Epigenetic landscapes reveal transcription factors that regulate CD8+ T cell differentiation

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Dynamic changes in the expression of transcription factors (TFs) can influence the specification of distinct CD8+ T cell fates, but the observation of equivalent expression of TFs among differentially fated precursor cells suggests additional underlying mechanisms. Here we profiled the genome-wide histone modifications, open chromatin and gene expression of naive, terminal-effector, memory-precursor and memory CD8+ T cell populations induced during the in vivo response to bacterial infection. Integration of these data suggested that the expression and binding of TFs contributed to the establishment of subset-specific enhancers during differentiation. We developed a new bioinformatics method using the PageRank algorithm to reveal key TFs that influence the generation of effector and memory populations. The TFs YY1 and Nr3c1, both constitutively expressed during CD8+ T cell differentiation, regulated the formation of terminal-effector cell fates and memory-precursor cell fates, respectively. Our data define the epigenetic landscape of differentiation intermediates and facilitate the identification of TFs with previously unappreciated roles in CD8+ T cell differentiation.

In response to infection, naive CD8+ T cells differentiate into a heterogeneous population of pathogen-specific effector CD8+ T cells. While the majority of these T cells undergo apoptosis after resolution of the infection, a small fraction persists as memory cells and provide lasting protection against re-infection1. Published studies have demonstrated that commitment to the effector or memory CD8+ T cell fate occurs early after infection, and differential expression of the activation marker KLRG1 (‘killer-cell lectin-like receptor G1’) and cytokine receptor IL-7R can be used to distinguish two effector subsets with distinct memory potential: terminally differentiated effector (TE) (KLRG1hiIL-7Rlo) CD8+ T cells and memory-precursor effector (MP) (KLRG1loIL-7Rhi) CD8+ T cells2-5. Numerous TFs have been identified as critical regulators of CD8+ T cell fate, including T-bet, Blimp-1, Id2, IRF4 and Zeb2 for TE and effector populations; and TCF-1, Eomes, Id3, E proteins, Bcl-6 and FOXO1 for MP and memory populations2-5. Notably, not all these factors exhibit differential expression in the TE subset relative to their expression in the MP subset, which suggests that additional mechanisms contribute to their activity in promoting cell fates. Furthermore, how these TFs function within a coherent regulatory network is unknown, and additional TFs relevant to CD8+ T cell differentiation remain unidentified.

We reasoned that integrated analysis of the expression and binding of TFs and expression of their target genes would provide additional insights for the identification of TFs with previously unappreciated involvement in CD8+ T cell differentiation. The ATAC-seq approach (assay for transposase-accessible chromatin with high-throughput sequencing) has been used to globally probe open chromatin to map TF-binding regions with high genomic resolution with a requirement for minimal material6-8. By scanning TF-binding motifs within accessible chromatin regions, it is possible to infer the binding of hundreds of TFs and identify potential gene targets of these TFs simultaneously, which has previously been technically impossible to achieve8. ATAC-seq has proven powerful for pinpointing TF-binding sites within regulatory elements characterized by active epigenetic marks such as promoters marked by trimethylation of histone H3 at Lys4 (H3K4me3) and enhancers associated with monomethylation of histone H3 at Lys4 (H3K4me1) and acetylation of histone H3 at Lys27 (H3K27ac)9-11. Additionally, trimethylation of histone H3 at Lys27 (H3K27me3) is associated with gene repression10. Published studies combining ATAC-seq and analysis of histone modifications have facilitated the prediction of TFs and enhancers that define tissue-specific macrophages and of lineage-determining TFs in hematopoiesis12,13. In naive CD8+ T cells, co-deposition of H3K4me3 and H3K27me3 at...
epromoter regions is a signature of genes encoding products important for cellular differentiation, suggestive of an epigenetic mechanism underlying CD8\(^+\) T cell differentiation\(^{14,15}\). However, those studies focused exclusively on promoters. Accumulating evidence suggests that enhancers also have a key role in ‘fine-tuning’ gene expression, providing better specificity than promoters\(^{12,16}\). However, the enhancer landscapes important for the differentiation of effector and memory CD8\(^+\) T cells remain largely unknown.

Here we characterized the epigenetic landscapes of naive, TE, MP and memory CD8\(^+\) T cells generated during bacterial infection to identify both enhancers and promoters important for CD8\(^+\) T cell differentiation. Using ATAC-seq to identify accessible regulatory regions, we predicted TF candidates and further constructed a transcriptional-regulatory network for each subset. To facilitate the identification of key TFs, we developed a new bioinformatics method using the PageRank algorithm to rank the importance of TF in each regulatory network. We identified TFs known to be central to CD8\(^+\) T cell differentiation and TFs not previously associated with specification to the CD8\(^+\) T cell fate. Among those, we experimentally confirmed that the TFs YY1 (‘yin and yang-1’) and Nr3c1 (‘nuclear receptor subfamily 3 group C member 1’; a glucocorticoid receptor) promoted the TE cell phenotype and MP cell phenotype, respectively. Together our results yielded a comprehensive catalog of the regulatory elements of CD8\(^+\) T cells and revealed unexpected regulators that control the fate of CD8\(^+\) T cells. Furthermore, our computational framework can be applied generally to any cell or tissue type to delineate regulatory networks and identify biologically important TFs.

