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Interferon regulatory factor 4 sustains CD8+ T cell expansion and effector differentiation

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SUMMARY

Upon infection, $CD8^+$ T cells undergo a stepwise process of early activation, expansion and differentiation into effector cells. How these phases are transcriptionally regulated is incompletely defined. Here, we report that interferon regulatory factor 4 (IRF4), dispensable for early $CD8^+$ T cell activation, was vital for sustaining the expansion and effector differentiation of $CD8^+$ T cells. Mechanistically, IRF4 promoted the expression and function of Blimp1 and T-bet, two transcription factors required for $CD8^+$ T cell effector differentiation, while repressed genes that mediate cell cycle arrest and apoptosis. Selective ablation of *Irf4* in peripheral $CD8^+$ T cells impaired anti-viral $CD8^+$ T cell responses, viral clearance and $CD8^+$ T cell-mediated host recovery from influenza infection. IRF4 expression was regulated by T cell receptor (TCR) signaling strength via mammalian target of rapamycin (mTOR). Our data reveal that IRF4 translates differential strength of TCR-signaling into different quantitative and qualitative $CD8^+$ T cell responses.

INTRODUCTION

$CD8^+$ T cells are an essential component of anti-viral and anti-tumor immunity (Zhang and Bevan, 2011). During an infection, naïve $CD8^+$ T cells rapidly undergo three stepwise stages of responses: early activation, clonal expansion and effector differentiation to generate a large number of antigen-specific effector T cells for pathogen clearance. During this process, $CD8^+$ T cells acquire the ability to express cytolytic molecules such as granzyme B (Gzmb) for direct cell killing and to produce effector cytokines such as interferon gamma (IFN-γ) for indirect activation of anti-viral and anti-tumor responses. Signals derived from antigen presenting cells including peptide-major histocompatibility complex (MHC), co-

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SUPPLEMENTAL INFORMATION
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stimulatory molecules and inflammatory cytokines ultimately control $CD8^+$ T cell expansion and effector differentiation. In particular, in the past several years, the strength (affinity) of T cell receptor (TCR) signaling has been shown to be critical for determining the size and duration of $CD8^+$ T cell expansion, and the functional differentiation of $CD8^+$ T cells (Denton et al., 2011; King et al., 2012; Vigano et al., 2012; Zehn et al., 2009). Currently, the underlying molecular mechanisms by which TCR signal strength influences the expansion and differentiation of $CD8^+$ T cells are not very well understood.

The expansion and effector differentiation of $CD8^+$ T cells are also subject to the regulation of various transcription factors. The transcription factor Id2 promotes the survival of activated $CD8^+$ T cells and controls the expansion size of antigen-specific $CD8^+$ effector T cells, while the transcription factors T-bet, Eomes, Runx3 and Blimp1 are required for the expression of effector molecules and thus are essential for the process of $CD8^+$ T cell effector differentiation (Kaech and Cui, 2012; Zhang and Bevan, 2011). Interferon regulatory factor 4 (IRF4) is a member of the IRF family of transcription factors and has been shown to play critical roles in orchestrating the effector differentiation of multiple lineages of $CD4^+$ T helper (Th) cells (Xu et al., 2012). Recent reports also have begun to shed light on the functions of IRF4 expression in $CD8^+$ T cells. In particular, IRF4 expression in the thymus has been implicated in the development of $CD122^+$ innate-like $CD8^+$ T cells (Nayar et al., 2012). Furthermore, IRF4 is required for the generation of interleukin-17 (IL-17) or IL-9 producing $CD8^+$ T cells in response to differential polarizing cytokines *in vitro* (Huber et al., 2013; Visekruna et al., 2013). However, the role of IRF4 in the development of conventional IFN-γ producing effector $CD8^+$ T cell responses *in vivo* is currently unknown.

In this report, using an *in vitro* model of dendritic cells (DC) and $CD8^+$ T cell co-culture as well as an *in vivo* model of influenza virus infection, we found that IRF4 was not required for the early activation of $CD8^+$ T cells, but was critical for controlling the expansion and effector differentiation of $CD8^+$ T cells in response to TCR signaling strength. We found that IRF4 repressed Bim and CDK inhibitors to prolong the survival and proliferation of activated $CD8^+$ T cells. In addition, IRF4 promoted Blimp1 and T-bet expression, and sustained active *Ifng* and *Gzmb* promoters, thereby enhancing effector differentiation of $CD8^+$ T cells. We showed that selective ablation of IRF4 in peripheral $CD8^+$ T cells impaired anti-viral $CD8^+$ T cell responses, viral clearance and $CD8^+$ T cell-mediated host recovery from influenza virus infection. These data reveal a critical role of IRF4 in translating the strength of TCR-signaling into the quantity and quality of effector $CD8^+$ T cell responses.

