Antigen-specific clonal expansion and cytolytic effector function of CD8+ T lymphocytes depend on the transcription factor Bcl11b

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CD8+ T lymphocytes mediate the immune response to viruses, intracellular bacteria, protozoan parasites, and tumors. We provide evidence that the transcription factor Bcl11b/Ctip2 controls hallmark features of CD8+ T cell immunity, specifically antigen (Ag)-dependent clonal expansion and cytolytic activity. The reduced clonal expansion in the absence of Bcl11b was caused by altered proliferation during the expansion phase, with survival remaining unaffected. Two genes with critical roles in TCR signaling were deregulated in Bcl11b-deficient CD8+ T cells, CD8 coreceptor and Plcγ1, both of which may contribute to the impaired responsiveness. Bcl11b was found to bind the E8I, E8IV, and E8V, but not E8II or E8III, enhancers. Thus, Bcl11b is one of the transcription factors implicated in the maintenance of optimal CD8 coreceptor expression in peripheral CD8+ T cells through association with specific enhancers. Short-lived Klrg1^{hi}CD127^{lo} effector CD8+ T cells were formed during the course of infection in the absence of Bcl11b, albeit in smaller numbers, and their Ag-specific cytolytic activity on a per-cell basis was altered, which was associated with reduced granzyme B and perforin.

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Abbreviations used: Ag, antigen; ChIP, chromatin immunoprecipitation; DP, double positive; EIU, egg infectious unit; Lm-Ova, *Listeria monocytogenes* expressing Ova; MFI, mean fluorescence intensity; PA, acidic polymerase; TSS, transcription start site.

CD8+ T cells are critical players of the immune response to viruses, intracellular bacteria, protozoan parasites, and tumor cells. Upon recognition of antigenic peptide–MHC-I complexes, CD8+ T cells are activated, undergo a profound clonal expansion and differentiate into effector CTLs with the ability to lyse a broad range of infected cells, and produce specific cytokines (van Stipdonk et al., 2001; Kaech et al., 2002). CTLs kill through granule exocytosis mediated by the membrane drilling protein perforin and the release of granzymes (Voskoboinik et al., 2006). After the expansion phase, the majority of effector cells die, leaving behind a small population of memory cells able to respond very efficiently by undergoing rapid proliferation and acquisition of effector function. The differentiation of naive CD8+ T cells into CTLs, the execution of the effector program, and the generation of the memory CD8+ T cells rely on several transcription factors. Elevated levels of the transcription factor T-bet are essential for the generation of the short-lived Klrg1^{hi} IL-7Rα^{lo} effector CD8+ T cells (Joshi et al., 2007). The related transcription factor Eomes plays a critical role in the expression of the effector molecules perforin, granzyme B and IFN-γ, whereas T-bet seems to have a more redundant function in this regard (Szabo et al., 2002; Pearce et al., 2003; Intlekofer et al., 2005). Eomes plays a complex role in the generation of memory CD8+ T cells, including the control of IL-15R expression (Intlekofer et al., 2005; Takemoto et al., 2006). Runx3 is another transcription factor that regulates the expression of perforin,

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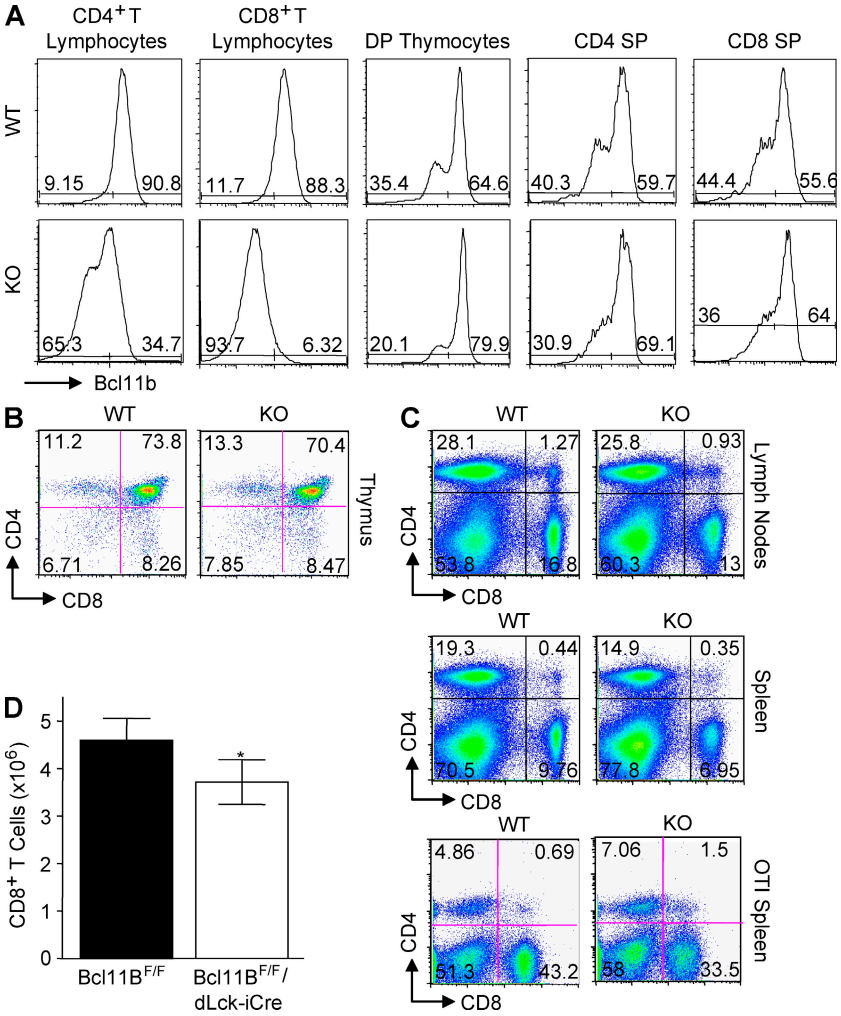

Figure 1. Removal of Bcl11b in mature T cells with the dLck-iCre deleter. (A) Flow cytometry analysis of Bcl11b in gated CD4+ and CD8+ T lymphocytes from spleen, and in CD4 and CD8 SP and DP thymocytes of Bcl11b$^{F/F}$/dLck-iCre (KO) and control mice (WT). Single cell suspensions of spleens and thymi were stained for CD4, CD8, and Bcl11b. This is a representative experiment out of three, with three pairs of mice. (B) FACS analysis of thymocytes of Bcl11b$^{F/F}$/dLck-iCre (KO) and control mice (WT). (C) FACS analysis of T cells from spleens and lymph nodes of Bcl11b$^{F/F}$/dLck-iCre (KO) and control mice (WT) and spleens from Bcl11b$^{F/F}$/dLck-iCre/OT-1 and OT-1 control mice. (D) Total cellularity of CD8+ T cells. Absolute numbers were derived based on the percentage of CD8+ T cells in total live cells. A two-tailed Student's *t* test was applied to determine statistical significance. P = 0.0133. In B–D, data are derived from 4–10 pairs of mice. Error bars indicate SD.

granzyme B, and IFN-γ (Chen et al., 2008; Cruz-Guilloty et al., 2009). Finally, Bcl6 and the related Bcl6b control the generation and maintenance of memory cells (Ichii et al., 2002; Manders et al., 2005), whereas Id2 is required for memory CD8+ T cells survival (Cannarile et al., 2006).

Bcl11b is a C_2H_2 zinc finger transcription factor that we initially identified as a mediator of the transcriptional repression function of the orphan nuclear receptors of the COUP-TF family (Avram et al., 2000). Bcl11b directly binds GC-rich response elements to mediate transcriptional repression of reporters driven by such elements (Avram et al., 2002; Cismasiu et al., 2005) and is a general transcriptional repressor of the HIV-I LTR in CD4+ T cells (Cismasiu et al., 2008). Bcl11b also participates in the transcriptional activation of specific target genes in the immune system, such as IL-2 and Cot kinase (Cismasiu et al., 2006; Cismasiu et al., 2009), findings which demonstrate its complex role in the regulation of gene expression in immune system. Conditional removal of Bcl11b at the double-positive (DP) stage of T cell development shows that this transcription factor is critical for positive selection of both CD4 and CD8 SP thymocytes and for survival of DP thymocytes, which is, in part, independent of Bcl2/Bcl-xl (Albu et al., 2007). Additionally, germ line removal of Bcl11b blocks β selection of thymocytes in relation with altered rearrangements at the TCR-β locus (Wakabayashi et al., 2003) and affects the nervous system development, namely, specification of the corticospinal motor neurons (Arlotta et al., 2005) and subcortically projecting axons (Chen et al., 2008).

