Cd8 enhancer $E8_I$ and Runx factors regulate CD8α expression in activated $CD8^+$ T cells

Hammad Hassan[a,1], Shinya Sakaguchi[a,1], Mari Tenno[b], Aglaja Kopf[a], Nicole Boucheron[a], Andrea C. Carpenter[c], Takeshi Egawa[d], Ichiro Taniuchi[b], and Wilfried Ellmeier[a,2]

[a]Division of Immunobiology, Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, 1090 Vienna, Austria; [b]Laboratory for Transcriptional Regulation, RIKEN Research Center for Allergy and Immunology, Yokohama, Kanagawa 230-0045, Japan; [c]Laboratory of Immune Cell Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; and [d]Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110

Edited by Matthias Merkenschlager, Medical Research Council Clinical Sciences Centre, Faculty of Medicine, London, United Kingdom, and accepted by the Editorial Board October 3, 2011 (received for review April 12, 2011)

***Cd8a* and *Cd8b1* coreceptor gene (*Cd8*) expression is tightly controlled during T-cell development by the activity of five *Cd8* enhancers ($E8_I$–$E8_V$). Here we demonstrate a unique transcriptional program regulating CD8 expression during $CD8^+$ effector T-cell differentiation. The *Cd8* enhancer $E8_I$ and Runx/core-binding factor-β (CBFβ) complexes were required for the establishment of this regulatory circuit, because $E8_I$-, Runx3-, or CBFβ-deficient $CD8^+$ T cells down-regulated CD8α expression during activation. This finding correlated with enhanced repressive histone marks at the *Cd8a* promoter in the absence of $E8_I$, and the down-regulation of CD8α expression could be blocked by treating $E8_I$-, Runx3-, or CBFβ-deficient $CD8^+$ T cells with the histone deacetylase inhibitor trichostatin A. Moreover, Runx/CBFβ complexes bound the *Cd8ab* gene cluster in activated $CD8^+$ T cells, suggesting direct control of the *Cd8a* locus. However, $CD8^+$ effector T cells maintained high levels of CD8α when CBFβ was conditionally deleted after activation. Thus, our data suggest an $E8_I$- and Runx3/CBFβ-dependent epigenetic programming of the *Cd8a* locus during T-cell activation, leading to Runx/CBFβ complex-independent maintenance of CD8α expression in effector T cells.**

epigenetic marks | transcriptional control | cytotoxic T lymphocytes

The expression of the CD4 and CD8 coreceptors is linked with the functional phenotype of mature T cells. On conventional T cells, CD8 usually consist of CD8α and CD8β heterodimers (encoded by the closely linked *Cd8a* and *Cd8b1* genes, respectively), and the expression of the *Cd8* genes during T-cell development is regulated by the activity of at least five different *cis*-regulatory elements (1). The first *Cd8* enhancer identified, designated $E8_I$, is active in mature CD8 single-positive thymocytes and in $CD8^+$ T cells, and in innate-like $CD8\alpha\alpha^+$ intraepithelial lymphocyte (IEL) of the gut (2, 3). The generation of $E8_I$-deficient mice revealed that $E8_I$ is essential for CD8αα expression in γδTCR (T-cell receptor) IEL, while CD8 expression on conventional T cells was not impaired (4, 5). The *Cd8* enhancer $E8_{II}$ directs expression of a reporter transgene in double-positive (DP) thymocytes and $CD8^+$ T cells (4), while $E8_{II}$-deficient mice have normal CD8 expression (6). Combined deletion of $E8_I$ and $E8_{II}$ leads to variegated expression of CD8 in DP thymocytes (6), and subsequent studies showed that CD8 variegation correlates with an epigenetic "off" state (7). A similar variegation phenotype is also observed in mice lacking the *Cd8* enhancer $E8_V$ (8). Another enhancer, $E8_{III}$, is active in DP thymocytes (4), and combined deletion of $E8_{II}$ and $E8_{III}$ resulted in a mild CD8 variegation phenotype in DP thymocytes, but $E8_{II}$,$E8_{III}$-deficient mice have normal levels of CD8 on peripheral T cells (9). Taken together, these studies revealed a complex network of *cis*-regulatory elements, and link *Cd8* enhancer functions with chromatin remodeling of the *Cd8ab* gene complex.

A new twist in the regulation of *Cd8* gene expression and an insight into a novel function of the *Cd8* enhancer $E8_I$ were obtained from a study showing that subsets of $CD8\alpha\beta^+$ T cells transiently express CD8αα homodimers upon activation (10). The expression of CD8αα homodimers on $CD8\alpha\beta^+$ T cells was linked to the survival and differentiation of memory precursor cells into memory cells and dependent on $E8_I$, because $E8_I^{-/-}$ $CD8\alpha\beta^+$ T cells failed to up-regulate CD8αα expression. It was shown that $E8_I$-deficient mice have impaired memory functions (10), although memory cell formation can also occur in the absence of CD8αα homodimer expression on $CD8\alpha\beta^+$ T cells in $E8_I$-deficient mice (11, 12). In one of the studies, a decrease in CD8αβ expression on splenic T cells in lymphocyte choriomeningitis virus (LCMV)-infected $E8_I$-deficient mice has been observed, providing the first indication that $E8_I^{-/-}$ $CD8^+$ T cells may have a defect in CD8αβ expression upon activation (11).

