to generate a signature of genes that were up-regulated during memory differentiation in OTI T cells (data not shown). This comparison mirrored the analysis performed in the human data and allowed the identification of all genes up-regulated by naive cells after Ag exposure during the time when commitment to a memory cell fate occurs (18, 27–30). The naive vs memory comparison therefore allowed the broadest evaluation of the transcriptional changes occurring in the naive to memory transition.

⁴ Abbreviations used in this paper: GSEA, gene set enrichment analysis; ES, enrichment score; LCMV, lymphocytic choriomeningitis.

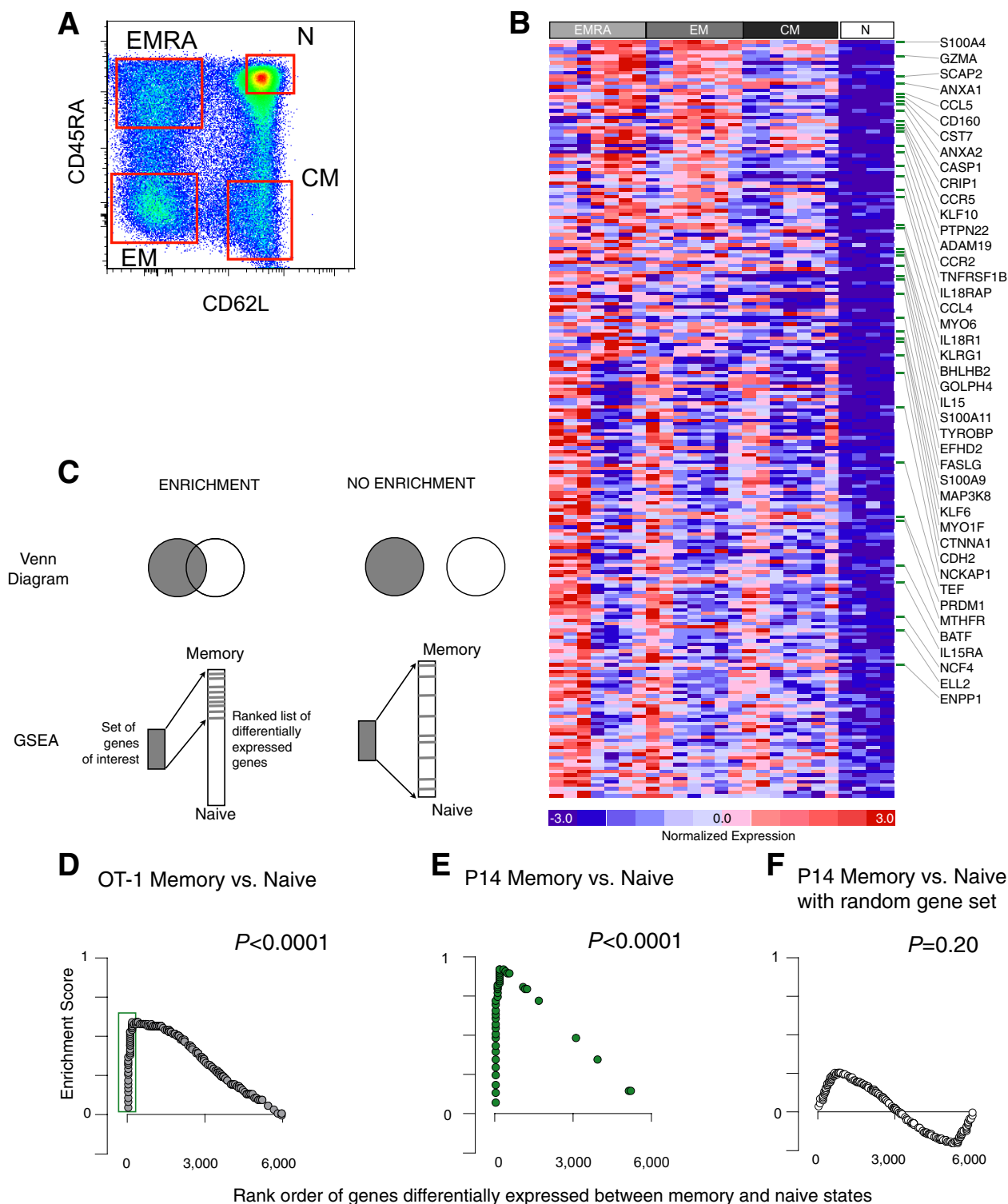


FIGURE 1. CD8 memory T cell differentiation signature is conserved between human and mouse. *A*, Human memory-phenotype and naive peripheral blood CD8 cells were sorted from healthy human donors using sort gates designed to capture central memory (CM), effector memory (EM), effector memory/CD45RA⁺ (EMRA), or naive T cells (N). *B*, Genes distinguishing memory phenotype (CM, EM, and EMRA) from naive samples were identified and ranked. Relative expression levels of all 220 genes significantly associated with the memory-phenotype class distinction ($p < 0.01$) are shown; column represents an individual donor and each row a gene. For each gene, relatively high expression is indicated in red, low expression in blue. Genes enriched in the OTI mouse model of CD8 memory differentiation are indicated by green bars on the right. *C*, Schematic representation of GSEA. A list of genes for a particular comparison of interest (e.g., human CD8 memory-phenotype vs naive) was tested for enrichment in the rank order of differentially expressed genes derived from an independently generated gene set (e.g., mouse CD8 memory vs naive). Gene sets that are related would be expected to be enriched at the top of the rank-ordered list. *D*, The 220-member set of genes defined in *B* was tested for enrichment in the expression profile of mouse OTI TCR transgenic memory CD8 T cells compared with naive OTI cells using GSEA. Each point represents an individual gene in the gene set, and its running enrichment score and position in the rank-ordered list of mouse memory vs naive genes are shown. Those most enriched in mouse memory, the leading-edge genes, are highlighted in green (*left panel*) and correspond to the genes marked in *B* with green bars. *E*, Enrichment of leading edge genes from *D* in LCMV memory vs naive expression profile. *F*, Enrichment of a random set of genes in LCMV memory vs naive expression profile shows no significant enrichment, confirming specificity of the analysis.

