BRIEF DEFINITIVE REPORT

Bromodomain protein BRD4 directs and sustains CD8 T cell differentiation during infection

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In response to infection, pathogen-specific CD8 T cells differentiate into functionally diverse effector and memory T cell populations critical for resolving disease and providing durable immunity. Through small-molecule inhibition, RNAi studies, and induced genetic deletion, we reveal an essential role for the chromatin modifier and BET family member BRD4 in supporting the differentiation and maintenance of terminally fated effector CD8 T cells during infection. BRD4 bound diverse regulatory regions critical to effector T cell differentiation and controlled transcriptional activity of terminal effector–specific superenhancers in vivo. Consequentially, induced deletion of Brd4 or small molecule–mediated BET inhibition impaired maintenance of a terminal effector T cell phenotype. BRD4 was also required for terminal differentiation of CD8 T cells in the tumor microenvironment in murine models, which we show has implications for immunotherapies. Taken together, these data reveal an unappreciated requirement for BRD4 in coordinating activity of cis regulatory elements to control CD8 T cell fate and lineage stability.

Introduction

CD8 T cells are critical mediators of host defense against intracellular pathogens and malignancy (Chang et al., 2014). Upon recognition of cognate antigen, CD8 T cells become activated, rapidly expand, and differentiate into effector cells critical for resolution of disease. Following pathogen clearance, a relatively small population of antigen-specific effector cells persists and gives rise to memory T cells. It is apparent that the CD8 T cell response to infection is accompanied by extensive functional heterogeneity at both the effector and memory phases (Jameson and Masopust, 2018). Understanding the molecular signals controlling T cell differentiation provides insight for harnessing discrete T cell states for therapeutic strategies.

Differential expression levels of KLRG1 and CD127 delineate effector populations with distinct fates during acute infections (Chang et al., 2014; Joshi et al., 2007; Kaech et al., 2003). Nascent cytotoxic effector CD8 T cells rapidly lose expression of CD127—expressed by naive cells—and form a transitional population of CD127loKLRG1lo early effector cells (EECs) before upregulation of KLRG1 or CD127 (Diao and Pipkin, 2019). KLRG1 expression correlates with terminal differentiation, and CD127 marks cells with a greater degree of memory potential (Chang et al., 2014; Joshi et al., 2007). As such, KLRG1hiCD127lo cells are referred to as memory precursor T cells (MP cells) and KLRG1hiCD127lo cells as terminal effector T cells (TE cells). MP cells display enhanced multipotency compared with TE cells and more efficiently give rise to central memory T cells (Tcm cells), effector memory T cells (Tem cells), and tissue-resident memory T cells (Trm cells; Kaech et al., 2003; Mackay et al., 2013; Milner and Goldrath, 2018); however, select TE cells are able to persist for several months after infection, forming a terminally differentiated Tem cell population (t-Tem cells) or long-lived effector population (Kurd et al., 2020; Milner et al., 2020a; Milner et al., 2020b; Olson et al., 2013). Canonical transcription factors known to regulate antiviral T cell differentiation include Id3 (Ji et al., 2011; Yang et al., 2011), TCF1 (Zhou et al., 2010), Bcl6 (Ichii et al., 2002; Liu et al., 2019), STAT3 (Cui et al., 2011), and Foxo1 (Kim...
et al., 2013; Hess Michelini et al., 2013; Utzschneider et al., 2018) as critical regulators of MP/T<sub>CM</sub> cells, and Id2 (Cannarile et al., 2006; Knell et al., 2013; Masson et al., 2013), Zeb2 (Dominguez et al., 2015; Omilusik et al., 2015; Omilusik et al., 2018), Blimp1 (Kallies et al., 2009; Rutishauser et al., 2009), STAT4 (Mollo et al., 2014), and T-bet (Joshi et al., 2007) are required for more terminally differentiated populations.

Analogous to infection responses, tumor-infiltrating CD8 T lymphocytes (TILs) also exist in a range of cell states, wherein multipotent progenitor exhausted cells can be distinguished as PD-1<sup>Int/hi</sup>SlamF<sup>hi</sup>Tim<sup>3lo</sup> and express elevated levels of the multipotent progenitor exhausted cells can be distinguished as lymphocytes (TILs) also exist in a range of cell states, wherein (Kallies et al., 2009; Rutishauser et al., 2009), STAT4 (Mollo et al., 2014), and T-bet (Joshi et al., 2007) are required for more terminally differentiated populations.

In vivo loss-of-function screen reveals chromatin modifier BRD4 as a critical regulator of CD8 T cell differentiation during infection

Chromatin modifiers and transcription factors can differentially regulate the formation of divergent cell states without varying expression levels, often limiting our ability to predict how key regulators of gene expression control T cell lineage specification (Milner et al., 2017; Yu et al., 2017). We have previously used a pooled in vivo RNAi screening strategy (Chen et al., 2014) to identify functional regulators of T<sub>EM</sub> cell formation (Milner et al., 2017). Using a similar miRNA-based shRNA (shRNA-mir) library and approach (Chen et al., 2014; Milner et al., 2017), we screened 215 shRNA-mirs to identify functional regulators of early memory T cell differentiation during lymphocytic choriomeningitis virus (LCMV) infection (Fig. 1 A and Fig. S1 A). Several transcription factors known to be important for long-lived memory or T<sub>CM</sub> cells were confirmed in this screening approach, including Id3 (Ji et al., 2011; Yang et al., 2011), Klf2 (Bai et al., 2007; Hart et al., 2012; Preston et al., 2013), and Bcl6 (Ichii et al., 2002; Liu et al., 2019), whereas Id2 (Cannarile et al., 2006; Knell et al., 2013; Masson et al., 2013) and Runx3 (Wang et al., 2018) were required for formation of CD62L<sup>lo</sup> t-T<sub>EM</sub>/T<sub>EM</sub> cells relative to T<sub>CM</sub> cells (Fig. 1 A and Table S1). A notable hit from this loss-of-function screen included chromatin modifier BRD4. BRD4 shRNA-mirs were enriched in the CD62L<sup>lo</sup> population relative to CD62L<sup>hi</sup> cells, identifying BRD4 as a putative mediator of t-T<sub>EM</sub>/T<sub>EM</sub> cell differentiation. BRD4 has emerged as a key regulator of cellular differentiation in multiple contexts, including diverse cancer types (Donati et al., 2018; Filippakopoulos et al., 2010; Ren et al., 2018), hematopoietic stem cells (Dey et al., 2019), CD4 T cell populations (Bandukwala et al., 2012; Cheung et al., 2017a; Cheung et al., 2017b; Mele et al., 2013), and CD8 T cells in vitro (Chee et al., 2020; Georgiev et al., 2019; Kagoya et al., 2016), but the role of BRD4 in regulating T cell differentiation in vivo and in the context of infection remains unclear.

