Dynamic regulation of permissive histone modifications and GATA3 binding underpin acquisition of granzyme A expression by virus-specific CD8\(^+\) T cells

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Numerous studies have focused on the molecular regulation of perforin (PFP) and granzyme B (GZMB) expression by activated cytotoxic T lymphocytes (CTLs), but little is known about the molecular factors that underpin granzyme A (GZMA) expression. In vitro activation of naïve CD8\(^+\) T cells, in the presence of IL-4, enhanced STAT6-dependent GZMA expression and was associated with GATA3 binding and enrichment of transcriptionally permissive histone posttranslational modifications (PTMs) across the Gzma gene locus. While GZMA expression by effector influenza A virus specific CTLs was also associated with a similar permissive epigenetic signature, memory CTL lacked enrichment of permissive histone PTMs at the Gzma locus, although this was restored within recalled secondary effector CTLs. Importantly, GZMA expression by virus-specific CTLs was associated with GATA3 binding at the Gzma locus, and independent of STAT6-mediated signaling. This suggests regulation of GZMA expression is underpinned by differentiation-dependent regulation of chromatin composition at the Gzma locus and that, given GATA3 is key for CTL differentiation in response to infection, GATA3 expression is regulated by a distinct, IL-4 independent, signaling pathway. Overall, this study provides insights into the molecular mechanisms that control transcription of Gzma during virus-induced CD8\(^+\) T-cell differentiation.

Keywords: Cytotoxic T cell · Epigenetics · GATA3 · Granzyme A · Influenza A virus

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Introduction

A cardinal feature of CD8\(^+\) T-cell immunity is the capacity to acquire lineage-specific effector functions that promote clearance of foreign pathogens and that are maintained into memory. Naïve CD8\(^+\) T cells are initially quiescent and need to undergo proliferation and differentiation upon activation to acquire effector functions and become effector cells [1, 2]. Optimal naïve T-cell activation requires the integration of multiple signaling cascades that include cross-linking of the antigen-specific T-cell receptor (signal 1); costimulation (signal 2) and soluble factors such as cytokines (signal 3) [3, 4]. In contrast, memory T cells are capable of triggering a more robust and rapid response upon subsequent exposure to the same antigen without the need to undergo extensive proliferation as their naïve counterparts [5, 6]. Importantly, differences in the functional capacity between these...
CD8+ T-cell subsets are underscored by coordinated changes in gene expression profiles [1, 7]. The incorporation of covalently modified histone proteins within specific gene regulatory elements is a key mechanism for modulating gene transcription [8]. For example, acetylation of the histone H3 at lysine 9 (H3K9ac) within gene promoters correlates with transcriptional activation [9], as does trimethylation of histone 3 at lysine 4 (H3K4me3) [10]. By contrast, trimethylation of lysine 27 (H3K27me3) is a correlate of transcriptional repression [9]. Recently, it has been demonstrated that acquisition of granzyme B (GZMB) and perforin expression after naïve CD8+ T-cell differentiation into effector CTLs was associated with enrichment at the gene promoters of histone modifications that promote gene transcription, namely H3K4me3 and H3K9Ac [7, 11–13]. Denton et al. [14] extended these observations by demonstrating that patterns of H3K4me3 (associated with active transcription) or H3K27me3 (associated with repression of transcription) were predictive of gene expression profiles at a number of effector gene loci across the distinct phases of CTL differentiation. Importantly, maintenance of histone PTMs that promote active transcription was observed at these effector loci in resting memory CTLs, and in the absence of active transcription. Thus, effector gene loci within memory CTL appear to be maintained in transcriptionally permissive state providing a molecular mechanism for the ability of memory T cells to rapidly express effector genes upon reactivation.

In this current study, we first utilized in vitro activation of naïve CD8+ T cells to demonstrate that the addition of IL-4 as a third signal during T-cell activation promoted Gzma transcription and this correlated with STAT6-dependent enrichment of a permissive epigenetic signature at the Gzma locus, and binding of GATA3 and the histone acetyltransferase p300. Interestingly, while GZMA expression by ex vivo derived influenza A virus (IAV) specific effector CTLs was associated with a similar permissive epigenetic signature and GATA3 binding to the Gzma locus, this was independent of STAT6. Moreover, in contrast to other effector gene loci, memory CTLs lacked enrichment of permissive histone PTMs at the Gzma locus, with a permissive signature restored within recalled effector CTLs induced after secondary infection. These data indicate that GZMA expression by virus-specific CTLs is regulated by differentiation-dependent modulation of chromatin composition at the Gzma locus that differs to other signature CTL effector gene loci and this signature can be influenced greatly by receipt of specific extracellular signals.

