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GATA-3 controls T cell maintenance and proliferation downstream of TCR and cytokine signals

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Abstract

GATA-3 is a master regulator for T_H2 differentiation. It is however poorly understood if GATA-3 controls mature T cell function beyond T_H2 determination. We show that TCR signals and cytokine stimulation promoted GATA-3 expression in CD8 T cells, which controlled cell proliferation. While GATA-3-deficient CD8 T cells were generated, their peripheral maintenance was impaired with reduced IL-7R expression. GATA-3-deficient T cells had defective responses to viral infection and alloantigen. c-Myc was a critical target of GATA-3 in promoting the proliferation of activated T cells. This study thus demonstrates an essential role of GATA-3 in controlling T cell maintenance and expansion beyond T_H2 differentiation, providing insights into immune regulation.

T cells are pivotal to controlling the immune response to fight infection, eradicate tumor and elicit autoimmunity. CD8 T cells are cytotoxic and mediate the killing of target cells[1], while CD4 T cells differentiate into helper T (T_H) cell subsets, such as T_H1, T_H2, T_H17 and T_{reg} cells, to modulate the functions of innate cells, B cells and T cells. To achieve effective T cell response, a series of events including cell activation, expansion and differentiation need to be accomplished following antigenic and mitogenic stimulation. Identifying the factors critical for these processes is a central question in T cell immunology.

T cell activation by TCR ligation is followed by T cell expansion. In addition, T cell expansion can be further promoted and sustained by cytokines produced by T and non-T cells. The TCR and cytokine receptors signal through largely discrete pathways with shared

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Author contribution statement

Y.W. designed and did cellular, molecular and biochemical experiments; Y.W., I.M, and J.K.W. did LCMV infection experiments and contributed to the writing of the manuscript; A.G. contributed to the GvH response experiments; T.A.C and L.S. provided critical genetic models and intellectual inputs; Y.Y.W. designed experiments, wrote the manuscript and provided overall direction.

Competing financial interests

The authors declare no competing financial interests.

components[2, 3]. Nevertheless, critical factors for T cell activation and expansion remain incompletely identified.

GATA-3 is a transcription factor highly expressed in T_H2 CD4 T cells[4–6]. It is required for the differentiation of T_H2 cells and is therefore regarded as a master regulator for these cells. GATA-3 also regulates T cell development[7, 8], NK cell generation and function[9, 10], Treg cell function[11–13], the generation of type-2 innate lymphoid cells[14, 15], as well as tumorigenesis[16, 17]. An outstanding question is whether GATA-3 is important for mature T cell function beyond T_H2 differentiation and whether a common mechanism can be used by GATA-3 to control the function of different cell types.

To address this question, we investigated GATA-3 expression in CD8 T cells. We found that GATA-3 expression was constitutive in CD8 T cells, was up-regulated by TCR activation and further increased by cytokine stimulation. Deletion of GATA-3 in CD8 T cells did not affect CD8 thymocyte development, but the long term peripheral maintenance of GATA-3-deficient CD8 cells was impaired, with reduced IL-7R expression and defective IL-7 response. TCR- and cytokine-promoted CD8 T cell expansion was virtually abolished in the absence of GATA-3. GATA-3-deficient CD8 T cells failed to expand in response to LCMV infection and during graft-versus-host responses *in vivo*. Mechanistically, we identified c-Myc as a critical target of GATA-3 for regulating T cell proliferation. Therefore, the present study reveals a fundamental role for GATA-3 in T cell function.

Results

TCR and cytokine stimulation induced GATA-3 in CD8 T cells

To study GATA-3-controlled T cell functions beyond T_H2 differentiation, we investigated GATA-3 expression in CD8 T cells. We first assessed if GATA-3 was expressed in naïve CD8 T cells under steady-state. Compared to B cells, where minimal GATA-3 expression was detected, substantial GATA-3 protein expression was detected in naïve CD8 T cells, albeit in lower amounts than in naïve CD4 T cells (Fig. 1a). Thus, GATA-3 is expressed constitutively in CD8 T cells. In addition, because TCR activation can promote GATA-3 expression in CD4 SP thymocytes[18], we evaluated the effect of TCR activation on GATA-3 expression in mature CD8 T cells. GATA-3 expression was increased upon TCR activation (Fig. 1b). We also investigated if GATA-3 expression in CD8 T cells could be further promoted by IL-2 and IL-4 stimulation, similar to CD4 T cells[4, 12, 19]. IL-4 and also IL-2 stimulation further enhanced GATA-3 expression in activated CD8 T cells (Fig. 1c).

In contrast to T_H2 cells, where enhanced GATA-3 expression leads to increased IL-4 production[4], induction of GATA-3 in CD8 T cells was not associated with expression of the T_H2-type cytokine IL-4 (Fig. 1d and Supplementary Fig. 1a). The numbers of recovered CD8 T cells were however significantly increased by both IL-2 and IL-4 stimulation (Fig. 1e). The cell number increase was predominantly due to enhanced cell proliferation (Fig. 1f) rather than improved cell survival (Supplementary Fig. 1b). These findings therefore suggest that GATA-3 is expressed in CD8 T cells and further induced by TCR and cytokine stimulation.

CD8 T cell development in the absence of GATA-3

We further investigated the role of GATA-3 in CD8 T cell function. Cd4Cre mice[20] were crossed with *Gata3*$^{fl/fl}$ mice[21] to generate Cd4Cre-*Gata3*$^{fl/fl}$ mice where the *Gata3* gene is deleted in the double positive (DP) developmental stage in the thymus. Efficient deletion of GATA-3 was confirmed at both protein and mRNA levels (Fig. 1a and Fig. 2a). In the absence of GATA-3, while the development of CD4SP thymocytes was virtually abolished, the generation of CD8SP thymocyte was not affected (Fig. 2a), in agreement with previous reports[6, 8, 21]. However, the expression of thymocyte maturation markers, such as CD5, CD24 and CD69 appeared slightly perturbed (Supplementary Fig. 2a). In the periphery of Cd4Cre-*Gata3*$^{fl/fl}$ mice, the numbers of mature CD4 T cells were drastically decreased, while the numbers of CD8 T cells were normal or slightly increased (Fig. 2a and Supplementary Fig. 2b). GATA-3-deficient CD8 T cells had an activated phenotype with increased percentage of CD62L^{lo}CD44^{hi} effector T cell population (Fig. 2b), associated with elevated IFN-γ production (Fig. 2c).