**RESULTS**

**Differential gene expression by TE and MP CD8\(^+\) T cells**

The effector CD8\(^+\) T cell population is characterized by extensive phenotypic and functional heterogeneity, including the TE and MP subsets\(^2\). Microarray analysis of the TE and MP subsets revealed genes expressed differentially by TE cells versus MP cells on day 8 of bacterial infection of mice, and comparison with gene-expression data for total effector and memory CD8\(^+\) T cell populations indicated that many genes upregulated in the TE subset relative to their expression in the MP subset also had higher expression by total effector cells than by memory CD8\(^+\) T cells\(^{17}\) (Supplementary Fig. 1a,b). This result indicated the unique transcriptional identities of effector and memory CD8\(^+\) T cells could be captured by analysis of the TE and MP subsets. Notably, the differences in abundance of mRNA and protein for the majority of TFs known to control the differentiation of the TE subset versus that of the MP subset were subtle (Supplementary Fig. 1c,d), which suggested that expression differences alone did not account for the differential dependence of distinct subsets on TFs. High-throughput RNA-based sequencing (RNA-seq) of TE and MP subsets was consistent with our microarray analyses: for genes upregulated in the TE subset relative to their expression in the MP subset, total effector CD8\(^+\) T cells showed higher expression of these genes than did memory CD8\(^+\) T cells, and many of the key TFs had similar expression by the TE subset and MP subset (Supplementary Fig. 1e-g and Supplementary Table 1). Thus, beyond TF expression, additional regulatory mechanisms, such as the control of TF binding, might contribute to the differentiation of these two subsets and the subsequent formation of long-lived memory cells.

![Figure 1](https://example.com/figure1.png)

**Figure 1** Epigenetic landscapes of CD8\(^+\) T cells in response to bacterial infection. (a) ChIP-seq analysis of H3K4me3 and H3K27me3 (key) at Tbx21 (left) and Tcf7 (right) in naive CD8\(^+\) T cells (N), TE CD8\(^+\) T cells (TE), MP CD8\(^+\) T cells (MP) and memory CD8\(^+\) T cells (M) (left margin). (b) Frequency of genes with increased H3K27me3 at the promoter regions, among genes with a decrease in expression at various stages of differentiation (horizontal axis). (c) ChIP-seq analysis (left three plots) of H3K4me1, H3K27ac and H3K27me3 (above plots) at Gzma (top left), Il7r (bottom left), Id2 (top right) and Id3 (bottom right) in cells as in a (left margin), and microarray analysis of the expression of those genes (far right bar plot). Each symbol (far right) represents a biological replicate of spleens pooled from three mice. Data are pooled from two independent experiments (a,b,c (left three plots); n = 10 mice) or three independent experiments (c (far right plots); n = 3 mice; mean ± s.e.m.).
Distinct enhancer repertoires of CD8+ T cell subsets

Spatial and temporal regulation of gene expression requires the specific binding of TFs at regulatory elements, which is affected by chromatin state and accessibility. We analyzed histone modifications (H3K4me1, H3K4me3, H3K27ac and H3K27me3) by chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) for characterization of potential enhancer and promoter elements, and combined that with ATAC-seq to integrate the chromatin state and accessibility of each CD8+ T cell subset; this allowed us to predict the binding of TFs at specific regulatory elements. We transferred

Figure 2 Dynamic use of enhancers is associated with differentially expressed genes during CD8+ T cell differentiation. (a) Quantification of enhancers gained or lost (key) during the differentiation of naive CD8+ T cells into TE, MP or memory CD8+ T cells (left margin). (b) Clustering (k-means analysis; k = 5) of H3K4me1 signal intensity (key) in total enhancers (52,331) across CD8+ T cell subsets (above plot) into clusters I–V (right margin); left margin, select genes associated with specific enhancers. RKPM, reads per kilobase per million mapped reads. (c) Gene-ontology (GO) analysis of the clusters in b (above plot), assessed with a binomial test, with the top two pathways for which the cluster showed enrichment presented (cut off binomial P value, <0.001). (d) Expression of mRNA from genes with one, two or three or more enhancers (horizontal axis) in clusters I–III (top) and cluster IV (bottom), presented as expression in effector CD8+ T cells relative to that in naive CD8+ T cells (EFF/N; top) and vice versa (N/EFF; bottom). * P < 0.0001 (unpaired two-tailed Student’s t-test). Data are pooled from two independent experiments (a–c; n = 10 mice) or three independent experiments (d; n = 3 mice; mean ± s.e.m. (symbols indicate outliers)).

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OT-I CD8^+ T cells (which have transgenic expression of a T cell antigen receptor that specifically recognizes a peptide fragment of ovalbumin (OVA) presented by major histocompatibility complex class I H-2K^b) into host mice, followed by infection of the host mice with *Listeria monocytogenes* engineered to express recombinant OVA (Lm-OVA)\(^7\). Naive, TE, MP and memory CD8^+ T cell populations were sorted for ChIP-seq and ATAC-seq (Supplementary Fig. 2a,b). Notably, OT-1 and polyclonal CD8^+ T cells responding to infection showed highly correlated gene expression throughout the immune response\(^7\) and OT-1 and polyclonal effector and memory CD8^+ T cells displayed similar ATAC-seq profiles (data not shown).

Published studies have shown that bivalent chromatin domains, comprising H3K4me3 and H3K27me3 modifications, exist in the promoters of genes encoding effector molecules in naive cells, and occupancy by H3K27me3 at these promoters is diminished when the promoters of genes encoding effector molecules in naive cells, and comprising H3K4me3 and H3K27me3 modifications, exist in the

| T-bet motif
|---|
| N | TE | MP |
| H3K4me1 | Zeb2 |
| TE | |
| MP | |
| M | |
| ATAC-peak |

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Focusing on distal regulatory regions of well-characterized genes in effector and memory CD8^+ T cells, we found both gains and losses of enhancer and repressive H3K27me3 marks. For example, *Gzmna*, a characteristic effector-molecule-encoding gene (that encodes granzyme A) with high expression in TE cells, was associated with increased deposition of H3K4me1 and H3K27ac after the differentiation of naive CD8^+ T cells into the TE subset (Fig. 1c). Conversely, *Il7r* (which encodes IL-7R) exhibited greater deposition of H3K4me1 and H3K27ac in MP and memory CD8^+ T cells than in the TE subset (Fig. 1c), consistent with its role promoting the long-term survival of memory CD8^+ T cells\(^3,18\). Alternatively, *Id2* and *Id3* (which encode established transcriptional regulators of CD8^+ T cell differentiation) exhibited substantial occupancy by H3K4me1 in all CD8^+ T cells but were associated with dynamic changes in the intensity of H3K27ac and H3K27me3 during differentiation\(^19,20\) (Fig. 1c). Thus, as expected, combinatorial epigenetic marks set the stage for gene expression.