Bcl11b is also expressed in mature T lymphocytes; however, its role in these cells has not been addressed because of the block at the DP stage of thymocyte development upon its removal with the CD4-Cre deleter (Albu et al., 2007). In this study, we conditionally removed Bcl11b in mature T cells, and its absence resulted in reduced antigen (Ag)-specific clonal expansion of CD8+ T cells during infection and in vitro. Furthermore, although the short-lived Klrg1^{hi}IL7Rα^{lo} effector CD8+ T cells were formed in the absence of Bcl11b, the execution of the effector program by CTLs, determined by their ability to kill Ag-specific targets and produce perforin and granzyme B, was altered. In addition, we found that Bcl11b controls Ag-specific clonal expansion of CD8+ T cells during infection by regulating maintenance of CD8 coreceptor expression in the periphery, through association with E8I, E8IV, and E8V enhancers and by controlling expression of Plcγ1. These results demonstrate, for the first time, that Bcl11b is a critical transcription factor for CD8+ T cell immunity by regulating genes of major importance for CD8+ T cells.

RESULTS

Removal of BCL11B in peripheral CD8+ T lymphocytes with the use of the dLck-iCre deleter

Bcl11b is expressed in mature CD4+ and CD8+ T cells (Fig. 1) in addition to thymocytes (Albu et al., 2007). Its removal

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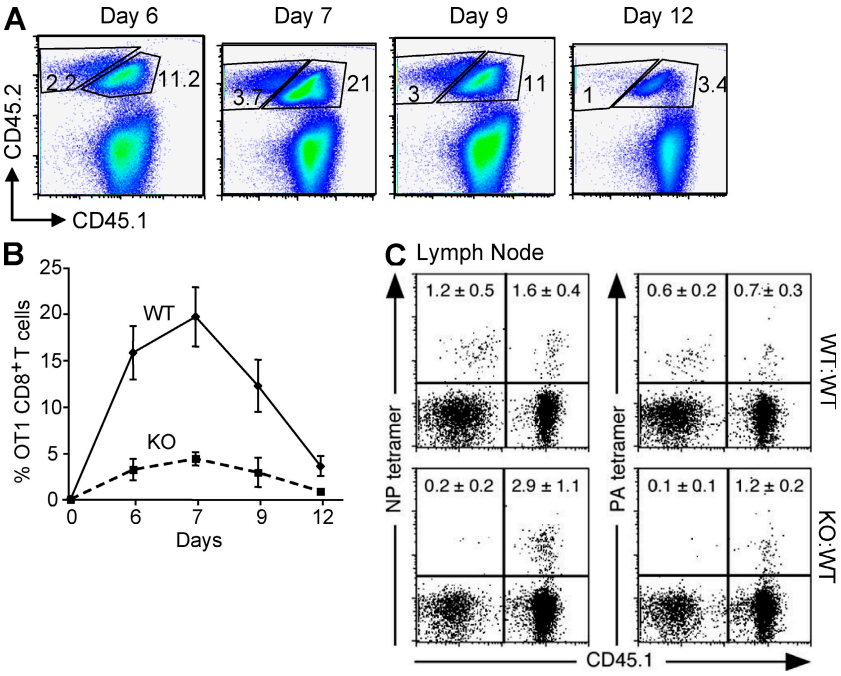

Figure 2. Bcl11b-deficient CD8+ T cells have a defect in expansion during the immune response to Lm-Ova and influenza infection. (A) Expansion of Bcl11b-deficient and WT OT-1 CD8+ T cells in spleen during the course of infection with LM-Ova. Naive CD62L^{hi}CD44^{lo} CD8+ T lymphocytes from CD45.2 Bcl11b$^{F/F}$/dLck-iCre/OT-1 and CD45.1/2/OT-1 mice (1:1; total 10^4) were cotransferred in CD45.1 recipient mice. The next day after transfer, mice were infected with 1.5×10^4 CFU Lm-Ova, and the immune response was monitored by evaluating the frequency of CD45.2 (Bcl11b deficient) and CD45.1/2 (WT) CD8+ T cells on days 6, 7, 9, and 12 after infection. This is representative data from four independent experiments in which cells were transferred from three mice from each group into three to four recipients. (B) Graph depicting the expansion of Bcl11b-deficient and WT OT-1 CD8+ T cells in spleen at the indicated time points after Lm-Ova infection, as described in A. Data are expressed as mean ± SD of the percentage of donor CD8+ T lymphocytes and a mean of four independent experiments. (C) Influenza-specific Bcl11b-deficient and WT CD8+ T cells in the mediastinal lymph nodes on day 10 after influenza infection. Mixed bone marrow chimeras were generated by reconstitution of lethally irradiated CD45.1 with either a 1:1 mix of CD45.1 and CD45.2 bone marrows (WT/WT chimeras) or a 1:1 mix of CD45.1 and Bcl11b$^{F/F}$/dLck-iCre bone marrows (WT/KO chimeras). After reconstitution, mice were infected with 1,000 EIU PR8. The frequencies of NP-specific and PA-specific CD8+ T cells derived from each donor type were determined by flow cytometry. The numbers in each plot refer to the mean percentage ± SD of CD8+ T cells that are either NP- or PA-specific. Data are representative for six pairs of donor mice, in which there were five mice reconstituted from each pair.

with the CD4-Cre deleter caused a block in positive selection with marked reduction of T cells in the periphery, which presented developmental abnormalities (Albu et al., 2007), therefore impeding the study of Bcl11b role in mature T cells. To circumvent this problem, we crossed Bcl11b$^{F/F}$ mice with the dLck (Lck distal) promoter–iCre mice, taking into account that the dLck promoter is active in mature T cells (Wildin et al., 1995; Zhang et al., 2005). We observed efficient removal of the floxed Bcl11b allele in CD8+ T cells, whereas the removal in CD4+ T lymphocytes was less efficient (Fig. S1), which is consistent with previous observations showing higher activity of the dLck-iCre in CD8+ T cells (Zhang et al., 2005; D'Souza et al., 2008). Quantitative evaluation by flow cytometry indicated that the floxed allele was removed in ~90% of the CD8+ T cells (Fig. 1 A), which is similar to previous results for other genes (Zhang et al., 2005; D'Souza et al., 2008). Bcl11b was still present in DP thymocytes, as well as in CD4 and CD8 SP thymocytes of Bcl11b$^{F/F}$/dLck-iCre mice (Fig. 1 A and Fig. S1).

CD8 and CD4 SP thymocytes were present in Bcl11b$^{F/F}$/dLck-iCre mice in similar percentages to those of WT (Fig. 1 B), and the TCR-β levels were similar to those of the WT cells, indicating that these cells reached maturity (Fig. S2). There was a modest but significant reduction in the percentage and absolute numbers of CD8+ T cells in the lymph nodes and spleens of Bcl11b$^{F/F}$/dLck-iCre mice, whereas the reduction in the CD4+ T cell numbers was less remarkable (Fig. 1, C and D; and not depicted).

Bcl11b-deficient CD8+ T cells have poor Ag-specific clonal expansion in vivo

The efficient removal of BCL11B in the CD8+ T lymphocytes of Bcl11b$^{F/F}$/dLck-iCre mice, and the fact that these mice still have substantial numbers of CD8+ T cells in the periphery which are not affected by developmental defects, makes Bcl11b$^{F/F}$/dLck-iCre mice an appropriate model to study the CD8+ T cell immune response in the absence of Bcl11b. For this purpose, we crossed the Bcl11b$^{F/F}$/dLck-iCre mice onto an OT-1 background (Fig. 1 C), in which the majority of CD8+ T cells express a TCR (Vα2Vβ5) which recognizes the OVA peptide residues 257–264 presented in the context of H-2K(b) (Hogquist et al., 1994). We further used an adoptive transfer system and infection with *Listeria monocytogenes* expressing Ova (Lm-Ova; Shen et al., 1995) to evaluate the immune response. Specifically, we cotransferred equal numbers of naive CD62L^{hi}CD44^{lo} CD45.2/OT-1 Bcl11b-deficient and CD62L^{hi}CD44^{lo} CD45.1/2/OT-1 WT CD8+ T cells in CD45.1 recipient mice. After the transfer, we infected the recipient mice with Lm-Ova and evaluated the expansion of Bcl11b-deficient and WT CD8+ T cells, based on the CD45.1 and CD45.2 allelic differences. We found that CD45.1/2 WT CD8+ T cells expanded significantly, reaching a peak at day 7 of infection, and then declined in number during the contraction phase (Fig. 2, A and B). The peak of expansion for Bcl11b-deficient CD8+ T cells was also at day 7; however, the magnitude of the expansion