In this study we investigated whether the expression of CD8 in activated $CD8^+$ T cells is differentially regulated compared with naive $CD8^+$ T cells. We could show that a unique transcriptional program regulates CD8 expression during $CD8^+$ effector T-cell differentiation that is distinct from naive T cells. The *Cd8* enhancer $E8_I$ and Runx/core-binding factor (CBF)β complexes were required for the establishment of this regulatory circuit, because $E8_I$- or Runx/CBFβ complex-deficient $CD8^+$ T cells down-regulated CD8α expression during activation. The down-regulation was specific for the *Cd8a* gene and correlated with enhanced repressive histone marks at the *Cd8a* promoter in the absence of $E8_I$. The down-regulation of CD8α expression could be blocked by treating $E8_I$-deficient $CD8^+$ T cells with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA). This finding demonstrates that CD8 expression can be maintained even in the absence of $E8_I$, and suggests that $E8_I$ might protect the *Cd8a* locus from HDAC-mediated repression upon activation. Moreover, Runx/CBFβ complexes bound the *Cd8ab* gene cluster in activated $CD8^+$ T cells, suggesting direct control of the *Cd8a* locus during $CD8^+$ T-cell activation. However, $CD8^+$ effector T cells maintained high levels of CD8α when CBFβ was conditionally deleted after activation. Thus, our data suggest that the induction of this effector T-cell–specific regulatory program for *Cd8a* gene expression requires $E8_I$-and Runx3/CBFβ-dependent epigenetic programming of the *Cd8a* locus during T-cell activation, lead-

Author contributions: H.H., S.S., and W.E. designed research; H.H., S.S., M.T., A.K., N.B., and A.C.C. performed research; T.E. and I.T. contributed new reagents/analytic tools; H.H., S.S., I.T., and W.E. analyzed data; W.E. wrote the paper; and T.E. provided experimental data.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. M.M. is a guest editor invited by the Editorial Board.

[1]H.H. and S.S. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: wilfried.ellmeier@meduniwien.ac.at.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1105835108/-/DCSupplemental.

ing to Runx3/CBFβ-independent maintenance of CD8α expression in effector T cells.

Results

Activated $E8I^{-/-}$ CD8$^+$ T Cells Down-Regulate CD8α Expression. In a previous study it has been reported that *Cd8* enhancer $E8_I$-deficient mice express lower levels of CD8αβ on peripheral CD8$^+$ T cells upon infection with LCMV (11). To investigate the role of $E8_I$ in regulating CD8α expression in more detail, peripheral wild-type and $E8_I$-deficient CD8$^+$ T cells were isolated and activated with anti-CD3/anti-CD28. Although $E8_I^{+/+}$ cells maintained high-levels of CD8α upon activation over a period of 14 d, $E8_I$-deficient T cells down-regulated CD8α expression already at day 3 and the expression remained low at day 14 (Fig. 1). A similar down-regulation of CD8α expression was also observed upon antigen-specific activation of $E8_I^{-/-}$,OT-I CD8$^+$ T cells in vitro and in vivo (Fig. S1 *A* and *B*). However, only $E8_I^{-/-}$ CD8$^+$ T cells, but no $E8_{II}$-deficient or $E8_{II}$,$E8_{III}$-deficient CD8$^+$ T cells down-regulated CD8α and CD8β expression on peripheral CD8$^+$ T cells upon activation (Fig. S1 *C* and *D*) (6, 9). Moreover, $E8_I$,$E8_{II}$-deficient CD8$^+$ T cells showed a similar reduction of CD8α expression as $E8_I^{-/-}$ CD8$^+$ T cells upon activation, indicating that $E8_{II}$ is not involved in the regulation of CD8α expression upon activation, even in the absence of $E8_I$ (Fig. S1*C*). Collectively, these data show that *Cd8* enhancer $E8_I$, but not $E8_{II}$ or $E8_{III}$, is essential for maintaining CD8α expression at high-levels upon activation.

$E8_I$ Regulates *Cd8a* but Not *Cd8b1* Gene Expression. Having determined that CD8α expression is affected in $E8_I^{-/-}$ CD8$^+$ T cells, we investigated whether CD8β (encoded by the *Cd8b1* gene) expression is impaired by loss of $E8_I$. To test for the expression of CD8β (which requires CD8α for surface expression), CD8$^+$ T cells were activated with anti-CD3/anti-CD28. After 5 d, CD8α$^-$ cells from the $E8_I^{-/-}$ T-cell cultures were isolated and the expression of the *Cd8a* and *Cd8b1* genes was determined by semiquantitative RT-PCR. As expected, *Cd8a* gene expression was terminated at the transcriptional level in CD8α$^-$ T cells (Fig. 2*A*). In contrast, CD8α$^-$ T cells still expressed normal levels of *Cd8b1*, indicating that loss of $E8_I$ selectively affects *Cd8a* expression upon activation. Loss of CD8α expression also did not interfere with the proliferation of CD8$^+$ T cells, because CFSE- [5-(and 6)-carboxyfluorescein diacetate succinimidyl ester] labeling experiments revealed a similar proliferation rate of $E8_I^{+/+}$ and $E8_I^{-/-}$ CD8$^+$ T cells upon activation (Fig. 2*B*). In addition, there was no difference in the cell death rate between $E8_I^{+/+}$ and $E8_I^{-/-}$ CD8$^+$ T cells (Fig. 2*C*). However, $E8_I^{-/-}$ CD8$^+$ T cells that underwent more cell cycles showed a lower level of CD8α expression at day 3 compared with cells in the same culture that proliferated less (Fig. 2*B*). In contrast, CD8α expression levels remained high in $E8_I^{+/+}$ CD8$^+$ T cells independent of the degree of proliferation (Fig. 2*B*). Thus, loss of CD8α expression upon T-cell activation is linked with cell proliferation.

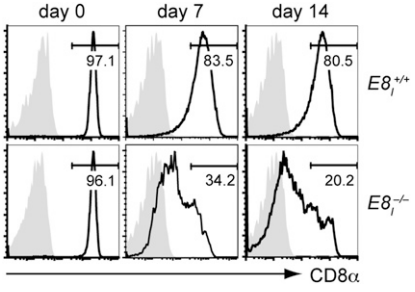

Fig. 1. Loss of CD8 expression upon activation of $E8_I^{-/-}$ CD8$^+$ T cells. $E8_I^{+/+}$ and $E8_I^{-/-}$ CD8$^+$ T cells were activated with anti-CD3/anti-CD28 and CD8α expression was assessed at the indicated time. Numbers show the percentage of cells in the respective region indicated by an interval gate. Filled histograms show CD8α expression levels on naive CD4$^+$ T cells. Data are representative of seven independent experiments.