To systematically identify putative enhancers, we applied the machine-learning algorithm RFECS (random forest-based enhancer identification from chromatin states)\(^21\). RFECS identified 27,236 enhancers, 26,561 enhancers, 23,302 enhancers and 21,883 enhancers in naive CD8^+ T cells, TE CD8^+ T cells, MP CD8^+ T cells and memory CD8^+ T cells, respectively; this constituted a non-redundant set of 52,331 putative enhancers. Upon the differentiation of naive CD8^+ T cells during infection with Lm-OVA, TE cells gained a greater number of newly formed enhancers than did MP cells or memory cells, while all populations lost a similar number of enhancers (Fig. 2a).

To understand the dynamics of the usage of enhancers during differentiation, we performed k-means clustering analysis of 52,331
Figure 4  Network analysis reveals subset-specific T-bet regulatory circuits. (a) Global regulatory network in the TE and MP subsets: red indicates genes regulated by T-bet (blue), and size indicates their expression. (b) Comparison of genes regulated by T-bet in the TE and MP subsets; numbers in plot indicate total genes in set. (c,d) Flow cytometry of sorted Tbx21+/+ (T-bet WT) and Tbx21−/− (T-bet KO) CD8+ T cell populations from recipient mice given co-transfer of Tbx21+/+ and Tbx21−/− OT-1 cells, followed by infection with Lm-OVA and analysis 9 d later, to identify KLRG1hiIL-7Rlo (MP) cells (outlined areas) (c), and RT-qPCR of mRNA encoding genes regulated by T-bet in total CD8+ T cells and the MP subset (key) from those mice (d); mRNA results are presented as expression in Tbx21+/+ cells relative to that in Tbx21−/− cells (dashed lines, onefold). (e,f) Flow cytometry of sorted Tbx21+/+ (T-bet WT) and Tbx21−/− (T-bet HET) CD8+ T cell populations from recipient mice as in c, analyzed 8 d after infection, to identify KLRG1hiIL-7Rlo (TE) cells (top left gate) and KLRG1loIL-7Rhi (MP) cells (bottom right gate) (e), and RT-qPCR of mRNA encoding genes regulated by T-bet in the TE and MP subsets (key) from those mice (f); mRNA results are presented as in d. (g) Frequency of MP cells among Tbx21+/+ and Tbx21−/− peripheral blood lymphocytes (key) during Lm-OVA infection as in c. NS, not significant (P > 0.05); *P < 0.05 and **P < 0.01 (paired two-tailed Student’s t-test).

Enhancers according to their H3K4me1 intensity. Enhancers were separated into five distinct clusters (I–V) (Fig. 2b). In cluster V, the intensity of H3K4me1 was maintained equivalently across the CD8+ T cell subsets, and genes associated with this cluster (Cd8a and Lck) had high expression in all subsets (Fig. 2b). The intensity of H3K4me1 increased in clusters I and II during differentiation, and the TE subset showed enrichment for H3K4me1 relative to its intensity in MP and memory CD8+ T cells (Fig. 2b). Genes associated with clusters I and II (Klrg1 and Tbx21) were associated with differentiation into the TE subset (Fig. 2b). In cluster III, the intensity of H3K4me1 was higher in all differentiated subsets than in naive CD8+ T cells, and genes associated with this enhancer cluster (such as Ptf1 (which encodes perforin 1)) encoded products involved in the activation of CD8+ T cells (Fig. 2b). Conversely, for cluster IV, the intensity of H3K4me1 decreased during the differentiation of naive CD8+ T cells into the TE subset and was higher in MP and memory CD8+ T cells than in the TE subset (Fig. 2b). Enhancers of genes encoding canonical regulators of memory potential and homeostasis (Il7r and Cxcr4 (which encodes the chemokine receptor CXCR4)) were in cluster IV (refs. 3,22) (Fig. 2b).

To determine if differential establishment of enhancers regulates subset-specific gene expression, we assigned enhancers to the nearest genes and compared gene expression during CD8+ T cell differentiation. Enhancers in clusters I, II and III were associated with genes upregulated in activated CD8+ T cells, and enhancers in cluster IV were associated with genes with high expression in naive CD8+ T cells (Supplementary Fig. 3a). Notably, around 66% of genes with enhancers in clusters I and II were upregulated in the TE subset relative to their expression in the MP subset, while 65% of genes with enhancers in cluster IV were upregulated in the MP subset relative to their expression in th TE subset (Supplementary Fig. 3b). We performed gene-ontology analysis using GREAT (genomic regions enrichment of annotations tool)23 with the whole genome as the background set and found that clusters I and II showed enrichment for enhancers of genes encoding components of the IL-12 signaling pathway (Fig. 2c), consistent with the role of IL-12 in promoting differentiation into the TE subset. In addition, cluster IV showed enrichment for enhancers of genes encoding components of the cytokine TGF-β and EGF signaling pathway (Fig. 2c), which suggested that these signaling pathways might favor the naive and/or memory T cell state, consistent with data showing TGF-β signaling is required for the differentiation of memory T cells24. We further observed that the association of genes with multiple enhancers correlated with higher expression than that of genes associated with a single enhancer (Fig. 2d).