Because failed CD4 T cell generation in Cd4Cre-*Gata3*$^{fl/fl}$ mice could create an aberrant environment for the expansion and activation of CD8 T cells[22, 23], we further investigated the cell-intrinsic effect of GATA-3 deletion in CD8 T cells. To do so, we generated mixed bone marrow chimeric mice by transferring equal numbers of bone marrow cells from Cd4Cre-*Gata3*$^{fl/fl}$ and wild-type mice bearing CD45.2 and CD45.1 congenic markers respectively into irradiated *Rag1*$^{-/-}$ recipient mice. In the fully reconstituted mixed bone marrow chimeric mice, comparable percentages of GATA-3-deficient and wild-type CD8 T cells were generated in the thymus and periphery (Fig. 2d). In addition, the expression of T cell maturation markers was comparable between GATA-3-deficient and wild-type CD8SP thymocytes from the same host (Supplementary Fig. 2c). Furthermore, the percentages of activated (CD62L^{lo}CD44^{hi}) T cells were similar between GATA-3-deficient and wild-type CD8 T cells (Fig. 2e) with minimal IFN-γ production (Fig. 2f) in the same host. These findings suggest that the perturbed expression of maturation markers in CD8SP thymocytes and the activated phenotype in CD8 T cells in the periphery of Cd4Cre-*Gata3*$^{fl/fl}$ mice is likely due to extrinsic mechanisms. Therefore, the development of CD8 T cell is largely unaffected by the deletion of GATA-3.

GATA-3 is required for the long term maintenance of CD8 T cells

GATA-3-deficient CD8 T cells efficiently populated the periphery of the Cd4Cre-*Gata3*$^{fl/fl}$: wild-type mixed bone marrow chimeric mice within 3 months after reconstitution. Starting at 4 months after reconstitution, we noticed a gradual decline in the numbers of GATA-3-deficient CD8 T cells (data not shown). To test if peripheral maintenance of CD8 T cells is impaired in the absence of GATA-3, we purified naïve (CD62L^{hi}CD44^{lo}) CD8 T cells from Cd4Cre-*Gata3*$^{fl/fl}$ (CD45.2) and wild-type (CD45.1) mice. Purified cells were mixed at a ratio of 1:1, labeled with CFSE as a tracker for live cells and then transferred into immune competent syngeneic wild-type mice (CD45.2). While GATA-3-deficient CD8 cell maintenance was comparable to wild-type during the first 2 weeks, virtually no GATA-3-deficient CD8 T cells were recovered by 4 weeks after transfer (Fig. 3a), suggesting that the long term peripheral maintenance of CD8 T cells is defective in the absence of GATA-3.

To test whether CD8 T cells isolated from Cd4Cre-*Gata3*$^{fl/fl}$ mice experienced developmental and homeostatic abnormalities (Fig. 2) that may have contributed to the defective maintenance of these cells in the recipient mice, we further investigated GATA-3 function in mature CD8 T cells. To do so, we generated ERCre-*Gata3*$^{fl/fl}$ mice by crossing ERCre mice[24] with *Gata3*$^{fl/fl}$ mice. In cells expressing ERCre, the Cre recombinase is not active until an estrogen receptor (ER) agonist such as tamoxifen or 4-hydroxy-tamoxifen (4-HT) is provided. Therefore, T cells in ERCre-*Gata3*$^{fl/fl}$ mice are functionally equivalent to those of wild-type mice before ER agonist treatment. We used this system to time the deletion of GATA-3 in mature T cells. Naïve CD8 T cells sorted from ERCre-*Gata3*$^{fl/fl}$ (CD45.2) and wild-type (CD45.1) mice were mixed at a ratio of 1:1, labeled with CFSE as a tracker for live cells, and then transferred into immune competent syngeneic wild-type mice (CD45.2). One group of recipient mice were mock-treated with vehicle (corn oil), the other group was treated with tamoxifen to induce the deletion of *Gata3* gene in transferred cells (Fig. 3b). We found that ERCre-*Gata3*$^{fl/fl}$ CD8 T cells were virtually absent by 4 weeks after transfer in tamoxifen-treated mice, but not in mock-treated mice (Fig. 3b). These findings suggest that GATA-3 is required for CD8 T cell peripheral maintenance.

IL-7 is crucial for T cell maintenance in the periphery[25]. We found that IL-7R (CD127) expression was significantly lower in GATA-3-deficient CD8 T cells than in wild-type counterparts (Fig. 3c, d). In addition, compared to wild-type CD8 T cells, GATA-3-deficient CD8 T cells were defective in responding to IL-7-promoted T cell survival *in vitro* (Fig. 3e). We further investigated whether GATA-3 regulates IL-7R expression by binding to the *Il7r* locus. Using PROMO, a transcription factor binding site prediction program, we identified multiple putative GATA-3 binding sites in the *Il7r* locus. To identify which putative sites bind to GATA-3, we performed chromatin-immuno-precipitation (ChIP) assays in sorted primary CD8 T cells. As a positive control, we detected enrichment of GATA-3 binding to the CGRE site[26] within the T_H2 locus using sorted primary CD8 T cells (Fig. 3f). GATA-3 bound to a conserved regulatory region of *Il7r* locus (Fig. 3f), suggesting that GATA-3 controls IL-7R expression directly in CD8 T cells. These findings therefore suggest that GATA-3 is required for the long-term peripheral maintenance of CD8 T cells, at least in part through controlling IL-7R expression and responses to IL-7.

Activated CD8 T cell function requires GATA-3

Because GATA-3 expression was promoted by TCR and cytokine stimulation, we examined TCR- and cytokine-induced CD8 T cell functions in GATA-3-deficient cells. Naïve (CD62L^{hi}CD44^{lo}) CD8 T cells were sorted from Cd4Cre-*Gata3*$^{fl/fl}$ (CD45.2) and wild-type (CD45.1) mice, mixed and labeled with CFSE as a cell division indicator. Labeled cells were then activated in the absence or presence of the cytokines IL-2, IL-4 or IL-15. Upon TCR ligation, although the T cell activation marker CD69 was efficiently up-regulated in GATA-3-deficient T cells, the up-regulation of CD44 and CD25 was less efficient in GATA-3-deficient T cells compared to wild-type T cells (Fig. 4a). TCR- and cytokine-promoted T cell proliferation was abolished in the absence of GATA-3 (Fig. 4b). GATA-3 deletion did not substantially affect the survival of activated T cells (Supplementary Fig. 3a), while the production of IFN-γ by activated CD8 T cells was only slightly affected by GATA-3 deletion (Fig. 4c).

To further study GATA-3 function in activated mature CD8 T cells, we used CD8 T cells purified from ERCre-*Gata3*$^{fl/fl}$ mice, which allow the acute deletion of GATA-3 in mature T cells. Naïve CD8 T cells were sorted from ERCre-*Gata3*$^{fl/fl}$ mice (CD45.2) and wild-type mice (CD45.1), mixed at the ratio of 1:1, and then labeled with CFSE as a cell division indicator. Labeled cells were activated in the absence or presence of IL-2, IL-4 and IL-15, and 4-HT was added to the culture to delete floxed *Gata3* alleles. Compared to wild-type counterparts, GATA-3-deficient CD8 T cell up-regulated activation markers efficiently (Fig. 4d), likely because the up-regulation of these markers occurred quickly after TCR signaling and preceded the functional deletion of GATA-3 in ERCre-*Gata3*$^{fl/fl}$ T cells. Nonetheless, GATA-3-deficient CD8 T cells proliferated poorly in response to TCR and cytokine stimulation (Fig. 4e). GATA-3 deletion did not lead to obvious change in T cell survival (Supplementary Fig. 3b), but resulted in a slight reduction in IFN-γ production by activated CD8 T cells (Fig. 4f). These findings therefore suggest that GATA-3 is critical for CD8 T cell proliferation in response to TCR and cytokine stimulation.