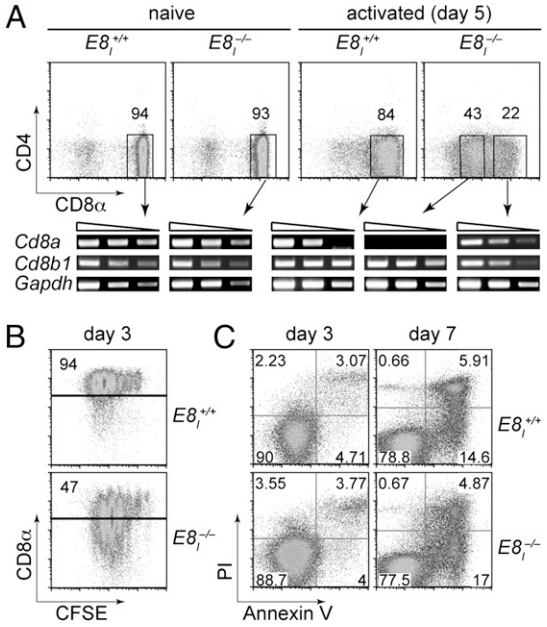

Fig. 2. $E8_I$ regulates selectively *Cd8a* gene expression. (*A*) Dot plots showing CD4 vs. CD8α expression on purified naive (*Left*) and anti-CD3/anti-CD28 activated (day 5; *Right*) $E8_I^{+/+}$ and $E8_I^{-/-}$ CD8$^+$ T cells. Rectangles indicate sorting gates for cell separation and subsequent isolation of RNA. Activated $E8_I^{-/-}$ cytotoxic T cells were sorted for CD8α$^-$ and CD8α$^+$ subsets. Semi-quantitative RT-PCR analysis shows *Cd8a* and *Cd8b1* expression in the various cell subsets. *Gapdh* expression was used as loading control. The triangle indicates fivefold dilutions of input. Data are representative of two independent experiments. (*B*) $E8_I^{+/+}$ and $E8_I^{-/-}$ CD8$^+$ T cells were labeled with CFSE and stimulated with anti-CD3/anti-CD28. Dot plots show CD8α expression vs. CFSE at day 3 after stimulation. Data are representative of two independent experiments. (*C*) $E8_I^{+/+}$ and $E8_I^{-/-}$ CD8$^+$ T cells were stimulated with anti-CD3/anti-CD28. Dot plots show Propidium Iodide (PI) uptake and Annexin V staining at day 3 and day 7 after stimulation. Data are representative of two independent experiments.

Altered Histone Marks at the *Cd8a* Gene-Promoter Region in Activated $E8_I^{-/-}$ CD8$^+$ T Cells. $E8_I$ may serve as a recruitment element for a transcription factor that is essential for maintaining CD8α expression after activation. Alternatively, but not mutually exclusive, $E8_I$ may be required to keep the *Cd8a* gene locus epigenetically "ON" to facilitate recruitment of transcription factors required for the continued transcription of the *Cd8a* gene. A similar role for $E8_I$ as a recruitment site for chromatin remodeling factors and epigenetic regulator of the *Cd8* loci has been shown already during thymocyte development (6, 7). Thus, we tested whether $E8_I$ regulates *Cd8a* gene expression at the epigenetic level. Naive and activated $E8_I^{+/+}$ and $E8_I^{-/-}$ CD8$^+$ T cells (sorted CD8α$^-$cells from activated $E8_I^{-/-}$ cells) were isolated and analyzed by ChIP experiments for differences in histone modifications at *Cd8a* and *Cd8b1* promoter regions, including the active marks histone 3 (H3), acetylation (H3Ac), and H3 lysine 4 trimethylation (H3K4me3), as well as the mark for silenced genes H3 lysine 27 trimethylation (H3K27me3) (13, 14). There were similar H3Ac levels at the *Cd8a* and *Cd8b1* promoter regions in nonactivated $E8_I^{+/+}$ and $E8_I^{-/-}$ CD8$^+$ T cells, correlating with similar expression levels of *Cd8a* and *Cd8b1* (Fig. 3, *Top*, and Fig. S2). In contrast, activated $E8_I$-deficient T cells display strongly reduced H3 acetylation levels at the *Cd8a* promoter compared with activated

IMMUNOLOGY

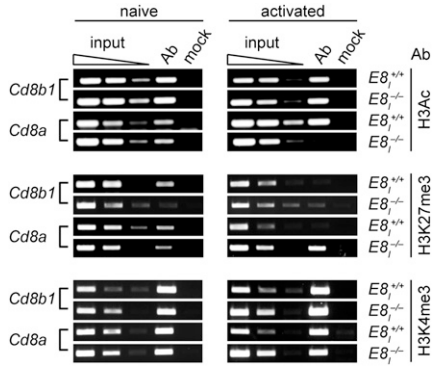

Fig. 3. Epigenetic status of the *Cd8a* promoter region is $E8I^{-/-}$ CD8$^+$ T cells. ChIP analysis of the *Cd8a* and *Cd8b1* promoter region. Chromatin from naive (*Left*) or anti-CD3/anti-CD28 activated (day 5, *Right*) $E8I^{+/+}$ and $E8I^{-/-}$ CD8$^+$ T cells (sorted CD8α-negative cells from activated $E8I^{-/-}$ cells) was immunoprecipitated with anti-H3Ac (*Top*), with anti-H3K27me3 (*Middle*), or with anti-H3K4me3 (*Bottom*) antibodies followed by PCR with primers specific for the *Cd8a* and *Cd8b1* promoter region. For the mock precipitations, no antibody was added. Input DNA was PCR amplified undiluted or at a dilution of 1:5 or 1:25 (wedges) to ensure PCR quantification in a nonsaturated amplification range. Data are representative of two independent experiments.