**Results**

**IL-4 induces GZMB expression following in vitro stimulation of CD8+ T cells**

GATA3- and STAT6-binding sites have been previously identified approximately 1 kb upstream of the transcription start site (TSS) of the Gzma gene (Fig. 1A), and it has been demonstrated that IL4 signaling promotes GZMA expression. In accordance with these earlier studies, in vitro activation of naïve CD8+ T cells (CD44+CD8+) under T422 conditions resulted in greater levels of Gzma transcription and protein when compared to CTLs cultured under neutral conditions (Fig. 1B, C, p < 0.006). Interestingly, GZMB production was similar between CTLs cultured under both neutral and T42 conditions (Supporting Information Fig. 2A and B), suggesting that GZMA and GZMB expression can be driven by distinct regulatory mechanisms.

**TCR signals alone induce greater chromatin accessibility around the Gzma locus**

We, and others, have demonstrated that acquisition of CTL effector gene transcription upon naïve T-cell activation is associated with increased chromatin accessibility within gene promoters [14–16]. We first assayed chromatin accessibility within a region encompassing 500 bp upstream of the Gzma TSS (primer sets A’C) to ~7 Kb into the Gzma gene body (primer sets S’, M, and S”; Fig. 1A) using FAIRE [17]. Naïve CD8+ T cells exhibited a degree of chromatin accessibility across the promoter region, relative to a noninducible gene Myod1 (Fig. 1D). In vitro T-cell activation further increased chromatin accessibility, particularly around the Gzma TSS, independent of culture conditions (Fig. 1D). Hence, TCR signaling is sufficient to mediate chromatin remodeling of the Gzma promoter to a more permissive confirmation.

**IL-4 signals promote deposition of active histone modifications across the Gzma gene locus**

Given increased chromatin accessibility at the Gzma promoter was independent of IL-4 signaling, we sought to investigate whether promotion of Gzma transcription by IL-4 signaling was associated with establishment of a permissive epigenetic signature at the Gzma locus. ChIP analysis demonstrated that activated CTLs displayed an overall reduction of H3K27me3 across the promoter (sets A–C) under both neutral and T42 culture conditions, compared with naïve cells (Supporting Information Fig. 2C versus Fig. 1E). While there was little change in the levels of H3K4me3, H3K9ac, and H3K27ac between naïve CD8+ T cells and CTLs cultured under neutral conditions (Supporting Information Fig. 2D–F versus Fig. 1F–H), there was an increase in these same marks within the proximal promoter and gene body of CTLs stimulated in the presence of IL-4 (Fig. 1F–H). Collectively, these findings show that removal of the repressive H3K27me3 is dependent on TCR ligation alone, and that IL4 promotion of GZMA expression correlates with greater deposition of permissive histone signatures at the Gzma locus.

**CD8 T cells cultured under T42 culture conditions upregulate pSTAT6 and GATA3**

We next compared the levels of Stat6 and Gata3 mRNA and protein within CTLs activated under neutral and T42 conditions (Fig. 2).
Figure 1. Chromatin accessibility and histone modification enrichment at the Gzma locus following in vitro stimulation in the presence or absence of IL-4. (A) The regulatory element upstream of the Gzma transcription start site (TSS) and the position of 2× GATA3 (WGATAR) and 1× STAT6 (TTC-N4-GAA) consensus binding sites; real-time PCR using primer sets spanning the promoter (sets A–C) and gene body (sets 5′, M, and 3′). (B–H) Sort-purified naïve CD8+ (CD44low CD62Lhi) T cells were in vitro activated in either presence or absence of IL-4 for up to 5 days. Cells were harvested at days 2 and 5 after activation and the (B) levels of Gzma mRNA and (C) proportion of CTLs expressing GZMA was determined by intracellular staining. CTLs were subjected to (D) FAIRE or (E–H) ChIP with antibodies directed against repressive mark H3K27me3, permissive marks H3K4me3, H3K9ac and H3K27ac. The enrichment was normalized to (D) reference gene Myod1 and (E–H) total histone H3. (B–H) Data are shown as mean ± SEM (n = 3 samples/group) and are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001; Student’s t-test.