TF-motif enrichment at subset-specific regulatory regions

We reasoned that accessible regulatory regions would show enrichment for TF-binding motifs relative to the abundance of such motifs in the whole genome (as background) and that we could use ATAC-seq to identify TFs important for CD8+ T cell differentiation. Thus, we identified subset-specific open enhancers and promoters and then scanned 761 unique known TF-binding motifs at the center of the ATAC peaks of these regulatory regions (Supplementary Fig. 4a,b). For example, the T-bet-binding motif appeared at a TE-specific
accessible enhancer near Zeb2 (encoding the TF ZEB2), which was expressed exclusively in the TE subset (Fig. 3a), in support of published findings showing that T-bet directly regulates Zeb2 to promote differentiation into the TE subset.25,26 Our motif-enrichment analysis predicted the enrichment or depletion of putative binding motifs for known TFs at promoters and enhancers relative to their abundance at randomly selected open chromatin.27–31 Naïve CD8⁺ T cell subsets showed depletion of binding motifs for T-bet, BATF, SREBP2 and AP-1, and differentiated CD8⁺ T cell subsets showed enrichment for these motifs (Fig. 3b and Supplementary Fig. 4c), consistent with the crucial role of these TFs in the activation and effector function of CD8⁺ T cells.27–29,32 TE CD8⁺ T cells showed depletion of binding motifs for TCF-1, Lef1 and E2A, and naïve, MP and memory CD8⁺ T cells showed enrichment for these motifs (Fig. 3b), which corresponded with the well-characterized roles of these TFs in regulating the differentiation of memory populations.4,30,31 Enrichment for binding motifs for some TFs (TCF-1 and T-bet) was highly correlated with gene expression and function;2,4; in contrast, enrichment for binding motifs for other TFs (enrichment for the SREBP2-binding motif in effector T cells and for the E2A-binding motif in MP and memory cells) was consistent with their demonstrated roles (SREBP2 maintains the activation of effector T cells, and E2A promoting MP and memory cell differentiation), yet their expression remained unchanged during CD8⁺ T cell differentiation.28,31 (Fig. 3c). These data indicated that subset-specific enhancers and promoters might be established by key TFs and that putative binding of TFs, in addition to differential expression, must be considered in the identification of TF involvement.

**Construction of TF regulatory networks in CD8⁺ T cell subsets**

To elucidate TF-mediated regulatory mechanisms underlying CD8⁺ T cell differentiation, we sought to construct a TF regulatory network in various CD8⁺ T cell subsets. Published studies have applied correlation of gene co-expression to construct regulatory networks;33,34 however, this approach does not consider direct TF-binding. We combined information on TF-binding motifs, chromatin states and chromatin accessibility to predict and link TF-binding sites to their potential gene targets (Supplementary Fig. 5). We reconstructed TF regulatory networks and identified critical regulatory circuits responsible for CD8⁺ T cell differentiation. For example, we identified a substantial number of putative targets regulated by T-bet in both the TE subset and MP subset (Fig. 4a and Supplementary Table 2). We compared the TE and MP subsets for genes predicted to be regulated by T-bet and found that 61.4% of the candidate genes were shared by these subsets; these included Ifng and Cxcr3, which are well-established targets regulated by T-bet that encode products important for effector function (interferon-γ (IFN-γ) and the chemokine receptor CXCR3, respectively).35,36 (Fig. 4b). Notably, on the basis of the subset-specific T-bet regulatory circuits, we predicted that T-bet uniquely controls the expression of Zeb2, Gzma and Klb1c in the TE subset with loss of T-bet relative to their expression in the wild-type TE subset (Fig. 4c). Notably, loss of T-bet affected the expression of Bcl2, Cmat and Pou6f1 in the MP subset (Fig. 4d), which suggested that T-bet regulated these genes in an MP-cell-specific manner. The absence of T-bet resulted in a defect in the accumulation of MP cells over the course of infection (Fig. 4g), consistent with the finding that T-bet also regulates memory differentiation.37. Thus, we demonstrated that T-bet positively regulated different genes in distinct CD8⁺ T cell subsets, which highlighted the proposal that this approach allows the prediction of potential gene targets unique to different CD8⁺ T cell subsets.

**Identification of key TFs from PageRank-based TF ranking**

Constitutively expressed TFs can exert cell-type-specific functions via regulation of the expression of distinct genes, but incorporating that knowledge for the identification of key TFs remains challenging because the TF targets are largely unknown. To overcome that limitation, we leveraged the TF regulatory network and developed a new bioinformatics method using the Personalized PageRank algorithm37 to assess the importance of each TF in the regulatory network...
YY1 is a transcriptional regulator of the differentiation of TE CD8⁺ T cells. (a) RT-qPCR analysis of YY1 mRNA in CD8⁺ T cells activated in vitro for 72 h (with antibody to the invariant signaling protein CD3 and antibody to the co-receptor CD28) and transduced with shCon or shYY1 (key); results are presented relative to those of shCon-transduced cells. (b) Flow cytometry of shRNA-transduced cells from host mice given co-transfer of OT-I CD8⁺ T cells (that had been activated in vitro (as in a) and transduced for 24 h with shCon or shYY1 (above plots)), followed by intravenous infection of the hosts with Lm-OVA and analysis, 7 d later, of the expression of KLRG1 and IL-7R. Numbers in quadrants indicate percent cells in each throughout (far right, gating of the TE and MP subsets). (c) Frequency (left half) and quantification (right half) of TE and MP CD8⁺ T cells as in b. (d) Expression of CD27, CXCR3 and TCF-1 (left) in cells as in b; right, summary of results at left. Numbers above and below bracketed lines (left) indicate mean fluorescent intensity (MFI) of the factor assessed (colors match key). (e) Frequency (far left) and quantification (middle right) of IFN-γ producing cells, mean fluorescent intensity of IFN-γ (middle left), and quantification of TNF producing cells (far right) among cells as in b stimulated in vitro for 4 h with OVA peptide, assessed by intracellular cytokine staining followed by flow cytometry. Each symbol (c-e) represents an individual mouse; small horizontal lines (c,e (far left)) indicate the mean (± s.e.m.). *P < 0.05, **P < 0.01 and ***P < 0.001 (two-tailed paired Student's t-test). Data are representative of two (a,d,e) or three (b,c) independent experiments (n = 3 mice; mean ± s.e.m. in d,e (middle and right)).

(FIG. 5a). The TF ranks determined by our method were influenced by the number of genes and the importance (determined from their expression) of genes regulated by the TF. Thus, TFs that regulate more important genes would receive higher ranks.