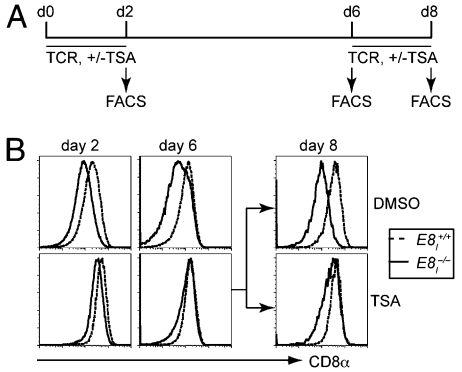

Fig. 4. Maintenance of CD8α expression in $E8I^{-/-}$ CD8$^+$ T cells in the presence of TSA. (*A*) Experimental outline: $E8I^{+/+}$ and $E8I^{-/-}$ CD8$^+$ T cells were CFSE-labeled and stimulated with anti-CD3/anti-CD28 in the presence of TSA for 2 d, and cultured for an additional 4 d without TSA as described in *Materials and Methods*. At day 6, $E8I^{+/+}$ and $E8I^{-/-}$ CD8$^+$ T cells that had been with TSA were restimulated in the presence or absence of TSA for 2 d. CD8α expression was assessed at days 2, 6, and 8. (*B*) Histograms show CD8α expression on $E8I^{+/+}$ and $E8I^{-/-}$ CD8$^+$ T cells that were treated as described in *A*. CD8α expression levels were compared on cell subsets that underwent a similar number of cell divisions (see Fig. S3*A* for gating regions). Data are representative of two independent experiments.

$E8I^{+/+}$ CD8$^+$ T cells, and H3Ac levels were readily detected at the *Cd8b1* promoter region (Fig. 3, *Top*, and Fig. S2). Reduced *Cd8a* expression correlated also with an enhanced appearance of the H3K27me3 repressive mark at the *Cd8a* gene promoter in activated $E8I^{-/-}$ CD8$^+$ T cells, but there was no difference at the *Cd8b1* promoter between $E8I^{+/+}$ and $E8I^{-/-}$ CD8$^+$ T cells (Fig. 3, *Middle*, and Fig. S2). Of note, the *Cd8a* gene promoter remained active H3K4me3 marks in activated $E8I^{-/-}$ CD8$^+$ T cells (Fig. 3, *Bottom*, and Fig. S2). This finding indicates the presence of active and repressive marks in $E8I$-deficient effector T cells that have down-regulated CD8α expression, and thus "bivalent" histone marks (15, 16), although it is possible that the marks could be on different alleles or could reflect a spectrum of histone modifications of residual CD8α expression across the population.

To test whether the impairment of CD8α expression can be overcome by inhibiting HDACs, $E8I^{+/+}$ and $E8I^{-/-}$ CD8$^+$ T cells were labeled with CFSE and activated for 2 d in the presence of TSA (Fig. 4*A*), a class I HDAC inhibitor (17). Subsequently, CD8α expression levels were compared at days 2 and 6 between $E8I^{+/+}$ and $E8I^{-/-}$ cells that underwent a similar number of cell divisions (see Fig. S3*A* for gating regions). In the presence of TSA, $E8I^{-/-}$ CD8$^+$ T cells did not down-regulate CD8α expression at day 2 (Fig. 4*B*), but in DMSO-supplemented control cultures CD8α expression was lost in the absence of $E8I$ (Fig. 4*B*). Moreover, the TSA-mediated rescue of CD8α expression was stable, because CD8α expression in $E8I^{-/-}$ T cells remained high even when the cells were cultured for an additional 4 d (day 6) in the absence of TSA (Fig. 4*B*). However, the restimulation of "TSA-rescued" $E8I$-deficient CD8$^+$ T cells led to a down-regulation of CD8α expression, indicating a requirement for $E8I$ if cells are reactivated via the TCR (Fig. 4*B*) (day 8). In contrast, restimulation of TSA-rescued $E8I^{-/-}$ CD8$^+$ T cells in the presence of TSA maintained high CD8α expression levels (Fig. 4*B*) (day 8). Of note, TSA could not rescue CD8α expression after $E8I^{-/-}$ T cells had lost already CD8α expression (Fig. S3 *B* and *C*). Taken together, these data indicate a TCR-signal–dependent epigenetic programming at the *Cd8a* gene and that $E8I$ influences the relative acetylation/deacetylation levels and thereby the expression of CD8α.

Runx-Complexes Mediated Control of CD8α Expression Is *E8I*-Dependent. The Runx transcription factor family has been implicated in the regulation of CD8 expression, and it has been shown that Runx3 binds to *Cd8* enhancer $E8I$, $E8II$, and $E8V$ in DP and CD8SP thymocytes (18). We observed that Runx/CBFβ complexes remain bound to the *Cd8ab* gene complex in activated CD8$^+$ T cells (Fig. S4*A*). In contrast, binding of Runx/CBFβ complexes to other *Cd8* enhancers, such as $E8_{II}$ and $E8_V$, was reduced in the absence of $E8I$ (Fig. S4*B*). Taken together, the ChIP data suggests a role for Runx/CBFβ complexes in the regulation of CD8α expression also upon activation.