Interestingly, CTLs cultured under both conditions demonstrated a decrease in Stat6 mRNA levels 2 days after activation, when compared to naïve CD8+ T cells (Fig. 2A). While Stat6 mRNA levels increased by day 7 under both culture conditions, levels were still lower than observed within naïve CD8+ T cells. Phosphorylated STAT6 (pSTAT6) was detected in CTLs cultured under Th2 conditions, compared to CTL activated with neutral conditions (Fig. 2B). Consistent with the activation of pSTAT6 within CTLs cultured under Th2 conditions, GATA3 mRNA and proteins levels were higher in Th2-stimulated CTLs, compared to CTL activated with neutral conditions (Fig. 2C and D). This suggests that STAT6 activation promotes GATA3 expression and together this promotes GZMA expression within Th2 cultured CTLs.
In an attempt to link STAT6 phosphorylation and GATA3 upregulation to establishment of a permissive histone signature at the Gzma locus, we utilized mice that have the STAT6 SH2 domain gene replaced with a neomycin cassette disrupting STAT6 function [18]. When compared to WT CTL, there was little difference in the levels of Gzma mRNA or induction of GZMA protein within STAT6−/− CTL, irrespective of the culture conditions (Supporting Information Fig. 3A and B). This demonstrates that pSTAT6 induced by IL-4 signaling is required for promoting GZMA protein expression. The lack of Gzma expression by STAT6−/− CTL cultured under Th2 conditions correlated with greater levels of H3K27me3, and lower levels of H3K4me3, H3K9ac, and H3K27ac deposition at the Gzma locus, and looked similar to patterns observed in WT CTL stimulated under neutral conditions (Supporting Information Fig. 3C–F, compare to Fig. 1). Overall, these data suggest that IL-4-dependent enhancement of Gzma expression within in vitro activated CTL is underpinned by STAT6-dependent deposition of a permissive histone signature at the Gzma locus.

Chromatin modifications at a regulatory region upstream of the Gzma promoter

An intergenic regulatory element that contains both GATA3- and STAT6-binding sites is located approximately 1 kb upstream of the Gzma TSS [19, 20]. Given that GATA3 was induced in CD8+ T cells cultured under Th2 conditions (Fig. 2), we examined whether similar chromatin remodeling events, and binding of GATA3 and STAT6 occurred at this region. When compared to naive CD8+ T cells, or CTLs activated under neutral conditions, CTLs cultured under Th2 conditions exhibited increased chromatin accessibility, significantly less total H3 enrichment (Supporting Information Fig. 4A and B), loss of the repressive mark H3K27me3 and enrichment of the permissive H3K4me3, H3K9ac, and H3K27ac marks at this noncoding regulatory region (Supporting Information Fig. 4C–F). Hence, IL-4-induced signaling promotes active chromatin remodeling and establishment of permissive histone signatures at both noncoding regulatory and TSS regions of the Gzma locus in activated CTLs.

To examine TF binding to this element, naive CD8+ T cells stimulated under either neutral or Th2 conditions were analyzed by GATA3 and STAT6 ChIP (Supporting Information Fig. 4G and H). Both GATA3 (Supporting Information Fig. 4G) and STAT6 (Supporting Information Fig. 4H) were enriched at the Gzma regulatory region in CTLs cultured under Th2 conditions, compared to CTLs cultured under neutral conditions. Remarkably, there was greater GATA3 enrichment at the Gzma locus than observed at the Il4 promoter, which acted as the positive control (Supporting Information Fig. 4G). Importantly, no enrichment of GATA3 binding was observed at the Ifng locus (Supporting Information Fig. 4G) or at the Gzma regulatory element in STAT6−/− CTL (Supporting Information Fig. 4H).
Histone modification patterns at the \( \text{Gzma} \) locus in OT-I cells following IAV infection.