BRD4 is a member of the BET protein family, functioning in many cases as a chromatin reader that binds acetylated lysine residues in enhancer regions and establishes a molecular scaffold for controlled transcription of critical genes (Dey et al., 2019; Lee et al., 2017; Lovén et al., 2013). We first sought to validate BRD4 as a putative positive regulator of TE cell and/or early t-T<sub>EM</sub> cell formation as observed in the in vivo screen. Two distinct BRD4 shRNA-mirs mediated 70%-80% knockdown efficiency, confirming their on-target activity (Fig. S1 B). Congenically distinct CD8 T cells expressing a transgenic TCR recognizing the LCMV GP<sub>33-41</sub> epitope presented by MHCI (P14 cells) were transduced with a control retrovirus—encoding Cdi9 shRNA-mir—or a BRD4 shRNA-mir encoding retrovirus and transferred into recipient mice subsequently infected with LCMV. Consistent with the loss-of-function screen results, we found that BRD4 RNAi impaired the formation of CD4<sup>lo</sup>CD62L<sup>lo</sup> t-T<sub>EM</sub>/T<sub>EM</sub> cells, resulting in a greater frequency of T<sub>CM</sub> cells (Fig. 1 B). We also assessed how depletion of BRD4 impacted CD8 T cell differentiation over the course of LCMV infection and detected a reduced frequency of KLRG1<sup>hi</sup> P14 cells at all time points, as well as an increased frequency of CD127<sup>lo</sup>KLRG1<sup>lo</sup> EECs on day 5 of infection (Fig. 1 C). CX3CR1 expression levels on CD8 T cells are reflective of the degree of terminal differentiation (Böttcher et al., 2015; Gerlach et al., 2016; Milner et al., 2020a), and knockdown of Brd4 resulted in a reduced frequency of CX3CR1<sup>hi</sup> P14 cells (Fig. 1 D). Taken together, informed through a pooled in vivo screening approach, we identified an essential role for BRD4 in the formation of TE cells as well as the more terminally differentiated populations.
fated memory population (i.e., CD127loCD62Llo)t-TEM cells. Additionally, we previously detected BRD4 as a top candidate in a TRM cell differentiation screen (Milner et al., 2017), and consistent with these findings, Brd4 RNAi impaired the early formation of CD69hiCD103hi cells in the intestinal epithelium (Fig. S1 C).

We next generated mixed bone marrow chimeric mice comprised of a 1:1 mixture of congenically distinct Brd4fl/flErt2Cre/+ bone marrow and Brd4+/+ bone marrow to further evaluate a CD8 T cell–intrinsic role for BRD4 and validate shRNAi knockdown studies (Fig. 1 E). Reconstituted chimeric mice were infected with LCMV and tamoxifen was administered on days 5–7 of infection to induce depletion of Brd4. Representative flow cytometry plots indicating the ratio of Brd4fl/flErt2Cre/+ and control tetramer+ CD8 T cells (left, similar to E) and the ratio of Brd4fl/flErt2Cre/+ and control cells among all tetramer+ KLRG1hiCX3CR1hi and KLRG1loCX3CR1lo CD8 T cell populations (right). Graphs show mean ± SEM of n = 7–10 mice pooled from two or three independent experiments (B and C), n = 4 from one representative of two independent experiments at days 5 or 7 of infection (D), n = 15 pooled from three independent experiments (E and F) or n = 11 pooled from two independent experiments (G and H). *, P < 0.05; ***, P < 0.005. Symbols represent an individual mouse (B–H).

Figure 1. In vivo RNAi screen reveals BRD4 as a critical regulator of CD8 T cell differentiation during infection. (A) Relative enrichment of shRNAmirs in splenic CD62Lhi and CD62Llo cells from an in vivo RNAi screen, reported as the average Z-score from three independent screens where each independent screen was performed by pooling DNA from sorted P14 populations from 15–18 mice. (B–D) Congenically distinct P14 cells were transduced with Brd4 shRNA-encoding or control shRNA-encoding retroviruses and transferred into recipient mice subsequently infected with LCMV. Frequency of TCM, TEM, t-TEM memory T cell populations (B), frequency of CD127hiKLRG1hi expressing cells (C), or frequency of CX3CR1hi cells in response to LCMV infection (D). (E) Bone marrow chimera mice were generated by adoptive transfer of a 1:1 mixture of bone marrow cells from CD45.1 Brd4+/+ controls (Ctrl) and CD45.2 Brd4fl/flErt2Cre/+ mice (inducible Brd4 [iBrd4]) into irradiated mice (left). Reconstituted mice were infected with LCMV and treated with tamoxifen on days 5–7 of infection to induce deletion of Brd4. Representative flow cytometry plots (left) and quantification (right) of the ratio of Brd4fl/flErt2Cre/+ CD8 T cells and control cells before infection and 8 d after infection (F and G) Frequency of CD127 and KLRG1 (F) or CX3CR1 and KLRG1 expressing tetramer+ cells from E (G). (H) Representative flow cytometry plots indicating the ratio of Brd4fl/flErt2Cre/+ and control tetramer+ CD8 T cells (left, similar to E) and the ratio of Brd4fl/flErt2Cre/+ and control cells among all tetramer+ KLRG1hiCX3CR1hi and KLRG1loCX3CR1lo CD8 T cell populations (right). Graphs show mean ± SEM of n = 7–10 mice pooled from two or three independent experiments (B and C), n = 4 from one representative of two independent experiments at days 5 or 7 of infection (D), n = 15 pooled from three independent experiments (E and F) or n = 11 pooled from two independent experiments (G and H). *, P < 0.05; ***, P < 0.005. Symbols represent an individual mouse (B–H).
Loss of BRD4 also resulted in a dramatically reduced frequency of KLRG1hiCX3CR1hi cells and a greater frequency of KLRG1hiCX3CR1lo cells (Fig. 1 G). Despite no change in the overall mean ratio of total WT control and mutant GP33-41 specific cells on day 8 of infection (Fig. 1 E), we detected a greater abundance of KLRG1hiCX3CR1lo cells with loss of BRD4 and a decreased overall abundance of KLRG1hiCX3CR1hi cells (Fig. 1 H). Further highlighting the critical and dynamic role of BRD4 in CD8 T cell immunity, we found that deletion of Brd4 at early infection time points—days 1–5 of infection—not only impacts cellular differentiation (as in Fig. 1 F) but also affects the overall accumulation of antigen-specific CD8 T cells (Fig. S1, E and F). Therefore, BRD4 is a critical mediator of CD8 T cell differentiation during acute viral infection and is required for optimal formation of a terminally differentiated cell state.

