NFAT1 and NFAT2 Differentially Regulate CTL Differentiation Upon Acute Viral Infection

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CD8+ T cell differentiation orchestrated by transcription regulators is critical for balancing pathogen eradication and long-term immunity by effector and memory CTLs, respectively. The transcription factor Nuclear Factor of Activated T cells (NFAT) family members are known for their roles in T cell development and activation but still largely undetermined in CD8+ T cell differentiation in vivo. Here, we interrogated the role of two NFAT family members, NFAT1 and NFAT2, in the effector and memory phase of CD8+ T cell differentiation using LCMVArm acute infection model. We found that NFAT1 is critical for effector population generation whereas NFAT2 is required for promoting memory CTLs in a cell intrinsic manner. Moreover, we found that mice lacking both NFAT1 and NFAT2 in T cells display a significant increase in KLRG1hi CD127hi population and are unable to clear an acute viral infection. NFAT-deficient CTLs showed different degrees of impaired IFN-γ and TNF-α expression with NFAT1 being mainly responsible for IFN-γ production upon ex-vivo stimulation as well as for antigen-specific cytotoxicity. Our results suggest that NFAT1 and NFAT2 have distinct roles in mediating CD8+ T cell differentiation and function.

Keywords: NFAT1, NFAT2, CD8+ T cells, differentiation, effector, memory, LCMV

INTRODUCTION

CD8+ T cells are pivotal in combating intracellular pathogens and for tumor immune surveillance (1, 2). Upon recognizing their cognate antigen presented by antigen-presenting cells (APCs), naïve CD8+ T cells are activated, rapidly proliferate and differentiate into a heterogeneous pool of effector cells which display cytotoxic activity (3). The heterogeneity of activated CD8+ T cells has been characterized by the expression of different surface markers and transcription factors. Conventionally, effector cells are further categorized into two main subsets: short-lived effector T cells (SLECs) as KLRG1hi CD127lo and memory precursor effector cells (MPECs) as KLRG1lo CD127hi (4). Upon clearance of the pathogen or tumor, most antigen-specific SLECs die due to lack of antigen stimulation. The remaining MPECs are responsible for providing long-term protection upon subsequent reinfections (4, 5). A recent study shows that SLECs could also contribute to part of the memory population pool, demonstrating CTLs' plasticity (6).

The dynamic CD8+ T cell terminal effector and memory differentiation balance and heterogeneity is orchestrated by transcription regulators, many of which are still unidentified. The transcription factors Tbet and Eomesodermin (Eomes) have long been known to directly control CD8+ T cell effector and memory differentiation, respectively (7, 8). In recent years, other
transcription factors have been identified to regulate CTL differentiation: Id2 and Blimp-1 are critical for effector CTL formation while Id3, Bcl-6, Tcf-1, and Runx3 are cardinal for memory CD8^+ T cell development (8–12). The b-zip transcription factor BATF is required for early TCR signaling amplification and partnering with other transcription factors, such as JUN and IRF4 (13–16). Runx3, a transcription factor responsible for mediating CD8^+ T cell commitment in the thymus by antagonizing ThPok, which drives CD4^+ Th cell fate (17), has been also recently implicated in the early TCR signaling events during priming to promote the accessibility of chromatin containing IRF, bZIP, and PRDM1 binding motifs resulting in the strengthening of memory population differentiation (18). Despite the knowledge on how these transcription factors regulate CTL differentiation in vivo, the exact mechanisms that set off the effector and memory CD8^+ T cells development are still unclear, especially those transcription factors downstream of early TCR signaling events.