(A–H) Naïve OT-I cells \((10^4)\) were adoptively transferred into naïve B6 mice and infected i.n. with \(10^4\) PFU x31-OVA virus 1 day later. Pre-infection ( naïve, black bars) and effector \((d7\) after infection, white bars) and memory \((>d60\) after infection, gray bars) time points spleens were harvested and OT-I cells \((\text{CD8}^+ \text{Ly5.1}^+)\) were sort purified. Sort-purified OT-I cells were probed for \( \text{Gzma} \) mRNA (A), \( \text{GZMA} \) protein (B), or to FAIRE (C) and ChIP with antibodies directed toward H3K4me3, H3K9ac, H3K27ac, H3K9me3, and H3K27me3 (D–H). ChIP analysis was conducted by real-time PCR using primers as described in Supporting Information Table 1 and expressed as enrichment ratio over histone H3. (A–H) Data are shown as mean ± SEM \((n = 3\) samples/group) and are representative of three to four independent experiments. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), ****\(p < 0.0001\); statistical significance showing naïve versus effector and memory versus effector; Student’s t-test.

### Information Fig. 4G, inset) under T\(_2\) conditions. Finally, binding of the histone acetyltransferase, P300 was also observed at the \( \text{Gema} \) regulatory region, the \( \text{Ib} \) promoter, but not at the non-inducible gene \( \text{Myod1} \), or within the \( \text{Gema} \) regulatory region of STAT6\(^{-/-}\) CTL (Supporting Information Fig. 4I). These data support the idea that GATA3 and STAT6 play a key role in chromatin remodeling of the \( \text{Gzma} \) locus establishing a transcriptionally permissive histone landscape that promotes gene transcription, potentially via recruitment of chromatin modifying enzymes including P300.

### IAV-specific memory CTL lacks a permissive histone signature at the \( \text{Gzma} \) locus

To explore whether similar changes in chromatin structure observed in in vitro activated CD8\(^+\) T cells were present in bonafide IAV-specific CTLs, we utilized an adoptive transfer model where B6 mice had received \(10^5\) naïve OT-I CTLs and were infected with a recombinant IAV virus engineered to express the SIINFEKL epitope [21]. As previously reported [22], \( \text{Gzma} \) mRNA and protein expression was greater at the effector phase (days 7 and 10
after infection), but was much lower in memory CTLs isolated 60 days after infection (Fig. 3A and B).

Given we observed different patterns of GZMA expression as OT-I cells progressed from naive to effector and memory stages, we asked whether there was an associated difference in chromatin accessibility and specific histone signature (Fig. 3). Both naive and memory OT-Is exhibited similar patterns of chromatin accessibility (Fig. 3C, black versus gray bars). In contrast, effector CTLs exhibited a different pattern with much greater levels of chromatin accessibility located closer to the Gzma TSS (Fig. 3C, white bars). Further, GZMA expression within effector OT-I CTLs correlated with enrichment for H3K4me3, H3K9Ac, and H3K27Ac (Fig. 3D–F). Effector OT-I CTLs demonstrated a loss of repressive histone modifications, namely H3K9me3 and H3K27me3, when compared to naive and memory CTLs (Fig. 3G and H). It was surprising to note that the histone signature within the Gzma locus of memory CTLs represented a more repressive pattern compared to effector OT-Is (Fig. 3C–H, compare white versus gray). It suggests that the lack of Gzma expression observed in memory CTL is due to the chromatin being less accessible and not permissive for transcription. This is contrast to other signature CTL effector gene loci, Ifng and Gzmb, where memory CTLs maintained a permissive histone signature characterized by loss of total H3, loss of H3K27me3, and gain of H3K4me3 and H3K9Ac, and similar to that observed within effector CTLs (Supporting Information Fig. 5A–D). Thus, in contrast to previous reports showing that memory CTLs maintain signature effector gene loci in a...
transcriptionally poised state, associated with a permissive epigenetic signature \[11, 14, 23\], the \textit{Gzma} locus is maintained in a more repressive state within memory CTLs.