Using PageRank analysis, we predicted TFs important for CD8⁺ T cell differentiation and compared our PageRank analysis with motif-enrichment analysis used by published studies⁵,¹¹ to determine how many TFs reported previously as essential regulators of CD8⁺ T cell differentiation could be recovered from predicted TF pools. We found that approximately half of the predicted TFs were shared by both analyses, and 25% of these shared TFs were identified in published studies (Supplementary Fig. 6a,b). PageRank analysis revealed more known TFs than did motif-enrichment analysis: 22% of TFs among the entire pool of predicted TFs were previously reported to regulate CD8⁺ T cell differentiation, and they were identified by PageRank analysis but not motif enrichment analysis, compared with 5% identified only by motif-enrichment analysis (Fig. 5b). For example, PageRank analysis assigned STAT3 a higher score in memory subsets than in the TE subset (Fig. 5c). That was consistent with the role of STAT3 in promoting the maturation and self-renewal of memory CD8⁺ T cells ³⁸. Additionally, more TFs with known roles in CD8⁺ T cell differentiation were identified by PageRank analysis than by another method, TF activity (TFA) analysis, which predicts the activity of TFs using the regulatory network constructed from gene-expression data ³⁹ (Supplementary Fig. 6c,d). These data highlighted the robustness of PageRank analysis and suggested that TFs predicted by PageRank analysis might be critical for CD8⁺ T cell differentiation.

Validation of PageRank-predicted TFs
To highlight the power of PageRank analysis, we focused on YY1 and Nr3c1, two regulators identified by PageRank analysis but not by the motif-enrichment analysis. Although the expression of YY1 and Nr3c1 did not change during CD8⁺ T cell differentiation (Supplementary Fig. 6e), YY1 was ranked highly in the TE subset while Nr3c1 was ranked highly in the MP subset (Fig. 5c). YY1 is a TF involved in transcriptional activation and repression and is important in immune-cell development, including the differentiation of B cells, the Tγ2 subset of helper T cells and regulatory T cells ⁴⁰–⁴². Nr3c1 encodes the glucocorticoid receptor Nr3c1, which translocates into the nucleus to regulate gene expression after binding to glucocorticoids in the cytosol. Nr3c1 has a critical role in development, metabolism and the immune response ⁴³–⁴⁵. The role of YY1 and Nr3c1 in the differentiation of effector or memory CD8⁺ T cells in response to infection is unknown.

On the basis of the PageRank predictions, we hypothesized that abolishing the expression of YY1 or Nr3c1 would affect formation of the TE subset or MP subset, respectively. To determine if YY1 is essential for differentiation of the TE subset, we transduced cognically distinct OT-I CD8⁺ T cells with retrovirus encoding short hairpin RNA (shRNA) targeting YY1 (shYY1) or shRNA targeting the control gene Cd19 (shCon) and co-transferred the cells into recipient mice, followed by infection of the recipients with Lm-OVA, then monitored the differentiation of effector T cells (Supplementary Fig. 7a). Knockdown of YY1 resulted in a 54% reduction in its expression relative to that in cells transduced with shCon (Fig. 6a). Flow cytometry of CD8⁺ T cell subsets on day 7 of infection showed a significantly lower frequency and number of the TE subset among shYY1-transduced cells than among shCon-transduced cells (Fig. 6b). Additional, the expression of MP-cell-associated molecules, including CD27, CXCR3 and TCF-1, was significantly higher in shYY1-transduced cells than in shCon-transduced cells (Fig. 6d). Furthermore, analysis of cytokine production showed that the mean expression of IFN-γ and the number of IFN-γ-producing cells were lower for shYY1-transduced cells than for shCon-transduced cells (Fig. 6e). Together, these data confirmed that YY1 was important for differentiation of the TE subset.

We used a similar method (transfer of OT-I cells and infection with Lm-OVA) to determine how lowering Nr3c1 expression, via shRNA targeting Nr3c1 (shNr3c1), affected the MP subset differentiation. Knockdown of Nr3c1 resulted in 86% reduction in its expression.

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Figure 7 Nr3c1 is essential for the formation of MP CD8+ T cells. (a) RT-qPCR analysis of Nr3c1 mRNA in CD8+ T cells activated in vitro for 72 h (as in Fig. 6a) and transduced with shCon or shNr3c1 (key); results are presented relative to those of shCon-transduced cells. (b) Flow cytometry of shRNA-transduced cells from host mice given co-transfer of OT-I CD8+ T cells (that had been activated in vitro (as in Fig. 6a) and transduced for 24 h with shCon or shNr3c1 (above plots)), followed by intravenous infection of the hosts with Lm-OVA and analysis, 7 d later, of the expression of KLRG1 and IL-7R. (c) Frequency (left) and quantification (right) of TE and MP CD8+ T cells as in b. (d) Expression of CD27, CXCR3 and TCF-1 (left) in cells as in b; right, summary of results at left. Numbers above and below bracketed lines (left) indicate mean fluorescent intensity (MFI) of the factor assessed (colors match key). (e) Frequency of MP cells among cells as in b, analyzed at various times (horizontal axis) after infection. (f) Flow cytometry of cells as in b, analyzed 30 d after infection (assessing expression of KLRG1 and IL-7R). *P < 0.05, **P < 0.01 and ***P < 0.001 (two-tailed paired Student’s t-test). Data are representative of two independent experiments (n = 3 mice (a–d) or n = 5 mice (e); mean ± s.e.m.).

