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Interplay between Chromatin Remodeling and Epigenetic Changes during Lineage-Specific Commitment to Granzyme B Expression

Torsten Juelich,* Elissa Sutcliffe,* Alice Denton,‡ Yiqing He,* Peter C. Doherty,‡ Christopher Parish,* Steven J. Turner,‡* David Tremethick,† and Sudha Rao2‡

The role of chromatin remodeling and histone posttranslational modifications and how they are integrated to control gene expression during the acquisition of cell-specific functions is poorly understood. We show here that following in vitro activation of CD4+/CD8+ T lymphocytes, both cell types show rapid histone H3 loss at the granzyme B (gzmB) proximal promoter region. However, despite the gzmB proximal promoter being remodeled in both T cell subsets, only CD8+ T cells express high levels of gzmB and display a distinct pattern of key epigenetic marks, notably differential H3 acetylation and methylation. These data suggest that for high levels of transcription to occur a distinct set of histone modifications needs to be established in addition to histone loss at the proximal promoter of gzmB. * The Journal of Immunology, 2009, 183: 7063–7072.

Upon primary activation, naive T lymphocytes undergo a program of proliferation and differentiation that results in the acquisition of lineage-specific T cell functions (1, 2). Naive CD4+ T cells are capable of differentiating into separate Th (Th) lineages characterized by specific patterns of cytokine production (2). For example, the Th1 lineage is characterized by IFN-γ production, the Th17 lineage by IL-17 and IL-21 secretion, and the Th2 lineage by IL-4, IL-5, and IL-13 production. There is increasing evidence that Th subset-specific functions are regulated and maintained by epigenetic mechanisms (3). Thus, compared with naive T cells, Th1 and Th2 cells exhibit significant alterations in both chromatin structure (4) and biochemical modifications within the IFN-γ and IL-4 loci (4, 5). For example, under sustained Th1 differentiating conditions, acetylation of the H3 and H4 histones is enriched within the IFN-γ locus and diminished within the IL-4 locus (5). In contrast, under continued Th2 differentiating conditions, this pattern is reversed, with enrichment of H3/H4 acetylation being observed within the IL-4, and not the IFN-γ locus (5). These data suggest a model of naive T cell differentiation where Th lineage-specific function is largely determined by the epigenetic marks within specific gene loci that control gene expression.

Following primary activation, naive Ag-specific CD8+ T cells differentiate into CTL, a process which results in extensive changes in transcriptional activity, signature gene expression, and CTL-specific functions (1). Although CTL can secrete a range of cytokines, such as IFN-γ, TNF-α, and IL-2 (6), a predominant function of CTL is lysis of virally infected cells or tumor cells (7). Granzyme B (gzmB)3 is known to play an integral part in the induction of programmed cell death in target cells, where it activates a cascade of events, ultimately leading to apoptosis of these cells and clearance of intracellular pathogens (8). The gzmB pathway is normally associated with NK cell and CD8+ T cell responses, but not usually with CD4+ T cell function (9). Intriguingly, early experiments indicated that granzymes can sometimes be expressed in both murine CD4+ and CD8+ T lymphocytes, albeit at differing levels, depending on the conditions of stimulation and developmental stages of these T cell subsets (10). Recently, gzmB was shown to possess a second function besides its role in target cell killing, being possibly involved in regulating the homeostasis of activated Th2 T cells (11). Specific transcription factors (TFs) required for the regulation of gzmB in NK cells and CD8+ T cells have been studied in some detail earlier (12–14). It has also been established that the proximal promoter region of up to 208 bp upstream of the transcriptional start site (TSS) is both necessary and sufficient for highly inducible gzmB expression. This region contains canonical TF binding sites for AP-1, Ikaros, CBFI family members, CREB, NFAT, as well as ETS proteins (reviewed in Ref. 15).

Despite these considerable information available about the TFs that control gzmB expression, surprisingly little is known about the consequences of chromatin-dependent processes involved in the transcriptional regulation of gzmB (16). In the nucleus of eukaryotic cells, gene transcription takes place in the context of a multicomponent DNA/protein structure known as chromatin. The fundamental unit of chromatin is the nucleosome, which is composed of an octamer of histone proteins enclosed in two helical turns of DNA (17). There are three key mechanisms by which chromatin structure can be modulated. First, chromatin remodeling where a promoter-bound histone octamer is removed or repositioned by ATP-dependent chromatin

3 Abbreviations used in this paper: gzmB, granzyme B; gzmN, granzyme N; TF, transcription factor; Pol II, polymerase II; ChIP, chromatin immunoprecipitation; CHART, chromatin accessibility and real time; Ct, threshold cycle; Mnase, micrococcal nuclease; H3K9/acet, H3K9 acetylation; TSS, transcriptional start site; PFM, posttranscriptional modification E-Pol II.