RESULTS

TCR strength determines IRF4 expression during $CD8^+$ T cell activation

IRF4 is required for the proper differentiation and function of regulatory T cells and many effector Th cell subsets (Xu et al., 2012). However, the role of IRF4 in antigen specific $CD8^+$ T cell responses remains unknown. We found that the *Irf4* expression was rapidly up-regulated *in vitro* in polycolonal $CD8^+$ T cells stimulated with bone marrow-derived DC (BMDC) and soluble α-CD3 (Figure 1A and B). Moreover, TCR re-stimulation of activated $CD8^+$ T cells could further increase *Irf4* expression (Figure S1 A). IRF4 also was highly expressed *in vivo* by OTI TCR-transgenic $CD8^+$ T cells following influenza A/PR8-OVA infection (Figure 1C). Furthermore, high affinity OTI TCR ligand (SIINFEKL, N4 peptide) induced stronger and prolonged IRF4 expression compared to low affinity altered peptide ligands (T4 peptide: SIITFEKL, Q4H7 peptide: SIIQFEHL (King et al., 2012; Zehn et al., 2009)) (Figure 1 D), suggesting that IRF4 expression in $CD8^+$ T cells correlates with TCR signaling strength. Consistent with this idea, IRF4 expression in activated $CD8^+$ T cells also

was correlated with the dose of peptide used in the culture (Figure S1B). We found that high TCR stimulation strength induced higher activities of the kinase, mammalian target of rapamycin (mTOR) signaling and rapamycin treatment impaired IRF4 expression in polyclonal $CD8^+$ T cells or OTI cells stimulated by N4 or T4 peptide (Figure 1 E – G and Figure S1 C). Together, these data suggested that IRF4 expression in $CD8^+$ T cells is determined by the strength of TCR signaling in a manner relying partially on, differential mTOR signaling. Recently, IL-2 inducible T-cell kinase (ITK) has been shown to regulate IRF4 expression in $CD8^+$ T cells (Nayar et al., 2012). We found that an ITK inhibitor in conjunction with rapamycin had synergistic effects in inhibiting IRF4 expression (Figure S1 D), suggesting that mTOR and ITK signaling cooperatively regulate IRF4 expression during $CD8^+$ T cell activation.

Selective IRF4 ablation in $CD8^+$ T cells impairs the magnitude of $CD8^+$ T cell responses

IRF4 expression in the thymus has been shown to regulate the development of innate-like $CD8^+$ T cells (Nayar et al., 2012). To bypass the effects of IRF4 deletion in thymic $CD8^+$ T cells, we crossed $Irf4^{fl/fl}$ mice to distal *Lck-cre* transgenic mice and generated peripheral T cell-specific conditional IRF4 mutant mice ($Irf4^{\Delta T}$) (Prlic and Bevan, 2011). Control ($Irf4^{fl/fl}$) and $Irf4^{\Delta T}$ mice were then infected with influenza and T cell responses were examined. We found that at day 7 post infection, the total number of T cells, in particular $CD8^+$ T cells, was dramatically diminished within the infected lung, where antigen specific effector T cells should be enriched (Figure S2 A). We also examined antigen-specific $CD8^+$ T cell responses by staining the NP_{366} and PA_{224} tetramers. We found that IRF4 deletion in T cells greatly impaired the generation of influenza-specific $CD8^+$ T cells in the lung, draining mediastinal lymph nodes (MLN) and spleen (Figure 2 A), suggesting that IRF4 is vital for robust anti-viral $CD8^+$ T cell responses during influenza infection.

To rule out the possibility that IRF4 expression in $CD4^+$ T cells may be responsible for the phenotype, we generated $CD8^+$ T cell-specific IRF4 conditional mutant mice ($Irf4^{\Delta CD8}$) by crossing $Irf4^{fl/fl}$ mice with *Cd8a-cre* transgenic mice (Maekawa et al., 2008). Utilizing GFP expression following cre expression as an indicator of excision (Klein et al., 2006), we confirmed that the deletion of the *Irf4* gene occurred specifically in peripheral $CD8^+$ T cells but not in $CD4^+$ T cells (Figure 2B). Furthermore, we found that cre-mediated *Irf4* deletion did not occur in the double-positive stage of thymocytes and only partially (~30%) occurred in single $CD8^+$ thymocytes, suggesting that *Irf4* deletion in $Irf4^{\Delta CD8}$ mice is a feature of mature $CD8^+$ T cells (Figure S2 B). We observed that splenic $CD8^+$ T cells isolated from $Irf4^{\Delta CD8}$ mice showed no signs of innate-like $CD8^+$ T cells (Figure S2 C – E). Furthermore, spleen or LN $CD8\alpha^+$ DCs from $Irf4^{\Delta CD8}$ mice, which were required for the optimal $CD8^+$ T cell responses following viral infection (Belz et al., 2004), were cre recombinase negative and expressed the same amount of IRF4 as $CD8\alpha^+$ DCs from control mice (Figure S2 F, G). Thus, this $CD8^+$ T cell-specific *Irf4* mutant mouse strain allows us to specifically examine IRF4 function in peripheral $CD8^+$ T cell responses following infection.