Nuclear factor of activated T cells (NFAT) is a well-known transcriptional regulator of T cell activation and plays an essential role in T cell development, activation and function. There are five NFAT family members, among them, NFAT1, NFAT2, and NFAT4 are expressed in T cells downstream of Ca^2+-Calcineurin signaling (19). All NFAT members share a consensus DNA binding motif which allows them to bind to the same DNA sequence (20–22). Upon T cell activation, NFAT is dephosphorylated and rapidly translocates into the nucleus to regulate gene expression via cooperation with other transcriptional regulators (21, 23). Recent studies have shown that different NFAT members have distinct properties and roles in CD4^+ T lymphocytes, especially in Th1, Th17, Th2, and Tregs (24–26). In vitro study of CD8^+ T cells selectively deficient in NFAT1, NFAT2, or NFAT4 and/or combination demonstrated a distinct role of these transcription factors regulating cytokines and inhibitory receptors expression (27). However, the distinction among NFAT members establishing CD8^+ T cell effector and memory differentiation in vivo is still undetermined.

In this study, we examined the role of NFAT1 and NFAT2 in CTL differentiation and function using an acute lymphocytic leukemia (ALL) model (28, 29). We characterized LCMV-specific CD8^+ T cell effector and memory population in mice deficient in NFAT1, mice with T cell-specific NFAT2 deficiency or with double deficiency of NFAT1 and NFAT2 in T cells. We found that NFAT1 is required for effector while NFAT2 is necessary for memory population generation. Mice deficient in both NFAT1 and NFAT2 have delayed memory differentiation and are unable to control an acute viral infection. Moreover, we also observed reduced cytokine production in all NFAT-deficient cells, with cells deficient in both transcription factors having the strongest effect, as well as an imbalanced Tbet and Eomes expression. The defect in CTL differentiation was cell-intrinsic, as evidenced by both mixed bone marrow chimera experiments and adoptive transfer of NFAT-deficient antigen-specific P14 TCR transgenic cells. These results suggest that NFAT1 and NFAT2 are indispensable and have distinct roles in initiating CD8^+ T cell effector and memory differentiation and function.

**MATERIALS AND METHODS**

**Mice**

All mice from C57BL/6 background used in the experiments were 6–8 weeks old, sex, and age matched. NFAT1^−/− and NFAT2^fl/fl^ CD4-Cre and NFAT1^−/− NFAT2^fl/fl^ CD4-Cre mice were obtained from La Jolla Institute for Allergy and Immunology (LJI, San Diego, CA) and have been described (24). NFAT1^−/− mice were crossed with NFAT2^fl/fl^ CD4-Cre^+^ to generate NFAT1^−/− NFAT2^fl/fl^ CD4-Cre^+^ (NFAT1/2 DKO) mice. P14 Thy1.1 or P14 TCRα−/− TCR transgenic mice were further crossed with NFAT deficient mice described above. For the mixed bone marrow chimera experiment, bone marrow cells were isolated from tibia and femur from B6.SJL CD45.1 mice, and mixed 1:1 ratio with bone marrow cells from C57BL/6 CD45.2 WT, NFAT1^−/−, NFAT2^fl/fl^ CD4-Cre^+, and NFAT1^−/− NFAT2^fl/fl^ CD4-Cre^+^ mice. Then, 7 million mixed bone marrow cells were transferred into lethally irradiated recipient B6SJL mice. All mice were maintained in specific-pathogen-free barrier facilities and used according to protocols approved by the Rosalind Franklin University of Medicine and Science Institutional Animal Care and Use Committee (IACUC).

**Lymphocytic Choriomeningitis Virus (LCMV) Models**

WT, NFAT1^−/− (NFAT1 KO), NFAT2^fl/fl^ CD4Cre^+^ (NFAT2 TKO), or NFAT1^−/− NFAT2^fl/fl^ CD4Cre^+^ (NFAT1/2 DKO), as well as mixed bone marrow chimera mice were infected intraperitoneally (i.p) with 2 × 10^5 PFU of LCMV Armstrong (LCMV^Arm^) kindly provided by Dr. Shane Crotty at LJI. After infection, spleenocytes, and serum were harvested. Serum viral titers were measured by plaque assay as described (29).