**Recall of memory OT-I cells results in chromatin remodeling of the Gzma locus**

Given IAV-specific memory OT-Is displayed a repressive histone modification signature within the \textit{Gzma} locus, we aimed to investigate whether this repressive histone signature prevented memory CTLs from expressing GZMA to any degree following secondary IAV challenge, or whether the \textit{Gzma} locus was capable of undergoing chromatin remodeling enabling GZMA expression. B6 mice that had previously received naïve OT-I were primed with X31-OVA for 60 days were challenged with a serologically distinct IAV (PR8-OVA) to recall the memory OT-I CTLs. Analysis of \textit{Gzm} mRNA and GZM protein levels demonstrated there was a...
~680-fold increase in Gama and Gzmb mRNA respectively, in secondary effector OT-1 CTLs compared with resting memory cells (Fig. 4A), and this correlated with greater GZMA and GZMB protein expression (Fig. 4B). Importantly, secondary effector OT-1 CTLs demonstrated significant enrichment for H3K4me3 and H3K9ac across the Gzma locus, as compared to resting memory cells (Fig. 4C–E).

Given naive and memory IAV-specific CD8+ T cells shared a similar histone landscape at the Gama locus, it was of interest to determine whether acquisition of GZMA was similar between both subsets in response to infection. Sort-purified naive and memory OT-1 cells were cotransferred into B6 recipients and effector CTLs were isolated 7 days after IAV infection (Fig. 4E and F). Interestingly, while there was no significant difference in the proportion of GZMA+ OT-I derived from either naive or memory OT-I cells (Fig. 4E), memory OT-I exhibited higher GZMA MFI indicative of higher levels of protein expression (Fig. 4F). Collectively, these findings demonstrate that while resting memory cells are capable of upregulating Gama transcription upon secondary challenge and this is associated with reestablishment of a permissive histone signature, they exhibit greater capacity to express GZMA on a per cell basis upon activation when compared to primary effector CTL.

GATA3 is bound at the Gzma locus within ex vivo isolated IAV-specific CTLs

Our earlier in vitro analysis demonstrated that IL4/STAT6 signaling promoted GZMA expression that correlated with establishment of a permissive histone signature. Given the Gama locus within ex vivo influenza-specific primary and secondary effector CTL demonstrated similar epigenetic signatures, we wanted to determine the in vivo role of the IL4/STAT6 pathway in driving GZMA expression by CTL after influenza infection. We were able to demonstrate that OT-I effector CTLs isolated 7 days after IAV infection expressed the IL4Ra both in the steady state, and could further upregulate IL4Ra expression after 5 h of peptide stimulation (Fig. 5A and B). Hence effector IAV-specific CTL could potentially signal via IL-4 to drive GZMA expression. Naive STAT6−/− OT-I s and WT OT-I s were adoptively transferred into naive B6 recipients followed by HKx31-OVA infection and in contrast to CTLs activated in vitro, the proportion of GZMA expressing WT and STAT6−/− effector CTLs was similar (Fig. 5C and D).

Given GZMA expression by OT-I effector CTLs was independent of STAT6 signaling, we sought to determine whether GATA3 could still potentially play a role in regulating GZMA expression. GATA3 mRNA transcription was observed in both in both WT and STAT6−/− effector OT-I CTLs isolated directly ex vivo 7 days after infection (Fig. 5E). Moreover, we performed ChIP and found that GATA3 was detected bound to the Gama promoter region in both WT and STAT6−/− OT-I effector CTLs, while there was no binding of GATA3 to the Myod1 promoter (Fig. 5F). These data demonstrate that GATA3 can be induced by IL4/STAT6-independent pathway/s and be bound to the Gama locus within IAV-specific effector CTL.

Discussion

Although GZMA is a signature CTL effector gene, little is known about the epigenetic regulation of GZMA upon CTL differentiation. Activation of naïve CD8+ T cells in the presence of IL-4 resulted in rapid acquisition of Gama transcription compared to CTLs cultured under neutral conditions, and this was associated with substantial enrichment of a permissive histone signature around the Gama promoter region. Interestingly, increased chromatin accessibility and removal of repressive H3K27me3 was independent of IL4 signals. Hence, we propose that activation of Gama transcription occurs in a stepwise manner whereby TCR-mediated signals drive initial chromatin remodeling of the Gama locus resulting in increased accessibility and exposure of potential transcription factor binding sites. IL-4 signaling provides a secondary signal that results in transcription factor dependent deposition of active histone modifications and subsequent transcriptional activation. Importantly, our findings are consistent with previous studies in CD4+ T cells that identified the presence of histone acetylation at T½ lineage specific gene loci, including Gama, after T-cell activation [20, 24]. Hence, the molecular mechanism that underpins acquisition of Gama transcription appears to be shared by CD4+ and CD8+ T cells.