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remodelers (17–21). Second, changes in histone composition by interchanging core histones with histone variants. Third, epigenetic modification of the N termini of the histone tails mediated by enzymes recruited to gene regulatory regions. Posttranscriptional modification E-Pol II (PTMs) can function either by directly changing chromatin structure or via recruitment of other factors (22–24).

From recent genome-wide studies in a broad range of different species, a picture is emerging about the complex interplay of histone modifications, nucleosome position, and recruitment of the general transcriptional machinery in regulating gene expression. Importantly, these studies indicate that transcriptional activation or repression of a gene is determined by a combination of distinct epigenetic events rather than by a single nucleosome modification (25–28) (25–27), as has often been proposed in previous studies. Despite this wealth of knowledge, the complex interplay between chromatin remodeling and the modification of histone tails is poorly understood with regard to their relative importance in the gene activation process.

Recently, a study has examined the epigenetic changes at the human granzyme B (gzmB) regulatory regions occurring in memory CD8+ T cells, correlating acetylation of H3K9 with an increase in gzmB expression in activated memory T cells compared with activated naive T cells (16). In this study, we show that major histone H3 loss occurs from the gzmB proximal promoter during the in vitro activation of both naive CD4+ and CD8+ T cells. However, despite this histone loss in T cell subsets, polymerase II (Pol II) recruitment and high levels of chromatin accessibility and corresponding gzmB transcription only occur in activated CD8+ T cells. Furthermore, to uncover the chromatin regulatory signatures associated with this efficient transcriptional activation, we performed a detailed analysis of specific histone modifications before and after in vitro stimulation. Specifically, we found that H3K9 acetylation (H3K9ac) is directly and strongly correlated with high levels of gzmB transcription in CD8+ T cells. Taken together, this work proposes a new step in the transcriptional activation process, a “prepoised” step, which involves substantial chromatin remodeling resulting in the loss of histones in both CD4+ and CD8+ T cells. We propose that it is the efficient recruitment of RNA Pol II and the acquisition of specific PTMs that drive high levels of gzmB transcription only in activated CD8+ T cells.

Materials and Methods

Primary T cell preparation and activation

All mice were maintained in a pathogen-free environment in barrier facilities. Spleens were isolated from C57BL/6 mice (4–6 wk old). The CD4 and CD8 cells were purified using MACS CD4 (LT34) beads and MACS CD8 beads, respectively, according to the manufacturer’s guidelines (Miltenyi Biotec). The cells were subsequently stained and analyzed by flow cytometry with T cell populations shown to be 90–95% pure using Abs against CD4 T cells and CD8 T cells.

To activate freshly isolated cells, 6-well plates were coated with anti-CD3ε (553058, 10 μg/ml; BD Pharmingen) for 24 h at 4°C and then washed three times with cold PBS before cells were added. Anti-CD28 Ab (553295; BD Pharmingen) was added directly to the cells at a concentration of 5 μg/ml before activation.

RNA extraction and cDNA synthesis

Total RNA was extracted from stimulated and unstimulated T cells using RNA TriZol reagent (Molecular Research Centre). The DNase I-treated total RNA was reverse transcribed using 50 U of Superscript II reverse transcriptase (Invitrogen) as detailed in the manufacturer’s guidelines.

SYBR Green PCR amplification and TaqMan gene expression assay

SYBR Green RealTime PCR for gDNA (chromatin immunoprecipitation (ChIP)/chromatin accessibility and real-time (CHART) assays) were performed in 96- or 384-well plates (PerkinElmer) with an Applied Biosystems PRISM 7900 Real-Time System (PerkinElmer/PE Biosystems) at the Biomolecular Resource Facility (John Curtin School of Medical Research, Australian National University, Canberra, Australia). To correlate the threshold cycle (Ct) values from the cDNA amplification plots to fold increases in starting material, the ΔΔCt method was applied, with addition of conversion into arbitrary copy numbers, using the formula: arbitrary copies = 2^{ΔΔCt}, after verification that amplification efficiencies were optimal and equal between primers used in one experiment.

For granzyme mRNA expression experiments, TaqMan gene expression assays were purchased and performed according to the manufacturer’s guidelines (Applied Biosystems).