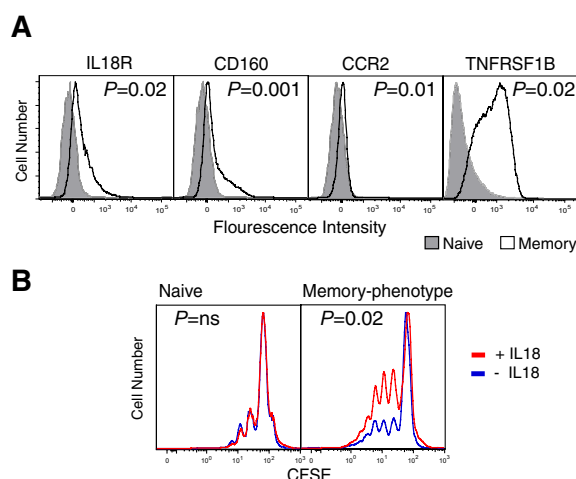


FIGURE 2. Functional validation of memory signature in human CD8 T cells. **A**, Validation of markers of memory differentiation in human CD8 T cells. Mean expression on the surface of naive or memory-phenotype CD8 human T cells ($n = 5$; representative donor shown) was measured by flow cytometry and significance was determined by paired t test. **B**, Functional validation of IL-18R as a marker of memory differentiation. CFSE-stained peripheral blood naive or memory CD8 T cells were cultured with suboptimal CD3 stimulation in the presence (red line) or absence (blue line) of IL-18 (50 ng/ml). The fraction of proliferating naive (left panel) or memory-phenotype (right panel) CD8 T cells was determined on day 6 ($n = 4$; representative donor shown) and significance was determined by paired t test.

Having identified genes up-regulated during CD8 memory differentiation in humans and mice, we next determined whether there was similarity between the two sets of genes using GSEA (Fig. 1C) (24). GSEA provides a general statistical method for testing whether a set of genes is enriched in an independent expression data set. Traditional strategies for gene expression analysis have focused on identifying individual genes that exhibit differences between two states of interest. Although useful, these strategies may fail to detect biological processes, such as transcriptional programs, that are distributed across an entire network of genes but are hard to distinguish at the level of individual genes. GSEA considers a priori defined gene sets comprised of biologically related genes (e.g., members of a metabolic pathway, genes at the same genomic locus, or, in this case, genes up-regulated in memory differentiation in a particular lineage or species). Given a data set in which genes can be rank ordered by the correlation of their expression levels in a collection of samples using a phenotype of interest (in this case, differentially expressed genes in memory vs naive cells from a second lineage or species), GSEA provides a score that measure the degree of enrichment of a given gene set at the top (highly correlated) or bottom (anti-correlated) of the second rank-ordered data set. GSEA uses the Kolmogorov-Smirnov statistic to estimate the degree of enrichment and to assess significance with a p value obtained by permutation testing. GSEA increases resolution and statistical power and can detect subtle but consistent changes that are often missed by inspection alone. GSEA has been previously used to test for the enrichment of coordinately regulated sets of genes representing anergic or effector T cell states (31, 32).