**Cell Staining and Flow Cytometry**

Single cell suspension isolated from spleens or heparinized blood were treated with RBC lysis buffer, washed and incubated with tetramer and antibody cocktails for surface staining. Single cell suspensions were initially incubated with LCMV tetramers H2D^b^-GP33-41 (KAVYNFATC) Alexa647, H2D^b^-GP276-286 (SGVENPGGYCL) BV421, and H2D^b^-NP396-404 (FQPQNGQFI) PE kindly obtained from the NIH Tetramer Facility, followed by staining of cell surface molecules including CD44, CD4, B220, CD8, KLRG1, CD127, and CXCR3. For intracellular transcription factor and cytokine staining, cells were then fixed, permeabilized and stained with antibody against Tbet, Eomes, IFN-γ, TNF-α, using eBioscience intracellular staining kits. Expression of these markers was assessed by flow cytometry using BD LSRII. The antibodies and reagents used are listed in Supplementary Table 1.

**T Cells Isolation, Culture, Cytokine Production, and Cytotoxicity Assay**

Spleen and lymph nodes were harvested, naïve CD8^+ cells were purified using Stem Cell EasySep kit from pooled spleen and...
RESULTS

**NFAT1 and NFAT2 Distinctively Regulate CD8+ T Cell Effector and Memory Differentiation During Acute LCMV^Arm Infection**

To examine the role of NFAT1 and NFAT2 in CD8+ T cell differentiation during acute viral infection, we used mice deficient in NFAT1 (NFAT1−/−, referred as NFAT1 KO) and mice with conditional T cell-specific deficiency of NFAT2 (NFAT2β/β CD4-Cre^+, referred herein as NFAT2 TKO). We bred NFAT1 KO mice with NFAT2 TKO mice to generate NFAT1−/− NFAT2β/β CD4-Cre^+ (hereafter mentioned as NFAT1/2 DKO) mice. Six to eight weeks old WT, NFAT1 KO, NFAT2 TKO, and NFAT1/2 DKO mice were intra peritoneally injected with LCMV^Arm. Eight days post-infection, corresponding to the peak of the T cell effector response, we characterized the generation of CD8^+ T cell effector and memory antigen-specific populations using LCMV H2D^b-GP33-41 tetramer staining (Figures 1A,B, S1). We found that NFAT1 KO mice have increased splenic cellularity, whereas NFAT2 TKO and NFAT1/2 DKO mice have significant fewer splenocytes compared to WT controls (Figure S1B). NFAT1/2 DKO mice also displayed significantly lower percentage and number of antigen-specific CD8^+ T cells (LCMV tetramer H2D^b-GP33-41+) compared to their WT counterparts (Figures 1A,B, S1E). Moreover, NFAT2 TKO and NFAT1/2 DKO mice exhibit lower number of CD8^+ T cells in spleen (Figures S1C,D).

To evaluate the effect of deficiency in distinct NFAT members in CTL differentiation, we determined the SLECs, MPECs and effector memory (KLRG1^hi CD127^hi) populations on antigen-specific CD8^+ T cells recognizing GP33-41 peptide of LCMV. NFAT1 KO mice exhibit a significantly decreased SLEC population and an increase in CD127^hi cells, with over 3-fold increase in effector memory and MPEC populations compared to WT controls (Figures 1C,D). On the contrary, NFAT2 TKO mice displayed an increase in KL RGL1-expressing cells, particularly the effector memory subset, while NFAT1/2 DKO mice had a significant increase in effector memory cells expressing both KLRG1 and CD127, suggesting an additive effect of NFAT1 and NFAT2 deficiency. To exclude that the phenotype observed was specific only to LCMV H2D^b-GP33-41 tetramer, we additionally evaluated the CTL differentiation in H2D^b-GP276-286 and H2D^b-NP396-404 specific CD8^+ T cells (Figures S1F,G). We observed that each and combined NFAT deficiency led to an altered effector/memory CTL differentiation as described above irrespective of the antigen specificity.