In vivo BET inhibition impairs CD8 T cell differentiation during viral infection

BET proteins bear characteristic tandem bromodomains (BDs; BD1 and BD2) that bind acetylated lysine residues facilitating protein–protein interactions (Shi and Vakoc, 2014). Widely used BET inhibitors JQ1 and OTX-015 potently impair the activity of BET proteins, especially BRD4, through competitively binding bromodomains (Boi et al., 2015; Filippakopoulos et al., 2010). Additionally, the small molecule MS402 selectively targets BD1 of BRD4, preferentially inhibiting BRD4-induced expression of genes key to lineage specification (Cheung et al., 2017a). BET protein inhibitors represent promising therapeutic modalities for a number of disease states ranging from cancer to autoimmunity. In vitro treatment with BET inhibitors has been shown to modulate T cell activation (Chee et al., 2020; Georgiev et al., 2019; Kagoya et al., 2016); however, the in vivo effects of BET inhibition and small-molecule targeting of BRD4 in CD8 T cell differentiation during infection is not known.

Given the striking impact of BRD4 deficiency on TE cell differentiation, we tested if this phenotype could be recapitulated through BET inhibition in vivo. P14 cells were adoptively transferred to recipient mice subsequently infected with LCMV and treated with JQ1, MS402, OTX-015, or corresponding vehicle controls from days 1–4 of infection (Fig. 2 A). On day 5 of infection, we assessed the phenotype of donor P14 cells and found that all BET inhibitors impaired the early formation of KLRG1hi TE cells, resulting in a greater proportion of KLRG1lo EECs (Fig. 2 A), consistent with RNAi and genetic deletion studies. Failure to optimally generate KLRG1hi cells was also reflected by reduced frequencies of CX3CR1hi, CD43lo, and CD27lo cells, but an elevated frequency of CD62Lhi cells (Fig. 2 B). Additionally, JQ1 treatment impaired the early formation of intestinal CD69+CD103hi T BM cells (Fig. S1 G), consistent with RNAi studies (Fig. S1 C). As there are four members of the BET protein family (BRDT, BRD2, BRD3, and BRD4), it was unclear what degree the observed phenotype of BET inhibition was solely due to BRD4 inhibition. Only robust expression of Brd2 and Brd4 was detected in CD8 T cell populations during LCMV infection (Fig. S2 A), with minimal variation in expression between CD8 T cell subsets. To clarify the effect of BET inhibitors on CD8 T cell fate, we evaluated a regulatory role for BRD2 using two distinct Brd2 shRNAmirs that result in ~60% to 70% Brd2 knockdown efficiency (Fig. S2 B). Brd2 RNAi resulted in a subtle loss of KLRG1hi cells on day 5 (Fig. 2 C) and day 7 of infection (Fig. S2 C); therefore, we concluded that, while both Brd2 and Brd4 regulated aspects of CD8 T cell differentiation, loss of BRD4 activity likely conferred the strongest BET-mediated contribution to impaired differentiation of TE cells during LCMV infection. Furthermore, BET proteins are broadly expressed in a wide range of cell types, and thus, indirect effects of BET inhibition on other cell types may also contribute to the observed phenotype in virus-specific CD8 T cells.

To further evaluate the degree to which BET inhibition impairs BRD4 activity in CD8 T cells in vivo, we profiled the transcriptome of KLRG1hiCD127lo EEC P14 cells from JQ1- or vehicle-treated mice as well as shBrd4 or shCtrl P14 cells in a mixed transfer setting (Fig. 2 D). Splenic KLRG1hiCD127lo EECs were sorted for RNA sequencing analyses rather than bulk P14 cells to avoid confounding transcriptional changes caused by differing frequencies of TE cells, as well as to further understand the perceived impairment in the transition from an EEC state to a TE state in BRD4 knockout cells (Fig. 1 C and Fig. 2 A). We found that 89% of the transcripts downregulated by Brd4 RNAi were also suppressed by in vivo JQ1 treatment, and nearly 80% of transcripts upregulated by Brd4 RNAi were similarly upregulated by JQ1 treatment (Fig. 2 D). Gene set enrichment analysis (Fig. 2 D) further confirmed that JQ1-mediated BET inhibition and Brd4 knockdown exerted similar transcriptional changes in KLRG1hi EEC P14 cells at early times after LCMV infection. Validation of key differentially expressed genes revealed elevated TCF1 (encoded by Tcf7) and Eomes, reduced GzB production, but similar expression of T-bet, consistent with mRNA levels for these targets (Fig. 2, E and F). These data suggest that BET inhibition might represent a therapeutic opportunity for targeting BRD4 activity and modulating CD8 T cell differentiation in vivo.