Deletion of Runx3 in the T-cell lineage (using *Cd4-Cre* × $Runx3^{F/F}$ mice or $Runx3^{-/-}$:RAG2 chimeras) leads to a partial loss of CD4 silencing in CD8$^+$ T cells. As a consequence, a fraction of peripheral CD8$^+$ T cells expresses CD4, but CD8 expression on peripheral CD8$^+$ T cells remains unchanged in Runx3-deficient T cells (19–21). To directly test whether Runx3 is required for CD8α expression during activation, CD8$^+$ T cells from $Runx3^{F/F}$ and *Cd4-Cre* × $Runx3^{F/F}$ mice were isolated and activated with anti-CD3/anti-CD28. Similar to $E8I$-deficient CD8$^+$ T cells, Runx3-deficient CD8$^+$ T cells down-regulated CD8 expression on day 7 upon activation, leading to low-level expression of CD8 (Fig. 5*A*). To test whether other members of the Runx family, such as Runx1, which is also expressed in CD8$^+$ T cells (19–21), might compensate for loss of Runx3, we determined CD8α expression in CBFβ-deficient CD8$^+$ T cells upon activation. Deletion of CBFβ with the *Lck-Cre* deleter strain causes a severe positive-selection defect of DP thymocytes, as well as derepression of T-helper-inducing POZ/Krüppel-like factor, leading to a loss of mature CD8$^+$ T cells in the periphery. However, in *Cd4-Cre* × $Cbfb^{F/F}$ mice there is still a significant number of peripheral CD4$^+$CD8$^+$ T cells (i.e., CD8$^+$ T cells which derepress CD4). These CD4$^+$CD8$^+$ T cells develop because of the stability and therefore a low turnover of the CBFβ protein after *Cbfb* gene inactivation (21). Although $Cbfb^{F/F}$ CD8$^+$ T cells displayed normal levels of CD8α expression upon activation, *Cd4-Cre* × $Cbfb^{F/F}$ CD4$^+$CD8$^+$ T cells down-regulated CD8α (Fig. 5*B*). Because loss of CD8 expression was similar in the absence of CBFβ compared with Runx3-deficient cells, Runx1 appeared not to compensate for loss of Runx3. Moreover, Runx3-deficient or CBFβ-deficient T cells that had down-regulated CD8α expression displayed reduced H3Ac at the *Cd8a* promoter (Fig. S5*A*) and the down-regulation could be blocked with TSA treatment (Fig. S5*B*), suggesting that the $E8I$ and Runx/CBFβ

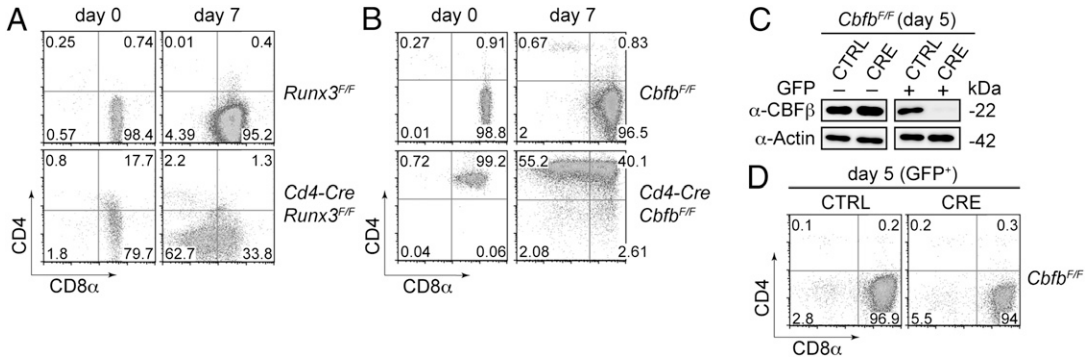

Fig. 5. Differential requirement for Runx complexes during activation of CD8$^+$ T cells and in CD8$^+$ effector T cells. (*A*) CD8$^+$ T cells from *Runx3*$^{F/F}$ and *Cd4-Cre* × *Runx3*$^{F/F}$ mice were activated with anti-CD3/CD28 and cultured for 7 d. Dot plots show CD4 vs. CD8α expression levels on day 0 (after purification) and on activated cells (day 7). Data are representative of four independent experiments. (*B*) CD8$^+$ T cells from *Cbfb*$^{F/F}$ mice and CD4$^+$CD8$^+$ T cells from *Cd4-Cre* × *Cbfb*$^{F/F}$ mice were activated and analyzed as described in *A*. Data are representative of four independent experiments. (*C*) Immunoblot analysis showing CBFβ expression at day 5 in sorted GFP$^-$ and GFP$^+$ M-SCV-pgk-GFP (CTRL) and M-SCV-CRE-pgk-GFP (CRE) transduced *Cbfb*$^{F/F}$ CD8$^+$ T cells. β-Actin was used as a loading control. Data are representative of two independent experiments. (*D*) *Cbfb*$^{F/F}$ CD8$^+$ T cells were activated with anti-CD3/CD28 and retrovirally infected at day 1 with M-SCV-pgk-GFP (CTRL) or M-SCV-CRE-pgk-GFP (CRE). CD8α expression on GFP$^+$ subsets was assessed at day 5. Gating areas are shown in Fig. S5*C*. Data are representative of four independent experiments.

deficiencies impair activation-mediated CD8α maintenance by the same mechanism.

To test whether Runx complexes are also required for the maintenance of CD8α expression in effector T cells, we conditionally deleted CBFβ with retroviral *Cre* after CD8$^+$ T-cell activation. Despite efficient deletion of CBFβ protein in CRE-transduced CD8$^+$ T cells (Fig. 5*C*), there were similar CD8α expression levels compared with mock-transduced *Cbfb*$^{F/F}$ CD8$^+$ T cells (Fig. 5*D* and Fig. S5*C*). Thus, Runx/CBFβ complexes were essential for sustained CD8 expression during activation, but CD8 expression in effector T cells was maintained in a Runx complex-independent manner.