CHART assay

The CHART assay was performed as described originally (24). For each batch of micrococcal nuclease (Mnase), digestion of gDNA was optimized as shown in supplemental Fig. S3. All samples were digested equally, a process that was verified by the bioanalyzer results obtained for each sample on multiple occasions (supplemental Fig. S3). Since there were no differences in the actual digestion efficiencies, the differences observed can only be explained by changes in nucleosomal positioning across the specific regions being measured. For Mnase accessibility time-course experiments, the data were calculated as a percentage of the accessibility observed in the unstimulated digested DNA sample, which was set to 100%. For basal accessibility studies, the data were graphed as percent genomic DNA signal observed in the undigested sample. Regarding the actual values, they are not calculated according to an end point of digestion (which was set at 100%), but against the accessibility of each region being examined in the unstimulated T cell samples.

ChIP assay

ChIP analysis was performed according to the standard Upstate Biotechnology ChIP protocol. After sonication, samples were precleared with 60 μl of salmon sperm DNA-protein A-agarose (Upstate Biotechnology) and subsequently incubated overnight with rotation at 4°C with one of the following Abs per 106 cells starting material: 20 μg of anti-histone H3 (Abcam ab7919), 5 μg of anti-H3K4-trimethyl (Upstate Biotechnology 06-942), 7 μl of anti-H3K3-dimethyl (Upstate Biotechnology 07-030), 5 μl of anti-H3K4-trimethyl (Upstate Biotechnology 07-473), 5 μg of anti-H3K9-trimethyl (Upstate Biotechnology 07-442), 4 μg of anti-TFIIId (Santa Cruz Biotechnology sc-204), and 4 μg of anti-Pol II (Abcam ab187) or no Ab as a specificity control. Briefly, Ct values from the PCR amplification plots were converted to arbitrary copy numbers. Sample data were then normalized to the corresponding total input before fold change above the average value of the control samples (without Ab) was calculated to give the ChIP enrichment ratio as previously described (29). ChIP enrichment ratios greater than 2 are considered to be significant binding above background. All ChIP assays were performed in duplicate or triplicates as indicated, and all PCRs were run in duplicate.

Flow cytometry

Naive and stimulated CD4+ and CD8+ T cells were harvested and washed with 0.1% BSA-containing PBS. For cell surface staining, cells were incubated with PBS and various Abs for 30 min on ice. The Abs used were PE-CD3, FITC-CD8, and allophycocyanin/Cy7-CD4. Cells were analyzed on a FACSCalibur (BD Biosciences).

Confocal microscopy

Primary mouse CD4+ and CD8+ T cells were isolated and cultured in MLC medium supplemented with 10% FCS and antibiotics. Cells were maintained at 37°C and in 5% CO₂. Cells were stimulated with anti-CD3ε (10 μg/ml; BD Pharmingen) and anti-CD28 Abs (5 μg/ml; BD Pharmingen) for 3 days. After rinsing in PBS briefly, cells were fixed with 2% formaldehyde in PBS for 10 min at room temperature. After washing cells in PBS three times, they were transferred to 0.1% poly-L-lysine (Sigma-Aldrich)-coated round coverslips (ø 13 mm). Coverslips were incubated with 1% Triton X-100 for 15 min and blocking solution (1% BSA and 0.1% Tween 20 in PBS) for 45 min at room temperature. Cells were incubated with FITC-conjugated anti-mouse gzmB-specific Ab as described previously (1/50; ebioscience 11-8822-80 (30)) for 60 min at 37°C and washed repeatedly with blocking solution. Nuclei were counterstained with 0.1 μg/ml 4',6-diamidino-2-phenylindole for 7 min and washed with blocking solution once and then twice with distilled water. Coverslips with

4 The online version of this article contains supplemental material.
cells were added with 5 μl of mounting medium (H-1000; Vector Laboratories) and sealed with nail polish. Immunofluorescent images were collected using a laser scanning microscopy (Olympus IX71). Merged images were generated in Adobe Photoshop CS.

**Tetramer and protein staining**

Purified T cells were stained with either anti-CD8-FITC, anti-CD3-PE, or anti-CD4-allophycocyanin-Cy7 (BD Pharmingen). Unstimulated and stimulated cells were stained as above but also fixed and permeabilized using a BD Pharmingen Cytofix/Cytoperm kit. Intracellular gzmB was detected using anti-human gzmB-allophycocyanin (clone GB12; Caltag Laboratories). The flow cytometry analysis utilized a FACScalibur (BD Immunocytometry Systems) and CellQuest software. For isolation of influenza A virus-specific CTL, splenic populations were stained with D^*NP366-PE or D^*PA224-allophycocyanin tetramers for 1 h in sort buffer (PBS/0.1% BSA). Cells were washed twice with sort buffer and stained with anti-CD8- FITC for 30 min on ice. The cells were then washed, resuspended in sort buffer, and transferred to polypropylene tubes for sorting. Isolation of CD8^* D^*NP366^+ and CD8^* D^*PA224^+ cells was conducted using a MoFlo high-speed cell sorter and summit software (DakoCytomation).