The expression of the chemokine receptor CXCR3 is upregulated during CD8^+ T cell activation, and maintained through the transition from effector to memory population, though it remains at higher levels in memory CD27^hi CTLs (31–33). CXCR3 is important for trafficking of CTLs to peripheral tissues and lymphoid compartments allowing for the interaction with antigen-presenting cells, and further sustaining a type-1 inflammatory response (32). Using this marker, we also found a differential generation of KLRG1^lo CXCR3^hi and KLRG1^hi CXCR3^hi populations upon NFAT1 or NFAT2 deficiency.
FIGURE 1 | NFAT1 and NFAT2 distinctively regulate CD8+ T cell effector and memory differentiation during acute LCMV Armstrong infection. Mice were infected with LCMV Armstrong (2 x 10^5 PFU/mouse) intraperitoneally (i.p.). Spleens were harvested on day 8 after infection, and phenotypic characterization of CD8+ T cells was performed.

(A,B) Frequency of H2D^b-gp33-41^+ (LCMV-specific) cells gated on CD8^+CD44^hiCD4^-B220^- splenocytes were determined, and presented as the combined data from three independent experiments (A) or a representative contour plot (B).

(C,D) Expression of KLRG1, CD127, and CXCR3 gated on H2D^b-gp33-41^+CD8^+CD44^hiCD4^-B220^- cells was determined as a representative contour plot (C) and pooled data from three independent experiments (D).

Statistical analysis was performed on three independent experiments combined using non-paired One-Way ANOVA followed by Tukey’s multiple comparisons. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
As expected, in WT mice, over 60% of the WT H2D\(^{b}\)-GP33-41 specific CD8\(^{+}\) T cells express the memory marker CD127, of which only around 25% retain KLRG1 expression suggesting the formation of a stable memory population (Figures 2C,D). Consistent with what we observed at the peak of the immune response on day 8, we found a significant further expansion of the memory population in NFAT1 KO mice on day 30 post-infection. Similarly, NFAT2 TKO mice displayed a reduction in the memory population in NFAT1 KO mice while NFAT1/2 DKO mice showed a trend of reduced frequency and significant decreased total numbers of H2D\(^{b}\)-GP33-41 specific CTLs compared to WT controls (Figures 2A,B, S2B–D).

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### Cell-Intrinsic Effect in the Generation of Effector And Memory Responses Mediated by NFAT Family Members

Given that NFAT1 germline KO mice, NFAT2 T cell knockout mice and NFAT1/2 DKO mice were used, the differences in the effector and memory CTL differentiation could be due to cell intrinsic or extrinsic effects. To address this, we performed a mixed bone marrow chimera transfer experiment (Figures 3A,B, S3A–D). WT bone marrow cells carrying CD45.1 marker were mixed in a 1:1 ratio with WT, NFAT1 KO, NFAT2 TKO, or NFAT1/2 DKO bone marrow cells carrying CD45.2 marker, and co-transferred to lethally irradiated CD45.1 recipient mice. After 6 weeks, bone marrow reconstituted mice were infected with LCMV\(^{Arm}\) and analyzed for the generation of effector and memory populations on day 8 post-infection. While there are no differences in total splenocytes or the percentage of H2D\(^{b}\)-GP33-41 specific CD45.1 CTLs among the different groups (Figures S3A,B), mice receiving NFAT1/2 DKO bone marrows showed a trend of decreased H2D\(^{b}\)-GP33-41 specific CTLs in the CD45.2 compartment compared to the control cells, similar to what we have observed in the germline KO mice. We analyzed the effector and memory CD8\(^{+}\) T cells differentiation by measuring KLRG1 and CD127 expression in both CD45.1 and CD45.2 population (Figures 3B, S3D). We found that cells lacking NFAT1 were skewed toward a memory CTL population, cells lacking NFAT2 showed higher effector memory cells, and cells with NFAT1/2 double deficiency displayed an additive effect (Figures 3B, S3D). In addition, we adoptively transferred NFAT-deficient naïve P14 TCR\(^{α/−}\) CD8\(^{+}\) T cells into congenic mice followed by LCMV\(^{Arm}\) infection (Figures 3C,D, S3E,F). Similar altered effector and memory differentiation was found upon NFAT deficiency (Figures 3C,D, S3E,F). Thus, these results indicate the NFAT1 and NFAT2 regulate CTL differentiation in a cell intrinsic manner.