BRD4 binds fate-specifying genes to coordinate effector CD8 T cell differentiation

Early inhibition of BRD4 activity with JQ1 or depletion of BRD4 through shRNAmir approaches limited the differentiation of terminally fated effector CD8 T cells, resulting in a greater frequency of the more multipotent EEC population (Fig. 2). Furthermore, induced Brd4 deletion resulted in a reduced abundance of terminally differentiated cells and enhanced accumulation of memory-like KLRG1hiCX3CR1lo tetramer+ cells (Fig. 1 H). These findings indicate that BRD4 is important for TE cell differentiation and may facilitate maintenance of the TE cell population. To further address this, we profiled the transcriptome of TE, EEC, and MP tetramer+ cells following induced deletion of Brd4 on days 5–7 of LCMV infection sorted from mixed bone marrow chimera mice (Fig. 3 A). Loss of Brd4 dramatically changed the gene-expression program in all three effector populations. Principle component analysis revealed that Brd4-deficient populations were transcriptionally distinct from control populations (Fig. 3 A); however, comparison of expression levels of TE-, EEC-, and MP-signature gene sets between control versus mutant subsets revealed that BRD4 was required for expression of lineage-specific genes in each cell type, as TE,
EEC, and MP signature transcripts were predominantly downregulated in each BRD4-deficient subset compared with the corresponding WT subset (Fig. 3 B). This finding was further exemplified through volcano plots (Fig. 3 C, top) and gene set enrichment analyses (Fig. 3 C, bottom), wherein BRD4-deficient effector T cells failed to upregulate $62\% - 82\%$ of the lineage-specific gene program. Notably, BRD4-deficient TE cells were enriched for MP and EEC gene expression signatures relative to control TE cells, demonstrating that induced loss of BRD4 resulted in enhanced expression of genes associated with less terminally fated T cell states (Fig. 3 D). Taken together, these data indicate that BRD4 was critical for promoting and/or
maintaining the identity of each of the specialized effector CD8 T cell populations (i.e., TE, EEC, and MP) during acute infection, especially TE cells (Fig. 3, C and D).

In diverse cell types, BET proteins coordinate expression of fate-determining molecules primarily through recruitment of transcriptional and chromatin-modifying complexes to regulatory enhancer regions (Cochran et al., 2019; Shi and Vakoc, 2014). Given that induced deletion of Brd4 resulted in an impairment in TE cell formation as well as a loss of TE cell transcriptional identity, we speculated that BRD4 binds to and promotes expression of key genes essential to effector T cell differentiation and the TE lineage. Genome-wide BRD4 binding was profiled in splenic P14 TE cells on day 8 of LCMV infection using chromatin immunoprecipitation sequencing (ChIP-seq).
Figure 4. **BRD4 enforces effector identity and regulates transcriptional activity of TE super-enhancers.** (A) Reconstituted mixed bone marrow chimeric mice were infected with LCMV and subsequently treated with tamoxifen daily on days 8–12 of infection (left). Frequency of tetramer* Brd4−/− KO and Brd4+/+
We detected BRD4 enrichment in gene bodies (comprising only ~1% to 2% of the genome), with 75.8% of BRD4 peaks located in promoter/transcription start sites, introns, untranslated regions, and transcription termination sites (Fig. 3 E). Examination of key loci bound by BRD4 revealed numerous molecules critical to effector CD8 T cell fate, function, and localization, and many of these relevant BRD4 targets exhibited failed upregulation with BRD4 deficiency (Fig. 3 F). Notable BRD4-sensitive targets included Id2, a transcriptional regulator essential for TE cell formation, and chemokine receptor Cx3cr1, which is markedly upregulated in TE cells (Fig. 3 F; Böttcher et al., 2015; Cannarile et al., 2006; Gerlach et al., 2016; Omilusik et al., 2018). BRD4 binding was observed at the Id2 and Cx3cr1 loci, including near promoter regions (Fig. 3 G). As a key function of BRD4 in other cell types is binding to enhancer regions to promote gene expression (Dey et al., 2019; Lee et al., 2017; Lovén et al., 2013), BRD4 binding patterns were contextualized with H3K27ac marks in TE cells (Yu et al., 2017). Indeed, we identified extensive overlap between BRD4 peaks and H3K27ac peaks (Fig. 3 G); however, the abundance of BRD4 at gene bodies suggests that an additional key role of BRD4 in CD8 T cells is direct transcription of genes in addition to regulation of transcriptional activity of enhancers. Certain genes were upregulated with loss of BRD4, and this could be attributed to failed upregulation of transcriptional repressors or alternative functions of BRD4 acting to repress select gene targets. In complementary experiments, later chemical inhibition of BRD4 on days 5-7 of infection also dramatically modified the gene expression program in TE, EEC, and MP cell populations compared with cells sorted from vehicle-treated mice (Fig. 3 H). TE cells sorted from mice treated with JQ1 exhibited impaired expression of characteristic TE cell transcripts, as determined through gene set enrichment analysis (Fig. 3 H), and resulted in rapid alterations in the phenotype of antigen-specific CD8 T cells (Fig. 3 I). Taken together, BRD4 binds to and promotes expression of genes central to CD8 T cell differentiation.

BRD4 enforces effector identity and regulates activity of TE super-enhancers

The robust occupancy of BRD4 at critical loci and the reduced expression of key TE cell-specific transcripts in BRD4-deficient cells foreshadowed an essential role for BRD4 in sequentially maintaining TE cell identity in addition to regulating differentiation. We sought to further clarify the role of BRD4 in supporting the maintenance of terminally differentiated effector cells following the peak of infection, after which the TE cell population is relatively stable (Chang et al., 2014). We induced Brd4 deletion in mixed bone marrow chimeric mice through tamoxifen administration on days 8-12 of infection (Fig. 4 A). Consistent with tamoxifen treatment on days 5-7 of infection, we detected no change in the overall accumulation of Brd4−/− tetramer+ cells on day 14 or later infection time points (Fig. 4 A); however, despite no change in the accumulation of antigen-specific CD8 cells, induced deletion of Brd4 resulted in a profound loss in the frequency of CD127+KLRG1hi and KLRG1hiCX3CR1hi cells and a greater frequency of CD127−KLRG1lo and KLRG1loCX3CR1lo cells (Fig. 4 B). Consistent with a role in supporting the maintenance and identity of terminally fated CD8 T cells, we found a reduced accumulation of KLRG1hiCX3CR1hi cells on day 25 of infection, with induced deletion of Brd4 and an enhanced abundance of multipotent KLRG1loCX3CR1lo cells (Fig. 4 C). These data indicate that induced deletion of Brd4 in TE cells resulted in a loss of TE identity, permitting transition to a KLRG1loCX3CR1lo memory-like phenotype; however, it is also possible that induced deletion of Brd4 results in the transition of EECs to KLRG1loCX3CR1lo cells.