DISCUSSION

The function and differentiation state of immune cells are controlled by TFs that relay environmental cues through regulation of gene expression. Efficient transcriptional regulation requires interaction between TFs and chromatin remodelers to control the binding of TFs with high fidelity. Key information is encoded in regulatory elements that contain TF-binding sequences and are associated with specific histone modifications that influence the accessibility, structure and location of those elements. To identify the TF-mediated regulatory circuits critical for CD8+ T cell differentiation, we systematically characterized the epigenome of CD8+ T cell subsets during pathogen infection. Our global map of regulatory elements revealed a dynamic pattern of enhancer establishment that foreshadowed specific gene-expression programs. Our network analysis of T-bet regulatory circuits in distinct effector-cell subsets revealed T-bet-targets that overlapped in and were distinct in the TE subset and the MP subset. This analysis suggested a previously unknown function for T-bet in maintaining the accumulation of MP cells, potentially through regulation of the anti-apoptotic protein Bcl-2 and additional targets. Studies of distinct targets will further elucidate nuanced functions of T-bet in driving effector and memory fates.

Numerous crucial TFs that modulate CD8+ T cell differentiation have been identified on the basis of differential gene expression and TF–gene co-expression correlation. However, alterations in the binding of TFs without changes in expression also result in the differential expression of downstream gene targets, which makes it clear that the identification of relevant TFs exclusively on the basis of gene-expression analysis provides only partial understanding of the TF networks involved. Indeed, our data demonstrated that gene expression alone could not fully explain the mechanisms behind cell-fate determination and supported the idea that the binding of TFs and gene expression should be considered together to facilitate the identification of important TFs. Differential TF binding can be achieved via numerous mechanisms, including variable chromatin state and accessibility, TF localization, the availability of co-factors, and post-translational modification of TFs. Our approach represents an advance in the efforts to achieve a comprehensive view of the regulatory networks that establish the effector and memory CD8+ T cell fates by integrating data describing mRNA expression as well as chromatin states and accessibility.

For prioritization of those data, it is essential to develop new methods that rank the potential importance of TFs on the basis of the quantity and quality of the TF-regulated genes. Here, we applied the Personalized PageRank algorithm to rank the absolute importance of TFs in each subset and their relative importance across cell types by considering both binding of TFs and gene expression. Notably, our method ranked TFs by integrating two features: distinct weights for TF-regulated genes, as assessed by differential gene expression; and a hierarchy of TF-to-TF circuitry. This strategy allowed the identification of TFs that regulate relatively few but important genes, which are often overlooked by other analyses. Future modifications of gene weights by gene ontology could facilitate identification of TFs important in specific functions or pathways.
We also confirmed the functions of two TFs identified by PageRank (YY1 and Nr3c1) and demonstrated their essential roles in differentiation of the TE subset and MP subset, respectively. YY1 has been shown to modulate long-range chromatin interactions of cytokine-encoding loci in Tπ2 cells. How YY1 regulates differentiation of the TE subset and if YY1 controls chromatin interactions in the TE subset remain to be determined. The glucocorticoid receptor Nr3c1 has been shown to regulate thymocyte apoptosis and inflammation responses. Here we found that Nr3c1 promoted differentiation of the MP subset, consistent with the role of glucocorticoids in inducing IL-7R expression. Treatment with dexamethasone increased the proportion of MP subset during differentiation, which demonstrated a previously unknown role for glucocorticoids in modulating CD8+ T cell differentiation and a potential strategy for manipulating memory-cell differentiation. Thus, using our framework, we were able to both identify critical TFs and predict microenvironmental signals involved in regulating the differentiation of CD8+ T cells.

Despite the successful confirmation of TFs predicted by our computational framework, additional factors could be integrated to refine our results. Global investigation of TF-binding motifs using new approaches, such as protein-binding microarrays, might be beneficial in broadening the database of known TF-binding motifs. Moreover, TFs function with co-factors to regulate specific gene expression; co-binding analyses could be incorporated into these analyses to improve our network construction. Furthermore, the assignment of enhancers to the nearest genes is a limited heuristic, and being able to better associate long-range enhancers with gene targets would enhance the power of our approach considerably. Published studies have shown that the interaction of enhancers and promoters is confined in topologically associated domains; thus, exploration of the chromatin organization of enhancer marks as well as the use of new computational methods should facilitate the assignment of enhancers to their targets. Here we have provided evidence for the involvement of many TFs in CD8+ T cell immunity that were previously overlooked in this context; future studies should aim to refine and resolve the transcriptional networks by incorporating these additional approaches.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

B.Y. designed and performed experiments, analyzed the data and wrote the paper; K.Z. performed computational analysis and wrote the paper; B.Y., J.J.M., C.T. and B.C. performed shRNA-mediated knockdown; J.P.-S.-B. and R.M.P. provided ATAC-seq data sets for polyclonal CD8+ T cell populations; S.C. and M.E.P. provided reagents, advice for the design of experiments and analysis of experiments and assisted in writing the paper; J.T.C. provided advice and assisted in writing the paper; W.W. supervised the computational analysis and wrote the paper; and A.W.G. supervised the project, designed the experiments, analyzed the data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice, cell transfer, infection, and drug treatment. All mice were maintained in specific-pathogen-free conditions according to the instructions of Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego (UCSD). OT-I mice (specific for OVA amino acids 257–264) were bred at UCSD or received from The Jackson Laboratory. We transferred 5 × 10^6 OT-I CD8+ T cells into congenically distinct mice by intravenous (i.v.) injection and then infected mice intravenously with 5 × 10^6 colony-forming units of L. monocytogenes expressing OVA (Lm-OVA) 1 d later. For T-bet-deficient experiments, we co-transferred 1 × 10^6 Tbx21−/− OT-I CD8+ T cells and Tbx21+/− OT-I CD8+ T cells into host mice and then infected the mice intravenously with 5 × 10^6 colony-forming units of Lm-OVA. For drug treatment, dexamethasone (Sigma-Aldrich) was dissolved in DMSO and diluted in PBS and then administered to mice by intraperitoneal (i.p.) injection at 10 mg/kg daily after i.v. infection with 5 × 10^6 colony-forming units of Lm-OVA.