IL-4 driven enrichment of permissive histone PTMs at the Gama locus of activated CD8+ T cells was dependent on STAT6 and involved GATA3 binding to the Gama promoter. Moreover, the recruitment of GATA3 coincided with high levels of the histone acetyltransferase, p300. Given that recruitment of P300 to the Il5 locus, and subsequent establishment of hyperacetylation within T½ cells is dependent on GATA3 [25], we propose that, IL4-dependent binding of GATA3 to the Gama locus recruits P300 to the Gama locus within activated CTL leading to a transcriptionally permissive conformation. Whether GATA3 alone is sufficient for induction of Gama transcription remains to be determined and it is possible that other transcription factors also play a role. For example, potential ETS1-binding sites, associated with GATA3-binding sites have been identified within the promoters of T½-associated gene loci including Gama [19]. Hence, GATA3 may work in conjunction with other TFs, such as ETS1, to promote Gama transcriptional regulation.

Similar to our in vitro observations, acquisition of GZMA expression by primary effector IAV-specific CTL correlated with deposition of permissive histone PTMs within the Gama locus. Surprisingly, the presence of these permissive marks was not maintained by long term, resting IAV-specific memory cells. This is in contrast to both the Gamba and Ifgn loci within IAV-specific memory CTL whereby memory IAV-specific CTL maintained the permissive histone PTM signature acquired during CTL differentiation during the primary response [11, 14]. RNA pol II was docked onto these effector gene loci within memory CTL suggesting that these loci are epigenetically maintained in a transcriptionally poised state that is able to facilitate rapid transcriptional activation upon secondary infection [11, 14]. Given this was not the case for the Gama locus, it points to the fact that transcriptional poising via epigenetic remodeling is not a
general mechanism characteristic of all memory CTL effector genes.

The nonpermissive state of the Gzma locus found in memory CTL was not fixed as secondary effector IAV-specific CTL upregulated GZMA expression and this was associated with enrichment of permissive histone PTMs within the Gzma locus. Thus, chromatin remodeling of the Gzma locus to a transcriptionally permissive state occurs upon a secondary differentiation of memory CTL. One possible explanation for why the Gzma locus is not transcriptionally permissive within memory CTL is that IAV-specific memory CTL can be established early during a primary response, without the need for extensive differentiation [26, 27]. A recent study of the epigenetic landscape of polyclonal human CD8+ T-cell populations demonstrated that memory T cells had a more similar landscape to naive, than effector T cells [28]. Moreover, this notion is supported by the observation that virus-specific CTL can exhibit transcriptional signatures indicative of memory subsets as early as one cell division after naive T-cell activation [27]. If this is indeed the case, our data suggest that memory CTL have not undergone sufficient differentiation to fully remodel the Gzma locus to a transcriptionally permissive state. That said, we demonstrated that secondary effector CTL exhibited higher levels of GZMA expression on a per cell basis, compared to primary effector CTL. This indicates that memory OT-I are intrinsically capable of increased transcriptional responsiveness, compared to primary activated OT-I. Given this was not at the level of maintenance of histone PTMs at the Gzma locus, it is tempting to speculate that potentiation of transcriptional activity by reactivated memory CTL is driven by expression of a specific factor/or factors, and/or engagement of novel regulatory elements, which can promote increased transcriptional responsiveness at specific gene loci upon secondary activation [29].