We next assessed if delayed BET inhibition paralleled findings from delayed Brd4 deletion. On days 7-12 of LCMV infection, mice were treated with JQ1 or vehicle (Fig. 4 D). Consistent with delayed deletion experiments, we found that BET inhibition resulted in a reduced frequency of TE cells (Fig. 4 D). Delayed treatment of JQ1 or tamoxifen also resulted in reduced GzB expression and a greater frequency of TCF1hi cells (Fig. 4 E). Finally, to clarify the lineage-specific role of BRD4 in maintaining TE identity, we sorted TE P14 cells, transferred the congenically distinct TE cells into infection-matched recipient mice, and subsequently treated recipients with JQ1 or vehicle from day 8 through day 13 of infection. On day 14 of infection, we evaluated the terminal phenotype of donor cells and found that BET inhibition resulted in a rapid loss of KLRG1 and CX3CR1 expression compared with vehicle-treated mice (Fig. 4 F); therefore, BRD4 is critical in enforcing a terminally differentiated state in TE cells.

Assessment of genome-wide occupancy of BRD4 revealed robust binding in a multitude of lineage-specifying genes critical to the identity of TE cells (Fig. 3 F). In other cell types, super-enhancers (i.e., large clusters of conventional enhancers) are known to control cell identity and can be regulated and
interpreted by BRD4 (Dey et al., 2019; Lee et al., 2017; Lovén et al., 2013); however, the regulation of super-enhancer activity in CD8 T cells remains unexplored. We next sought to understand if BRD4 occupied and regulated super-enhancer regions, potentially explaining the critical role of BRD4 in enforcing TE identity. Similar to previous reports (He et al., 2016), we called 554 TE super-enhancers through the ROSE algorithm using H3K27ac ChIP-seq data of sorted TE cells (Yu et al., 2017), which was processed by the ENCODE ChIP-seq pipeline (Fig. 4 G). Genes nearest to identified super-enhancer regions were characteristically upregulated in TE cells compared with naive cells, supporting the biological relevance and overall robustness of the super-enhancer peak calling (Fig. 4 G, top). Next, we evaluated the extent to which BRD4 occupied these identified super-enhancer regions and found that, remarkably, 549 of 554 (>99%) super-enhancers overlapped with BRD4 binding (Fig. 4 G, bottom). We detected impaired expression of genes associated with BRD4-occupied super-enhancers in BRD4-deficient TE cells (Fig. 4 G, bottom right). Notable super-enhancer regions occupied by BRD4 with failed upregulation of the nearest genes in Brd4−/− TE cells included TE-associated genes Zeb2, Gzma, Kdm6b, as well as Bhlhe40, Gzmb, Klrgh1, and Cd5, highlighted in Fig. 4 H, implicating BRD4 as a robust regulator of the TE gene expression program.

**BRD4 regulates CD8 T cell differentiation in response to tumors**

Analogous to CD8 T cell subsets responding to infection, TILs display a range of multipotent to terminally fated states within the tumor microenvironment (Fig. S1 A; Kallies et al., 2020). Given that BRD4 was central to the initiation and maintenance of a terminally differentiated state during infection, we next assessed the role of BRD4 in supporting the differentiation of terminally exhausted cells, characteristically marked by elevated Tim3 expression (Kallies et al., 2020; Miller et al., 2019; Siddiqui et al., 2019), in a mouse model of melanoma. BRD4 was required for the formation of terminally exhausted cells in an antigen-specific CD8 T cell–intrinsic manner, evidenced by a reduced frequency of Tim3hi TILs with Brd4 knockdown using two distinct shRNAmirs (Fig. 5 A and Fig. S3 A). Consistent with these results, we also found that daily in vivo treatment with JQ1 impaired the formation of Tim3hi tumor-specific TILs (Fig. 5 B). Impaired generation of Tim3hi terminally fated TILs with Brd4 RNAi or JQ1 treatment emphasizes the central role of BRD4 in coordinating the expression of genes critical to terminal CD8 T cell differentiation (as in Fig. 3 F and Fig. 4 H), and this fate-specifying activity is further underscored by a BRD4-occupied super-enhancer region near Havcr2 encoding Tim3 (Fig. 5 C).

BET inhibition is an emerging treatment for diverse malignancies and is currently under investigation in >25 clinical trials (Khandekar and Tiriveedhi, 2020); however, the focus of BET inhibition in cancer therapies has been predominantly limited to direct effects on malignant cells (Boi et al., 2015; Delmore et al., 2011; Filippakopoulos et al., 2010; Khandekar and Tiriveedhi, 2020; Ott et al., 2012; Shi and Vakoc, 2014; Shu et al., 2016), whereas the impact of in vivo BET inhibition on tumor immunity (Hogg et al., 2017; Mao et al., 2019; Zhu et al., 2016), especially antitumor CD8 T cells, remains unclear. Given that Brd4 knockdown and JQ1 treatment constrained CD8 T cell differentiation during infection and within tumors, we tested if we could leverage these findings to modulate the efficacy of promising immunotherapy approaches, such as adoptive cell therapy and immune checkpoint blockade. We first tested how JQ1 treatment impacted the efficacy of adoptive cell therapy in a widely used and characteristically immunosuppressive B16 melanoma tumor model (Fig. 5 D; Chen et al., 2015; Juneja et al., 2017; Kleffel et al., 2015). Upon adoptive transfer of tumor-specific T cells into immunocompetent mice bearing established melanoma tumors, we found that tumor growth was delayed in vehicle-treated mice that received antitumor P14 cells compared with mice that did not receive P14 T cells, as expected (Fig. 5 D and Fig. S3 B); however, JQ1 treatment resulted in diminished efficacy of transferred tumor-specific P14 cells, ultimately yielding similar tumor growth rates to that of recipient mice without P14 cells (Fig. 5 D and Fig. S3 B). Further evaluation of the phenotype of adoptively transferred cells in JQ1-treated mice or P14 cells deficient for BRD4 revealed failed production of the essential cytolytic molecule GzB (Fig. 5 E), a direct target of BRD4 (Fig. 4 H) characteristically expressed by terminally exhausted cells (Miller et al., 2019; Milner et al., 2020b; Siddiqui et al., 2019). Furthermore, daily JQ1 treatment resulted in nearly 10-fold fewer P14 cells in the tumor microenvironment (Fig. S3 C). These findings highlight a scenario wherein BET inhibition of terminal CD8 T cell differentiation may be problematic in an adoptive cell therapy setting or perhaps in patients with a robust antitumor CD8 T cell response. These results can be partially explained by the finding that terminally exhausted cells produce more GzB and exhibit superior antitumor cytotoxicity compared with progenitor-exhausted cells (Miller et al., 2019), despite their short-lived and terminal phenotype. It is important to note that, although the phenotype of tumor-specific T cells in JQ1-treated mice is similar to the phenotype detected with cell-intrinsic Brd4 RNAi studies, BET inhibition may also indirectly impact CD8 T cell responses through modulating the activity of BET proteins in other cell types.