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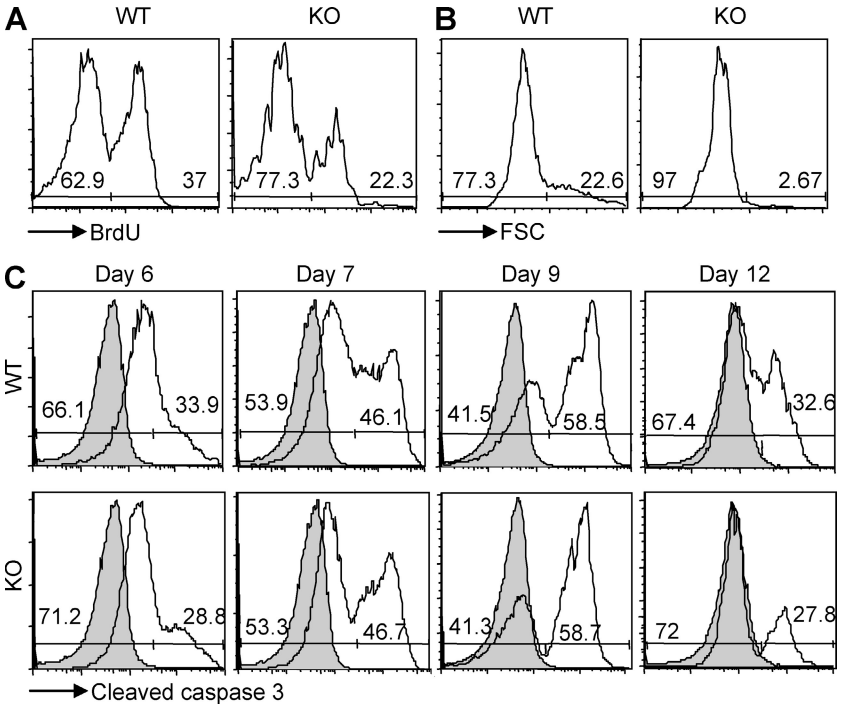

Figure 3. Proliferation and survival of Bcl11b-deficient OT-1 CD8+ T cells in vivo after Ag-specific activation. (A) Proliferation at day 6 after infection was evaluated by BrdU incorporation 16 h after the i.p. injection of BrdU in mice cotransferred with KO and WT cells and then infected with Lm-Ova, as described in Fig. 2. BrdU incorporation was evaluated within the donor Bcl11b-deficient CD45.2 and WT CD45.1/2 CD8+ T cell populations. (B) Forward scatter of the CD8+ T cells was evaluated in the same conditions as in A. (C) Survival of Bcl11b-deficient and WT CD8+ T cells during the course of infection with Lm-Ova. Cleaved caspase-3 was evaluated within the donor Bcl11b-deficient CD45.2 and WT CD45.1/2 CD8+ T cell populations at the indicated time points. Bcl11b-deficient CD45.2 and WT CD45.1/2 CD8+ T cells were transferred in CD45.1 recipient mice further infected with Lm-Ova, as described in Fig. 2. Histograms show levels of cleaved caspase-3 on gated CD45.2 (Bcl11b-deficient) or CD45.1/2 (WT) CD8+ T cell populations versus isotype control (filled histogram). Numbers indicate the percentage of cells within the gated populations. Levels of cleaved caspase-3 in recipient CD8+ T cells are indicated in Fig. S3 as an internal control. Data in A–C are representative of three independent experiments.

was dramatically reduced (Fig. 2, A and B). In addition, the numbers of CD45.2 Bcl11b-deficient CD8+ T cells remained lower compared with the numbers of WT cells during the contraction phase of the immune response (Fig. 2, A and B). Similar results were observed when knockout and WT OT-1 CD8+ T cells were transferred separately in recipient mice (unpublished data), suggesting that the defect in expansion was not a result of the competition by WT cells.

To determine whether similar defects in expansion were also observed in a polyclonal CD8+ T cell response, we next investigated the ability of Bcl11b-deficient CD8+ T cells to expand in response to infection with influenza virus. In these experiments, irradiated CD45.1 recipient mice were reconstituted with Bcl11b$^{F/F}$/dLck-iCre (CD45.2) and WT (CD45.1/2) bone marrow cells, and the mixed chimeras were infected with a sublethal dose (1,000 egg infectious units [EIU]) of A/PR8/34 influenza 8 wk after reconstitution. The frequency of influenza nucleoprotein NP-specific, as well as influenza acidic polymerase (PA)–specific, CD8+ T cells was evaluated in mediastinal lymph nodes. The results show that the frequency of NP-specific or PA-specific Bcl11b-deficient CD8+ T cells was >10-fold decreased compared with WT (Fig. 2 C). These results show that even on a polyclonal background, Bcl11b-deficient CD8+ T cells have a poor expansion in response to infection, which is similar to the results obtained with the OT-1/Lm-Ova model.

Bcl11b-deficient CD8+ T cells have reduced Ag-specific proliferation, but they do not die more during the expansion or contraction phases of the immune response

To further investigate the cause of the reduced expansion of the Bcl11b-deficient CD8+ T cell, we first evaluated the incorporation of BrdU in transferred OT-1 cells on day 6 after the Lm-Ova infection to determine whether their Ag-specific proliferation was altered. We found that the percentage of CD8+ T cells that had incorporated BrdU was reduced in Bcl11b-deficient OT-1 CD8+ T cells compared with the WT OT-1 cells (Fig. 3 A). In addition, we evaluated the forward scatter of the CD8+ T cells as a measurement of T cell size, which increases after activation and before division (Grumont et al., 2004). Our results clearly indicate that the population with high forward scatter was reduced in the Bcl11b-deficient CD8+ T cells compared with WT CD8+ T cells (Fig. 3 B), which is consistent with fewer proliferating cells in this population.

To determine whether the reduction in the CD8+ T cell numbers during the course of infection was caused by enhanced cell death, we evaluated the apoptosis of Bcl11b-deficient and WT CD8+ T cells by measuring the levels of cleaved caspase-3. We found that a similar percentage of Bcl11b-deficient CD8+ T cells stained positively for cleaved caspase-3 compared with WT on days 6, 7, 9, or 12 after infection (Fig. 3 C and Fig. S3), demonstrating that the reduction in the number of Bcl11b-deficient CD8+ T cells during the course of infection is not a result of increased cell death.

Reduced proliferation of Bcl11b-deficient CD8+ T cells is caused by multiple alterations in TCR signaling

To directly measure the proliferative response of the OT-1 CD8+ T cells to antigenic stimulation, we labeled Bcl11b-deficient and WT naive OT-1 CD8+ T cells with CFSE, incubated them with APCs loaded with OVA$_{257-264}$ peptide, and monitored their proliferation by dilution of CFSE. Bcl11b-deficient CD8+ T cells showed decreased proliferation

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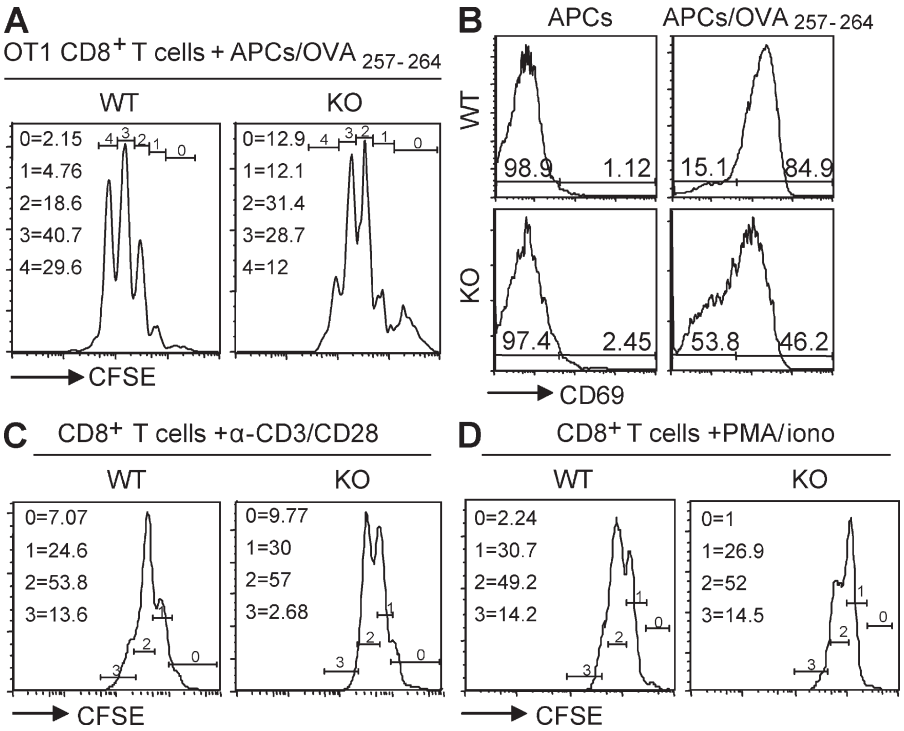