Discussion

Our study reveals a unique transcriptional program that regulates CD8α expression during CD8$^+$ effector T-cell differentiation in a manner distinct from naive T cells, and demonstrates an essential role for *Cd8* enhancer $E8_I$ and Runx3/CBFβ in this regulatory circuit. It has been shown that $E8_I^{-/-}$ CD8$^+$ T cells have normal expression levels of CD8 on DP thymocytes and peripheral CD8$^+$ T cells, demonstrating that $E8_I$ is dispensable for the establishment of CD8 expression in thymocytes and in naive T cells (4). However, $E8_I$,$E8_{II}$-deficient mice display variegated expression of CD8 in DP thymocytes (6), showing that the combined activity of $E8_I$ and $E8_{II}$ is necessary for the activation of the *Cd8ab* gene complex in DP thymocytes. Our current study revealed a unique and non-redundant role of $E8_I$ in maintaining *Cd8a* gene expression during CD8$^+$ T-cell activation. There are two important differences in how $E8_I$ is used at different developmental stages. First, the function of $E8_I$ in DP thymocytes and naive CD8$^+$ T cells is only revealed in the absence of $E8_{II}$, but in activated CD8$^+$ T cells deletion of $E8_I$ alone is sufficient to alter CD8 expression. Second, in activated CD8$^+$ T cells $E8_I$ controls only *Cd8a* but not *Cd8b1* gene expression, but in $E8_I$,$E8_{II}$-deficient DP thymocytes both *Cd8* genes are variegated (6). There are several potential binding sites of the insulator protein CTCF at the *Cd8ab* gene complex (22). It will be interesting in future studies to investigate whether CTCF recruitment to the *Cd8ab* gene complex leads to differential regulation of *Cd8a* and *Cd8b1* gene expression in activated T cells.

Another important finding of our study is that *Cd8a* gene expression in CD8$^+$ effector T cells might be maintained independently of factors required for high-level expression of CD8α during CD8$^+$ T-cell activation. Our data indicate that Runx transcription factors contribute to the regulation of *Cd8a* gene expression upon activation. Runx/CBFβ complexes have been shown to regulate *Cd4* gene silencing (20). In addition, they have been implicated in the activation of CD8 expression during T-cell development, although the molecular details of how Runx factors regulate CD8 expression are not fully understood (18). Among the Runx factors, distal promoter-derived Runx3 is the dominant form expressed in CD8$^+$ T cells (19, 23). However, although conditional deletion of Runx3 in the T-cell lineage leads to a partial loss of CD4 silencing in CD8$^+$ T cells, CD8 expression on peripheral naive CD8$^+$ T cells is not altered in Runx3-deficient T cells, possibly because of the compensatory function of Runx1 (19, 21). This finding clearly demonstrates that Runx3 is not essential for CD8 expression in naive T cells. In contrast, we found that Runx3 and CBFβ are indispensable for high-level expression of CD8 during CD8$^+$ T-cell activation. Because Runx3 expression levels were not changed in activated $E8_I^{-/-}$ CD8$^+$ T cells that have down-regulated CD8α expression (Fig. S5*D*), it is likely that the loss of CD8α expression in the absence of $E8_I$ is caused by impaired recruitment of Runx/CBFβ complexes to the *Cd8ab* gene complex. This theory is supported by the observation that there is less Runx/CBFβ recruited to other *Cd8* enhancers in the absence of $E8_I$ (Fig. S4*B*). Interestingly, Runx3/CBFβ recruitment appears to be specific for the CD8 lineage because in Th1 cells (that express Runx/CBFβ complexes) CBFβ did not bind the *Cd8* loci (Fig. S4*C*). In contrast to the initial T-cell activation during CD8$^+$ T-cell differentiation, deletion of CBFβ after T-cell activation (using retroviral Cre-mediated deletion) did not result in loss of CD8α expression. This finding indicates that once *Cd8a* gene expression is established in CD8$^+$ effector T cells, CD8 expression is maintained independently of Runx/CBFβ complexes and potentially also of *Cd8* enhancer $E8_I$.

It has been shown that CD8 expression is also regulated at an epigenetic level during thymocyte development (7). Our study also indicates a role for epigenetic regulatory mechanisms during the activation of CD8$^+$ T cells. We observed that the down-regulation of the *Cd8a* gene in E8$_I$-deficient cells correlated with reduced H3 acetylation. In addition, in $E8_I^{-/-}$ cells that had down-regulated CD8α expression, the *Cd8a* gene promoter contained both active histone H3K4me3 and repressive histone H3K27me3 marks. This finding indicates bivalent chromatin modifications resulting in a silenced state of the *Cd8a* gene (15, 16), although it remains possible that the active and repressive marks are on different alleles. Remarkably, the addition of the HDAC inhibitor TSA facilitated CD8α expression upon

activation, although H3K27me3 marks at the *Cd8a* promoter are still present in the absence of $E8_I$ (Fig. S6). This finding indicates the dominant role of histone acetylation marks over H3K27me3 marks in *Cd8a* gene regulation upon activation. Because TSA-treated $E8_I$-deficient CD8$^+$ T cells maintained CD8α expression even when cultured for additional 4 to 5 d, it appears that $E8_I$ is essential only at the onset of activation. In line with this hypothesis, we found that CD8 expression was not restored by TSA treatment if cells had already down-regulated CD8. Taken together, these data indicate that recruitment of a histone acetyltransferases (HAT) to the *Cd8a* gene locus is necessary for the maintenance of CD8 expression during T-cell activation, although one cannot formally exclude that the effect of TSA is indirect (e.g., by altering the expression of a chromatin modifying factor). However, restimulated TSA-rescued $E8_I$-deficient CD8$^+$ T cells down-regulated CD8α expression, suggesting that TCR triggering might lead to the induction of an $E8_I$-dependent transcriptional "maintenance" program for CD8α expression. One possible mechanistic explanation for the loss of CD8α expression upon TCR triggering in $E8_I$-deficient CD8$^+$ T cells is that Runx/CBFβ complexes facilitate the recruitment of HATs necessary to keep the *Cd8a* gene promoter in an open configuration. A similar role for Runx proteins in the recruitment of HATs has been described in Runx1-dependent transcription during myeloid differentiation (24). Thus, in the absence of Runx/CBFβ complexes or $E8_I$, regulatory complexes containing HAT activity and potentially other epigenetic modifying factors cannot assemble properly at the *Cd8a* gene locus, and as a consequence *Cd8a* gene expression is lost upon activation. Although the HAT p300 appears to be not recruited to the *Cd8ab* gene complex (Fig. S7), future studies including ChIP-seq approaches will help to reveal which members of the HAT and HDAC family are recruited to the *Cd8a* gene locus during T-cell activation.