We infected $Irf4^{\Delta CD8}$ mice with influenza and examined $CD8^+$ T cell responses. We found that $Irf4^{\Delta CD8}$ mice exhibited diminished $CD8^+$ but not $CD4^+$ T cell lung infiltration at day 7 and 9 post infection (Figure S2 H, I). *Irf4* deletion in $CD8^+$ T cells resulted in diminished percentages (Figure 2 C–D) and numbers (Figure 2 E) of influenza-specific NP_{366}^+ and PA_{244}^+ T cells in the lung, MLN and spleen at day 7 and 9 post infection. $Irf4^{\Delta CD8}$ mice also exhibited diminished percentages of antigen-specific T cells in the memory phase (day 42) after virus was cleared (Figure S2 J). These data together suggested that IRF4 expression in mature $CD8^+$ T cells is essential for the development of robust anti-viral $CD8^+$ T cells during influenza infection.

IRF4 sustains CD8$^+$ T cell expansion

We next used an *in vitro* DC and CD8$^+$ T cell co-culture system to examine the underlying mechanisms by which IRF4 regulates CD8$^+$ T cell responses. To this end, control or IRF4-deficient CD8$^+$ T cells were stimulated with DC plus soluble α-CD3 and T cell activation and expansion were followed. We found that IRF4-deficient CD8$^+$ T cells were able to acutely up-regulate CD25 (IL-2Rα) and CD69, expressed similar IL-2Rβ and IL-2Rγ as well as to produce comparable IL-2 at the early time point of T cell activation (day 1) (Figure 3 A, B and Figure S3 A, B). However, IRF4-deficient CD8$^+$ T cells failed to expand and accumulate compared with control CD8$^+$ T cells (Figure 3C). Furthermore, in correlation with high and prolonged expression of IRF4, CD8$^+$ T cells stimulated with strong TCR signaling induced greater and prolonged expansion of CD8$^+$ T cells following stimulation (Figure 3 D and Figure S3 C). Thus, these data suggested that IRF4 is essential for the expansion of CD8$^+$ T cells. The failed expansion of IRF4-deficient T cells was not due to the difference of IL-21 expression (a cytokine controlled by IRF4 in Th cells (Huber et al., 2008)) (Figure S3 D). Furthermore, provision of IL-21 or provision of human IL-2 in the presence of mouse IL-2 neutralizing Ab (so we can accurately control the amount of bioactive IL-2) did not reverse the expansion defects of IRF4-deficient CD8$^+$ T cells (Figure S3 E, F), formally ruling out the possibility that the failed expansion of IRF4-deficient CD8$^+$ T cells was due to the lack of endogenous IL-2 or IL-21 production.

The expansion of CD8$^+$ T cells is controlled by cell proliferation and death (Ream et al., 2010). IRF4-deficient CD8$^+$ T cells exhibited enhanced cell death as evidenced both by increased proportions of 7-AAD$^+$ dead cells in the culture (Figure 3 E, F) and enhanced percentages of active caspase-3$^+$ (early apoptosis marker) in the gated live cells (Figure 3 G). Furthermore, consistent with their IRF4 expression, T cells stimulated with weak TCR signals showed enhanced cell death following activation (Figure S3 G). These data suggested that IRF4 expression in CD8$^+$ T cells is critical for the survival of the activated CD8$^+$ T cells. We next examined the proliferation of IRF4-deficient CD8$^+$ T cells following activation. We found that IRF4-deficient CD8$^+$ T cells were able to initiate several rounds of division following activation (Figure 3 H), which is consistent with the idea that IRF4 is not required for the early activation of CD8$^+$ T cells. However, IRF4-deficient CD8$^+$ T cells failed to efficiently sustain their proliferation (Figure 3 H). Furthermore, CD8$^+$ T cells stimulated with weak TCR signal also showed diminished cell proliferation following activation (Figure S3 H). Thus, IRF4 is essential to sustain the expansion of CD8$^+$ T cells following activation by promoting both the proliferation and survival of activated CD8$^+$ T cells.