Antibodies and flow cytometry. Antibodies to KLRG1 (2F1), CD127 (A7R34), CD45 (53–6.7), CD45.1 (A20–1.7), CD45.2 (104), CXCR3 (CXCR3–173), CD27 (LG–7F9), T–bet (B10) and Bcl-6 (K112–91) were purchased from eBioscience. Antibodies to FOXP3 (C09H4), TCF-1 (C639F), IFN-γ (XMG1.2) and TNF (MP6–XCT22) were from Cell Signaling Technology. All antibodies for flow cytometry were used at a dilution of 1:200, except Bcl6, used at a dilution of 1:50. Antibodies for ChIP-seq, to H3K4me3 (Ab8580), H3K4me1 (Ab8895) and H3K27ac (Ab4729), were from Abcam. Antibody to H3K27me3 (07–449) was from Millipore. All antibodies for ChIP-seq were used at a concentration of 5 µg per 2 × 10^6 cells. For intracellular staining of cytokines, splenocytes were in vitro restimulated with 1 µg/ml OVA peptide (SIINFEKL) with Protein Transport Inhibitor (eBioscience) for 4 h and then fixed and permeabilized using BD cytofix/cytoperm kit (BD Biosciences). Foxp3–transcription factor staining buffer kit (eBioscience) were used for intracellular staining of transcription factors. For intracellular staining of shRNA-transduced cells containing Ametrine-reporter, cells were fixed using freshly made 2% paraformaldehyde for 45 min on ice and then permeabilized. All flow cytometry data were acquired by BD LSRFortessa X-20 and all cell sorting was performed on a BD FACSDaria.

shRNA-mediated knockdown by retroviral transduction. The detailed protocol was described previously.20 PLAT-E cells were transduced with shRNAmir using TransIT-LT1 Reagent (Mirus). Retrovirus-containing supernatant was harvested after 48 h and mixed with 2-mercaptoethanol and polyethylene glycol (Millipore) for subsequent transductions. Purified naive OT-I CD8+ T cells were in vitro activated by anti-CD3 (145–2C11) and anti-CD28 (37.51) (1 µg/ml for each; both from eBioscience) for at least 18 h and then ‘spininfected’ with retrovirus for 1 h at 37 °C. After 4 h of incubation, the retrovirus-containing medium was replaced by T cell medium. Transduction efficiency was measured by flow cytometry analyzing the ametrine reporter after 24 h, and 1 × 10^6 shRNA-transduced cells were transferred into host mice, followed by Lm-OVA infection. For Noor1 shRNA knockdown, purified P14 CD8+ T cells were in vitro activated and transduced by shRNA retrovirus similarly to OT-I CD8+ T cells. Transduced P14 CD8+ T cells (5 × 10^6) were transferred into host mice, followed by i.p. infection with 1.5 × 10^8 plaque-forming units of LCMV-Ci3, which results in acute infection.21 The full hairpin sequence for shRNA was as follows: shYy1, 5'-TGCTGTTGACAGTGAGCGCCCTCCTGA TTATTCTGAATAATGTGAACCCACAGTATTATCTGAAAAAATCA ATGGAGGTTGTCTCAGTCTCCGTA-3'; shNr3l1-1, 5'-TGCTGTTGACAGTGAGCGCCCTCCTGA-3'; and shNr3l1-2, 5'-TGGCTCGTAAATAAATAGGCTTG-3'. Humanized MHC class II tetramers with KLRG1, CD127, CD45.1 and sequence alignment.

Chromatin immunoprecipitation (ChIP), ChIP-seq library construction and sequence alignment. Cells were fixed in 1% formaldehyde for 10 min and then quenched with 125 mM glycine for 5 min. Cells were lysed for 5 min on ice and sonicated to generate 200–500 bp fragments by Bioruptor sonicator (Diagenode). Sonicated DNA was used as input control. Magnetic-dynabeads (30 µl) were washed with blocking buffer twice and then mixed with 5 µg antibody in 500 µl blocking buffer and rotated at 4 °C. The sonicated lysates were first diluted to a final 0.1% SDS concentration. The diluted lysates were added to antibody–conjugated Dynabeads incubated at 4 °C. Beads were washed by Wash Buffer I, II and III for 5 min and then washed twice by TE buffer for 5 min. The beads were resuspended in 200 µl Elution Buffer and reverse-crosslinked at 65 °C overnight and then treated with RNaseA for 30 min at 37 °C and Proteinase K at 55 °C for 1 h. DNA was purified by Zymo DNA Clean & Concentrator kit (Zymo Research). The purified DNA was end-repaired using End-it End-repair kit (Epipore) and then added an ‘A’ base to the 3′ end of DNA fragments using Klenow (NEB). Then DNA was ligated with adaptors using quick DNA ligase (NEB) at 25 °C for 15 min followed by size selection of 200–400 bp using AMPure SPRI beads (Beckman Coulter). The adaptors ligated DNA was amplified using NEBNext High-Fidelity 2X PCR master mix (NEB). To prevent PCR overamplification, 1 µl DNA was first quantitatively amplified using Syber Green I master mix to determine the best amplification cycle. Then the amplified library was size-selected as 200–400 bp using SPRI beads and quantified by Qubit dsDNA HS assay kit (ThermoFisher). Finally, the library was sequenced using HiSeq 2500 for single-end 50 bp sequencing to obtain around 20 million reads for each sample. We used BWA to map raw reads to the Mus musculus genome (mm10) with following parameters: --q 5 -s 2 -2. All reads with low quality (MAPQ < 30) were filtered out. If multiple reads were mapped to the same location, only one read was kept.