The observation that $E8_I$ is required for *Cd8a* gene expression upon activation is in part reminiscent to the function of the proximal *Cd4* enhancer (*E4p*) in T cells (25). *E4p* has been shown to be essential for CD4 expression in DP thymocytes, and *E4p* is required to establish an epigenetic pattern at the *Cd4* locus that allows the propagation of CD4 expression in dividing mature CD4$^+$ T cells, even in the absence of E4p (26). $E8_I$ appears to have a very similar function at the *Cd8a* locus, with respect to its role in the establishment of CD8 expression during TCR stimulation. Thus, both *Cd4* and *Cd8a* gene expression is regulated by *cis*-regulatory elements that mediate the generation of active histone marks at the respective gene loci to maintain coreceptor expression upon activation.

Our observations raise the interesting question as to why CD8$^+$ effector T cells regulate CD8α expression differently when compared with naive CD8$^+$ T cells. It is conceivable that the switch in the regulation of CD8α expression upon T-cell activation is part of an effector program that is induced during cytotoxic T-lymphocyte (CTL) differentiation. Preliminary results indicate that $E8_I$-deficient CD8$^+$ T cells display reduced CTL activity, most likely because of reduced CD8α expression, despite normal expression of activation markers and CTL effector molecules (Fig. S8). It has been shown that CD8 expression levels can be modulated in vivo upon Vaccinia virus infection (27), and certain cytokines, such as IL-4, can lead to the down-regulation of CD8 expression (28). It will be interesting to investigate whether there is a (patho)physiological condition under which wild-type CD8$^+$ T cells down-regulate CD8α expression via modulation of $E8_I$ enhancer (or Runx/CBFβ activity).

Finally, our finding that $E8_I$ is essential for *Cd8a* gene expression in activated CD8$^+$ T cells may also provide an alternative explanation for the observation that $E8_I$-deficient mice show impaired expression of CD8αα homodimers on γδTCR as well as on αβTCR IEL of the gut (4). It has been shown that IEL share characteristics of partially activated lymphocytes (29, 30). Thus, it is tempting to speculate that CD8αα expression on $E8_I^{-/-}$ IELs is severely impaired because the cells are at least partially activated, and not because of a distinct mode of *Cd8a* gene regulation in IELs compared with conventional naive CD8$^+$ T cells.

Taken together, our data reveal a unique and unexpected role for the *Cd8* enhancer $E8_I$ and Runx/CBFβ complexes in the regulation and maintenance of *Cd8a* gene expression in CD8$^+$ effector T cells, and indicate different mechanisms of how CD8 expression is regulated in naive and effector T cells.

Materials and Methods

Mice. $E8_I$, $E8_{I\!I}$, $E8_I$,$E8_{I\!I}$, and $E8_{I\!I}$,$E8_{I\!I\!I}$-deficient mice have been previously described (4, 6, 9). OT-I mice were kindly provided by Maria Sibilia (Medical University of Vienna, Vienna, Austria). All mice were used between 6 and 12 wk of age. All mice were bred and maintained in the animal facility of the Medical University of Vienna, and animal experiments were approved by the animal committee of the Medical University of Vienna and by Federal Ministry for Science and Research.

Antibodies and Flow Cytometry. The antibodies used for the analysis are described in *SI Materials and Methods*. Samples were acquired on FACSCalibur and LSRII (BD Biosciences) and data were analyzed with FlowJo software (Treestar).

Isolation of Splenic T Cells and CFSE-Labeling of T Cells. The purification protocols of the T cells are described in detail in *SI Materials and Methods*. For some experiments cells were labeled with CFSE, as described in *SI Materials and Methods*.

CD8$^+$ T-Cell Stimulation. Purified CD8$^+$ T cells were stimulated as described in *SI Materials and Methods*.

cDNA Synthesis, Quantitative Real-Time PCR, and Semiquantitative RT-PCR. Total RNA was isolated from sorted splenic naive or activated (day 5) CD8$^+$ T cells (5×10^5 cells) using TRI reagent (Sigma) and cDNA synthesis was performed as described in *SI Materials and Methods*.

ChIP Assays. Chromatin of naive or activated CD8$^+$ T cells was precipitated as described in *SI Materials and Methods*. The primers used for PCR are shown in Table S1.

TSA Inhibitor Experiments. Purified CFSE-labeled CD8$^+$ splenic T cells were stimulated with plate-bound anti-CD3ε (1 μg/mL) and anti-CD28 (2 μg/mL) on 48-well plates (0.5×10^6 cells per well) in 500-μL T-cell medium (supplemented with 20 U/mL rhIL-2) in the presence of trichostatin A (16-nM final concentration) or DMSO as carrier for the control group. Two days later, cells were washed once with PBS to remove TSA, and CD8$^+$ T cells were split 1:2 and cultured for additional 4 d in the presence of 100 U/mL rhIL-2. After a total of 6 d in culture, the cells were restimulated on 48-well plates with plate-bound anti-CD3ε (1 μg/mL) and anti-CD28 (2 μg/mL) for an additional 2 d, either in the presence or absence of TSA (or DMSO). In one experimental approach, CD8$^+$ T cells were stimulated with anti-CD3/CD28 for 2 d, split 1:2 and cultured for an additional 4 d in the presence of 100 U/mL rhIL-2. At day 6, cells were treated with TSA or DMSO for 2 d. The cells were analyzed by flow cytometry at the indicated time points.