Fig. 1C illustrates two approaches for determining enrichment of a related set of genes of interest in a second data set. The simplest approach to this analysis would be to look for overlap in the corresponding data sets using a Venn diagram (Fig. 1C, upper panels). But this approach is limited by the arbitrary cutoff of

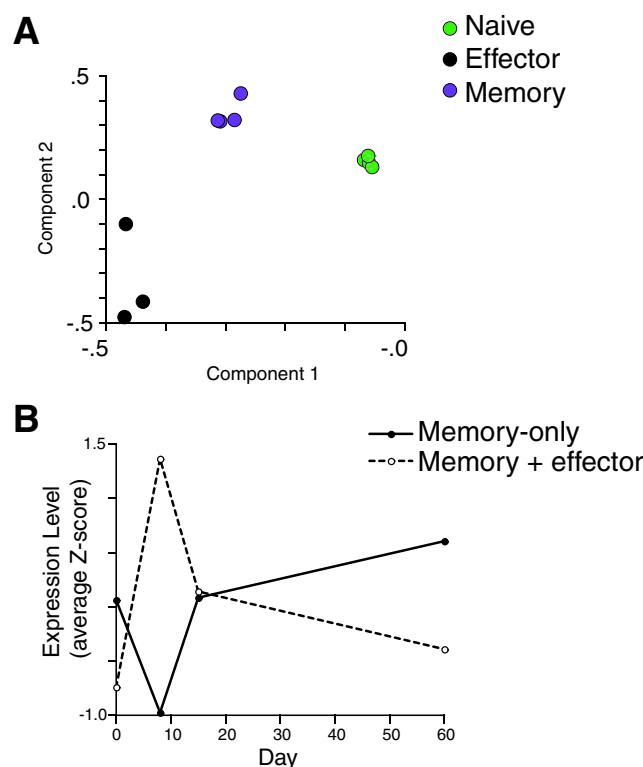
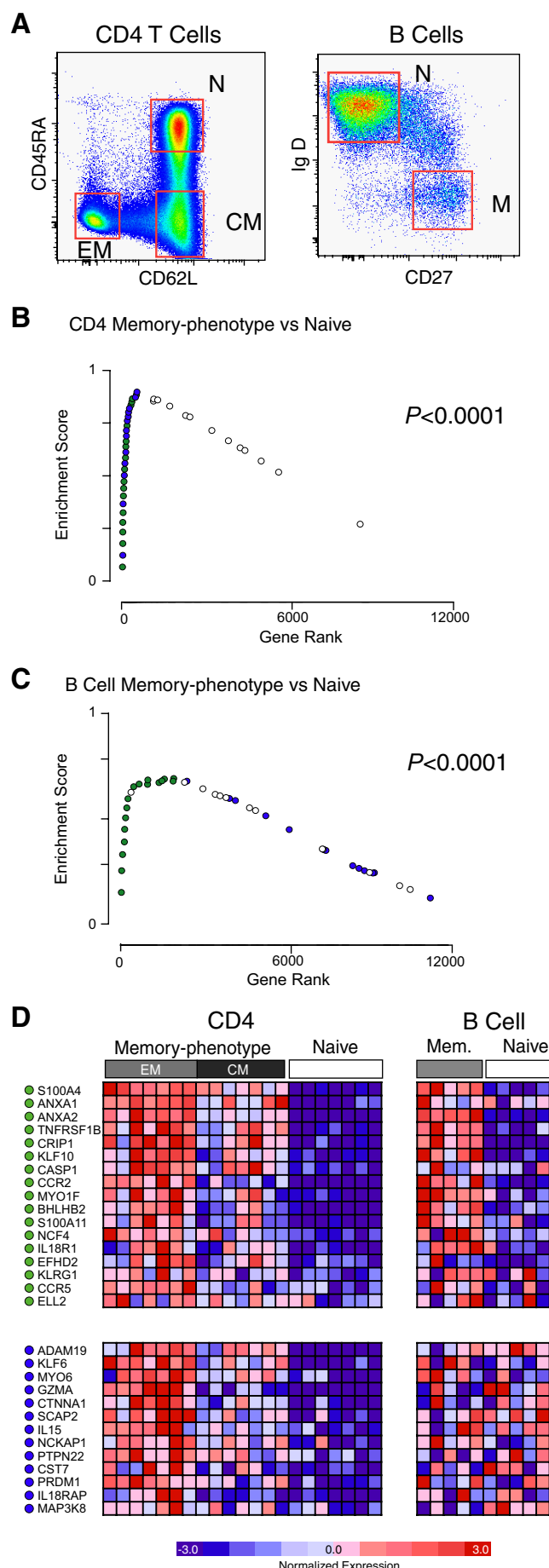


FIGURE 3. Memory differentiation signature is distinct from T cell effector signature. **A**, Analysis using clustering by principal components of naive (green), effector (black), and memory (purple) LCMV-specific T cells in the space of the conserved CD8 differentiation signature. **B**, The relative expression of the genes in the memory or effector component of the CD8 signature determined in P14 CD8 T cells before, during, and after acute LCMV infection. Composite expression level of all genes in signature calculated as z-score to adjust for wide variation in baseline expression levels of genes in the signature. Each symbol represents mean z-score from naive (day 0), effector (day 8), and memory cells (day 60).

genes defined as differentially expressed and by the loss of information about the relative position of genes when comparing the two lists. GSEA (Fig. 1C, lower panels) determines whether a list of genes for a particular comparison of interest (e.g., human CD8 memory vs naive) is enriched in the rank order of differentially expressed genes derived from an independently generated gene set (e.g., mouse CD8 memory vs naive).