RT–PCR and qPCR. For RT–PCR, RNA was extracted using Trizol (Life Technologies), followed by precipitation of isopropanol. CDNA was synthesized using Superscript II kit (Life Technologies) following the manufacturer’s instruction. For qPCR, cDNA was quantitatively amplified using Stratagene Brilliant II Syber Green master mix (Agilent Technologies). The abundance of transcripts was normalized to that of the housekeeping gene Hprt. The following primers were used: Zeb2 forward, 5'-CATGAAACCATTATGTGCCA-3', and Zeb2 reverse, 5'-AGCAACCTCGTCTGAAGTCT-3'; Bcl2 forward, 5'-ACTCTGGACAGTGATGTCGTC-3' and Bcl2 reverse, 5'-TGGGCAGAAGCTCCCTC-3'; Gzma forward, 5'-TGCTGCCACATCTAAC-3' and Gzma reverse, 5'-GGTACGTTGAAAGTATCCAC-3'; Klf4 forward, 5'-GACACAGAAGATTCTTACCT-3' and Klf4 reverse, 5'-TACTA AGACTGCACTTATGAC-3'; Pou5f1 forward, 5'-GTCAGATCCCGC AACGTC-3' and Pou5f1 reverse, 5'-GAGTACGGCTTGGACCTG-3'; Crtam forward, 5'-CCTTTTCTACATCTGTCGACCT-3' and Crtam reverse, 5'-GGAGCTGTTTCCTGGCTCTACATA-3'; Nr3c1 forward, 5'-CGGCCGCTCAGGATTTAAAGA-3' and Nr3c1 reverse, 5'-TGCTCGGTAAATAAATAGGCTTG-3'; and Hprt forward, 5'-GGCCGAGATGGCIATTGATT-3' and Hprt reverse, 5'-CAACCTGCGCTACTTCTAAG-3'.

Microarray analysis. The protocol was described previously,27, KLRG1−/−Thr− T cells and KLRG1−/−Thr− MP CD8+ T cells (× 200) were sorted to TRizol on day 8 of Lm-OVA infection. RNA was amplified and labeled with biotin, followed by hybridized to Affymetrix Mouse Gene Set 1.0 microarrays (Affymetrix). Microarray analysis was performed using GenePattern Multiplot Studio module. All data was generated in collaboration with the Imgen project (http://www.imgen.org) and passed ImmGen quality-control pipeline. The gene-expression data of naive and memory CD8+ T cells were used from a published study28 and were normalized with the gene-expression data of TE and MP subsets by RNA normalization. Given that the TE and MP subsets are highly similar ‘effector’ populations on day 8 of infection and the finding that no genes showed a significant difference under the 1% false-discovery rate using the Student’s t-test, we used a cutoff of a 1.5-fold change in expression to identify genes expressed differentially in TE and MP subsets.

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The microarray, ATAC-seq and ChIP-seq data are available in the GEO database with the accession codes GSE8903 (ATAC-seq) and GSE89037 (microarray). Other public available data sets are described in subsections above. The source data published as supplementary items and data support the findings of this study are available from the corresponding author upon request.

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**Corrigendum:** Quantifying the shifting landscape of B cell immunodominance

Gordon A Dale, Jessica R Shartouny & Joshy Jacob

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In the version of this article initially published, a spelling error was made in the citation list. The error has been corrected in the HTML and PDF versions of the article.

**Erratum:** Epigenetic landscapes reveal transcription factors that regulate CD8+ T cell differentiation

Bingfei Yu, Kai Zhang, J Justin Milner, Clara Toma, Runqiang Chen, James P Scott-Browne, Renata M Pereira, Shane Crotty, John T Chang, Matthew E Pipkin, Wei Wang & Ananda W Goldrath

*Nat. Immunol.*; doi:10.1038/ni.3706; corrected online 27 March 2017

In the version of this article initially published online, some labels in Figure 2 were illegible or incorrect. Those should read “Enhancers (× 10^3)” along the top and “TE, MP and M” (top to bottom) along the left margin of Figure 2a; “N, TE, MP and M” (left to right) above the plot in Figure 2b; and “GO” below the plot in Figure 2c. Also, in the third sentence of the final paragraph of the final subsection of Results (Validation of PageRank-predicted Tfs), the description of the control cells (“shCon-transfected”) was incorrect. The correct text is “...lower among shNr3c1-transduced cells than among shCon-transduced cells...”. The errors have been corrected in the print, PDF and HTML versions of this article.

**Erratum:** THEMIS enhances TCR signaling and enables positive selection by selective inhibition of the phosphatase SHP-1

Seeyoung Choi, Claude Warzecha, Ekaterina Zvezdova, Jan Lee, Jérémy Argenty, Renaud Lesourne, L Aravind & Paul E Love

*Nat. Immunol.*; doi:10.1038/ni.3692; corrected online 7 March 2017

In the version of this article initially published online, in the second sentence of the first paragraph of the third subsection of Results (‘Deletion of Ptpn6 restores T cell development in Themis−/− mice’), the TCR chain is identified incorrectly as ‘CD3’; that phrase should read “...antibody to the TCR invariant chain CD3ε (anti-CD3ε)...” instead. In the legend to Figure 3, the P value (< 0.005) was incorrect; the correct value is P < 0.05. Also, in the final sentence of that legend, the directions ‘(left)’ and ‘(right)’ are incorrect; that should read “Data are representative of (top) or (bottom) four experiments...” instead. In Figure 4a, the numbers along the horizontal axes are incorrectly vertical; they should be horizontal instead. In Figure 4b, the labels along the vertical axes of the second and fourth plots incorrectly include ‘(%)’; the correct label is ‘CD8SP cells (×10^6)’ only. In the third sentence of the first paragraph of the fifth subsection of Results (‘p-SHP-1 does not correspond with PTP activity’), the word ‘of’ is missing; this should read “The lower abundance of p-SHP-1...” instead. In the legend to Figure 5c, the antibody is incorrectly set off in commas; that should read “...immunoprecipitated with anti-SHP-1 from...” instead. Finally, Figure 7e is too large and should be the same size as all other panels in that figure. The errors have been corrected in the print, PDF and HTML versions of this article.

**Erratum:** CCL19-CCR7–dependent reverse transendothelial migration of myeloid cells clears *Chlamydia muridarum* from the arterial intima

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In the version of this article initially published, the label along the horizontal axis of the graph in Figure 1a (‘Dose (mg)’) is incorrect. The correct label is ‘Dose (μg)’. The error has been corrected in the HTML and PDF versions of the article.