Figure 4. In vitro proliferation of Bcl11b-deficient and WT CD8+ T cells in response to TCR stimulation. (A) Bcl11b-deficient and WT naive OT-1 CD8+ T cells were labeled with CFSE and incubated for 3 d with 0.1 µM $OVA_{257-264}$ peptide-loaded APCs. Numbers of cell divisions of WT or knockout cells were measured by CFSE dilution. The frequency of cells in each CFSE dilution peak is given. Data are representative of three independent experiments. (B) Response of Bcl11b-deficient and WT naive OT-1 CD8+ T lymphocytes to TCR stimulation. Bcl11b-deficient and WT naive OT-1 CD8+ T cells were cultured with $OVA_{257-264}$ peptide-pulsed APCs for 5 h. Cells were then stained for CD8 and CD69 and analyzed by flow cytometry. Histograms represent surface levels of CD69 within the gated CD8+ T cells. Data are representative of three independent experiments, in which two to three pairs of mice were used. (C and D) In vitro proliferation in response to stimulation with α-CD3/CD28 antibodies (C) and PMA/ionomycin (D), evaluated as in A. Data are representative of two independent experiments with two pairs of mice.

in response to Ag-specific stimulation compared with WT (Fig. 4 A). In addition, the up-regulation of the early activation marker CD69, a measurement of TCR activation, was reduced in response to stimulation with $OVA_{257-264}$ peptide-loaded APCs (Fig. 4 B). We further investigated the ability of Bcl11b-deficient CD8+ T cells to proliferate in response to stimulation with α-CD3/CD28 antibodies and found that though the proliferation was substantially improved, it did not reach the same levels as in WT (Fig. 4 C). However, when the cells were stimulated with PMA/ionomycin, proliferation of Bcl11b-deficient CD8+ T cells reached the WT levels (Fig. 4 D). These results suggest that the reduced proliferation of Bcl11b-deficient CD8+ T cells is likely to be caused by altered TCR signaling, with a potential defect upstream of CD3 engagement, and a second defect upstream of PKC-Θ. The levels of both TCR and CD28 were equivalent in Bcl11b-deficient OT1 CD8+ T cells (unpublished data), although on a polyclonal background we observed a slight reduction in TCR and CD3 levels (unpublished data). Interestingly, the CD8 coreceptor levels were reduced in Bcl11b-deficient CD8+ T cells (Fig. 5 A and Table S1). The reduction in the CD8 coreceptor levels was not only observed in $Bcl11b^{F/F}$/dLck-iCre mice but also when Bcl11b was removed from CD8+ T cells with the use of the ER-Cre deleter in vivo (Fig. S4). We conducted quantitative (q) RT-PCR and demonstrated that CD8α and β coreceptor mRNAs were lower in the absence of Bcl11b (Fig. 5 B), suggesting that the reduction in the coreceptor levels is caused by a decrease in the coreceptor gene expression.

It has been demonstrated that the CD8 coreceptor plays a critical role in CD8+ T cell activation and immune response by amplifying the Ag-specific TCR response and Ag-specific proliferation (Veillette et al., 1989; Arcaro et al., 2001; Holler and Kranz, 2003). To further demonstrate that that the altered expression of CD8 coreceptor causes diminished TCR signaling, we activated the CD8+ T cells by treatment with α-TCR/CD8/CD28 and evaluated the phosphorylation of Zap70. Our results demonstrate that Zap70 phosphorylation was diminished after TCR/CD8 corecptor/CD28 engagement in Bcl11b-deficient CD8+ T cells (Fig. 5 C), suggesting that one of the causes of the reduced proliferation of Bcl11b-deficient CD8+ T cells is decreased CD8 coreceptor levels.

The reduced levels of CD8 coreceptor could not be the sole cause of the reduced proliferation of Bcl11b-deficient CD8+ T cells because stimulation with α-CD3/CD28 antibodies also resulted in reduced proliferation but substantially improved. Considering that PMA/ionomycin treatment brought the expansion of Bcl11b-deficient CD8+ T cells to WT levels (Fig. 4 D), we hypothesized that there might be a second defect in Bcl11b-deficient CD8+ T cells, upstream of PKC-Θ. We found that an additional gene with central role in TCR signaling presented altered mRNA levels in Bcl11b-deficient CD8+ T cells, specifically Plcγ1 (phospholipase C gamma 1; Wilde and Watson, 2001; Bonvini et al., 2003; microarray analysis, unpublished data), and demonstrated by qRT-PCR that the Plcγ1 mRNA levels were reduced in Bcl11b-deficient CD8+ T cells (Fig. 5 D). Calcium flux was attenuated in Bcl11b-deficient CD8+ T cells after activation of CD8+ T cells with α-CD3/CD28 antibodies, supporting the observation with regard to Plcγ1 expression (Fig. 5 E). All these data collectively demonstrate that deregulated expression of Plcγ1, together with the reduced CD8 coreceptor

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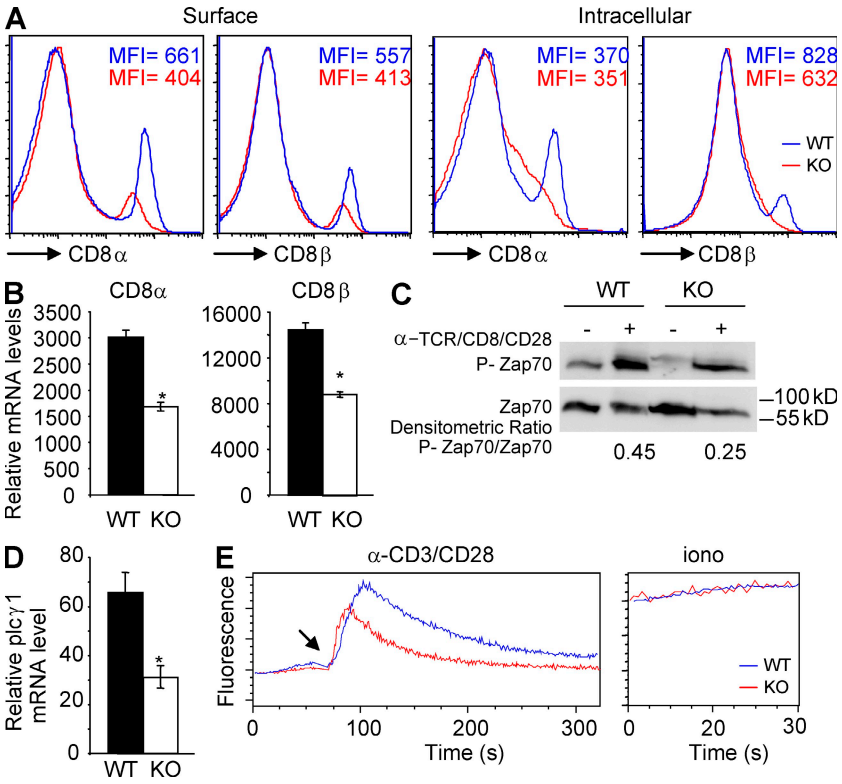

Figure 5. Bcl11b regulates expression of CD8 coreceptor and Plcγ1 genes. (A) Surface and intracellular levels of CD8α and CD8β coreceptors. Single cell suspensions from lymph nodes of Bcl11b$^{F/F}$/dLck-iCre and control mice were stained for surface or intracellular CD8α and CD8β. Overlayed histograms indicate surface or intracellular CD8α and CD8β. CD8α and CD8β MFIs are also indicated. Representative data for five pairs of mice are shown. (B) Relative mRNA levels of the CD8 coreceptor in sorted naive CD44^{lo} Bcl11b-deficient and WT CD8$^+$ T lymphocytes from four (CD8a) and three (CD8b) pairs of mice, normalized to actin. A two-tailed Student's *t* test was applied to determine statistical significance. $P = 3.66 \times E^{-4}$, and 0.006. (C) Immunoblot analysis of Zap70 phosphorylation after TCR, CD8, and CD28 cross-linking of purified CD8$^+$ T cells from OT1 mice. CD8$^+$ T cells were purified by depletion, and CD8$^+$ T cells were preincubated with the indicated biotinylated antibodies, followed by addition of streptavidin. 10^6 cells were used in each treatment. P-Zap70 was quantified by densitometric analysis versus total Zap70. Data are representative for two pairs of mice. (D) Relative Plcg1 mRNA levels in Bcl11b-deficient (KO) and WT (WT) CD8$^+$ T cells from four pairs of mice, normalized to actin. * indicates significant difference. Error bars indicate SD. P = 0.05. (E) Calcium flux analysis in Bcl11b-deficient (KO) and WT (WT) CD8$^+$ T cells. Lymphocytes were loaded with Fluo4 and surface stained for CD4 and CD8. Cells were preincubated with biotinylated α-CD3/CD28, after which streptavidin was added (arrow), and the fluorescence was measured for the indicated period of time. The histogram on the right indicates the fluorescence after addition of ionomycin to record the maximal calcium response. Calcium flux was analyzed using the kinetics platform of FlowJo and represented as Fluo4 fluorescence over time. This is a representative experiment out of three.

expression in Bcl11b-deficient CD8$^+$ T lymphocytes, are likely to contribute to the reduced Ag-specific proliferation and expansion in vivo and in vitro.