CD4+ T cells can produce IL-4 after IAV infection [30]. Surprisingly, despite surface expression of the IL-4 receptor on IAV-specific effector CTL, the same in vivo analysis demonstrated that STAT6−/− IAV-specific CTLs were still capable of expressing GZMA and demonstrated upregulation and binding of GATA3 to the Gzma locus. While not totally discounting a role for IL4 signals in regulating GZMA expression, these data suggest that IL-4 is not necessary for promoting IAV-specific GZMA expression during infection and that alternate signaling pathways are able to drive GATA3 expression. It has been reported that signals from the IL-2R [31], Notch [32, 33], and the Wnt/β-catenin/T-cell factor 1 [34] pathways can all upregulate GATA3 expression within CD4+ T cells. In particular, NOTCH signaling can act directly on the Gata3 locus to increase GATA3 expression and is therefore important for promoting T1/2 differentiation in response to in vivo physiological challenges [32, 33]. Two independent studies have shown that GATA3 [31] and NOTCH [35] each play a key role in virus-specific CD8+ T-cell differentiation. Given the importance of each of these pathways in regulating CTL differentiation, it is tempting to speculate that NOTCH signals interact with GATA3 by driving GATA3 expression in virus-specific CTL in response to infection and we are currently investigating this possibility. Taken together, this study provides insights into the molecular mechanisms that are involved in the acquisition of CD8+ T-cell effector genes, particularly Gzma during CD8+ T-cell differentiation and the role that GATA3 may have in this process.

Materials and methods

Animals, viruses, and infections

Female Ly5.2+ C57BL/6 (B6), B6.129S2(C)-Stat6tm1Gru/J (STAT6−/−), congenic Ly5.1+ OT-I (OT-I), and Ly5.1+ OT-I STAT6−/− (STAT6−/− OT-I) mice were bred and housed under specific-pathogen free conditions in the Department of Microbiology and Immunology Animal Facility, the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Parkville, Victoria. For primary infections, naive mice (6–8 weeks of age) were anaesthetized and infected intranasally (i.n.) with 10^4 plaque-forming units (PFU) of recombinant Hkx31 (H3N2) IAV engineered to express the OVA257-264 peptide (x31-OVA) in the neuraminidase stalk. For secondary infections, mice were primed as described above at least 60 days prior to i.n. challenge with 600 PFU of recombinant PR8-OVA (H1N1) IAV. All experiments were conducted according to approval obtained from the Institutional Animal Ethics Committee.

Tissue sampling, adoptive transfer, and cell sorting

Spleen and lymph nodes (LNs) were harvested from naive and infected mice. For adoptive transfer, pooled LNs from naive OT-I or STAT6−/− OT-I mice were counted and resuspended in PBS at a concentration of 5 × 10^6 CD8+ OT-I cells/mL. B6-recipient mice were intravenously (i.v.) injected with 1 × 10^6 cells/200 µL WT or STAT6−/− OT-I cells via the tail vein. For cell sorting, spleen and/or LN lymphocyte preparations from B6, STAT6−/−, OT-I, and STAT6−/− OT-I mice were adjusted to a concentration of 20 × 10^6 cells/mL in PBS/0.1% BSA. Cells were surface stained with anti-CD8α and anti-CD44 antibodies to isolate naïve cells (CD8+CD44lo), or anti-CD8α and anti-CD45.1 antibodies to isolate transferred OT-I cells. Naïve (CD8+CD44lo) cells and transferred OT-I, STAT6−/−, OT-I (CD8+Ly5.1+) cells were sort purified to a minimum purity of 95% using a FACSARia cell sorter (BD Biosciences). For comparison of naive and memory T-cell responses, naive (day 0) and memory OT-I cells (>30 post primary IAV infection) were sort purified and 4 × 10^4 of each subset were cotransferred into B6-recipient hosts. Recipients were infected i.n. with A/x31-OVA infection a day later and OT-I cells were isolated from the bronchoalveolar lavage (BAL) and spleens of mice 7 days later and GZMA expression determined.
In vitro CD8⁺ T-cell activation

Freshly sorted naïve CD8⁺ T cells were activated in plates coated with anti-CD3ε (145-2C11; 10 μg/mL), anti-CD8α (53.6.7; 10 μg/mL), and anti-CD11a (L21.7/7; 10 μg/mL) [36] in complete RPMI media with 10 U/mL of human recombinant IL-2 (Roche) and 5 μg/mL anti-CD28 (Biolegend; neutral conditions). To skew cells under Th1/2 conditions, 25 ng/mL of mouse recombinant IL-4 (ProSpec-Tany TechnoGene) and 1 μg/mL of anti-IFN-γ Ab (XMG1.2) were added. Cells were stimulated for 2 days at 37°C with 5% CO₂, removed from stimulation and further cultured in the same neutral or Th1/2 skewing medium at 37°C with 5% CO₂ for a further 3 days.