GATA-3 is important for activated CD8 T cell expansion *in vivo*

To address the requirement for GATA-3 in CD8 T cell function *in vivo*, we used a lymphocytic choriomeningitis virus (LCMV) infection model, in which the expansion of LCMV-specific CD8 T cells can be detected by tetramer staining of CD8 T cells specific for the LCMV-antigen gp_{33-41}[27]. Splenocytes isolated from young wild-type (CD45.1) and Cd4Cre-*Gata3*$^{fl/fl}$ (CD45.2) mice were mixed at the ratio of 1:1 and then transferred into syngeneic wild-type recipient mice (CD45.1xCD45.2), followed by infection with LCMV Amstrong strain. Eight days after infection, CD8 T cells reactive to the LCMV antigen gp_{33-41} were detected by tetramer staining. While gp_{33-41}-specific wild-type donor CD8 T cells were readily detectible after LCMV infection, very few gp_{33-41}-specific Cd4Cre-*Gata3*$^{fl/fl}$ donor CD8 T cells were found (Fig. 5a). In the same experiments, the percentage of IFN-γ producing gp_{33-41}-specific CD8 T cells was much smaller in GATA-3-deficient than in wild-type donor CD8 T cells (Fig. 5b). Therefore, GATA-3 is required for the expansion of CD8 T cells in response to specific viral antigen stimulation during LCMV infection.

We also investigated GATA-3-deficient CD8 T cells expansion in an acute graft-versus-host (GvH) response model, in which transferred CD8 T cells reacting to MHC-mismatched host cells are activated and expanded[28, 29]. Splenocytes isolated from ERCre-*Gata3*$^{fl/fl}$ (CD45.2) and wild-type (CD45.1) mice, which are both on a C57BL/6 background were mixed at a ratio of 1:1 and then transferred into immuno-deficient *Rag2*$^{-/-}\gamma_c^{-/-}$ recipient mice that are on an allogeneic Balb/c background. Immediately after transfer, recipient mice were treated with tamoxifen to delete floxed *Gata3* alleles in transferred cells or mock-treated with vehicle. CD8 T cells of different donor origins were monitored 3–4 weeks post transfer. In mock-treated recipient mice the percentages of CD8 T cell originated from ERCre-*Gata3*$^{fl/fl}$ and from wild-type mice were comparable (Fig. 5c), while in tamoxifen-treated recipient mice the percentage of ERCre-*Gata3*$^{fl/fl}$ donor CD8 T cell was reduced compared to wild-type donor CD8 T cells (Fig. 5c).

The defects observed in GATA-3-deficient CD8 T cells in this setting could be partly due to failed lymphopenia-driven proliferation[29], because GATA-3-deficient CD8 T cells have reduced responses to IL-7. To minimize the effects of lymphopenia-driven proliferation, we treated recipient mice with tamoxifen two weeks after cell transfer, to allow efficient T cell reconstitution before GATA-3 deletion. While the numbers of ERCre-*Gata3*$^{fl/fl}$ and wild-type donor CD8 T cell were comparable in mock-treated mice, the numbers of ERCre-*Gata3*$^{fl/fl}$ donor CD8 T cell were much reduced compared to wild-type donor CD8 T cells in tamoxifen-treated mice (Fig. 5d). In addition, compared to wild-type CD8 T cells, GATA-3-deficient CD8 T cells expressed much less Ki67, an indicator for cell proliferation (Fig. 5e). Collectively, these findings suggest that GATA-3 is critical for the expansion of CD8 T cells under both infection and inflammation conditions.

GATA-3 controls c-Myc expression

To gain insight into the mechanisms by which GATA-3 controls CD8 T cell activation and proliferation, we investigated which T cell activation and proliferation signaling pathways were affected by GATA-3 deletion. The activation of NF-κB, JNK and Erk pathways was normal in the absence of GATA-3 (Supplementary Fig. 4a). Nevertheless, we consistently observed that the cell size of activated GATA-3-deficient CD8 T cells was significantly smaller than that of GATA-3-sufficient cells (Fig. 6a). Because c-Myc is critical for T cell metabolism, growth and proliferation[30, 31], we assessed c-Myc expression in GATA-3-deficient CD8 T cells. While c-Myc was promptly and robustly up-regulated in wild-type T cells following TCR stimulation, GATA-3 deletion abolished c-Myc up-regulation (Fig. 6b). In addition, while the expression of a CDK-inhibitor p27^{Kip1} in wild-type cells was downregulated at the time of cell-cycle entry, p27^{Kip1} expression remained high in GATA-3-deficient T cells (Supplementary Fig. 4b), consistent with the observation that T cell proliferation was defective in the absence of GATA-3,

To investigate if c-Myc is a critical functional target of GATA-3 in CD8 T cells, we tested whether ectopic expression of c-Myc in GATA-3-deficient T cells ameliorated the proliferation defects. We transduced wild-type and GATA-3-deficient CD8 T cells with either control MSCV-IRES-EGFP (MIG) virus or with a c-Myc-expressing (MIG-Myc) retrovirus (Fig. 6b) and assessed the proliferation of transduced T cells. While control virus-transduced GATA-3-deficient T cells proliferated poorly compared with control virus-transduced wild-type T cells, c-Myc expression greatly promoted the proliferation of GATA-3-deficient T cells (Fig. 6c). Therefore, c-Myc is an important downstream target of GATA-3 to control T cell proliferation. Using PROMO we identified multiple putative GATA-3 binding sites in the *Myc* locus. To identify which putative sites bind to GATA-3, we performed ChIP assays using purified CD8 T cells. As a positive control for ChIP assay, we detected enrichment of GATA-3 binding to the CGRE site[26] within the T_H2 locus using purified CD8 T cells (Fig. 6d). GATA-3 bound to at least two conserved nucleotide sequence in the *c-Myc* locus in CD8 T cells (Fig. 6d). These findings collectively indicate that GATA-3 controls T cell function by controlling c-Myc expression.

Discussion

CD8 T cells are central to mediating the immune response to clear pathogen, eradicate tumor and elicit inflammation[1]. Normal generation, maintenance and function of CD8 T cells are required to achieve effective immune response. Therefore, identification of factors controlling CD8 T cell function is important for understanding immune regulation and treating immune diseases. This study reveals that GATA-3, a well-studied factor controlling T_H2 differentiation of CD4 T cells[4], is required for the maintenance and function of CD8 T cells.

In this study, the long term peripheral maintenance of CD8 T cells was found defective upon GATA-3 deletion. Such a defect could be attributed to reduced IL-7R expression and impaired IL-7 response. Compared to IL-7R knockout mice[32], the defects observed in Cd4Cre-*Gata3*$^{fl/fl}$ mice was less severe however. It suggests that other factor(s), e.g. Foxo1[33, 34], may compensate for the loss of GATA-3 to promote IL-7R expression in GATA-3-deficient T cells.