We speculated that the fairly aggressive regimen of daily 50 mg/kg JQ1 treatment in Fig. 5 D may excessively limit T cell differentiation and effector capacity, and we next evaluated a lower dose of 10 mg/kg JQ1. In vivo treatment with 10 mg/kg JQ1 resulted in a reduced frequency of Tim3hi tumor-specific cells (Fig. 5 F) and a greater frequency of TCF1hi cells (Fig. S3 D) compared with vehicle-treated mice, but not to the same extent as that for 50 mg/kg JQ1. Given that terminally differentiated CD8 T cells exhibit a fixed epigenetic state that limits longevity and responsiveness to immunotherapies, such as immune checkpoint blockade (Fig. S1 A; Pauken et al., 2016; Philip et al., 2017; Siddiqui et al., 2019), we next tested if a 10-mg/kg JQ1 treatment course may allow for enhanced efficacy of α-PD-1 therapy through the blunting of terminal differentiation of P14 cells in tumors. Here, we used an MC38 colon carcinoma model with known sensitivity to α-PD-1 therapy (Juneja et al., 2017). Indeed, we found a 10-mg/kg JQ1 treatment regimen enhanced the efficacy of α-PD-1, wherein only ~17% of vehicle-treated mice responded to α-PD-1 therapy compared with nearly 50%
of mice responding to α-PD-1 in combination with 10-mg/kg JQ1 treatment (Fig. 5 G). These data highlight that resolving the roles BET proteins and their degree of activity in antitumor CD8 T cells may yield insight into enhancing BET therapy efficacy and synergy, which is particularly relevant given that early findings from clinical studies of BET inhibitors as a cancer monotherapy leave room for improvement (Khandekar and Tiriveedhi, 2020).

This investigation provides insight into how complex chromatin dynamics accompanying CD8 T cell differentiation are interpreted and conveyed during infection. We established that BRD4 binds to gene bodies, traditional enhancers, and super-enhancers to regulate the expression of genes critical to effector differentiation and identity, including pro-effector transcription factors (Id2, Bhlhe40, Zeb2, and Runx1), effector molecules (Gzma, Gzmb, and Fasl), and canonical TE-surface molecules or mediators of cell trafficking (Klrg1, Cx3cr1, Itgam, Ccr5, and Ly6c2). Brd4 expression is relatively uniform among diverse CD8 T cell populations, highlighting the utility of our RNAi screening strategy and indicating that the fate-specifying activity of BRD4 is likely dictated by the cis regulatory landscape as CD8 T cells gain nearly twofold more de novo enhancer
regions during their transition from a naive cell to a TE cell state compared with an MP state (Yu et al., 2017). BRD4 activity may also be regulated by post-translational modifications (Shu et al., 2014; Jang et al., 2005; Yang et al., 2005). BRD4 is widely classified as a chromatin reader, but has diverse functions ranging from kinase activity (Devaiah et al., 2012) to binding nonhistone acetylated molecules, such as p65 (Huang et al., 2009). While it is possible that BRD4 controls T cell fate during infection and cancer through a range of actions, assessment of genome-wide binding patterns indicates that a primary function of BRD4 in CD8 T cells is regulating expression of key genes through supporting transcription of gene bodies directly and promoting activity of super-enhancers. Taken together, we identify BRD4 as a critical regulator of CD8 T cell differentiation and function during infection and cancer.

Materials and methods

Mice

All mice were bred and housed in specific pathogen-free conditions in accordance with the Institutional Animal Care and Use Guidelines of the University of California San Diego or the National Institute of Child Health and Human Development. P14 mice (with transgenic expression of H-2Db-restricted TCR specific for LCMV glycoprotein GP33-41, Brd4fl/flErt2Cre+/− (Dey et al., 2019), and CD45.1 and CD45.2 congenic mice were bred in-house. Male and female mice were used with sex-matched T cell donors and recipients or female cells were transferred into male recipients.

T cell transfers, bone marrow chimeras, infections, and treatments

For BET inhibition studies, naive P14 cells (5 × 10⁴) were transferred into congenically distinct recipient mice subsequently infected with 2 × 10⁶ PFU of LCMV by i.p. injection. The following BET inhibitors were used in this study: JQ1 (prepared by the laboratory of Jun Qi), OTX-015 (MedChemExpress), and MS402 (prepared by the laboratory of Ming-Ming Zhou). A concentrated stock of JQ1 in DMSO was prepared at 50 mg/ml and diluted 1:10 in 10% cyclodextrin, and mice received 50 mg/kg or 10 mg/kg via i.p. injection as indicated. A concentrated stock of OTX-015 in DMSO was prepared at 50 mg/ml and diluted in sunflower oil, and 50 mg/kg was administered via oral gavage. A concentrated stock of MS402 in DMSO was prepared at 20 mg/ml and diluted in 10% cyclodextrin, and 40 mg/kg was administered i.p. Administered vehicle control for all inhibitors was DMSO in 10% cyclodextrin and 40 mg/kg was administered via oral gavage. A concentrated stock of JQ1 in DMSO was prepared at 50 mg/ml and diluted in sunflower oil, and 50 mg/kg was administered via oral gavage. A concentrated stock of MS402 in DMSO was prepared at 20 mg/ml and diluted in 10% cyclodextrin, and 40 mg/kg was administered i.p. Administered vehicle control for all inhibitors was DMSO in 10% cyclodextrin (i.p injections) or sunflower oil (oral gavage treatments).