**Results**

**Comparison of gzmB expression after in vitro stimulation of CD4^* and CD8^* T cells**

The cellular differentiation pathways as well as the effector functions of committed CD4^+ and CD8^+ subsets have been studied in great detail (31), thus making them an ideal system to study chromatin-based mechanisms associated with lineage commitment. For the full manifestation of CTL activity by CD8^* T cells, expression of the effector function gene gzmB is generally regarded as crucial (7–9). We first assessed the gzmB expression patterns within purified CD4^+ and CD8^+ T cell populations after in vitro stimulation with Abs directed against CD3 and CD28. As expected, both nonstimulated CD4^+ and CD8^+ T cells exhibited similar low amounts of gzmB mRNA (supplemental Fig. S1b) and no protein expression (Fig. 1, a and b) as measured by quantitative PCR and immunofluorescence, respectively. Interestingly, increased levels of gzmB mRNA were first detected in both CD4^+ and CD8^+ T cells 12 h after initial activation (Fig. 1a). gzmB mRNA levels continued to increase up to 48 h after stimulation, with levels in the CD8^+ T cells increasing ~1200-fold compared with nonstimulated cells (Fig. 1a). Surprisingly, CD4^+ T cells also showed an ~400-fold increase in gzmB mRNA content at the 48-h time point compared with nonstimulated cells. This increase was significantly smaller than in activated CD8^+ T cells (Fig. 1a) and cannot be attributed to any differences in mRNA recovery due to normalization with the L32 control gene (supplemental Fig. S1a). This suggests that CD8^+ T cells possess specific regulatory mechanisms for high-level gzmB mRNA expression, either at the single-cell or cell population level.

To examine gzmB protein expression at the cellular level, flow cytometry (Fig. 1b) and immunofluorescence microscopy (Fig. 1c) studies were performed. gzmB mRNA production was mainly associated with high levels of protein expression, with the majority (~90%) of 48-h activated CD8^+ T cells producing gzmB, but only 10% of CD4^+ T cells expressing gzmB at levels detectable above the background autofluorescence of the cells (Fig. 1b). Close examination of the anti-gzmB staining of the 48-h activated CD4^+ T cells revealed, however, a small fluorescence shift in the entire T cell population, suggesting that low-level production of gzmB occurs in all of the activated CD4^+ T cells. Taken together, these data suggest that gzmB expression is likely to be regulated at the transcriptional level in CD8^+ T cells, which is the focus of the present study.

**Mnase accessibility changes across the gzmB proximal promoter in CD4^+ and CD8^+ T cells**

Chromatin accessibility is now well established as playing a crucial role in transcriptional regulation of inducible genes (18, 32–35). To investigate changes in the chromatin structure of the gzmB gene in activated CD4^+ and CD8^+ T cells, a Mnase-based CHART-PCR assay was performed (33). Mnase digestion is an ideal tool to measure chromatin accessibility, since it cuts preferentially in regions of DNA that are nucleosome free. Primer sets were designed across the proximal gzmB promoter, spanning a region of ~400 bp upstream of the TSS (Fig. 2a). Initial basal chromatin accessibility measurements revealed that there was comparable low-level (~10–15%) accessibility of the gzmB...
proximal promoter in both resting CD4+ and CD8+ T cells (supplemental Fig. S1c).

CD4+ and CD8+ T cells were then stimulated with anti-CD3 and anti-CD28 Abs and harvested at different time points to establish the kinetics of chromatin accessibility. Following stimulation, increased DNA accessibility was observed in both the CD4+ and CD8+ T cells across the entire 400-bp region of the gzmB proximal promoter (Fig. 2b). Closer examination revealed that regions B and C, corresponding to the regions within 300 bp of the TSS, exhibited the greatest relative increase in accessibility in both CD4+ and CD8+ T cells (Fig. 2b). Changes in chromatin structure were observed as early as 12 h after activation (Fig. 2b), correlating with the early onset of transcriptional activity (Fig. 1a). However, there were differences in both the extent and rapidity of chromatin accessibility between the CD4+ and CD8+ T cells (Fig. 2b). Maximum chromatin accessibility was observed 24–48 h after activation of the CD8+ T cells, whereas changes in chromatin structure within the gzmB promoter of activated CD4+ T cells was highest at 72 h after stimulation. Although, the same regions of the proximal gzmB promoter within both CD4+ and CD8+ T cells were altered following activation, the overall extent of chromatin accessibility was 3- to 4-fold higher in activated CD8+ T cells compared with CD4+ T cells. This difference in chromatin accessibility closely parallels the differential mRNA levels observed in CD4+ and CD8+ T cells.