Bcl11b controls expression of the CD8 coreceptor by association with specific enhancers

Regulation of CD8 expression by Bcl11b raised the question of whether Bcl11b directly controls the expression of CD8 coreceptor genes by binding to the CD8 enhancers. We conducted chromatin immunoprecipitations (ChIPs) to determine whether Bcl11b associates with any of the CD8 enhancers, including E8I, E8II, E8III, EIV, and E8V. Our ChIP assays demonstrated that Bcl11b associated with E8I, EIV, and E8V enhancers but not with E8II and E8III enhancers (Fig. 6). E8I and E8II control the CD8 coreceptor levels in mature CD8$^+$ T cells. In addition, E8II and E8III together control the CD8 coreceptor levels in DP thymocytes (Ellmeier et al., 1997, 1998; Hostert et al., 1997; Kioussis and Ellmeier, 2002; Feik et al., 2005). However, maintenance of the CD8 coreceptor genes expression in the periphery seems to be complex, as even in the absence of E8I and E8II, CD8$^+$ T cells still maintain much of their coreceptor expression (Ellmeier et al., 2002). It has been suggested that other enhancers, such as EIV and EV, and/or other mechanisms, might be involved in the maintenance of CD8 expression in the periphery (Kioussis and

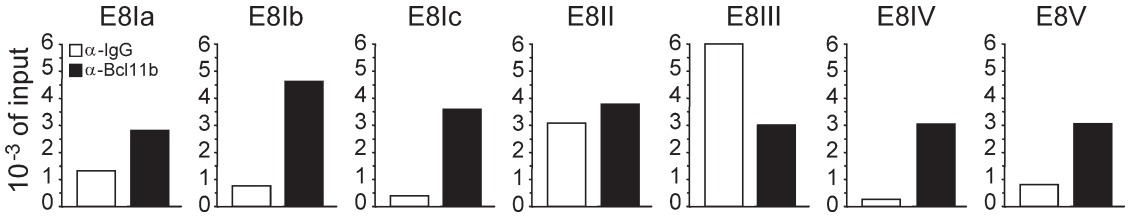

Figure 6. BCL11B associates with the E8I, E8IV, and E8V enhancers. ChIPs with anti-Bcl11b or IgG antibodies on purified CD8$^+$ T cells. The association of Bcl11b with the CD8 enhancers was evaluated by qPCR using previously described primers (Sato et al., 2005), which cover the indicated regions of the enhancers. Binding is expressed relative to the input and calculated as indicated in Materials and methods. Data are representative of four independent experiments with four independent pairs of mice.

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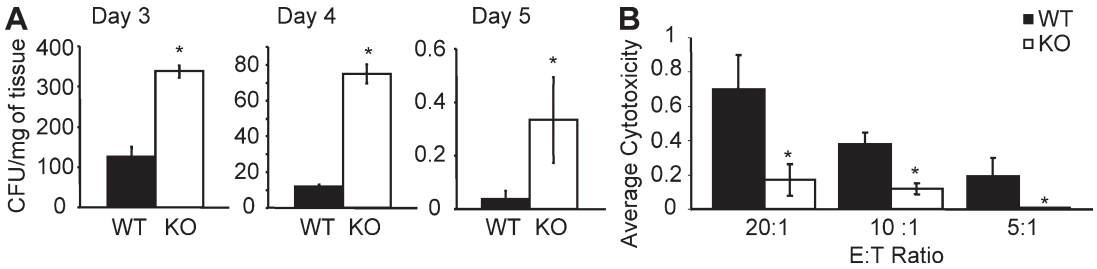

Figure 7. Evaluation of bacterial burden and Ag-specific cytolytic activity in the absence of Bcl11b from CD8+ T cells. (A) Bacterial burden was assessed in the livers of Lm-Ova–infected mice, separately transferred with Bcl11b-deficient and WT OT-1 CD8+ T cells, on days 3, 4, and 5 after infection. CFUs were normalized to milligrams of tissue. Three independent experiments were conducted, each with three pairs of mice. (B) Ex vivo killing of Ag-specific targets by Bcl11b-deficient and WT OT-1 CD8+ T cells was evaluated by measuring lactate dehydrogenase activity released into the media after killing of target cells, as described in the Materials and methods. Bcl11b-deficient and WT OT-1 CD8+ T cells were purified from spleens of Lm-Ova–infected mice on day 5 after infection and incubated with MEC.B7.SigOva cells expressing $OVA_{257-264}$ peptide via MHC class I (van Stipdonk et al., 2001). Data are presented as means ± SD of cytotoxicity (lactate dehydrogenase activity) of triplicate samples and are representative of three independent experiments.

Ellmeier, 2002). Our results show that Bcl11b is present on several enhancers of the CD8 coreceptor genes, including E8I, E8IV, and E8V and through these multiple associations perhaps contributes to the maintenance of optimal expression levels of the CD8 coreceptor genes in the periphery. The remaining expression of CD8 coreceptor in the absence of Bcl11b may be attributed to the E8II enhancer, to which Bcl11b did not bind (Fig. 6), as well as to other transcription factors that control the E8I, E8IV, and E8V enhancers. Overall, our data demonstrate that Bcl11b is one of the transcription factors implicated in the maintenance of CD8 coreceptor expression in peripheral CD8+ T cells, potentially through direct association with some of the CD8 gene enhancers.

Bcl11b-deficient CTLs fail to clear LM-Ova and have poor Ag-specific killing activity

Because effective CTLs are required for the control of *L. monocytogenes*, we next evaluated the bacterial clearance mediated by Bcl11b-deficient CD8+ T cells. In these experiments Bcl11b-deficient and WT OT-1 CD8+ T cells were transferred to separate recipient mice, which were then infected with Lm-Ova. We found that bacterial titers were much higher in recipients of Bcl11b-deficient OT-1 CD8+ T cells than in recipients of normal OT-1 CD8+ T cells (Fig. 7 A). Bacterial clearance was also impaired when $Bcl11b^{F/F}$/dLck-iCre/OT-1 and WT OT-1 mice were directly infected with Lm-Ova (unpublished data).

Because reduced bacterial clearance may be caused by the reduced Ag-specific expansion of Bcl11b-deficient CD8+ T cells, we further evaluated ex vivo, on a per cell basis, the Ag-specific killing activity of CTLs purified from LM-Ova–infected mice. Specifically, we compared equal numbers of Bcl11b-deficient and WT OT-1 CTLs purified from Lm-Ova–infected OT-1 mice for Ag-specific cytotoxic activity against MEC.B7.SigOva target cells, expressing $OVA_{257-264}$ peptide via MHC class I (van Stipdonk et al., 2001). Our results clearly show that in the absence of Bcl11b, CTLs killed $OVA_{257-264}$-expressing target cells less efficiently on a per cell basis (Fig. 7 B), suggesting that Bcl11b-deficient CD8+ T cells might have defects in CTL activity.

The effector population is formed in the absence of Bcl11b during the Lm-Ova infection, albeit in smaller number, and the effector cells produce less perforin and granzyme

One cause for the poor Ag-specific cytolytic activity of Bcl11b-deficient CTLs might be defective differentiation into short-lived effector cells, which up-regulate Klrg1 and down-regulate CD127, during the immune response (Kaech et al., 2003). Our results show that Bcl11b-deficient OT-1 CD8+ T cells differentiated normally into $Klrg1^{hi}CD127^{lo}$ effector population (Fig. 8 A); however, the absolute numbers of effector cells was reduced overall (not depicted). Indeed, the reduced numbers of effector cells may account for the increased bacterial burden. However, in the ex vivo killing assays, equal numbers of CD8+ T cells from LM-Ova–infected $Bcl11b^{F/F}$/dLck-iCre/OT-1 and WT OT-1 mice were used. Even in these conditions, Bcl11b-deficient CTLs presented reduced Ag-specific killing activity, demonstrating that the reduced killing activity is not only a result of reduced numbers of CTLs. Thus, the reason for poor cytolytic activity could be caused by a reduction in the cytolytic effector molecules, perforin, and granzyme B. We found that the levels of granzyme B were reduced in Bcl11b-deficient CTLs at the peak of infection with LM-Ova (Fig. 8 B). In addition, the CD8+ T cells purified from LM-Ova–infected $Bcl11b^{F/F}$/dLck-iCre/OT1 mice presented a major reduction in the perforin levels, compared with equal numbers of OT1 WT cells (Fig. 8 C). We also investigated the perforin and granzyme B mRNA levels in equal numbers of CD8+ T cells purified from LM-Ova–infected $Bcl11b^{F/F}$/dLck-iCre/OT1 and OT1 mice and found lower levels of both mRNAs in the absence of Bcl11b (Fig. 8 D).