Apart from being essential for the peripheral maintenance of CD8 T cells, IL-7 signal is required for CD8 thymocyte development[32]. We found that CD8 T cells developed normally in the absence of GATA-3, suggesting that GATA-3 controls IL-7 response in a cell-type specific manner: it is important for IL-7 response in mature CD8 T cells but not in thymocytes. This may contribute to the observation that, although the long term peripheral maintenance of GATA-3-deficient CD8 T cells was impaired, the numbers of peripheral CD8 T cell did not decrease in Cd4Cre-*Gata3*$^{fl/fl}$ mice, because CD8 T cells were efficiently generated from the thymus in these mice. Cd4Cre-*Gata3*$^{fl/fl}$ mice could thus serve as a model where IL-7 signal is defective in peripheral naive CD8 T cells without the complications associated with profound CD8 T cell development defects.

Besides controlling T cell development and peripheral maintenance, IL-7 signal regulates the differentiation of long term (central) memory CD8 T cells[35]. In the current study, we noticed that, compared to the wild-type counterparts, GATA-3-deficient CD8 T cells had less $CD62L^{hi}CD44^{hi}$ central memory CD8 T cells. These findings indicate that GATA-3 regulates the generation and differentiation of CD8 memory T cells through IL-7 signal, which requires further investigation.

Upon activation, naïve T cells grow, exit quiescent state and proliferate rapidly. The cell growth and expansion are essential for CD8 T cells to achieve sufficient numbers to carry out its function. Transcription factors, e.g. c-Myc[30, 31], TOB1[36] and KLF2[37], are important for these biological processes. In this study, we found that GATA-3 is required for CD8 T cell to growth and to proliferate in response to activation. c-Myc is an important functional target of GATA-3 to promote CD8 T cell proliferation. Nonetheless, c-Myc may not be the sole downstream of GATA-3 in controlling CD8 T cell proliferation, because ectopic expression of c-Myc did not fully restore the proliferation of GATA-3-deficient CD8 T cells. In agreement with this observation, it is reported that GATA-3 regulates N-myc expression[38]. And we found that the expression of TOB1 and KLF2 was deregulated in GATA-3-deficient CD8 T cells (our unpublished data). How these factors contribute to

GATA-3-controlled T cell proliferation and how they functionally related to c-Myc need further study.

We found that GATA-3-deficient CD8 T cells failed to grow in size after activation. Abolished c-Myc expression in these cells may contribute to such an observation because c-Myc is essential for T cell metabolism and growth[30, 31]. Nonetheless, it remains to be addressed as whether GATA-3-controlled T cell growth, similar to GATA-3-controlled T cell proliferation, is dependent on c-Myc.

While the growth and proliferation of CD8 T cell required GATA-3, GATA-3 deletion had minimal effect on the IFN-γ production of activated CD8 T cells, suggesting that GATA-3, albeit essential for CD8 T cell expansion, is dispensable for the effector function of CD8 T cells. Nonetheless, further investigation is required to address if GATA-3 is needed for the effector functions of CD8 T cells, such as the production of granzyme and perforin and the cytotoxic T lymphocyte activity. In addition, the finding that GATA-3 deletion did not lead to increased IFN-γ production in CD8 T cells suggests that, unlike in CD4 T cells[39], GATA-3 does not suppress IFN-γ production in CD8 T cells. This agrees with the observation that IL-2 and IL-4 stimulation, albeit promoted GATA-3 expression, did not lead to reduced IFN-γ production by CD8 T cells.

The function of CD8 T cells is influenced by the micro-environment and cytokine milieu. We found that GATA-3 controlled CD8 T cell function, suggesting that factors enhancing GATA-3 expression, such as IL-4, Wnt-TCF-β-catenin[40], Notch-RBPjκ[21, 41] may regulate CD8 T function. Indeed, these signaling pathways were found important for CD8 T cell function during immune response[42–44]. Therefore, it is of interest to investigate if and how GATA-3 is involved in IL-4-, Wnt- and Notch- mediated effects on CD8 T cell during these immune responses.

This study reveals that GATA-3 is essential for CD8 T cell proliferation through c-Myc. However GATA-3 controlled proliferation may not be limited to CD8 T cells. Inhibition of GATA-3 leads to reduced proliferation of T_H2 and hematopoietic stem cells[4, 45], although the underlying mechanism is unclear. These observations suggest that controlling proliferation is a conserved function of GATA-3 in different immune cell types. Therefore, such a GATA-3 function needs to be taken into consideration when we evaluate how immune function, besides T_H2 response, is affected after GATA-3 has been interfered with by genetic or biochemical approaches[5, 46–48].

Despite intense interest in GATA-3 function in T cells, GATA-3 function beyond T_H2-differentiation is poorly defined. This study reveals previously unappreciated GATA-3-IL-7R and GATA-3-c-Myc signal axis fundamental for T cell function and demonstrates that GATA-3 is critical for T cell maintenance and expansion. In addition, it sheds new lights on the mechanism through which T cell function and immune response are controlled.

Online Methods

Mice

Gata3$^{fl/fl}$, CD4Cre, ERCre, *Rag1*$^{-/-}$, and CD45.1 congenic wild-type mice were on C57BL/6 background. *Rag2*$^{-/-}\gamma_c^{-/-}$ mice were on Balb/c background. All mice were housed and bred in specific-pathogen-free conditions in the animal facility at the University of North Carolina at Chapel Hill. ERCre mediated deletion of floxed *Gata3* alleles was induced by intra-peritoneal injection of 2mg of Tamoxifen dissolved in 100μl of corn oil every day for three consecutive days. For LCMV infection, mice were infected by intra-peritoneal administration of 2×10^5 plaque forming units (PFU) of LCMV, Armstrong strain. All mouse experiments were approved by Institution Animal Care and Use Committee of the University of North Carolina.

Flow cytometric analysis and cell sorting

Lymphocytes were isolated from the various organs (as described) of age- and sex-matched mice of 8–16 weeks of age. Fluorescence-conjugated antibodies for CD4, CD8, CD5, CD24, CD25, CD44, CD69, CD62L, CD45.1, CD45.2, IFN-γ, IL-4 (eBioscience) and Annexin V and 7-AAD (BD biosciences) were purchased. APC-conjugated gp33 tetramer was kindly provided by NIH tetramer core facility. Surface and intracellular staining was performed per manufacturer's protocols. For intracellular cytokine staining, lymphocytes were stimulated with 50ng/ml of phorobol-12-myristate 13-acetage (PMA) and 1μM of ionomycin for 4 hr in the presence of Brefeldin A. Stained cells were analyzed on a LSRII station (BD biosciences) or sorted on Moflow cell sorter (Dako cytomation, Beckman coulter).

Cell isolation, culture, activation and proliferation

Lymphocytes were isolated from peripheral lymph nodes and/or spleens from mice. Cells were then activation by TCR stimulation, either alone or in combination with IL-2 (10 U/ml), IL-4 (20 ng/ml), or IL-15 (40 ng/ml). For proliferation assay, cells were labeled with CFSE dye and then cultured under different conditions as indicated. Cell proliferation was assessed by CFSE dilution and flow cytometry 72–96 hours after T cell activation. For co-culture experiment, naïve (CD62L^{hi}CD44^{lo}) T cells were either sorted from *CD4:Gata3*$^{fl/fl}$ mice (CD45.2) or from *ER:Gata3*$^{fl/fl}$ mice (CD45.2) and then mixed with naïve T cells sorted from wild-type mice (CD45.1) at a ratio of 1:1. Cells were activated with anti-CD3 and anti-CD28, either alone or in combination with IL-2 (10 U/ml) or IL-4 (20 ng/ml), or IL-15 (40 ng/ml). For cells from ERCre mice, 1μM of 4-OH-Tamoxifen (4-HT) was added to the culture to induced GATA-3 deletion currently with T cell activation.