The primary result of GSEA is the enrichment score (ES) (Fig. 1, D–F), which reflects the degree to which a gene set is over-represented at the top or bottom of a ranked list of genes. GSEA calculates the ES by walking down the ranked list of genes (plotted on the x-axis), increasing a running sum statistic when a gene is in the gene set and decreasing it when it is not. The magnitude of the increment depends on the correlation of the gene with the phenotype. The ES is the maximum deviation from zero encountered in walking the list. A positive ES indicates gene set enrichment at the top of the ranked list; a negative ES indicates gene set enrichment at the bottom of the ranked list.

Using this method, we found that the human memory phenotype signature was highly enriched in the mouse OTI CD8 memory T cell profile ($p < 0.001$, false discovery rate < 0.001 ; Fig. 1D). This analysis demonstrates that a common set of genes is coordinately up-regulated during the differentiation of naive CD8 T cells into memory lymphocytes in both mouse and humans. To confirm that up-regulation of this signature is a feature of CD8 T cell memory differentiation, we repeated GSEA with the conserved memory



signature in a second, independent mouse model of memory development, the P14 TCR transgenic model of acute lymphocytic choriomeningitis (LCMV) infection (18). Again, we found that the conserved CD8 T cell memory signature was highly enriched in the P14 memory profile ($p < 0.001$; false discovery rate < 0.001 ; Fig. 1E). Thus the coordinate up-regulation of the same set of genes is a characteristic of CD8 T cell memory differentiation between species. By comparison, a random set of human genes of the same size showed no significant enrichment (Fig. 1F). This suggests that transcriptional changes coordinately regulated during memory differentiation in CD8 T cells are highly evolutionarily conserved.

Phenotypic and functional validation of the signature

We next validated selected genes in the signature by confirming that their protein levels were increased in human memory-phenotype CD8 T cells. We studied the surface expression of four genes in the signature not usually assumed to be markers of CD8 memory differentiation: *IL18R*, *CD160*, *CCR2*, and *TNFRSF1B* (Fig. 2A). We found that all were expressed at higher levels on human memory phenotype CD8 T cells than on naive CD8 cells. Consistent with increased IL-18R expression, we found that recombinant human IL-18 significantly augmented proliferation in memory phenotype CD8 T cells but not in naive cells (Fig. 2B), demonstrating that members of the signature can be validated at the protein and functional level.

The differentiation signature contains both genes unique to memory cells and genes shared with effector cells

If this molecular signature accurately represents the memory state, we would predict that the expression of this pattern of genes should distinguish memory T cells from cells in other differentiation states. Inspection of genes in the CD8 T cell differentiation signature revealed that many have known roles in effector T cells, e.g., *KLRG1* and *GZMA*. We therefore tested whether the expression pattern of genes in the signature was different in memory and effector cells (Fig. 3). The evolutionary conservation of the signature allowed us to use the mouse model of memory development to analyze the pattern of gene expression in naive, effector, and memory T cells from the LCMV model of CD8 memory differentiation. Clustering of LCMV-specific naive, effector, and memory T cells in the space of the memory signature revealed that each T cell population formed a separate group, indicating that the expression pattern of the signature in each differentiation stage was distinct (Fig. 3A).

To further characterize the differences in the pattern of expression of the signature genes between memory and effector cells, we

FIGURE 4. Components of the CD8 memory T cell differentiation signature are shared by T and B cell lineages. **A**, Naive (N), central memory (CM), or effector memory (EM) CD4 T cells were identified and sorted using the gates shown (left panel). Naive or memory (M) B cells were identified within the CD19⁺ compartment and sorted using the gates shown (right panel). **B** and **C**, The conserved CD8 memory signature was tested by GSEA in CD4 (**B**) or B cell (**C**) memory-phenotype vs naive profiles. Green symbols indicate genes enriched in both CD4 and B memory-phenotype cells, blue symbols indicate enrichment-only CD4 memory-phenotype cells, and white symbols indicate enrichment only in CD8 memory phenotype profile. **D**, The relative expression of genes from the CD8 memory signature in CD4 (left panels) or B cell (right panels) memory signatures are shown with genes ranked by signal-to-noise metric. Upper panels correspond to genes in the CD8 memory signature enriched in CD4 and B memory (Mem.) cells, and lower panels correspond to genes enriched in CD4 memory but not B cell memory. Green and blue symbols correspond to points in **B** and **C**.

identified which genes of the memory signature were also increased in expression in effector cells relative to naive cells (data not shown and Fig. 3B) and analyzed their expression during the development of CD8 memory in the LCMV model. Genes in the memory signature that were shared with effectors were found at low levels in naive cells, peaked in effector populations, and fell to lower levels in memory cells. In contrast, unique memory genes in the signature were reduced in expression in effector cells compared with naive and memory cells. Thus, the memory differentiation signature contains genes that are both unique to the memory state and genes that are initially expressed in effector cells before becoming down-regulated in memory cells.