To determine whether the extent of chromatin accessibility across the proximal gzmB promoter was dependent on TCR-mediated signal strength, naive CD8+ T cells were stimulated, as described earlier, with increasing concentrations of anti-CD3 Ab (0.5–10 μg/ml) in the presence of a constant concentration of anti-CD28 Ab (5 μg/ml). Increasing levels of anti-CD3-mediated TCR stimulation resulted in increasing levels of chromatin accessibility within the proximal gzmB promoter (Fig. 2c). As observed earlier, the greatest overall increase in accessibility was observed in the −34 bp to −300 bp region of the proximal promoter, with no significant change in accessibility being observed in the distal region of the proximal promoter (−300 bp to −420 bp). These results suggest that the degree of chromatin accessibility of the gzmB promoter depends upon TCR signaling strength.

**Loss of histone H3 from the gzmB promoter following activation of CD4+ and CD8+ T cells**

It has been shown for both yeast (36) and mammalian genes (37) that loss of the core histone H3 at the proximal promoter region of genes is often associated with chromatin remodeling and transcriptional activation. To examine whether histones are lost from the gzmB promoter during activation of naive CD4+ and CD8+ T cells, a ChIP assay was performed using a specific Ab raised against the C-terminal region of histone H3. As shown in Fig. 3a, nonstimulated CD4+ and CD8+ T cells showed comparable levels of H3 enrichment using three primer sets which span the 420 bp to 400-bp region of the proximal promoter, with no significant change in DNA accessibility being observed in the distal region of the proximal promoter (−34 bp to −300 bp). Changes in chromatin accessibility closely parallels the differential mRNA levels observed in CD4+ and CD8+ T cells.

**H3K9acet and RNA Pol II recruitment**

Epigenetic modifications across the regulatory regions of inducible genes, including acetylation of lysine 9 in histone H3 (H3K9acet),
are well-established hallmarks of transcriptional activation (20). Acetylation of this residue is associated with lineage-specific gene expression within Th1 and Th2 cells (5). Given that there was no apparent difference in the extent of histone H3 loss at the proximal promoter region, it was of interest to determine whether the difference in gzmB transcription between activated CD4+/H11001 and CD8+/H11001 T cells was associated with changes to the level of H3K9acet. ChIP analyses utilizing an Ab specific for H3K9acet revealed a striking difference in the level of H3K9acet within the proximal gzmB promoter between the two T cell subsets (Fig. 3b). Activated CD8+ T cells displayed substantial levels of H3K9acet across the proximal gzmB promoter, especially within the region 300 bp upstream of the TSS (primer B, Fig. 3b). It is important to note that this same promoter region exhibited a substantial increase in chromatin accessibility following T cell activation (Fig. 2). Furthermore, H3K9acet was sustained in activated CD8+ T cells as it was maximal 24 h after activation. In contrast, activated CD4+ T cells exhibited minimal levels of H3K9acet across the gzmB promoter (Fig. 3b). Therefore, efficient gzmB transcription in CD8+ T cells is associated with a high level of H3K9acet across the gzmB proximal promoter region. These findings are in line with a previous study conducted in yeast that showed histone loss occurs before or independently of histone acetylation (38).

To ascertain whether the increase in H3K9acet is associated with transcriptional activity, a ChIP assay was performed using an Ab against the active form of Pol II. Similar to the large differences observed in H3K9acet between CD4+ and CD8+ T cells, Pol II was essentially only recruited to the gzmB promoter in activated CD8+ T cells, but not in activated CD4+ T cells (Fig. 3c). Specifically, activated CD8+ T cells displayed rapid recruitment of Pol II to the proximal gzmB promoter, with the greatest enrichment being at 4–12 h after activation (Fig. 3c). Furthermore, after 24 h of activation, Pol II recruitment decreased to levels equivalent to those before induction (Fig. 3c); a result that is consistent with the decrease in Mnase accessibility at this time point in CD8+ T cells (Fig. 2b).

Collectively, these data demonstrate that H3 histone loss occurs in a comparable manner for both CD4+ and CD8+ T cells, whereas substantial H3K9acet and Pol II recruitment appear to be hallmarks of the gzmB promoter in activated CD8+ T cells.

Histone H3 methylation patterns and transcriptional activation
Next, we investigated the relationship between specific histone H3 methylation marks with the significantly different gzmB mRNA expression patterns observed in CD4+ and CD8+ T cells. To this end, we performed ChIP assays utilizing specific Abs against the
methylating marks H3K4me3, H3K4me2, and H3K9me3, with subsequent quantitative PCR analysis using primers for both promoter and transcribed regions (Fig. 4a). Previously, it was shown that H3K4me2 marks active as well as inactive euchromatin genes, while H3K4me3 directly correlates with the transcriptional process (39). In contrast, H3K9me3 has traditionally been associated with repression of transcription, involving the recruitment of the heterochromatin protein 1 (40).