We further determined, by ChIP assays, whether Bcl11b associates with the genomic regions upstream of the transcription start sites (TSSs) of perforin and granzyme B genes in CD8+ T cells purified from LM-Ova–infected mice. Our data demonstrated that Bcl11b associated with the genomic

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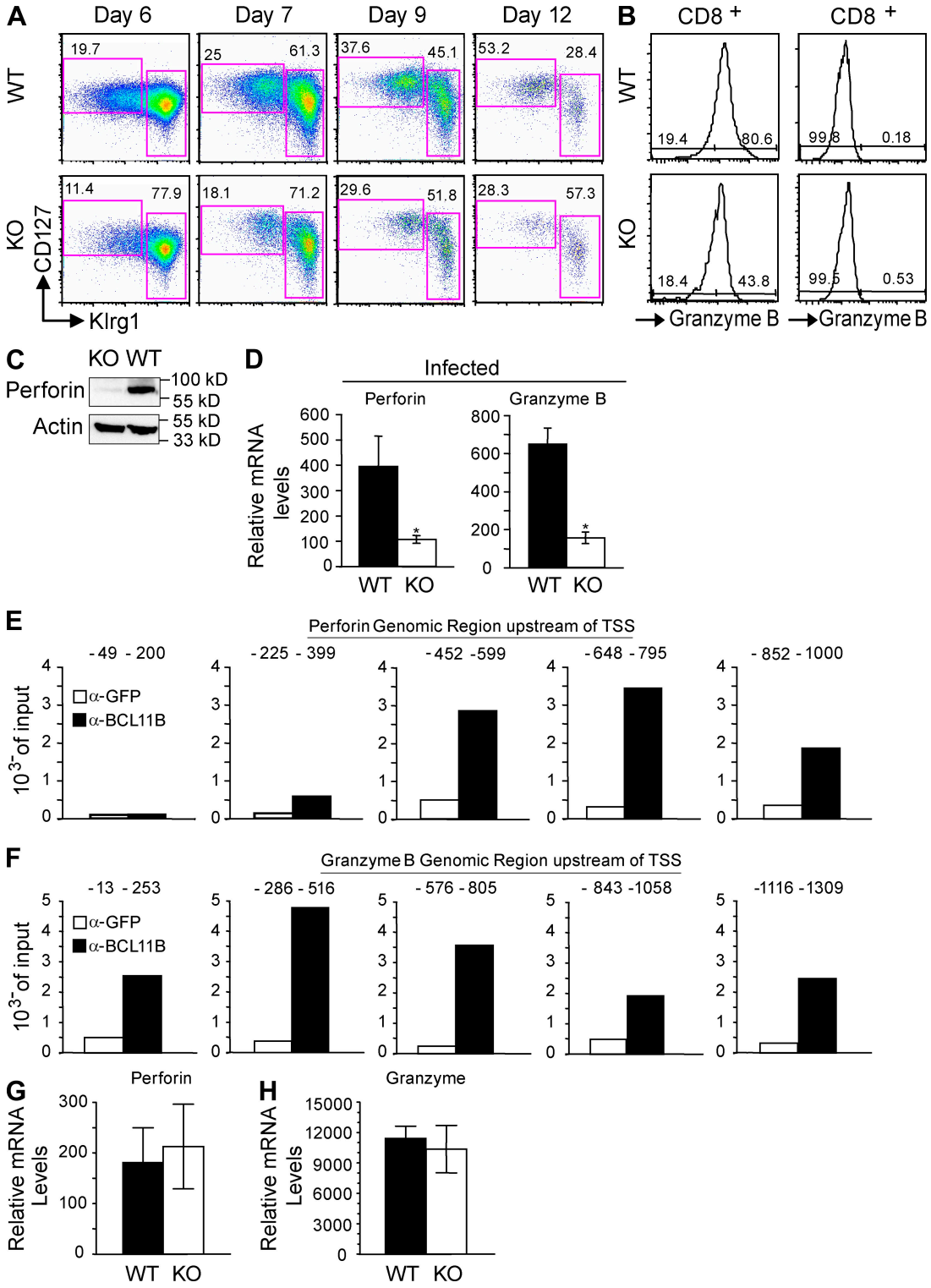

Figure 8. Evaluation of effector population and effector molecules in the absence of Bcl11b from CD8+ T cells. (A) The formation of effector population during the immune response to Lm-OVA infection was evaluated in Bcl11b-deficient and WT donor OT-1 CD8+ T cells on days 6, 7, 9, and 12 after infection by staining for Klrg1 and CD127. The boxes indicate the Klrg1^{hi}CD127^{lo} and Klrg1^{lo}CD127^{hi} populations gated on the donor CD45.2 Bcl11b-deficient and CD45.1/2/ WT CD8+ T cells. Data are representative of three independent experiments. (B) Granzyme B was evaluated on day 7 after the Lm-Ova infection within the donor Bcl11b-deficient CD45.2 and WT CD45.1/2 CD8+ T cell populations. Experiments were conducted as described in Fig. 2. Numbers indicate the percentage of cells in the gated populations. The histograms on the right show granzyme B in CD8+ T cells from uninfected mice. Data are representative of three independent experiments in which cells were transferred from two to three mice from each group in three to four recipients.

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region between −452 and −1000 upstream of the perforin gene TSS (Fig. 8 E) but not to the distal CTL-specific DNase I hypersensitive site (Pipkin et al., 2007; Cruz-Guilloty et al., 2009; Fig. S5). Bcl11b also associated with the granzyme B genomic region from −13 to −1309 upstream of TSS (Fig. 8 F) but not with a genomic region localized 1 kb downstream of the granzyme B gene stop codon (Fig. S5). The ratios of the CD8 mean fluorescence intensity (MFI) between Bcl11b-deficient and WT CD8$^+$ T cells during T cell activation, as well as in CD8$^+$ T cells isolated from LM-Ova–infected mice, remained similar to the ratio of CD8$^+$ T cells from naive mice (Table S2), suggesting that it is unlikely that further reduction in CD8 levels during the activation might contribute to the reduction in effector molecules.

All these results collectively demonstrate that Bcl11b-deficient CD8$^+$ T cells have reduced cytolytic activity in vivo in response to infection and, ex vivo, against Ag-specific targets. The reduced cytolytic activity in vivo correlates with decreased ability of Bcl11b-deficient CTLs to produce perforin and granzyme B during LM-Ova infection.

We further used the ER-Cre system for inducible removal of Bcl11b in an in vitro culture system for CTL differentiation (Cruz-Guilloty et al., 2009). Specifically, we first activated CD8$^+$ T cells from Bcl11b$^{F/F}$/ER-Cre and ER-Cre mice, after which the cells were grown in media containing OH-tamoxifen. Under these in vitro conditions, and with removal of the gene after the activation of the CD8$^+$ T cells, the perforin and granzyme B mRNA levels were similar in Bcl11b$^{F/F}$/ER-Cre and ER-Cre CD8$^+$ T cells (Fig. 8, G and H). Thus, removal of Bcl11b after in vitro activation of CD8$^+$ T cells does not result in down-regulation of perforin and granzyme B mRNA levels, suggesting that the in vivo reduction of perforin and granzyme B and the reduced cytolytic activity might be secondary to suboptimal TCR activation, at least in part.