The memory differentiation signature is shared by memory-phenotype human CD8, CD4, and B cells

We next tested whether expression of this transcriptional signature was an integral feature of the memory differentiation process for other lymphocyte populations. The cross-species comparison allowed us to use functionally defined memory cells from a mouse model as a “biologic filter” to focus exclusively on the subset of genes in the differentiation signature in mice that were also up-regulated during memory differentiation in human memory CD8 T cells. We refined the CD8 T cell memory signature to include only those genes that were most differentially expressed in both species by focusing on the leading edge subset of 43 genes (of the initial 220 differentially expressed in human CD8 memory-phenotype T cells) indicated by the green box in Fig. 1D. These genes are of the subset that contributes most to the ES. Although this signature of genes is highly conserved in the CD8 lineage in humans and mice, CD8 memory T cells show considerable functional and phenotypic differences from those in the CD4 and B cell lineages. Conservation of some or all of the transcriptional changes found in CD8 T cells in CD4 T cells or B cells would suggest that coordinated up-regulation of this set of genes is central to the process of memory lymphocyte differentiation. We therefore tested whether genes in the CD8 T cell memory differentiation signature were also up-regulated during memory differentiation in CD4 T cell and B cell lineages. We purified memory phenotype and naive CD4 and B cells from human peripheral blood (Fig. 4A), and generated differentiation signatures for each lineage by comparing memory phenotype with naive cells.

We found that the conserved CD8 memory gene signature was highly significantly enriched not only in CD4 (Fig. 4B) memory differentiation but also in the B cell memory signature (Fig. 4C). Approximately 40% of genes in the conserved CD8 memory differentiation signature were up-regulated during the differentiation of memory cells in all three lineages. The genes that were not up-regulated in B cell differentiation (i.e., only up-regulated in CD4 and CD8 memory development; shown in Fig. 4, B and C, blue symbols) tended to be the genes most associated with T cell-specific functions, such as *GZMA* and *FASLG*. However, our analysis identified a subset of genes (Fig. 4, B and C, green symbols) that were highly significantly up-regulated ($p < 0.001$) during memory development in all three lineages (Fig. 4, B and C, green symbols). Indeed, 94% of genes in the leading edge of enrichment in B cell memory were enriched in the CD4 memory signature. These results demonstrate that the core signature of CD8 T cell memory differentiation that is conserved between mouse and human includes a common transcriptional program that is a general feature of memory differentiation in both B and T lymphocytes.

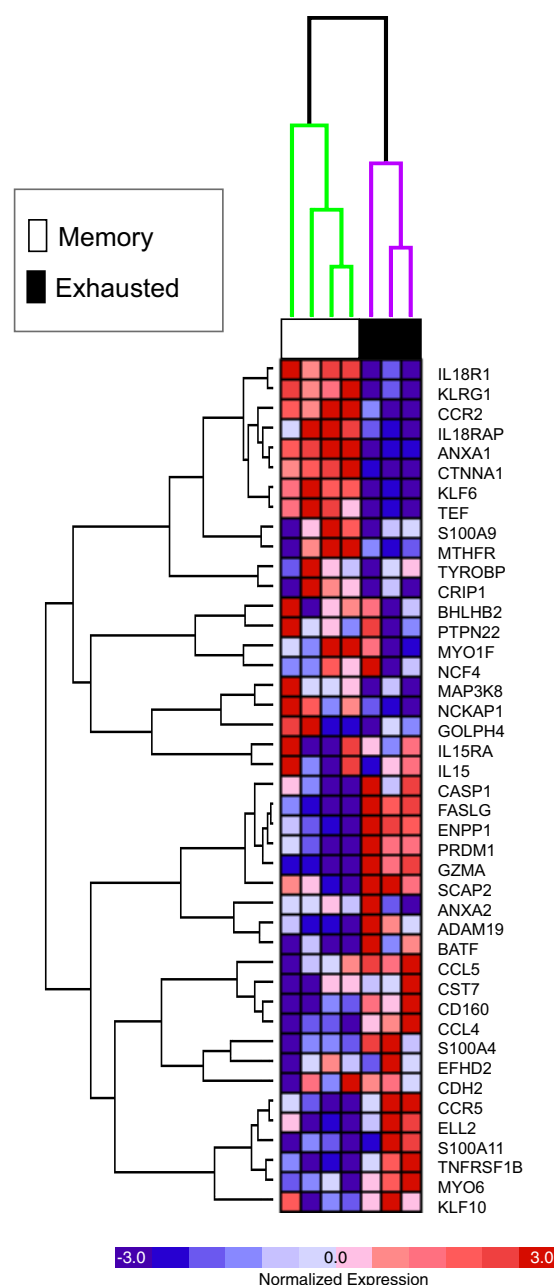


FIGURE 5. T cell exhaustion disrupts memory differentiation in mouse CD8 T cells. Unsupervised hierarchical clustering discriminates between exhausted and functional memory LCMV-specific CD8 T cells in the space of the conserved memory signature. Expression levels are normalized across each row, i.e., expression levels of samples are presented relative to each other.