Consistent with the histone H3K9acet results (Fig. 3b), higher levels of H3K4me3 in the transcribed region of the gzmB gene were observed in activated CD8+ T cells (Fig. 4b), in particular downstream of the TSS (primer 5', Fig. 4a), which is in agreement with recent genome-wide studies (25, 26). In CD8+ T cells, maximum levels of H3K4me3 recruitment within the promoter region were reached within the first 24 h following stimulation, whereas H3K4me3 levels in the gzmB-transcribed region kept increasing up to 3 days after stimulation, reaching maximum levels of ~500-fold enrichment. (Fig. 4b). A similar H3K4me3 pattern was observed for CD4+ T cells, but the average levels of H3K4me3 enrichment were 10-fold lower (Fig. 4b). To compare H3K4me3 levels with that of an inactive gene, the promoter and transcribed region of the granzyme N (gzmN) gene were included in the analysis. On average, the H3K4me3 levels were ~10 in both CD4+ and CD8+ T cells (Fig. 4b), consistent with the view that this histone modification does indeed play an activation-specific role.

Next, we investigated the distribution of H3K4me2 across the promoter and transcribed region of the gzmB gene (Fig. 4c). For both CD4+ and CD8+ T cells, H3K4me2 enrichment at the gzmB promoter and transcribed regions was already observed in nonstimulated cells, with comparable levels of methylation in both regions for both cell types. Following activation of CD8+
T cells, significant enrichment of H3K4me2 occurred in the promoter, in the 5′, and in particular in the 3′-transcribed regions of the gzmB gene. Such major changes were not observed in activated CD4+ T cells. In contrast, the silent gene gzmN exhibited less pronounced differences. Moreover, increases in H3K4me2 only occurred on the gzmB promoter in CD8+ T cells upon transcriptional activation.

Finally, we investigated whether H3K9me3 played a role in the transcriptional regulation of gzmB in CD4+ and CD8+ T cells. The H3K9me3 levels were generally higher in CD4+ compared with CD8+ T cells (Fig. 4d), in particular across the gzmB promoter region. Following activation, H3K9me3 enrichment levels increased to appear across the gzmB promoter in CD4+ T cells, whereas this mark increased in the transcribed region of gzmB in CD8+ T cells (Fig. 4d). These findings are reminiscent of a recent report indicating that H3K9me3 may influence the termination of inducible transcription (41). In contrast, low levels of H3K9me3 were detected across the gzmN promoter and transcribed regions in both T cell subsets (−2 to 3-fold), despite gzmN being a transcriptionally inactive gene. This raises the possibility that H3K9me3 plays only a minor role in the long-term silencing of this gene.

Taken together, lineage-specific epigenetic changes mark the gzmB gene in activated CD8+ T cells, which include increases in H3K9acet, H3K4me3, and H3K4me2. In contrast, there is an increase in H3K9me3 in CD4+ T cells, which may help keep the gzmB gene in a more repressed state.

Chromatin remodeling and epigenetic modification of the gzmB in vivo

Our in vitro data suggest that the acquisition of gzmB transcription by CD8+ T cells is associated with increased Mnase accessibility followed by a dramatic increase in H3K9acet within the proximal promoter and transcribed region of gzmB. To examine whether the observed in vivo chromatin changes also occur in an in vivo context, we used a well-characterized model of influenza A virus infection to induce a de novo virus-specific CTL response. Influenza A virus infection induces CTL responses via a number of different peptide Ags, with the predominant responses being directed against H-2Db-binding peptides derived from the viral nucleoprotein (aa NP366–374; termed NP366) (42) and the acid polymerase A subunit (aa PA224–233; termed PA224) (43). To obtain the required number of Ag-specific CD8+ T cells for CHART and ChIP analysis, we used H-2Db tetramer staining to isolate DNP366- and DPA224-specific CTL from mice that were initially primed with the A/PR8 virus strain and then challenged 8 days later with the A/HKx31 influenza strain. We have previously demonstrated that CTL isolated at this time point exhibit potent cytotoxic capacity (44) and significant gzmB expression (45). It should be noted that the virus-specific CTL are derived from memory CD8+ T cells that have been re-exposed to specific Ag 8 days earlier. Thus, only chromatin changes that are required for long-term gzmB transcription will be evident. As was observed with in vitro-activated CD8+ T cells, DNP366-specific CTL exhibited an extensive increase in Mnase accessibility within the proximal gzmB promoter region (Fig. 5a) when compared with naive (tetramer negative) CD8+ T cells. Importantly, the same regions of the gzmB proximal promoter that showed increased accessibility after in vitro activation were also more accessible in DNP366-specific CTL (i.e., −34 to −281 bp upstream of the TSS; Fig. 5a).