DISCUSSION

In this paper, we provide evidence that Bcl11b is a critical transcription factor for CD8$^+$ T cell immune response by controlling two critical aspects of the CD8 immunity: Ag-specific expansion in response to infection, and cytolysis. Specifically, we demonstrate that Bcl11b-deficient CD8$^+$ T cells failed to efficiently expand in response to infection with *L. monocytogenes* or influenza and produced less perforin and granzyme B. Bcl11b-deficient CD8$^+$ T cells presented reduced Ag-specific proliferation, but not increased apoptosis, during the course of the immune response. TCR signaling after Ag-specific activation is required for naive CD8$^+$ T lymphocytes to enter cell cycle and expand. After infection, the majority of Bcl11b-deficient CD8$^+$ T lymphocytes were found blocked in G0/G1 (unpublished data). Furthermore, Bcl11b-deficient CD8$^+$ T lymphocytes presented reduced up-regulation of the early activation marker CD69 after TCR activation, which is suggestive of altered TCR signaling. In terms of the mechanism of the reduced expansion during the immune response, we found that the CD8 coreceptor levels were reduced in the absence of Bcl11b. CD8 coreceptor genes not only play a critical role in the CD8 lineage development (Fung-Leung et al., 1991; Crooks and Littman, 1994) but also in proliferative response of CD8$^+$ T cells (Cai and Sprent, 1994). CD8 coreceptor contributes to TCR binding to peptide–MHC class I complexes on APCs (Garcia et al., 1996), and it induces a conformational change in the CD3, which is important for TCR signaling (Gil et al., 2008). In addition, the coreceptor amplifies TCR signaling, through partitioning, together with the TCR complex, to the lipid rafts and association with p56lck (Arcaro et al., 2001). Bcl11b regulated CD8 coreceptor gene expression through association with the E8I enhancer, which is known to play a role in the CD8 coreceptor expression in mature CD8$^+$ T cells (Ellmeier et al., 1997, 1998; Hostert et al., 1997; Kioussis and Ellmeier, 2002; Feik et al., 2005). In addition, Bcl11b was found to be present on E8IV and E8V enhancers, whose role in the control of CD8 expression is less characterized. Although the regulation of CD8 coreceptor expression during development has been well characterized (Ellmeier et al., 1997, 1998; Hostert et al., 1997; Kioussis and Ellmeier, 2002; Feik et al., 2005), not much is known about the regulation and maintenance of its expression in the peripheral CD8$^+$ T cells. In the absence of Bcl11b, the levels of the CD8 coreceptor were reduced but not abrogated, which is consistent with the idea that other transcription factors play roles in controlling the activity of the E8I, E8IV, and E8V enhancers. In addition to this, Bcl11b might also regulate expression of CD8 coreceptors indirectly by regulating other transcription factors implicated in this process, such as MAZR (Bilic et al., 2006).

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(C) Western blot analysis of perforin in purified CD8$^+$ T cells from mice infected with Lm-Ova. Bcl11b$^{F/F}$/dLck-iCre/OT1 and WT OT1 mice were infected with Lm-Ova, and 7 d after infection total CD8$^+$ T cells were purified. Extracts were prepared from equal numbers of cells and Western blot analysis was conducted for evaluation of perforin. Data are representative of two independent experiments. (D) Relative mRNA levels of perforin and granzyme B (normalized to actin) in equal numbers of total CD8$^+$ T cells purified form LM-Ova–infected Bcl11b$^{F/F}$/dLck-iCre/OT1 and WT OT1 mice. Data are derived from multiple experiments with, respectively, 10 and 11 pairs of mice and, respectively, P = 0.0039 and 0.0001. * indicates significant difference. (E and F) ChIP assays with anti-Bcl11b or anti-GFP antibodies on purified CD8$^+$ T cells from infected mice at day 6 after infection. Association of Bcl11b with perforin (E) and granzyme B (F) genomic regions was evaluated by qPCR using primers covering the indicated regions upstream of TSS, and values were expressed relative to the input as described in Materials and methods. Data are representative of two independent experiments on two pairs of mice. (G and H) Evaluation of perforin and granzyme B mRNA levels in CD8$^+$ T cells from Bcl11b$^{F/F}$/ER-Cre and ER-Cre mice in vitro stimulated for 2 d, before the initiation of the treatment with OH-tamoxifen, according to a protocol for in vitro differentiation to effector cells (Cruz-Guilloty et al., 2009). After activation, cells were grown in the presence of 100 U/ml rIL-2 and 100 nM OH-tamoxifen for 4–5 d and RNA was purified followed by qRT-PCR. This is representative of two independent experiments, each with two to three pairs of mice. P = 0.64 and 0.46, respectively. Error bars indicate SD.

Such a possibility is currently under investigation. The remaining CD8 coreceptor levels in the absence of Bcl11b may be attributed to the E8II contribution, which is also known to control CD8 coreceptor expression in $CD8^+$ T cells, in addition to the DP thymocytes (Ellmeier et al., 1997), to which Bcl11b did not bind. In the absence of Bcl11b in DP thymocytes, CD8 coreceptor levels were not altered, suggesting that Bcl11b does not control expression of CD8 coreceptor in DP thymocytes (Albu et al., 2007). Therefore, our results demonstrate that Bcl11b is one of the transcription factors critical for the maintenance of the CD8 coreceptor levels in the periphery. In addition to the CD8 coreceptor, we found that Bcl11b regulates the expression of Plcγ1 in mature $CD8^+$ T cells, which is another critical gene for TCR signaling (Wilde and Watson, 2001; Bonvini et al., 2003). In conclusion, our results suggest that TCR signaling in Bcl11b-deficient $CD8^+$ T cells is impaired by at least two mechanisms: reduced expression of CD8, and probably, therefore, less optimal activation of $CD8^+$ T cells, and reduced expression of Plcγ1 and, hence, reduced $Ca2^+$ mobilization.

After Ag-specific activation, naive $CD8^+$ T lymphocytes expand and differentiate into effector cytotoxic T cells. The effector $Klrg1^{hi}IL7R^{lo}$ CTL population was formed in the absence of Bcl11b, although in reduced numbers. The production of perforin and granzyme B, evaluated in equivalent numbers of CTLs from infected mice, was altered. Moreover, the perforin and granzyme B mRNAs were reduced in $CD8^+$ T cells purified from LM-Ova–infected mice, and ChIP experiments demonstrated that Bcl11b bound to both perforin and granzyme B genomic regions upstream of their TSS in CTLs from infected mice. Unexpectedly, removal of Bcl11b after in vitro activation did not result in reduced perforin and granzyme B mRNA levels, suggesting that perhaps the reduction in perforin and granzyme B levels during infection is caused in part by suboptimal TCR activation. However, the fact that Bcl11b-deficient CTLs on a per cell basis had a reduced capacity to kill Ag-specific MEC.B7.SigOva target cells suggests that the reduced clonal expansion is not solely responsible for the altered cytolytic activity because in this assay equal numbers of Bcl11b-deficient and WT CTLs were compared. Thus, other possibilities for the differences that we observed between the in vivo versus the in vitro conditions include: growth of Bcl11b-deficient $CD8^+$ T cells in vitro for several days might select a specific population, and/or might only favor survival of certain cells; the absence of specific conditions which are only present in vivo, but not in vitro; or in vivo removal of the gene with the dLckD-iCre before TCR activation, as opposed to in vitro removal after activation, could have different effects on $CD8^+$ T cell activation/differentiation.

With regard to the presence of Bcl11b on genomic regions upstream of TSS of perforin and granzyme B genes, there are multiple possibilities: the presence of a transcription factor on promoter/enhancer does not always result in regulation of expression; or the presence of Bcl11b on genomic regions upstream of perforin and granzyme B TSS genes might only regulate perforin and granzyme B expression under specific conditions, which might differ between in vivo and in vitro experiments. Although at the moment it is hard to have a clear answer because of the discrepancy between the in vivo and in vitro results, it is possible that in certain conditions Bcl11b might regulate expression of perforin and granzyme B. Other transcription factors, such as Eomes and Runx3, were also found to control the expression of the CTL effector molecules (Pearce et al., 2003; Intlekofer et al., 2005; Chen et al., 2008; Cruz-Guilloty et al., 2009). Runx3 was found to associate with perforin and granzyme B promoters (Cruz-Guilloty et al., 2009). There are several Runx3 consensus sequences in the perforin promoter. One such site is closely located to a Bcl11b consensus site (unpublished data), suggesting the possibility that these two transcription factors may function together by binding to adjacent sites. The granzyme B promoter has a region of TGTG repeats which may potentially constitute binding sites for Runx3, and a Bcl11b consensus binding site resides very close to that (unpublished data), also suggesting the possibility that the two transcription factors may bind to the adjacent sites on the granzyme B promoter. Such possibilities are under investigation.

Bcl11b is expressed during T cell development, as well as in mature T cells, which suggests the possibility that it may regulate common genes in T cells and implicitly common genetic programs. Our observations suggest that, at least in DP thymocytes and $CD8^+$ T cells, this is the case for certain genes (unpublished data); however, Bcl11b seems to differentially regulate specific genes in $CD8^+$ T cells versus DP thymocytes, such as the CD8 coreceptor. How can Bcl11b regulate different genes in different T cell types, despite the fact that it is expressed at similar levels in these cells, rather than only regulating common genes? Bcl11b may cooperate with stage-specific transcription factors, and/or Bcl11b function may be modulated by specific signals, including TCR signaling, such that it can play a differential role in specific T cell subtypes. Such possibilities are under investigation. In conclusion, our data demonstrate that Bcl11b is a critical transcription factor for the CD8 immune response, controlling the expansion and execution of the effector function.