Creating mixed bone marrow chimeric mice

Bone marrow cells were isolated from the femur bones of sex- and age-matched Cd4Cre-*Gata3*$^{fl/fl}$ (CD45.2) and wild-type (CD45.1) mice. $1x10^6$ bone marrow cells from each donor were mixed and transferred into irradiated (500 cGy) *Rag1*$^{-/-}$ recipient mice. T cell populations contributed from each donor origin in the recipients was determined 8–10 weeks after transfer.

Peripheral maintenance experiments

Naïve ($CD62L^{hi}CD44^{lo}$) CD8 T cells were sorted from wild-type (CD45.1), Cd4Cre-*Gata3*$^{fl/fl}$ (CD45.2) and ERCre-*Gata3*$^{fl/fl}$ (CD45.2) mice. Sorted wild-type (CD45.1) CD8 T cells were mixed either with *Gata3*$^{fl/fl}$ (CD45.2) CD8 T cells or with ERCre-*Gata3*$^{fl/fl}$ (CD45.2) CD8 T cells at the ratio of 1:1. Cell mixtures were labeled with CFSE and then transferred into immune competent congenic recipient mice. Mice received ERCre-*Gata3*$^{fl/fl}$ T cells were injected with vehicle control (Mock) or tamoxifen to delete floxed *Gata3* alleles at the time of cell transfer. Different time points after cell transfer, the transferred CD8 T cells were detected by CFSE label and donor cells of different origins were further distinguished by CD45.1 and CD45.2 expression by flow cytometry.

IL-7 response

Naïve ($CD62L^{hi}CD44^{lo}$) CD8 T cells were sorted from wild-type (CD45.1) and Cd4Cre-*Gata3*$^{fl/fl}$ (CD45.2) mice. Sorted CD8 T cells were mixed at the ratio of 1:1. Cell mixtures were then cultured in the presence of IL-7 (20ng/ml). At different time points, the contribution of CD8 T cell from different origins was detected by CD45.1 and CD45.2 staining and flow cytometry.

LCMV infection and re-stimulation of CD8 T cells with gp_{33-41} peptide

For LCMV infection, splenocytes isolated from wild-type (CD45.1) and Cd4Cre-*Gata3*$^{fl/fl}$ (CD45.2) mice were mixed at the ratio of 1:1. Cell mixtures were transferred into immune competent congenic recipients (CD45.1xCD45.2). One day after cell transfer, recipient mice were infected by intra-peritoneal administration of 2×10^5 plaque forming units (PFU) of LCMV, Armstrong strain. 8 days after LCMV injection, the gp_{33-41} specific CD8 T cells of different origins in the spleens of recipient mice were detected by gp33-tetramer, CD45.1 and CD45.2 staining and flow cytometry. After LCMV infection, a portion of recipient splenocytes were cultured with gp_{33-41} peptide in the presence of Brefeldin A for 6 hours. CD8 T cells of different origins re-stimulated by gp_{33-41} peptide were detected by IFN-γ intracellular staining and flow cytometry.

GvH response mouse model

Total splenocytes from sex- and age-matched ERCre-*Gata3*$^{fl/fl}$ (CD45.2) and wild-type (CD45.1) mice of C57BL/6 background were mixed at a ratio of 1:1 and then transferred into sub-lethally irradiated *Rag2*$^{-/-}\gamma_c^{-/-}$ mice of Balb/c background via retro-orbital injection. Tamoxifen or vehicle control (mock) was injected into recipient mice either immediately or 2 weeks after cell transfer. Recipient mice were monitored and euthanized at indicated time. T cells from the spleens of recipient mice were harvested and subjected to immunological analysis.

Immuno-blotting analysis

Protein extracts were resolved on 4–12% SDS-PAGE gels (Invitrogen), transferred to a polyvinylidene fluoride membrane (Millipore) and analyzed by immuno-blotting with the following antibodies: anti-GATA-3 (L50-823, BD biosciences), anti-c-Myc (D84C12, Cell signaling), anti-p27 (F-8, Santa Cruz), and anti-β-actin (I-19, Santa Cruz).

Quantitative RT-PCR

Total RNA was extracted from T cells using TRIzol reagent (Invitrogen) per manufacturer's instructions, and was reverse-transcribed into c-DNA with Superscript III reverse transcriptase (Bioline). Quantitative PCR was performed on ABI9700 real-time PCR system with primer-probe sets purchased from Applied Biosystems.

Chromatin Immuno-precipitation (ChIP) assay

ChIP assay was performed per Upstate Biotechnology's protocol. Briefly, cells were cross-linked by 1% formaldehyde and lysed in lysis buffer. The lysates were sonicated with a Bioruptor sonicator to shear genomic DNA into 200–500 bp fragments. Chromatin prepared from $2x10^6$ cells were subjected to immuno-precipitation overnight at 4^oC using goat anti-GATA-3 (D-16, sc-22206, Santa Cruz) or normal goat IgG (sc-2028, Santa Cruz) antibodies. Quantitative real-time PCR was performed to determine the relative abundance of target DNA. Specific primers for analysis of GATA-3 binding to Foxp3 and other target loci are listed in Supplementary Table 1.