### NFAT1 Deficiency Results in Reduced Effector Function, Which Is Further Compromised Upon Compound NFAT2 Deficiency

To assess if the abnormal CD8\(^{+}\) T cell differentiation upon deficiency of distinct NFAT family members also results in further transcriptional and functional changes, we initially determined the expression of two transcription factors Tbet and Eomes, which have been referred to as master regulators of CTL differentiation controlling the generation of effector and memory cells, respectively (34–36), on both day 8 and 30 post-LCMV infection (Figure 4). We found that Tbet expression was significantly reduced in NFAT2 TKO as well as NFAT1/2 DKO mice, with a slight, but not statistically different decrease in NFAT1 KO mice on day 8 p.i (Figures 4A,B). On the contrary, Eomes expression was significantly increased in NFAT1/2 DKO, and showed a consistent trend toward an increase, although not statistically significant, in NFAT2 TKO CD8\(^{+}\) T cells compared to WT controls. To delineate the relative expression between these two transcription factors, we calculated the fold change of Eomes vs. Tbet expression (using MFI) relative to WT cells (Figure 4B). CTLs lacking NFAT2 showed a slight increase in the Eomes:Tbet ratio, while cells with NFAT1 and NFAT2 double deficiency displayed an average of more than 5-fold increase in Eomes:Tbet ratio compared to WT controls (Figure 4B). This dysregulated Eomes:Tbet expression was observed irrespective of the subpopulation analyzed (effector or memory cells based on KLRG1 and CD127 expression), and thus is not due to differences in frequencies of effector or memory CTLs (data not shown). Similar changes in Eomes:Tbet ratio were observed on day 30, a time point in which Tbet expression is reduced due to a memory transition (Figures 4C,D). The altered Tbet vs. Eomes ratio in NFAT1/2 DKO mice was a result of not only diminished Tbet but also increased Eomes expression (Figures 4B,D). However, at this time point, single NFAT deficiency did not show significant differences in expression of these two transcription factors (Figure 4D). Our results suggest that NFAT transcription factors regulate expression of Tbet and Eomes in vivo. This is in fact consistent with the presence of direct NFAT1, and to a lesser
extent NFAT2, binding in both Tbx21 and Eomes loci as shown using previously published ChIP-seq data (27, 37) (Figure S4).

To determine the impact of NFAT deficiency on CTL function, we initially determined cytokine production in antigen-specific CD8$^+$ T cells after ex vivo stimulation with PMA and ionomycin (Figures 5A,B). The percentage of CTLs producing both IFN-γ and TNF-α were reduced in all NFAT-deficient cells, with NFAT1/2 double deficient cells displaying almost complete lack...
of cytokine production compared to WT controls both on day 8 and 30 (Figures 5A, B). Given that NFAT deficiency results in defective cytokine production, and Eomes levels are increased, which is also characteristic of exhausted cells (38), we hypothesized that these mice were unable to properly control the viral infection. For this purpose, we determined serum viral load in our experimental animals both on day 8 and 30 (Figure 5C). We observed that NFAT1/2 DKO mice were unable to control acute LCMV infection and the viral load increased over time, suggesting that NFATs are crucial for eliciting a proper immune response preventing viral persistence. This is consistent with a previous publication in which mice lacking both STIM1 and STIM2, which are upstream of NFAT signaling, were reported to have viral reoccurrence in the serum upon acute LCMV infection (39). Thus, our results pinpoint NFATs, within the calcium signaling pathways, as vital regulators of CTL function. Moreover, we observed a positive correlation between the viral load and the percentage of KLRG1hi CD127lo population suggesting that a delayed transition to a memory phenotype is associated with viral persistence (Figure 5D).