Intracellular staining and flow cytometry

Cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) and Foxp3 fix/perm kit (Biolegend), according to the manufacturer’s instructions. Intracellular gzm staining was performed using anti-human gzmB-APC (GB12; Invitrogen) or isotype control anti-IgG1-APC (Invitrogen); and anti-mouse GZMA-FITC (3G8.5; Santa Cruz Biotechnology) or isotype control anti-IgG1-APC (Invitrogen); and anti-mouse GATA3 was stained with anti-GATA3- APC (TWAJ; eBioscience). Intracellular GZMA was stained with anti-GZMA3- APC (TWAJ; eBioscience). Data were collected on a FACSCantoII flow cytometer (BD Biosciences) and analyzed with FlowJo analysis software (TreeStar). The gating strategy for all analysis is outlined in Supporting Information Figure 1. Mean fluorescence intensity (MFI) was determined as the geometric mean of staining within the positive population.

Western blot

Sorted naïve CD8⁺ T cells were stimulated under neutral and Th1/2 conditions for 15 min. Cells were harvested and lysed in RIPA (0.05M Tris-HCL, 0.15M NaCl, 1% NP-40 (Sigma), 0.5% sodium deoxycholate (Sigma), 0.1% SDS (Bio-Rad), 1 x protease inhibitor cocktail (Roche) buffer). Cell lysate was harvested and protein was separated on 10% acrylamide/bis (Bio-Rad) gel electrophoresis, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blotted with anti-phosphoSTAT6 (Millipore; Tyr641), anti-STAT6 (Cell signaling Technology, Danvers, MA, USA) and anti-GAPDH (Santa Cruz Biotechnology) following by anti-Rabbit-HRP (Bio-Rad, Hercules, CA, USA). Visualization was done using Western Lightning Plus-Enhanced Chemiluminescence (PerkinElmer, Shelton, CA, USA).

RNA extraction and cDNA synthesis

RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions, and followed by chloroform extraction and ethanol precipitation. RNA (250–500 ng) was reverse transcribed using the Omniscript kit (Qiagen). Ten to twenty-five nanograms of equivalent cDNA was used as template in a real-time PCR by using TaqMan® Gene MGB primer/probes (FAM labeled). Samples were run on an IQ™5 Multicolor Real-Time PCR Detection System using iQ5 software (Bio-Rad) and normalized against mitochondrial ribosomal protein, L32.

Chromatin immunoprecipitation (ChIP)

ChIP was carried out as previously described [15]. Approximately, 5 × 10⁵–1 × 10⁶ starting cells equivalent were incubated with the following antibodies; 4 μg of anti-histone H3 (Abcam ab1791), 5 μg of anti-H3K9ac (Millipore 06–942), 5 μg of anti-H3K4-me3 (Abcam 07–473), 5 μg of anti-H3K27-me3 (Millipore 07–449), anti-H3K27ac (ab4729, Abcam), 5 μg of anti-H3K9me3 (ab8898, Abcam), 10 μg of anti-GATA3 (H-48, Santa Cruz), anti-p300 (C20, Santa Cruz), anti-STAT6 (M-20, Santa Cruz), or no Ab as a specificity control. The chromatin was sonicated to yield fragment sizes of 200–500 bp. Quantitative PCR was performed using a SYBR Green Mix (Applied Biosystems). The primer sequences used are outlined in Support Information Table 1. Each primer pair was subjected to PCR with serially diluted, sonicated genomic DNA as template.

Formaldehyde-assisted isolation of regulatory elements (FAIRE)

FAIRE was carried out as previously described [17]. Open chromatin sites were phenol–chloroform extracted and assayed using the same primer sets as outlined in ChIP. Samples were normalized against either total input DNA or a reference gene.

Statistical analyses

Statistical analyses were conducted using Student’s t-test, one-way, and two-way ANOVA on Microsoft Excel and GraphPad Prism.

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Abbreviations: BIAV: influenza A virus · GZMB: granzyme · PFP: Perforin · TSS: transcription start site

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