The extent of H3K9acet was then characterized for both DNP366- and DPA224-specific CTL (Fig. 5b). As was observed for in vitro-activated CD8+ T cells, both Ag-specific CTL populations showed enrichment of H3K9acet (Fig. 5b) within the proximal gzmB promoter (primer C, region −34 to −191). This supports the notion that H3K9acet is intricately linked with activation of inducible genes such as gzmB and can persist in CD8+ T cells for long periods after activation.

Discussion
gzmB, unlike many other inducible genes in T cells, is not required to be expressed immediately following T cell activation because it is an effector molecule involved in target cell lysis. Furthermore, gzmB exhibits specific expression, being predominantly activated in CD8+ compared with CD4+ T cells. Thus, we proposed that chromatin regulatory mechanisms may play a key role in this differential expression of gzmB. Indeed, this is the case, with a number of important transcriptional features being identified, which are depicted in Fig. 6. We show that histone H3 loss at the proximal promoter region of gzmB appears to be an essential event that accompanies gzmB transcription in both CD4+ and CD8+ T cells (prepoised chromatin state). Significant chromatin accessibility and the corresponding high levels of gzmB transcription observed specifically in CD8+ T cells appear to require 1) the recruitment of substantial Pol II on the promoter of this gene (poised chromatin state) and 2) the existence of a specific epigenetic signature, namely, H3K9acet, H3K4me3, and H3K4me2 (active chromatin state).
Our findings concur with previous studies which demonstrated that histone loss accompanies inducible gene transcription (35–37, 46, 47). Although both activated CD4+ and CD8+ T cells undergo extensive histone loss at the gzmB proximal promoter, only CD8+ T cells are able to establish and maintain high levels of gzmB transcription. Indeed, chromatin accessibility correlated well with the gzmB transcriptional profiles in both CD4+ and CD8+ T cells. This finding is consistent with previous observations with inducible genes, such as IL-2 and IL-12p40 (35, 48). Furthermore, an earlier study detected a hypersensitivity site at approximately −150 bp from the TSS of the gzmB gene (12), which overlaps with the region covered by primers sets B and C in this study. Our finding that chromatin accessibility changes coincide well with gene transcription in both CD4+ and CD8+ T cells is not surprising, given that accessibility changes can be attributed to an array of factors. These include histone variant exchange (46), nucleosome sliding, the binding of transcriptional activators/loss of repressors, and chromatin conformational alterations as a consequence of histone modifications (35, 48).

Many early inducible genes have been shown to have Pol II already bound to their regulatory regions before gene transcription, a state termed “poised” to indicate the ability for rapid transcriptional activation (49–51). Interestingly, we show here that Pol II is recruited rapidly and robustly in a transient manner to the gzmB promoter in activated CD8+ T cells, but not CD4+ T cells. This event appears to be closely coupled to histone loss across this promoter following activation, suggesting that this chromatin configuration represents a prepoised state, which may have to be established before the recruitment of Pol II and establishment of a “poised chromatin state.” This additional mechanistic step could account for the delay in the activation of the gzmB gene. This step from a prepoised to a poised state may occur in a lineage-dependent manner and is likely to be inefficient in cell types not appropriately primed for high-level gzmB transcriptional activation, such as CD4+ T cells (Fig. 6). One possible explanation for this lineage-specific behavior could be the lack of cell-specific gzmB transcriptional activators, such as Eomesodermin (52), or the presence of gzmB transcriptional repressors, including Bcl-6 (53). Taken together, these results lend support to a model whereby the prepoised state before the recruitment of Pol II. Conversion of this prepoised state to an “active state” appears to require changes in the modification status of histone H3 in CD8+ T cells. Specifically, our findings illustrate that lineage-specific epigenetic changes mark the gzmB gene in activated CD8+ T cells.