Statistical analysis

Data from at least three sets of samples were used for statistical analysis. Statistical significance was calculated by Student's t test. A *P* value of less than 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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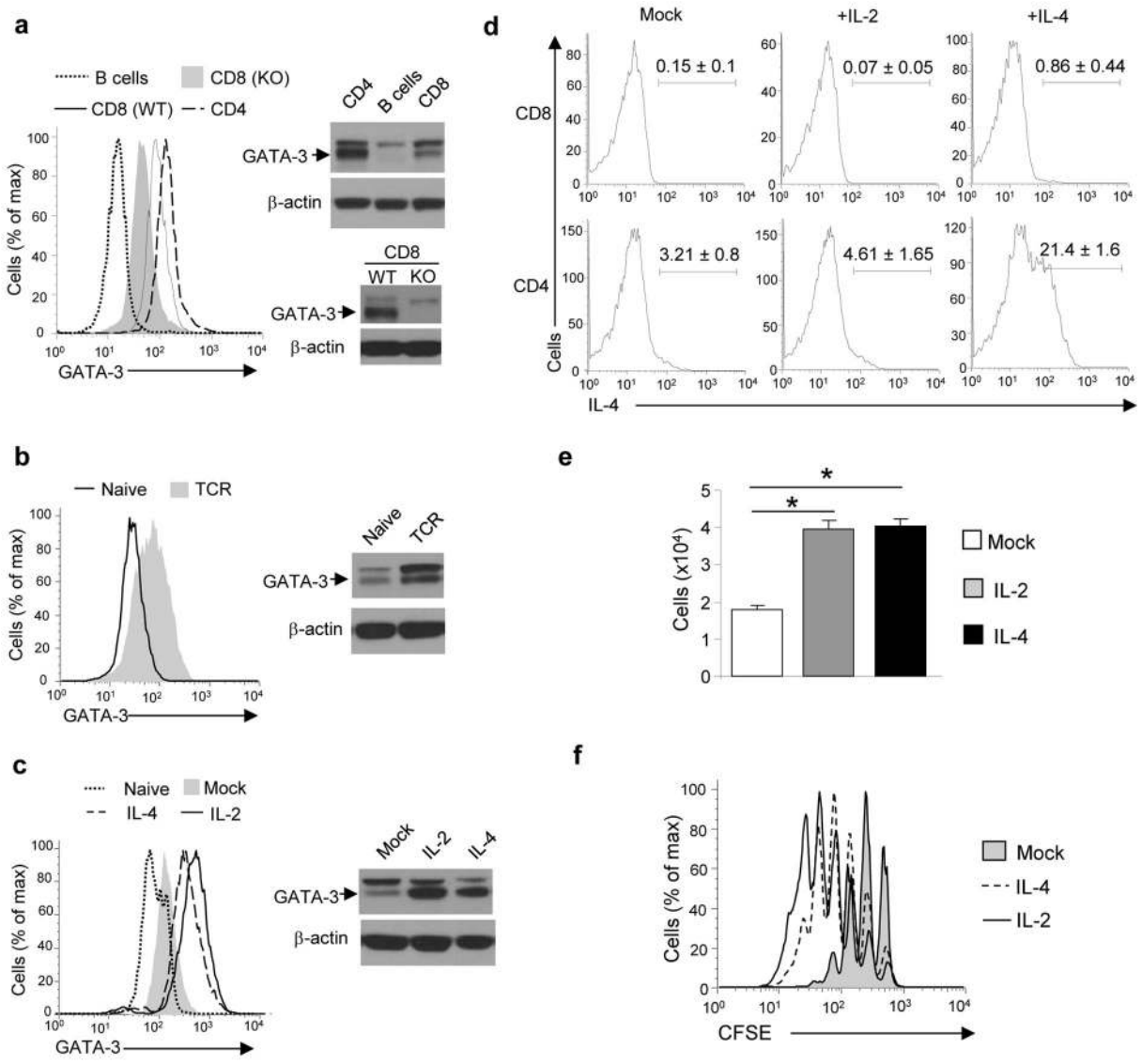

Fig. 1. TCR and cytokine stimulation promoted GATA-3 expression in CD8 T cells

(a) GATA-3 expression in CD4 T cells (dashed line), CD8 T cells (solid line) and B cells (dotted line) purified from wild-type mice and CD8 T cells purified from Cd4Cre-*Gata3*$^{fl/fl}$ mice (KO, shaded) as assessed by intra-cellular staining and immuno-blotting. Results shown are representative of at least three experiments. (**b**) GATA-3 expression in naïve CD8 T cells and CD8 T cells stimulated with anti-CD3 for 72 hours as assessed by intracellular staining and immuno-blotting. Representative results of at least three experiments are shown. **(c)** GATA-3 expression in CD8 T cells stimulated with anti-CD3 for 72 hours, in the absence (Mock) or presence of IL-2 or IL-4 as assessed by intracellular staining and immuno-blotting. Results shown are representative of at least three experiments. **(d)** Flow cytometry of IL-4 production in CD8 and CD4 T cells isolated from wild-type mice and activated with anti-CD3 and anti-CD28, in the absence (Mock) or presence of IL-2 or IL-4 for 72 hours. Representative results of at least three experiments are shown. (**e**) Numbers of live CD8 T cells recovered after 72 hours of culture as in **d**. Means ± SD were shown. (*

$P<0.05$). **(f)** CFSE dilution in CD8 T cells labeled with CFSE assessed 72 hours after stimulation with anti-CD3, in the absence (Mock) or presence of IL-2 or IL-4. Results shown are representative of at least three experiments.

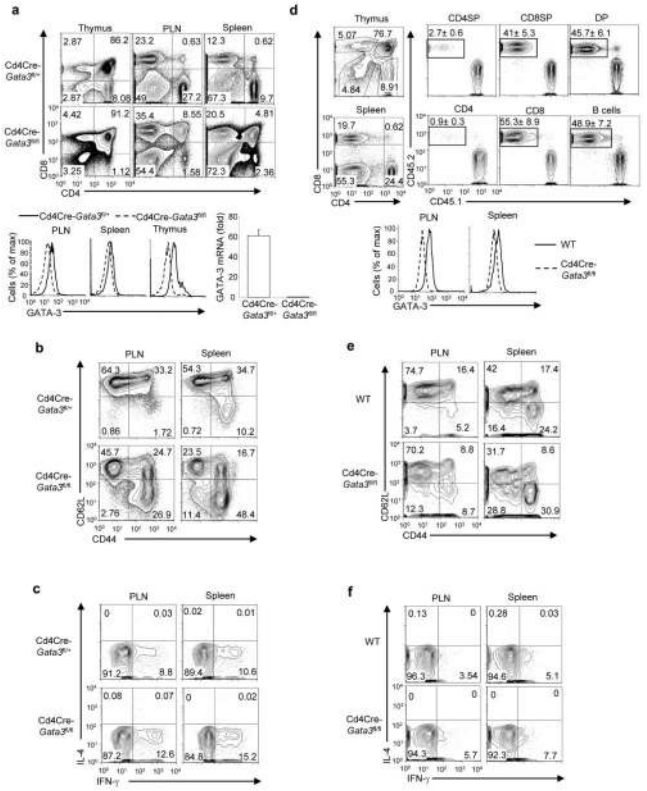

Fig. 2. CD8 T cell development in the absence of GATA-3

(a) Flow cytometry of T cell distribution in the thymus, peripheral lymph-nodes (PLN) and spleens of Cd4Cre-*Gata3*$^{fl/fl}$ and Cd4Cre-*Gata3*$^{fl/+}$ mice (top panels). GATA-3 expression assessed by intracellular staining and qRT-PCR (lower panels). Flow-cytometric results are representative of at least three experiments. qRT-PCR results are means ± SD of triplicates done in one experiment representative of at least three. (**b)** Flow cytometry of CD44 and CD62L expression in CD8 T cells isolated from PLN and spleen of Cd4Cre-*Gata3*$^{fl/fl}$ and Cd4Cre-*Gata3*$^{fl/+}$ mice. Representative results of at least three experiments are shown. **(c)** Flow cytometry of IFN-γ and IL-4 production in CD8 T cells isolated from PLN and spleen of Cd4Cre-*Gata3*$^{fl/fl}$ and Cd4Cre-*Gata3*$^{fl/+}$ mice. Results are representative of at least three experiments. (**d)** Flow cytometry of T cell populations in the mixed bone marrow chimera containing wild-type (CD45.1) and Cd4Cre-*Gata3*$^{fl/fl}$ (CD45.2) T cells (top panels). Means ± SD of five mice from one experiment representative of two are shown. GATA-3 expression assessed by intracellular staining (lower panels). **(e)** Flow cytometry of CD44 and CD62L expression in CD8 T cells from the mixed bone marrow chimera described in **d**. Results are representative of at least three experiments. **(f)** Flow cytometry of IFN-γ and IL-4 production in CD8 T cells from the mixed bone marrow chimera described in **d**. Results are representative of at least three experiments.