To further evaluate the role of NFAT members in CTL function, we utilized an in vitro antigen-specific cytotoxicity assay (Figures 5E, F, S5A–C). For this purpose, WT or NFAT-deficient P14+ TCR transgenic naïve T cells were activated and
differentiated into memory-like CTLs, as previously described (30). These cells were then incubated at different ratios with parental breast cancer cell line EO771 expressing GFP (as negative controls) or EO771-GFP cells expressing GP33-41 (target cells) for 12h. Cytotoxicity was determined as the frequency of CD8− GFP− cells. We did not observe any significant differences in antigen-specific killing between NFAT2-deficient and WT cells at the different ratios, however, cells lacking only NFAT1 or both NFAT1 and NFAT2 showed reduced killing ability (Figure 5F). To further determine the mechanism behind this reduced cytotoxicity, we measured the expression of cytokines or lytic molecules. We found a reduction in Pym1, Ifng, and Il2 mRNA levels in cells with NFAT1 deficiency (either single KO or NFAT1/2 DKO cells) (Figure S5D). Moreover, we also observed a reduction in IFN-γ, IL-2, and TNF-α cytokine production upon restimulation of these NFAT1-deficient memory-like CTLs compared to WT controls (Figures S5D–F). Thus, our data suggests that NFAT1 is mainly responsible for proper effector CTL differentiation and function, which is further strengthened upon NFAT2 deficiency.

FIGURE 4 | NFAT1 and NFAT2 regulate Tbet and Eomes expression. Expression of Tbet and Eomes was determined on antigen-specific cells from day 8 and 30 experiments depicted in Figures 1, 2. A representative histogram showing Tbet and Eomes MFI is shown on H2Dg-gp33-41+ CD8+ T cells from day 8-infected mice (A) and day 30-infected mice (C). Eomes and Tbet MFI fold change ratio as well as the fold change relative to WT mice was calculated by comparing the average Eomes or Tbet MFI fold change from different genotypes in each individual experiment on day8 (B) and day 30 (D). Statistical analysis was done using non-paired One-Way ANOVA followed by Tukey’s multiple comparisons. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

DISCUSSION

NFAT family members have redundant and distinct (sometimes even opposite) roles in lymphocytes development and function. Studies performed in vitro have demonstrated the requirement of different NFATs in CTL cytokine production but their roles in CD8+ T cell differentiation has yet to be determined in vivo (27). In our study, we found that NFAT1 and NFAT2, despite showing similar DNA binding motif specificity and belonging to the same transcription factor family, distinctively regulate CD8+ T cell differentiation in vivo. NFAT1 is needed to generate a proper CD8+ T cell effector population whereas NFAT2 is required for promoting memory CTL formation, indicating distinct and even opposing roles in CD8+ T cell differentiation. Compound deletion of both NFAT1 and NFAT2 in CTLs resulted in a significant increase in effector memory cells (expressing high levels of both KLRG1 and CD127) suggesting a delayed transition to memory cells, and a possible additive effect upon deficiency of these two transcription factors. NFAT1/2 DKO antigen-specific CD8+ T cells are significantly less compared to WT counterparts at the peak of the immune response, suggesting an impaired proliferation, which is in accord with NFAT controlling cell-cycle entry and proliferation of activated T cells (40). However, CTLs lacking either NFAT1 or NFAT2 are sustained differently upon LCMV Armstrong infection: NFAT1 deficiency led to an increased CD44hi CD8+ T cell population in contrast to lack of NFAT2, which resulted in reduced CD44hi CD8+ cells compared to WT controls. Our data is in line with a recent report where expression of NFAT2 in Store-Operated Calcium Entry (SOCE)-deficient T cells restores T cell proliferation in vivo (40). Similarly, a previous report showed that NFAT1 represses cell proliferation whereas NFAT2 promotes cell proliferation in NIH 3T3 cells (41).