High levels of gzmB transcription in activated CD8+ T cells correlated with dramatic increases in H3K9acet upstream of the gzmB TSS, a result that is in agreement with a recently published study of human CD8+ T cells (16). The H3K9acet findings also appear to be relevant for long-term in vivo-activated CD8+ T cells, since H3K9acet is also enriched within the gzmB promoter of virus-specific CTL generated during a secondary immune response to a viral infection, a result which is in agreement with a recently published study (16). In addition, distinct and specific H3 methylation changes occur at different regions of the genome, which correlate well with the extent of gzmB transcription. Methylation of histone H3K4 is currently thought to be exclusively associated with active transcription of inducible genes (39). Indeed, we observed significant enrichment of H3K4me3 in the promoter and transcribed region of the gzmB gene after in vitro activation of CD8+ T cells. A similar pattern was observed for H3K4me2, albeit with the greatest enrichment detected in the 3′-transcribed region. These patterns are in agreement with previous studies on the genome-wide distribution of modifications of activated genes (25–27). When CD4+ and CD8+ T cells exist in a basal state, the level of H3K4me2 at the promoter region of gzmB in both T cell populations was similar. Interestingly, the H3K4me3 and H3K4me2 marks across the gzmB gene were generally higher than those in the inactive gzmN gene, indicating that these PTMs may have a role in facilitating lineage-specific gzmB transcription.
The H3K9me3 PTM has typically been associated with transcriptional repression (40). Our in vitro analysis demonstrated that H3K9me3 was enriched across the gzmB promoter and transcribed region of both activated CD4+ and CD8+ T cells, compared with naïve T cells. In general, CD4+ T cells displayed greater enrichment of H3K9me3 following induction, raising the possibility that these repressive marks may be specifically targeted to ensure that gzmB is not expressed.

Our study may provide a molecular explanation for previous observations showing that under certain circumstances, CD4+ T cell have the potential to express gzmB (11, 54). Upon in vitro activation, the proximal gzmB promoter of both CD4+ and CD8+ T cells undergoes significant H3 loss and an increase in chromatin accessibility. Since these events are occurring in a lineage-independent fashion, this proposed prepoised chromatin state supports the notion that CD4+ T cells have the potential to express gzmB depending upon additional intrinsic signals (11, 55, 56).

In conclusion, our study provides new insights into the mechanisms underpinning lineage-specific transcriptional activation. We propose that high levels of gzmB transcription in CD8+ T cells are driven by the coordinated interplay of histone loss, substantial promoter-bound Pol II, together with specific PTM signatures distributed across the gzmB gene.

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Disclosures
The authors have no financial conflict of interest.

References


Corrections


The middle initials were omitted for the second, sixth, and eighth authors. In addition, the first name of the seventh author was published incorrectly. The corrected author line is shown below.

Torsten Juelich, Elissa L. Sutcliffe, Alice Denton, Yiqing He, Peter C. Doherty, Christopher R. Parish, Stephen J. Turner, David J. Tremethick, and Sudha Rao

In addition, one of the corresponding authors was omitted from the second footnote. The corrected footnote is below.

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Supplemental Figure Legends

**Fig. S1.** Basal gzmB mRNA levels and basal chromatin accessibility across the proximal promoter region of gzmB in CD4⁺ and CD8⁺ T cells.

(a) Comparison of Ct values for the control gene L32 in resting CD4⁺ and CD8⁺ T cells as measured by Taqman qPCR. Data shown are the mean ± SE of three replicate experiments.

(b) mRNA expression of gzmB as measured by Taqman qPCR. GzmB mRNA expression levels were measured in resting CD4⁺ and CD8⁺ T cells. mRNA levels for gzmB are expressed as arbitrary copy numbers after normalization with the control gene L32. Data shown are the mean ± SE of three replicate experiments.

(c) CHART assays were performed using the accessibility agent micrococcal nuclease (MNase) and nuclei generated from unstimulated CD4⁺ and CD8⁺ T cells. Equivalent amounts of total genomic DNA (20 ng/reaction) were subsequently analysed by SYBR Green real-time PCR using primer sets A, B, and C (see Fig. 2a) that amplify the proximal promoter region of gzmB. Data is graphed as % genomic DNA signal of the undigested sample. Data is representative of three independent experiments.

**Fig. S2.** Primer details for gzmB CHART and ChIP analysis

(a) GzmB primer sequences presented in the 5' to 3' direction, with the relative location from the TSS, and amplicon size indicated. (b) Location of the gzmB primers according to the UCSC Genome Browser (http://genome.ucsc.edu/) using the Mouse July 2007 Assembly. (c) GzmB primer location coordinates in bed file format for UCSC Genome browser custom track presentation.

**Fig. S3.** Bioanalyzer analysis of micrococcal DNA digest (CHART assay)

Serial dilutions of MNase were used to optimize gDNA cutting such that about 80% of gDNA occurred into mononucleosomes, shown in (a) gel format and (b) as electropherogram. MNase digestion analysis was performed using the Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA, USA).
## Supp. Figure 2

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### (b)

UCSC Genome Browser on Mouse July 2007 Assembly

### (c)

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