Mixed bone marrow chimera mice were established by transferring a 1:1 mix of Brd4fl/flErt2Cre+/−-bone marrow cells with congenically distinct WT Brd4fl/fl bone marrow cells into irradiated congenically distinct recipient mice. After reconstitution (~12 wk after transfer), chimeric mice were then infected with LCMV. For ER-Cre-mediated deletion of floxed alleles, 2 mg tamoxifen (Cayman Chemical Company) was administered daily by i.p. injection for 3–5 consecutive days at various time points after infection.

In vivo RNAi screen

The pooled in vivo shRNAmir screen was performed similarly as previously described (Milner et al., 2017). Briefly, P14 cells were activated in 96-well plates, individually transduced with 215 distinct shRNAmir encoding retroviruses, pooled, and 5 × 10⁴ total P14 cells were transferred into 15–18 recipient mice subsequently infected with LCMV. Twelve days after infection, CD62Lhi and CD62Llo splenic P14 cells were sorted and genomic DNA was harvested. Proviral passenger strand shRNAmir sequences were PCR amplified, and a minimum of 2.5 million reads per sample were generated and retained after filtering low-quality reads. shRNAmir representation in CD62Lhi cells relative to CD62Llo cells was calculated by normalizing the total number of reads in each of the samples, and then the number of reads for each shRNAmir was scaled proportionally. Subsequently, the normalized number of reads in the CD62Llo cells for a given shRNAmir was divided by the normalized number of reads for the same shRNAmir in the CD62Lhi cell sample and then log₂ transformed. Mean and SD of the ratios of 25 negative control shRNAmir constructs (targeting Cdh9, Cd4, Cd7, Ms4a1, Cd22, Hesi, Kif12, Mafq, Plag1, Pou2af1, and Smarca1) were used to calculate the Z-score for each shRNAmir. The in vivo screen was repeated three times and the Z-score of each construct from each individual screen was averaged. All constructs were screened two to three times (except for 13 constructs, which are marked by an asterisk in Table S1) as certain constructs were added after the first screen or were not detectable in one of the replicate experiments.

In vivo Brd2 and Brd4 RNAi during LCMV infection

For in vivo Brd2 and Brd4 RNAi experiments, two distinct shRNAmir clones for each gene were used in our previously described pLMPd-Amt vector (Chen et al., 2014; Wang et al., 2018), and retroviral supernatant was produced as described previously (Milner et al., 2017). For transfections, Plat-E cells were seeded in 10-cm dishes at a density of 2.5 × 10⁶ cells/plate 1 d before transfection in serum-free media. Transfections were performed with 100 µg plasmid DNA from each pLMPd-Amt clone and 50 µg pCL-Eco with TransIT-LTI (Mirus). Retroviral supernatant was harvested 48 h and 72 h after transfection. For transductions, negatively enriched naïve CD8 T cells from spleen and lymph nodes were activated in 6-well plates coated with 100 µg/ml goat anti-hamster IgG (H+L; Thermo Fisher Scientific), 1 µg/ml anti-CD3 (145-2C11; eBioscience), and 8 µg/ml anti-CD28 (37.51; eBioscience). T cell culture media was removed 18 h after activation and replaced with retroviral supernatant supplemented with 50 µM β-mercaptoethanol (Gibco) and 8 µg/ml polybrene (Millipore) followed by a 1 h spinfection centrifugation at 2,000 rpm and 37°C. One day after transduction, congenically distinct ametrine+ T cells were mixed 1:1 and 5 × 10⁵ total cells were transferred into recipient mice subsequently infected with LCMV.

Tumor models

Tumors were established by transplanting 5 × 10⁶ B16-GP33-41 cells or 2.5–5 × 10⁵ MC38-GP33-41 cells subcutaneously. Cell lines were treated for mycoplasma contamination and authenticated in
Antibodies, intracellular staining, flow cytometry, and cell sorting

Single-cell suspensions were prepared from spleen or lymph node by mechanical disruption, and intestinal tissue and tumor were processed as described previously (Milner et al., 2017). RBCs were lysed with ACK buffer (140 mM NH₄Cl and 17 mM Tris-base, pH 7.4). The following antibodies were used for surface staining (all from eBioscience unless otherwise specified): CD8 (53–6.7), CD27 (LG–7F9), CD43 (eBioR2.60), CD44 (IM7), CD45.1 (A20–1.7), CD45.2 (104), CD69 (H1.2F3; BioLegend), CD103 (2E7), Tim3 (RMT3–23), CD127 (A7R34), CD62L (MEL–14), KLRG1 (2F1), PD–1 (43F), and CX3CR1 (SA011F11; BioLegend); or intracellular staining: GzB (GB12; Invitrogen), Eomes (Dan11-mag), TCF1 (C63D9; Cell Signaling Technology), T-bet (4B10), KLRG1 (2F1), Tim3 (RMT3-23), CD127 (A7R34), CD62L (MEL–14), CD103 (2E7), and CD19 (1D3). Intracellular staining was performed on BD FACSAria or BD FACSAria II sorter equipped with a 488 nm laser and a 561 nm laser. Fixation, permeabilization, and staining were performed as described (eBioscience). Cells were acquired on a BD LSRFortessa X-20 or a BD LSRFortessa. Data were acquired at least 100,000 cells for each sample.

Quantitative PCR and RNA sequencing

For validation of Brd2 and Brd4 knockdown with shRNAmir constructs, enriched CD8+ T cells were activated, transduced, and expanded for 4–6 d in 100 mg/ml IL-2. Ametrine+ cells (Brd2 shRNAmir, Brd4 shRNAmir, or control Cd19 shRNAmir) were sorted directly into TRIzol (Life Technologies) and RNA was extracted per manufacturer specifications. cDNA was then synthesized using Superscript II (Life Technologies) and quantitative PCR was performed using the Stratagene Brilliant II Syber Green master mix (Agilent Technologies). Brd2 and Brd4 expression levels were normalized to the housekeeping genes Hprt1 or Gapdh. The following primers were used for quantitative PCR: Brd2 forward: 5’-GCTGACCGCGCCGGGTCC-3’, and Brd2 reverse: 5’-GTAAAG CTGGTACAGAAGCC-3’; Brd4 forward: 5’-TTCCAGCACCCTACCTCCA CC-3’, and Brd4 reverse: 5’-CTCGTG TTTTGGCTCCTGC-3’; Hprt forward: 5’-GGCCAGACTTTTGTTTGATT-3’, and Gapdh forward: 5’-CAGATGAAGCT CACTCAG-3’, and Gapdh reverse: 5’-GACTCCAGACATACAGC-3’.