NFAT deficiency results in dysregulated expression of Tbet and Eomes, two essential transcription factors that can determine CD8+ T cell fate commitment. While single NFAT deficiency had an increased Eomes:Tbet ratio, cells lacking both NFAT1 and NFAT2 showed the highest Eomes:Tbet ratio. Both NFAT1 and NFAT2 have been shown to directly bind to two regulatory elements in Tbx21 loci, one 11kb upstream and another one in the intronic region, which could account for the decrease in Tbet expression upon NFAT deficiency (37) (Figure S4). NFAT1 and NFAT2 also bind to Eomes regulatory elements. Other transcriptional regulators could also compete for NFAT binding and/or Eomes expression regulation. Eomes expression has been shown to be upregulated in exhausted CD8+ T cells during chronic infections and cancer (2); mice with dual deficiency...
FIGURE 5 | NFAT1 and NFAT2 differentially contribute to CTLs’ effector function. (A,B) Cytokine production upon re-stimulation of total splenocytes was determined on antigen-specific cells from day 8 and 30 experiments depicted in Figures 1, 2. Expression of IFNγ and TNFα is shown in H2Dβ-gp33-41+ CD8+ T cells from day 8 (A) and day 30 (B) infections analyzed by One-Way ANOVA. (C) Viral titers from serum of each individual mouse collected from day 8 and 30 experiments (Figures 1, 2) was determined by plaque assay as Plaque Forming Units (PFU) per ml of serum. N.D., not detectable. (D) Spearman non-parametric correlation (Continued)
in NFAT1 and NFAT2 are unable to clear LCMV even in an acute infection model, and similarly express the highest Eomes levels. This viral persistence has also been observed in mice with dual deficiency of STIM1 and STIM2, which are upstream of NFAT signaling pathway. Moreover, these Stim−/− Stim2fl/fl CD4Cre+ mice also displayed similar CD8+ T cell differentiation defects as observed in the absence of both NFAT1 and NFAT2 (39). The viral persistence also correlates with a significant decrease in frequency of cells recognizing the immunodominant NP396–404 epitope, suggesting chronic antigen stimulation and exhaustion of CTLs in these mice (29, 42). The positive correlation between viral titers and KLRG1hi CD127lo population on day 30 again echoes chronic antigen stimulation. Given that expression of inhibitory receptors associated with exhaustion are directly regulated by NFAT family members (27), the assessment of exhaustion cannot be determined using these markers. Our phenotypic analysis in germline KO mice was also confirmed using P14 adoptive transfer and mixed bone marrow chimera experiments, demonstrating that NFAT transcription factors regulate CTL differentiation in a cell-intrinsic fashion.

NFAT1, but not NFAT2, controls effector CTL differentiation and cytotoxicity function. Our results show that cells lacking NFAT1 have an enhanced memory CTL commitment in vivo, and show decreased cytotoxicity against mammary carcinoma cells expressing cognate antigen. On the contrary, NFAT2-deficient cells showed increased terminal effector CTL commitment in vivo and normal cytotoxicity function compared to WT counterparts. Surprisingly, it has been recently reported that NFAT2 control cytotoxicity in CD8+ T cells in a study utilizing MHC mismatch killing of MOPC 315 plasacyoma cells and A20j cells (37). Our in vitro killing assay is antigen-specific, which resembles more of CTL in vivo function compared to MHC mismatch-induced killing. Another report recently found a role for NFAT2 in antitumor function by controlling advanced-stage non-small cell lung cancer (NSCLC); regional NFAT2 expression correlated with tumor prognosis (43). To support their argument, Heim et al. deleted NFAT2 in both CD4+ and CD8+ T cells which resulted in severe tumor development. Given that NFAT2 is essential for supporting T cell proliferation (40), the loss of tumor control could be due to a reduction in T cell numbers. Moreover, a defect in both CD4+ and CD8+ T cell function was observed, which impedes the understanding of cell-intrinsic effects. Therefore, the differences in NFAT1 and NFAT2 deficient CTL killing capacity need further illustration in vivo. Additionally, the dysregulated effector and memory CD8+ T cell differentiation upon NFAT1 and NFAT2 deficiency could also have differential effects in the effector and memory phase. Nevertheless, our ex vivo and in vitro stimulation of NFAT deficient CD8+ T cells recapitulate previous findings that NFAT1 mainly regulates production of IFN-γ while other NFATs might have compensatory roles upon re-stimulation in vitro (27).