Signature is disrupted in dysfunctional virus-specific CD8 T cells in the mouse

One implication of a defined gene expression signature corresponding to CD8 memory differentiation is that it might be useful as a surrogate marker for memory T cells with the greatest potential to confer immunologic protection. To address this issue, we next applied it as test to discriminate between functional and dysfunctional CD8 T cells. We compared the signatures of T cells from acute and chronic LCMV infection in the mouse model. In contrast to acute LCMV infection, chronic LCMV infection results in exhausted CD8 cells that fail to manifest the cardinal properties of memory: robust response to Ag, and Ag-independent persistence (33, 34). Comparison of T cell differentiation after acute or

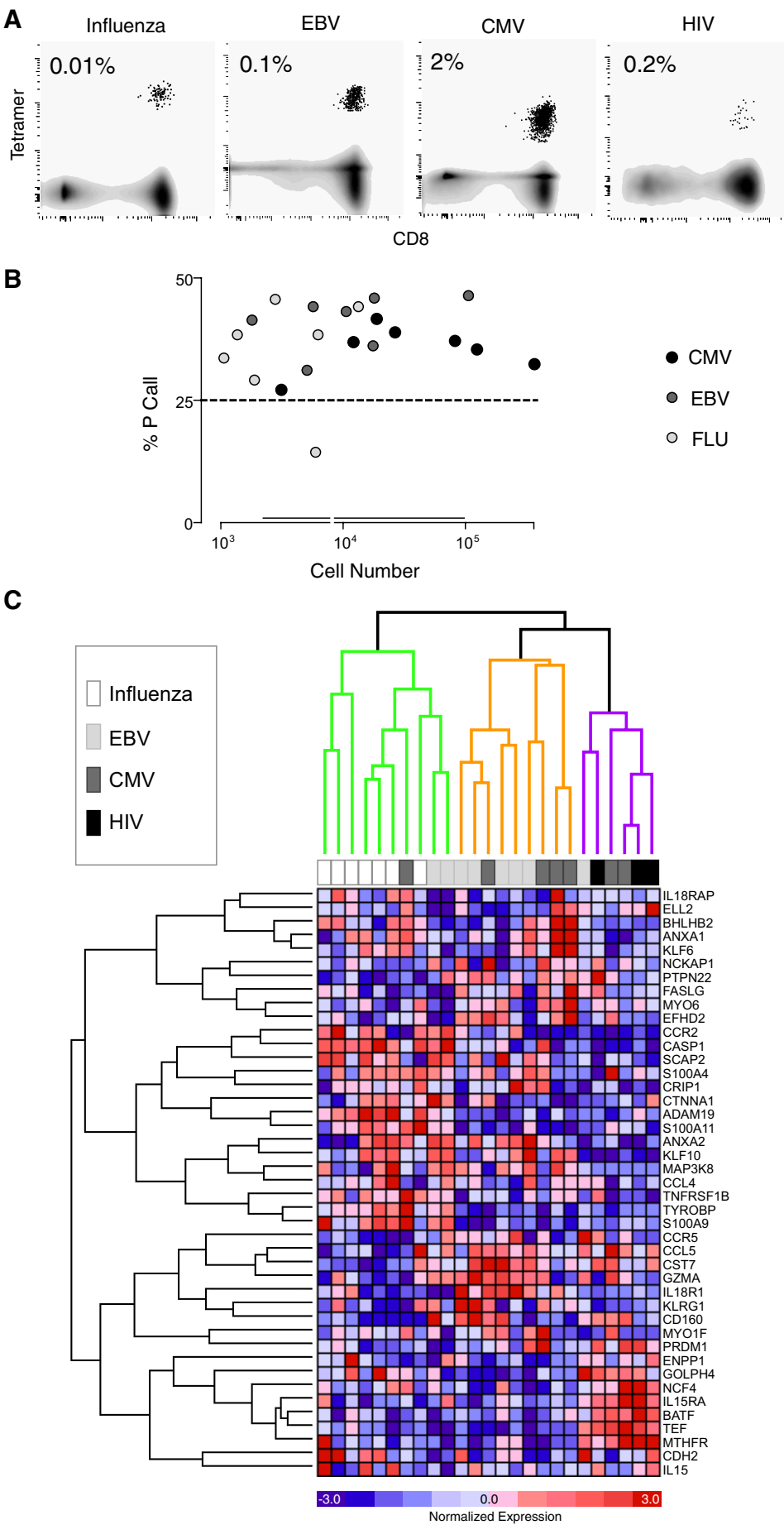


FIGURE 6. Differentiation signature distinguishes human Ag-specific T cells from acute and chronic viral infection. **A**, Human CD8 T cells specific for HLA-A*0201-restricted immunodominant epitopes from influenza, EBV, CMV, and HIV were identified and sorted with MHC-peptide tetramers as shown. Percentages refer to fraction of CD8 T cells stained with tetramer in these representative plots. Gray contours represent total CD8 T cells and the black dot plots represent tetramer-positive cells in the sort gate. **B**, High quality microarray data were generated from small cell numbers. Percentage of transcripts assessed as “present” (P Call) vs cell number for samples of tetramer sorted CMV, EBV, or influenza-specific T cells. Dotted line represents adequate data quality, the double line on *x*-axis represents interquartile range, and the line break on *x*-axis represents the median. **C**, Unsupervised hierarchical clustering of samples and genes in human Ag-specific T cells in the space of the conserved CD8 memory differentiation signature.