For RNA sequencing studies, 10⁵ P14 cells were sorted on day 5 of infection or tetramer+ cells were sorted on day 8 of infection. RNA sequencing was performed in duplicate or triplicate. RNA sequencing was performed in duplicate or triplicate wherein day 5 Q1/shBrd4 or shCtrl P14 replicates or day 8 tetramer+ replicates were sorted from cells pooled from two mice (i.e., each replicate was composed of cells from two separate mice). For all samples, polyA+ RNA was isolated and RNA sequencing library preparation and RNA sequencing analysis were performed as described (https://www.immgen.org/Protocols/11Cells.pdf). Heatmaps were generated using Morpheus (https://software.broadinstitute.org/morpheus).

Gene set enrichment analysis was performed with gene set enrichment analysis v4.0.3: Number of permutations = 1,000, permutation type = gene_set, enrichment statistic = weighted, metric for ranking genes = Signal2Noise. MP, TE, and EEC signature gene sets were generated by identifying genes differentially expressed between each subset in WT tetramer+ cells on day 8 of infection (1.5-fold change, expression threshold ≥ 10).

BRD4 ChIP-seq

For ChIP-seq studies, 10⁵ naive P14 cells were transferred to recipient mice subsequently infected i.p. with 2 × 10⁸ PFU of the Armstrong strain of LCMV 1 d after cell transfer. Negatively enriched splenocytes were sorted for TE cells (KLRG1+CD127–) on day 8 after infection. Duplicate samples were prepared for subsequent ChIP-seq analyses, wherein each replicate consisted of 1.5 × 10⁸ TE cells pooled from five mice. Cells were then fixed by adding 1/10 volume of 37% formaldehyde (αF–8779; Sigma–Aldrich) for 10 min. Fixation was then stopped by adding 1/20 volume of 2.5 M glycine for 5 min. Cells were washed with chilled 0.5% Igepal/PBS and 1 mM PMSF, and then cell pellets were flash frozen and shipped to Active Motif for anti-BRD4 (Bethyl A301-985A100) ChIP-seq. For BRD4 ChIP reactions, 25 μg chromatin and 6 μl antibody were used. Fastq files for H3K27ac ChIP-seq in TE OT-1 cells were downloaded from the Gene Expression Omnibus (accession no. GSE89036). The Fastq files for BRD4 from Active Motif and for H3K27ac ChIP-seq were analyzed through the Chip-seq pipeline from Encode (https://www.encodeproject.org/pipelines/), and the reproducible peak sets along with the bigwig tracks of fold change over background were used. The ROSE algorithm (Lovén et al., 2013; Whyte et al., 2013) was used to identify super-enhancers from the H3K27ac peak set. Homer was used to annotate the peak sets and compare BRD4 binding and super-enhancer locations (http://homer.ucsd.edu/homer/).

Statistics

Statistical analysis was performed using GraphPad Prism software. Two-tailed paired or unpaired t test was used for comparisons between two groups. P values of <0.05 were considered significant. Log-rank (Mantel–Cox) test was used to compare survival curves.
Online supplemental material
Fig. S1 provides supporting information on the efficacy of Brd4 RNAi and inducible flexed models, as well as further phenotyping of Brd4-deficient CD8 T cells. Fig. S2 illustrates expression patterns of BET proteins in CD8 T cell populations during LCMV infection and provides information on the phenotype of Brd2-deficient CD8 T cells. Fig. S3 lists details on the phenotype of Brd4-deficient T cells in mouse melanoma tumors. Table S1 shows summary Z-scores from the in vivo RNAi screening approach.

Data availability
All RNA sequencing and ChIP-seq datasets have been deposited in the Gene Expression Omnibus under accession no. GSE173515 (and subseries GSE173510, GSE173511, GSE173512, and GSE173513).

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Fig. 1 A schematic was generated with Biorender.com.
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Figure S1. BRD4 regulates CD8 T cell differentiation during infection. (A) Model of effector and memory T cell populations during infection and their corresponding characteristics (top). Spectrum of CD8 T cell states during infection and cancer (bottom). (B) Gene expression analysis of Brd4 in sorted CD8 T cells transduced with shBrd4- or shCtrl-encoding retroviruses after in vitro culturing for 4–5 d. (C) Congenically distinct P14 cells were transduced with Brd4 shRNA–encoding or control shRNA–encoding retroviruses and transferred into recipient mice that were subsequently infected with LCMV. The frequency of CD103- and CD69-expressing P14 cells in the epithelium of the small intestine on day 7 of infection was evaluated. (D) RNA sequencing expression levels of the floxed Brd4 region based on the experimental schematic in Fig. 3 A. (E) Bone marrow chimera mice were generated by adoptive transfer of 1:1 mixed bone marrow cells from CD45.1 Brd4+/+ control mice (Ctrl) and CD45.2 Brd4fl/flErt2Cre/+ mice (inducible Brd4 [iBrd4]) into irradiated mice (left). Reconstituted mice were infected with LCMV and treated with tamoxifen on days 1–5 of infection to induce deletion of Brd4. Representative flow cytometry plots (right) and quantification (bottom left) of the ratio of Brd4fl/flErt2Cre/+ and control cells before infection and 8 d after infection. (F) Frequency of CD127- and KLRG1-expressing tetramer+ cells from D. (G) Naive P14 CD8 T cells were transferred into congenically distinct recipient mice that were subsequently infected with LCMV. Infected mice were treated daily with 50 mg/kg JQ1 or vehicle (left). Frequency of CD103- and CD69-expressing P14 cells in the epithelium of the small intestine on day 7 of infection (right). Graphs show mean ± SEM of n = 5–8 mice pooled from two independent experiments (C–E); n = 4 from one representative of two independent experiments (F); or data pooled from three independent experiments (B). *, P < 0.05; ***, P < 0.005. Symbols represent an individual mouse (C–F). IEL, intraepithelial lymphocyte.
Table S1 is provided online and shows summary Z-scores from in vivo RNAi screening approach.