The early transcriptional regulation of CD8+ T cell differentiation that set the epigenetic tone for gene expression prior to the proliferative burst is not fully understood (44). BATF and IRF4 have also been shown to function as pioneer factors required for CTL differentiation (13, 15). However, recently Pipkin and colleagues demonstrated that Runx3 functions upstream of these factors, working as a pioneer transcription factor that induces chromatin accessibility of cis-regulatory landscapes crucial for memory CTL formation (18). NFATs are among the primary regulators downstream of TCR signaling, being activated and translocated to the nucleus within minutes of TCR stimulation (23), and are therefore expected to have profound roles during priming. This idea is consonant with the loss of chromatin accessible regions containing NFAT binding sites in Runx3 knockout CD8+ T cells within the first 24 h of stimulation (18). Furthermore, it has also been suggested that NFATs directly regulate IRF4 expression and form an IRF4-NFAT-BATF transcriptional circuit (45). Similarly, NFAT factors have also been reported to control expression of transcription factor HIF1α as well as metabolic genes, pathways that can also affect CD8+ T cell fate upon activation (40, 46–48). Thus, NFAT family members could control the transcriptional induction of several genes that can coordinately regulate CTL differentiation in vivo, suggesting that they could function as non-redundant pioneer transcription factors driving effector and memory CTL commitment. Future work on better understanding the role of NFAT family members can help elucidate these distinct mechanisms.

We have identified that NFAT1 and NFAT2 have opposite functions in CTL differentiation. These differential roles of transcription factors belonging to the same family have been explored. For example, Id2 is essential for generating SLEC population whereas Id3 is crucial for long-lived memory CTLs (10). IL-10-IL-21-STAT3 axis promotes CD8+ T cell memory differentiation and preserve cell stemness (49), while IL-12-STAT4 axis downregulates TCF1 expression to promote effector cell differentiation (50). Another example is the Egr family members, which also display opposing functions: Egr-2 and –3 play as TCR-induced negative regulators of T cell function, whereas Egr-1 promotes T cell function (51, 52). While the induction of Egr-2 and Egr-3 expression by NFAT can be independent of AP-1 (27), Egr-1 requires both NFAT and AP-1 to induce its expression in CD4+ T cells (53).

NFAT1 and NFAT2 could distinctly control gene expression, driving CTL differentiation through distinct mechanisms. Three possible mechanisms are: (i) the structural differences between NFATs, especially in the transactivation region (TAD) domain (54); (ii) the timing of activation, where NFAT2 short isoform expression is induced by NFAT1 activation (55); (iii) the unique binding partners of different NFATs even in different NFAT isoforms. Recent mass spectrometric analysis showed a
higher number of NFAT1 unique associated proteins compared to NFAT2, suggesting these family members could distinctly regulate gene expression to induce CTL differentiation by cooperating with different transcription partners (56). The challenge of understanding the mechanism behind the distinct function of NFAT members should be further investigated during early T cell activation. How NFATs cooperate with other transcriptional regulators immediately after priming to form the chromatin landscape that eventually leads to effector and memory differentiation still remains unclear. Nonetheless, our work demonstrates the differential regulation of CD8+ T cell differentiation by NFAT1 and NFAT2 in acute viral infection, and provides a framework for understanding the roles of NFATs in early priming, effector and memory CD8+ T cell function.

AUTHOR CONTRIBUTIONS

TX and GM designed the experiments and wrote the article. TX performed experiments, analyzed and plotted the data. AK maintained the mouse colony and helped with in vivo experiments. GM supervised the project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00184/full#supplementary-material
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