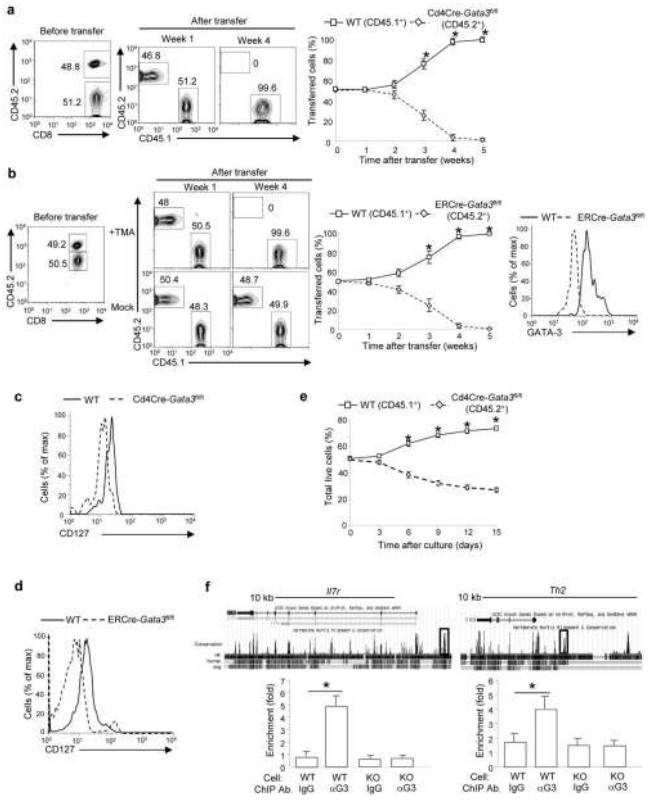

Fig. 3. Defective peripheral maintenance of GATA-3-deficient CD8 T cells

(a) Flow cytometry of CD8 T cell distribution during peripheral maintenance experiments using wild-type (CD45.1) and Cd4Cre-*Gata3*$^{fl/fl}$ (CD45.2) donor cells. Representative results and means ± SD of five mice from one experiment of two are shown (* P<0.05). **(b)** Flow cytometry of CD8 T cell distribution during peripheral maintenance experiments using wild-type (CD45.1) and ERCre-*Gata3*$^{fl/fl}$ (CD45.2) donor cells. Representative results and means ± SD of five mice from one experiment of two are shown (* P<0.05). GATA-3 expression was also assessed by flow-cytometry one week after tamoxifen (TMA) treatment. **(c)** Flow cytometry of CD127 expression in CD8 T cells of wild-type (solid line) and Cd4Cre-*Gata3*$^{fl/fl}$ (dashed line) origins in the recipient mice described in **a. (d)** Flow-cytometry of CD127 expression in CD8 T cells of wild-type (solid line) and ERCre-*Gata3*$^{fl/fl}$ (dashed line) origins in recipient mice described in **b. (e)** Flow cytometry of CD8 T cell distribution during IL-7 response experiment. Means ± SD of triplicate in one experiment representative of two are shown (* P<0.05). **(f)** GATA-3 binding to a conversed non-coding sequence in *Il7r* locus and to the CGRE site within the T_H2 locus in CD8 T cells isolated from wild-type and Cd4Cre-*Gata3*$^{fl/fl}$ (KO) mice detected by chromatin-immunoprecipitation (ChIP). Means ± SD of triplicates done in one experiment representative of at least three are shown. (* P<0.05)

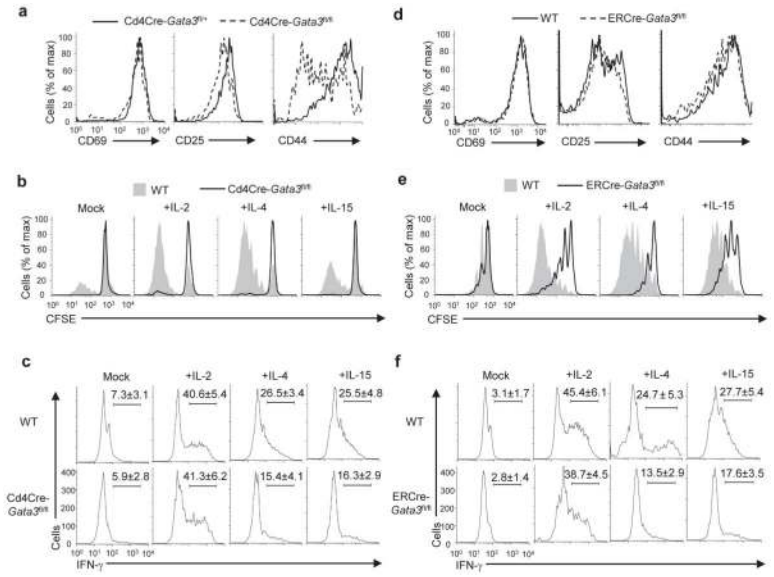

Fig. 4. Activated CD8 T cell function requires GATA-3

(a) Flow cytometry of CD25, CD44 and CD69 expression on wild-type (CD45.1) and Cd4Cre-*Gata3*$^{fl/fl}$ (CD45.2) CD8 T cells that were co-cultured and activated for 24 hours. Results are representative of at least three experiments. **(b)** The proliferation of wild-type (CD45.1) and Cd4Cre-*Gata3*$^{fl/fl}$ (CD45.2) CD8 T cells that were co-cultured and activated for 72 hours in the absence (Mock) or presence of indicated cytokines as assessed by CFSE dilution and flow cytometry. Results representative of at least three experiments are shown. **(c)** Flow cytometry of IFN-γ production by CD8 T cells described in **b**. Means ± SD of three experiments are shown. **(d)** Flow cytometry of CD25, CD44 and CD69 expression on wild-type (CD45.1) and ERCre-*Gata3*$^{fl/fl}$ (CD45.2) CD8 T cells that were co-cultured and activated for 24 hours in the presence of 4-HT. Results are representative of at least three experiments. **(e)** The proliferation of wild-type (CD45.1) and ERCre-*Gata3*$^{fl/fl}$ (CD45.2) CD8 T cells that were co-cultured and activated in the absence (Mock) or presence of indicated cytokines for 72 hours while 4-HT was added, as assessed by CFSE dilution and flow-cytometry. Results are representative of at least three experiments. **(f)** Flow cytometry of IFN-γ production by CD8 T cells described in **e**. Means ± SD of three experiments are shown.