Retroviral Infection of CD8$^+$ T Cells. Retroviral infection was performed as described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dagmar Stoiber-Sakaguchi, Olivia Simma, and Winfried Pickl (Medical University of Vienna) for technical help. This study was supported by the Austrian Science Fund (research Grants P16708, P19930, and I698), and by the START Program (Project Y-163) of the Austrian Ministry of Science and Research (BM:WF) (to W.E.); a PhD fellowship of the High Education Commission of Pakistan (to H.H.); a Leukemia and Lymphoma Society Special Fellowship (to T.E.); and a RIKEN Research Center for Allergy and Immunology International collaboration award (to I.T. and W.E.).

1. Kioussis D, Ellmeier W (2002) Chromatin and CD4, CD8A and CD8B gene expression during thymic differentiation. *Nat Rev Immunol* 2:909–919.
2. Ellmeier W, Sunshine MJ, Losos K, Hatam F, Littman DR (1997) An enhancer that directs lineage-specific expression of CD8 in positively selected thymocytes and mature T cells. *Immunity* 7:537–547.
3. Hostert A, et al. (1997) A region in the CD8 gene locus that directs expression to the mature CD8 T cell subset in transgenic mice. *Immunity* 7:525–536.
4. Ellmeier W, Sunshine MJ, Losos K, Littman DR (1998) Multiple developmental stage-specific enhancers regulate CD8 expression in developing thymocytes and in thymus-independent T cells. *Immunity* 9:485–496.
5. Hostert A, et al. (1998) Hierarchical interactions of control elements determine CD8alpha gene expression in subsets of thymocytes and peripheral T cells. *Immunity* 9:497–508.
6. Ellmeier W, Sunshine MJ, Maschek R, Littman DR (2002) Combined deletion of CD8 locus cis-regulatory elements affects initiation but not maintenance of CD8 expression. *Immunity* 16:623–634.
7. Bilic I, et al. (2006) Negative regulation of CD8 expression via Cd8 enhancer-mediated recruitment of the zinc finger protein MAZR. *Nat Immunol* 7:392–400.
8. Garefalaki A, et al. (2002) Variegated expression of CD8 alpha resulting from in situ deletion of regulatory sequences. *Immunity* 16:635–647.
9. Feik N, et al. (2005) Functional and molecular analysis of the double-positive stage-specific CD8 enhancer E8III during thymocyte development. *J Immunol* 174:1513–1524.
10. Madakamutil LT, et al. (2004) CD8alphaalpha-mediated survival and differentiation of CD8 memory T cell precursors. *Science* 304:590–593.
11. Chandele A, Kaech SM (2005) Cutting edge: memory CD8 T cell maturation occurs independently of CD8alphaalpha. *J Immunol* 175:5619–5623.
12. Zhong W, Reinherz EL (2005) CD8 alpha alpha homodimer expression and role in CD8 T cell memory generation during influenza virus A infection in mice. *Eur J Immunol* 35:3103–3110.
13. Jenuwein T, Allis CD (2001) Translating the histone code. *Science* 293:1074–1080.
14. Ruthenburg AJ, Li H, Patel DJ, Allis CD (2007) Multivalent engagement of chromatin modifications by linked binding modules. *Nat Rev Mol Cell Biol* 8:983–994.
15. Azuara V, et al. (2006) Chromatin signatures of pluripotent cell lines. *Nat Cell Biol* 8: 532–538.
16. Bernstein BE, et al. (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* 125:315–326.
17. Yoshida M, Kijima M, Akita M, Beppu T (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *J Biol Chem* 265:17174–17179.
18. Sato T, et al. (2005) Dual functions of Runx proteins for reactivating CD8 and silencing CD4 at the commitment process into CD8 thymocytes. *Immunity* 22:317–328.
19. Egawa T, Tillman RE, Naoe Y, Taniuchi I, Littman DR (2007) The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. *J Exp Med* 204:1945–1957.
20. Taniuchi I, et al. (2002) Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell* 111:621–633.
21. Naoe Y, et al. (2007) Repression of interleukin-4 in T helper type 1 cells by Runx/Cbf beta binding to the Il4 silencer. *J Exp Med* 204:1749–1755.
22. Parelho V, et al. (2008) Cohesins functionally associate with CTCF on mammalian chromosome arms. *Cell* 132:422–433.
23. Egawa T, Littman DR (2008) ThPOK acts late in specification of the helper T cell lineage and suppresses Runx-mediated commitment to the cytotoxic T cell lineage. *Nat Immunol* 9:1131–1139.
24. Kitabayashi I, Yokoyama A, Shimizu K, Ohki M (1998) Interaction and functional cooperation of the leukemia-associated factors AML1 and p300 in myeloid cell differentiation. *EMBO J* 17:2994–3004.
25. Taniuchi I, Ellmeier W (2011) Transcriptional and epigenetic regulation of CD4/CD8 lineage choice. *Adv Immunol* 110:71–110.
26. Chong MM, et al. (2010) Epigenetic propagation of CD4 expression is established by the Cd4 proximal enhancer in helper T cells. *Genes Dev* 24:659–669.
27. Xiao Z, Mescher MF, Jameson SC (2007) Detuning CD8 T cells: Down-regulation of CD8 expression, tetramer binding, and response during CTL activation. *J Exp Med* 204: 2667–2677.
28. Kienzle N, et al. (2005) Progressive differentiation and commitment of CD8+ T cells to a poorly cytolytic CD8low phenotype in the presence of IL-4. *J Immunol* 174: 2021–2029.
29. Montufar-Solis D, Garza T, Klein JR (2007) T-cell activation in the intestinal mucosa. *Immunol Rev* 215:189–201.
30. van Wijk F, Cheroutre H (2009) Intestinal T cells: Facing the mucosal immune dilemma with synergy and diversity. *Semin Immunol* 21(3):130–138.