chronic LCMV infection therefore allowed us to test whether the CD8 T cell memory signature could distinguish between protective vs defective memory T cells. Unsupervised hierarchical clustering within the conserved CD8 T cell memory signature showed that memory and exhausted samples were partitioned in separate branches of the dendrogram, indicating marked differences in expression of signature genes in exhausted cells (Fig. 5). We found two primary clusters of genes in this analysis: one with expression levels higher in exhausted T cells than in memory cells, and a second with genes that were decreased in exhausted cells compared with memory T cells. This suggests that it is not the presence or absence of expression of signature genes per se that discriminates between the two populations. Rather, it is the correct balance of genes in the differentiation signature that can distinguish between functional and dysfunctional virus-specific T cells.

Signature is disrupted in dysfunctional human virus-specific CD8 T cells

We next sought to determine whether this signature could be applied to human disease states. Distinguishing between functional and dysfunctional T cell responses in humans represented a considerably more stringent test of the signature compared with the LCMV model, because the degree of genetic variability in human samples is considerably higher than in the TCR transgenic LCMV model. Moreover the molecular basis of dysfunction in the LCMV model may be dissimilar to different pathogens in an unrelated species.

To address this question, we studied T cell responses to representative human viral infections, i.e., influenza A virus (influenza), EBV, CMV, and HIV, that represent a range of functional states; T cells specific for influenza are considered highly functional whereas HIV-specific T cells from patients with chronic infection are characterized by significant functional defects (35, 36). T cells specific for EBV and CMV were also evaluated to represent persistent viral infections with low Ag load. To be useful as a test of T cell immunity, the signature should be capable of discriminating between functional (influenza) and dysfunctional (HIV) responses. Starting with ~50 ml of peripheral blood, 1,000–50,000 Ag-specific cells were purified by flow-sorting populations of MHC-peptide tetramer-positive CD8 T cells (Fig. 6A) from 25 donors. Using a highly efficient cDNA amplification process, high-quality microarray data were generated in all but one sample (Fig. 6B).

Hierarchical clustering of Ag-specific T cells in the space of the conserved CD8 memory signature showed marked differences between T cells specific for different viruses (Fig. 6C). HIV-specific and influenza-specific T cells samples partitioned to entirely separate clusters. Most EBV- and CMV-specific samples formed a third cluster more related to the HIV cluster (shown in yellow). Interestingly, some CMV samples clustered with HIV samples, consistent with reports of increasing CMV-specific T cell dysfunction in some individuals (37). Thus, in humans as in mice, alterations in the CD8 memory differentiation signature could distinguish T cells in functional responses to acute viral infections from those in dysfunctional responses to chronic viral infection.

Discussion

In this study we have used cross-species gene expression analysis to identify a molecular signature of memory CD8 differentiation that is conserved between humans and mice. We found that elements of this CD8 memory differentiation signature were shared by CD4 and B cell memory differentiation, suggesting that coordinated up-regulation of this set of genes is common to all memory lymphocyte lineages. Alterations in this signature could distinguish T cells from functional and dysfunctional responses to viral

infection. These data indicate a central role for this common differentiation signature in memory development in each lymphoid lineage and suggest that the genomic classification of memory differentiation could be used to assess functional immunity.

It is surprising that lymphocytes with such distinct functions and diverse transcriptional regulation as T cells and B cells should share a common transcriptional program during memory development. Indeed, the formation of discrete populations of mature lymphocytes depends on discrete, lineage-specific transcription factors that impart functions unique to that lineage (10–14). However, our data suggest that in addition to these lineage-specific mechanisms, memory lymphocytes in multiple lineages use a common transcriptional program during differentiation. These findings differ from previous studies that have evaluated the genome-wide changes during memory differentiation. Working in the mouse, Luckey et al. showed a similarity between the signature of genes up-regulated in CD8 memory T cells and the signature of those in hematopoietic stem cells, and also between memory B cells and hematopoietic stem cells. However, that study found only limited similarity in the genes up-regulated during memory differentiation in both T and B cell lineages (15). Appay et al., studying human CD4 and CD8 memory-phenotype cells in humans, showed similarity in gene expression profiles between terminally differentiated cells in each lineage, but none in earlier stages (16). Our study, in contrast, which encompassed both functionally defined mouse memory CD8 T cells and phenotypically defined human CD4, CD8 T, cells and B cells (sorted with different markers in T and B lineages), identified a transcriptional program up-regulated during memory differentiation in both species and all three lineages.