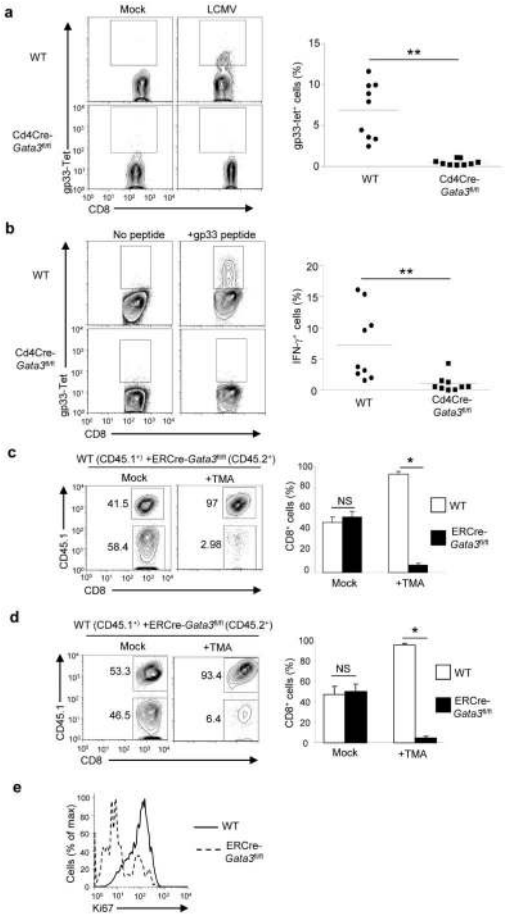

Fig. 5. GATA-3 is important for activated CD8 T cell expansion *in vivo*

(a) Flow cytometry of gp33-tetramer$^+$ wild-type (CD45.1) and Cd4Cre-*Gata3*$^{fl/fl}$ (CD45.2) CD8 T cells in the recipients (CD45.1xCD45.2) after LCMV infection. Representative flow-cytometric plots and combined results from multiple mice from two experiments are shown (** P<0.01). **(b)** Flow cytometry of IFN-γ production by wild-type (CD45.1) and Cd4Cre-*Gata3*$^{fl/fl}$ (CD45.2) CD8 T cells (described in **a**) upon gp_{33-41} peptide re-stimulation. Representative flow-cytometric plots and combined results from multiple mice from two experiments are shown (** P<0.01). **(c)** Flow cytometry of CD8 T cell distribution in *Rag2*$^{-/-}\gamma_c^{-/-}$ recipient mice (Balb/c) where GvH response was induced by transferred splenocytes from both wild-type (CD45.1, C57BL/6) and ERCre-*Gata3*$^{fl/fl}$ (CD45.2, C57BL/6) mice. Tamoxifen (TMA) or vehicle (Mock) was injected at the time of cell transfer. Representative flow-cytometric plots and means ± SD of five mice from one experiment representative of two are also shown. (NS, non-significant; * P<0.05). **(d)** Flow cytometry of CD8 T cell distribution in *Rag2*$^{-/-}\gamma_c^{-/-}$ recipient mice (Balb/c) where GvH response was induced by transferred splenocytes from both wild-type (CD45.1, C57BL/6) and ERCre-*Gata3*$^{fl/fl}$ (CD45.2, C57BL/6) mice. Tamoxifen (TMA) or vehicle (Mock) was injected two weeks after cell transfer. Representative flow-cytometric plots and means ± SD of five mice from one experiment representative of two are shown (NS, non-significant; * P<0.05). **(e)** Flow cytometry of Ki67 expression in wild-type and ERCre-*Gata3*$^{fl/fl}$ CD8 T

cells during GvH response as described in **c**. Result is representative of at least three experiments.

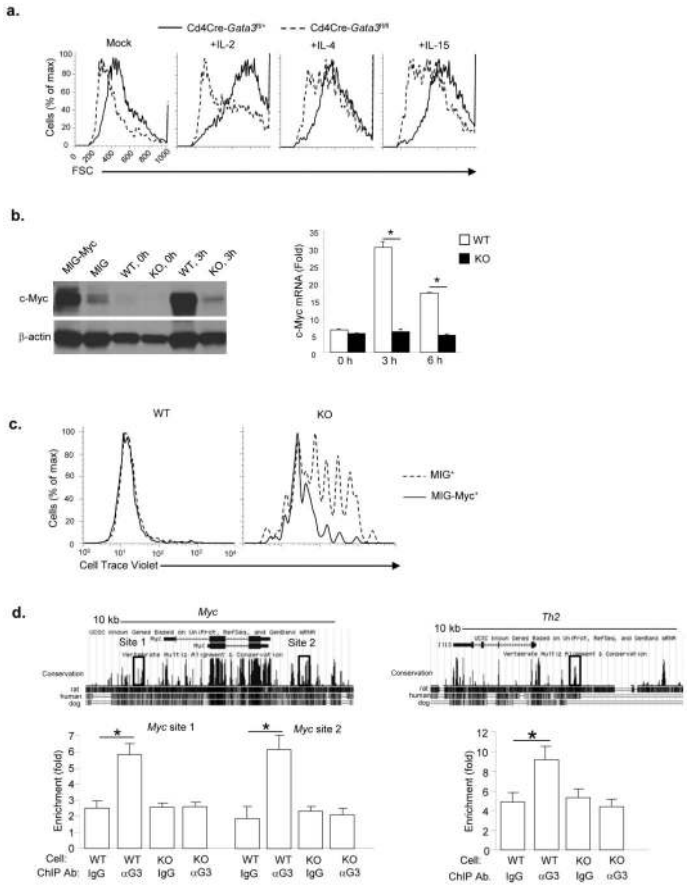

Fig. 6. GATA-3 controls c-Myc expression

(a) Flow cytometry of the cell size of Cd4Cre-*Gata3*$^{fl/fl}$ and Cd4Cre-*Gata3*$^{fl/+}$ CD8 T cells that were sorted naïve (CD62L^{hi}CD44^{lo}) and stimulated with anti-CD3 and anti-CD28 in the absence (Mock) or presence of indicated cytokines for 24 hours. Results are representative of at least three experiments. **(b)** The protein and mRNA expression of c-Myc in wild-type and Cd4Cre-*Gata3*$^{fl/fl}$ (KO) CD8 T cells that were sorted naïve (CD62L^{hi}CD44^{lo}) and stimulated with anti-CD3 and anti-CD28 as assessed by immuno-blotting and qRT-PCR respectively. Immuno-blotting results are representative of at least three experiments. qRT-PCR results are means ± SD of triplicates done in one experiment representative of three (*P<0.05). Immune-blotting for c-Myc in wild-type CD8 T cells transduced either with MSCV-IRES-EGFP (MIG) control virus or with MSCV-Myc-IRES-EGFP (MIG-Myc) viruses. Results are representative of at least two experiments. **(c)** Comparison of the proliferation of MIG and MIG-Myc -transduced (GFP^{+}) wild-type and ERCre-*Gata3*$^{fl/fl}$ (KO) CD8 T cells that were stimulated with anti-CD3 and anti-CD28 in the presence of IL-2 and 4-HT for 4 days, as assessed by Cell Trace Violet dye dilution with flow cytometry. Representative results of at least three experiments are shown. **(d)** GATA-3 binding to conversed non-coding sequences in *Myc* locus and the CGRE site within the *Th2* locus in CD8 T cells isolated from wild-type and Cd4Cre-*Gata3*$^{fl/fl}$ (KO) mice detected by ChIP. Means ± SD of triplicates done in one experiment representative of at least three are shown. (* P<0.05)