MATERIALS AND METHODS

Mice. Mice carrying the floxed Bcl11b alleles ($Bcl11b^{F/F}$) were backcrossed on C57BL/6 background for nine generations and crossed with dLck-iCre C57BL/6 mice (gift from N. Killeen, University of California, San Francisco, San Francisco, CA) to generate conditional $Bcl11b^{F/F}$/dLck-iCre mice. To obtain OT-1 TCR/ $Bcl11b^{F/F}$/dLck-iCre, $Bcl11b^{F/F}$/dLck-iCre mice were crossed to OT-1 TCR transgenic mice (The Jackson Laboratory). CD45.1 mice were purchased from NCI or The Jackson Laboratory. Experiments were conducted with 5–10-wk-old mice unless specified. All mice except for the infection model were kept under specific pathogen-free conditions in the Albany Medical Center (AMC) Animal Facility, and all animal procedures were approved by the AMC Animal Care and Use Committee.

Cell culture, antibodies, and flow cytometry. Naive $CD8^+$ T lymphocytes were either sorted as $CD62L^{hi}CD44^{lo}$ population or purified by depletion, using the $CD8^+$ T cell depletion kit (Miltenyi Biotec). When using the Miltenyi depletion kit, the antibody cocktail was supplemented with 50 ng biotinylated anti–mouse CD25 and anti–mouse CD44 antibodies per 10^7 cells to

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isolate mostly naive cells. The purity of $CD44^{lo}CD8^+$ T cells after purification was always >95%. The following antibodies (eBioscience) were used for surface and intracellular staining: anti-CD4 (clone GK1.5), -CD8α (clone 53–6.7), -CD8β (clone eBioH35-17.2), -CD45.1 (clone A20), -CD45.2 (clone 104), -CD69 (clone H1.2F3), -CD127 (clone A7R34), -Klrg1 (clone 2F1), –TCR-β (clone H57-597), -BrdU (clone PRB-1), –granzyme B (clone 16G6), and -perforin (clone eBioOMAK-D). Intranuclear staining of Bcl11b was conducted after staining for surface CD4 and CD8, followed by fixation and permeabilization with 1% Triton X-100 (Albu et al., 2007). Determination of apoptosis was conducted by intracellular staining with anti-cleaved caspase 3 antibodies (Cell Signaling Technology) after surface staining. Antibodies for Western blot analysis were: anti-Bcl11b (Bethyl Laboratories, Inc.), -perforin (Abcam), and P-Zap70 and Zap70 (Cell Signaling Technology).

Granzyme staining for flow cytometry analysis. $1–2 \times 10^6$ immune cells were stained with antibodies against surface markers for 30 min on ice. Cells were then fixed with freshly prepared Fixation/Permeabilization working solution (eBioscience) at 4°C for 30–60 min. After washing twice with permeabilization buffer (eBioscience), cells were incubated in permeabilization buffer at 4°C for 15 min, followed by addition of fluorophore-labeled anti–mouse granzyme antibodies (clone 16G6; eBioscience). Cells were then incubated on ice for 30 min in the dark, after which the cells were washed in the same permeabilization buffer and resuspended in staining buffer for flow cytometry analysis.

Flow cytometry analysis. Flow cytometry analysis was conducted on FACSCalibur or FACSCanto flow cytometers (BD). Flow cytometry data were analyzed using FlowJo software (Tree Star, Inc.).

Bone marrow reconstitution. For influenza infection experiments, mixed bone marrow chimeras were generated by lethally irradiating CD45.1 recipient mice and reconstituting them with either a 1:1 mix of CD45.1 and CD45.2 C57BL/6 bone marrow (WT:WT chimeras) or a 1:1 mix of CD45.1 and CD45.2 $Bcl11b^{F/F}$/dLck-iCre bone marrow (WT:KO) chimeras. After reconstitution, mice were infected with 1,000 EIU of PR8. The frequencies of NP-specific and PA-specific CD8 T cells derived from each donor type were determined by flow cytometry in the mediastinal lymph nodes on day 10 after infection.

Adoptive transfers, Lm-Ova infection, and bacterial burden. Sorted naive $CD44^{lo}$ $CD8^+$ T lymphocytes from CD45.2 $Bcl11b^{F/F}$/dLck-iCre/OT-1 and CD45.1/2/OT-1 mice (1:1; total 10^4) were intravenously injected either together or separately into CD45.1 recipient mice. The next day after transfer, recipient mice were injected with 1.5×10^4 CFU Lm-Ova (Shen et al., 1995). Bacterial burden was assessed in the livers and spleens of Lm-Ova–infected mice, separately transferred with Bcl11b-deficient or WT $CD8^+$ OT-1 T cells on days 3, 4, and 5 after infection by plating homogenized liver and spleen tissues on tryptic soy broth agar plates. The next day, individual colonies were counted and bacterial burden was expressed as CFU/mg of tissue.

In vitro killing. Bcl11b-deficient and WT $CD8^+$ T lymphocytes were purified from spleens of LM-Ova infected mice at day 5 after infection and incubated with MEC.B7.SigOva cells expressing $OVA_{257-264}$ peptide via MHC class I (van Stipdonk et al., 2001). The lactate dehydrogenase activity released into the media after killing of the target cells was evaluated with the CytoTox96 kit (Promega).

In vivo and in vitro proliferation assays. Allelically different Bcl11b-deficient (CD45.2) and WT (CD45.1/2) naive $CD8^+$ T lymphocytes were co–adoptively transferred in CD45.1 recipient mice which were then infected with LM-Ova. On day 6 after infection, mice were i.p. injected with 1 mg BrdU and sacrificed 16 h later for tissue harvesting. After surface staining, cells were permeabilized with ice-cold 95% ethanol and fixed, and then resuspended in freshly prepared DNase I (Roche) solution (50 Kunitz U/ml), stained with anti–BrdU-FITC antibody (eBioscience), and analyzed by flow cytometry.

In vitro proliferation was evaluated by CFSE dilution. Bcl11b-deficient and WT naive $CD8^+$ T lymphocytes were purified from OT-1 mice as shown in the previous paragraph. The naive $CD8^+$ T cells were labeled with 5 μM CFSE and incubated for 3 d with APCs pulsed with 0 or 0.1 μM $OVA_{257-264}$ peptide, for 2 d in media with 2 μg/ml of soluble α-CD28 and 0.5 μg/ml of cross-linked α-CD3, or in media with 10 nM PMA and 1 μM ionomycin. Cell divisions were assessed by tracking the dilution of CFSE by flow cytometry.

ChIP. ChIP was conducted using $CD8^+$ T cells purified on beads (Miltenyi Biotec), according to our previously published protocol (Cismasiu et al., 2006), with the difference that anti-Bcl11b antibodies were preloaded on anti–rabbit IgG-Dynabeads (Invitrogen), and the precipitated DNA was analyzed by qPCR. As a control, we used either anti-GFP antibodies or IgG. The primers for CD8 enhancers I, II, III, IV, and V were previously described (Sato et al., 2005). The transcription factor–bound DNA was calculated based on the following formula: 2^{-ct} of the immunoprecipitated sample divided by 2^{-ct} of the input, where ct is the threshold cycle, followed by corrections for the input dilution.

qRT-PCR. qRT-PCR from total RNA purified from naive or total $CD8^+$ T lymphocytes, from naive mice, or from LM-Ova–infected mice was conducted as previously described (Cismasiu et al., 2006), always using primers that cross introns.

Statistical analysis. Differences between $Bcl11b^{F/F}$/dLck-iCre and control mice under all experimental conditions were analyzed by the two-tailed Student's *t* test and expressed as the mean ± the SD. $P \leq 0.05$ was considered significant for all analyses.

Online supplemental material. Fig. S1 shows conditional removal of Bcl11b in mature T cells with the use of the dLck-iCre system. Fig. S2 shows TCR-β on CD4 and CD8 SP thymocytes of $Bcl11b^{F/F}$/dLck-iCre mice. Fig. S3 shows histograms showing cleaved caspase-3 levels on gated CD45.1 recipient $CD8^+$ T cell populations. Fig. S4 shows that acute removal of Bcl11b in vivo with the use of ER-Cre deleter results in reduced CD8 coreceptor levels. Fig. S5 shows that Bcl11b does not bind actin promoter, the distal CTL-specific DNase I hypersensitive site in the perforin locus, or a genomic region located 1 kb downstream of the granzyme B stop codon. Table S1 shows CD8 MFI in SP thymocytes and $CD8^+$ T lymphocytes of $Bcl11b^{F/F}$/dLck-iCre and $Bcl11b^{F/F}$ mice. Table S2 shows ratios of CD8 MFI of $Bcl11b^{F/F}$/dLck-iCre and $Bcl11b^{F/F}$ $CD8^+$ T lymphocytes. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092136/DC1.

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