Several reasons may account for the difference between the previous studies and our own. First, we used a cross-species genomic comparison of memory differentiation. Focusing only on the signature of genes up-regulated during CD8 differentiation in two species would be more likely to identify genes critical to the differentiation process by virtue of their evolutionary conservation. Second, we used a sensitive analytic technique (GSEA) well suited to detecting the coordinated up-regulation of correlated sets of genes that could have been missed by other analyses, and to determining its statistical significance. GSEA has proven a powerful analytic tool to evaluate coordinately regulated patterns of genes occurring in cellular differentiation in stem cell biology (38), and immunology (31, 32).

The transcripts identified in our analysis include many that are not known to be involved in memory differentiation. However, many have functions consistent with the functional characteristics common to the memory state in each lineage. For example, the kinetics of proliferation in memory lymphocytes is different than that in their naive precursors; memory lymphocytes show a higher rate of division and shorter lag time after Ag stimulation than do naive cells (39). This finding has been attributed to the increased expression of cell-cycle components necessary for G₀-G₁ transition (39, 40). How memory cells remain quiescent but “poised” to divide rapidly remains unclear. Our data show that a common feature of memory lymphocytes in all lineages is the elevated expression of transcription factors that could serve to enforce quiescence e.g., KLF10 and BHLHB2. Several of the Krüppel-like transcription factors have been implicated in blocking cell cycle passage (41) and the absence of KLF2 leads to spontaneous T cell activation and abnormal trafficking (42–44). Similarly, mice deficient in the transcription factor Stra-13/BHLHB2 also show spontaneous activation of T cells and develop autoimmunity (45, 46). Increased expression of these transcription factors in memory cells compared with naive cells may be a critical component of the quiescence that is a common feature of memory differentiation.

The ability of memory lymphocytes to migrate to sites of inflammation is crucial to their function, a finding that is consistent with the increased expression of transcripts for S100 family members (47), MYO1F (48), and chemokine receptors by both T and B memory lymphocytes. S100A4, a member of the calcium-binding EF-hand motif superfamily, enhances motility of cancer cells through its interaction with myosin-IIA (47), suggesting that it may have a similar role in augmenting the ability of memory T and B lymphocytes to migrate. However, it is more than the up-regulation of individual genes that characterizes memory differentiation in each lineage. Rather, the coordinated up-regulation of a set of genes suggests the existence of a common developmental program shared by memory cells. Such a program would provide an efficient mechanism by which common attributes such as quiescence and migratory potential could be acquired in different lineages.

Some of the genes contained in the memory differentiation signature are not uniquely expressed by memory cells, (e.g., *GZMA* and *KLRG1*) and are known to be up-regulated in effector T cells (18). We found that expression levels of these effector genes is greatly reduced in memory cells compared with effector cells, consistent with previous observations, allowing clear distinction of effector and memory states (Fig. 3A) (18). This illustrates that the differentiation signature of memory cells represents a composite of genes uniquely expressed by memory cells and those retained from prior stages of differentiation, possibly to allow rapid re-expression upon re-exposure to Ag. Thus, it is not necessarily the genes themselves that are unique to the memory T cells as it is the expression pattern of the signature of a whole that defines the memory state.

Our findings have implications for the diagnosis of T cell dysfunction in humans. Impressive progress has been made on defining the phenotypic and functional characteristics of memory T cells in different infectious settings, but it has been difficult to use these properties as a measure of protective immunity in humans (7, 35). There is a significant need for accurate correlates of immunity to develop effective immunotherapies for chronic viral diseases and cancer. We used the “gold-standard” mouse model of memory differentiation as a biological filter to identify the corresponding differentiation signature in human memory-phenotype CD8 T cells. Alterations in the conserved memory signature distinguished between T cells specific for effective and ineffective viral responses in mice (acute vs chronic LCMV) and in humans (influenza vs HIV). This suggests that the pattern of gene expression in this memory differentiation signature correlated with T cell functional status. Ultimately, larger studies of clinical outcomes in viral infection or vaccination will be required to refine this signature and determine how well the presence of this optimal differentiation signature predicts clinical endpoints such as viral load or protection from infection in humans. However, measuring the integrity of a validated signature of genes corresponding to a defined differentiation state in Ag-specific T cells may be a useful tool for interrogating the human immune response.

Our data used cross-species genomic analysis to identify, for the first time, a common signature of genes up-regulated during memory differentiation in CD4, CD8, and B cell lineages that is conserved between humans and mice. These findings support the hypothesis that the shared attributes of memory cells are achieved in different lineages by common transcriptional programs. The ability to identify these critical differentiation signatures in Ag-specific T cells could allow the genome-wide assessment of memory differentiation as a correlate of functional immunity in humans and as a target for therapeutic intervention.

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Disclosures

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