BATF, a basic leucine zipper transcription factor, was recently found to be an important binding partner of IRF4 and is required for many aspects of IRF4 function in CD4$^+$ T cells (Ciofani et al., 2012; Li et al., 2012). However, while BATF-deficient CD8$^+$ T cells exhibited enhanced active caspase-3 and cell death following activation (Figure 3 I and Figure S3 I), the cells proliferated comparably to WT CD8$^+$ T cells (Figure 3 J). This indicates that BATF-deficient T cells show only a partial expansion defect as compared to IRF4-deficient CD8$^+$ T cells (Figure S3 J) and suggest that IRF4 regulates the expansion of CD8$^+$ T cells by BATF-dependent and -independent mechanisms.

We next activated WT CD8$^+$ T cells and transduced them with IRF4-expressing retroviruses. The growth of virus-transduced cells (human (h)-CD4$^+$) was monitored between days 1 and 3 following transduction (days 2 and 4 following the culture). We predicted that if the expression of IRF4 provided selective advantage, the proportion of hCD4$^+$ cells within the live gate would increase. Consistent with this expectation, while the percentages of hCD4$^+$ cells remained constant between day 2 and day 4 in the control group, the percentages of hCD4$^+$ cells dramatically increased in the group of CD8$^+$ T cells

transduced with IRF4-expressing retrovirus (Figure 3 K, L). The increased percentages of IRF4-transduced cells were associated with increased proliferation of transduced cells (Figure S3 K). Together, these data suggest that ectopic expression of IRF4 promotes polyclonal $CD8^+$ T cell expansion following activation. We also expressed IRF4 ectopically in OTI T cells and observed T cell expansion following high or low affinity peptide ligand stimulation. We found that IRF4 had a moderate effect in promoting the expansion of OTI T cells stimulated by high affinity peptide ligand N4, presumably due to the high endogenous expression of IRF4 (Figures 3 M and 1 D). In contrast, ectopic expression of IRF4 strongly promoted the expansion of OTI T cells when stimulated by low-affinity peptide ligand T4 and Q4H7 (Figures 3 M). The enhanced expansion of IRF4 in transduced T cells also was associated with the enhanced proliferation (Figure S3 L). Taken together, these data suggest that a high IRF4 expression is critical for sustaining the expansion of $CD8^+$ T cells following activation.

IRF4 sustains $CD8^+$ T cell expansion by repressing CDK inhibitors and Bim

We next sought to examine the underlying molecular mechanisms by which IRF4 sustains the expansion of $CD8^+$ T cells. Myc and Id2 were previously shown to regulate $CD4^+$ and $CD8^+$ T cell expansion respectively (Cannarile et al., 2006; Guy et al., 2013). However, IRF4-deficient $CD8^+$ T cells expressed comparable *Myc* and *Id2* as control $CD8^+$ T cells, suggesting that IRF4 regulates $CD8^+$ T cell expansion independently of Myc and Id2 (Figure S4 A, B). Following activation, IRF4-deficient $CD8^+$ T cells exhibited enhanced expression of multiple cyclin-dependent kinase (CDK) inhibitors, including *Cdkn2a*, *Cdkn1a* and *Cdkn1c* (Cicenas and Valius, 2011) (Figure 4 A). Consistent with their ability to proliferate normally, BATF-deficient $CD8^+$ T cells expressed CDK inhibitors similar to WT $CD8^+$ T cells (Figure 4 B). IRF4 can act as either transcription activator or repressor (Biswas et al., 2010). We hypothesized that perhaps IRF4 binds directly to DNA to repress the expression of these *Cdkn* genes. In support of this idea, the *Cdkn2a* locus contains a potential IRF4 binding site (Figure S4 C) and IRF4 was shown to bind directly to the *Cdkn2a* locus by a ChIP assay (Figure 4 C). Collectively, these data indicate that IRF4 sustains $CD8^+$ T cell proliferation by repressing the production of CDK inhibitors. The underlying mechanisms by which IRF4 controls the survival of activated $CD8^+$ T cells also was investigated. IRF4-deficient $CD8^+$ T cells expressed equivalent anti-apoptotic genes (Figure S4 D, E). However, IRF4-deficient $CD8^+$ T cells expressed increased pro-apoptotic gene *Bcl2l11* (encodes Bim) following activation (Figure 4 D, E). Similarly, BATF-deficient $CD8^+$ T cells expressed higher Bim (Figure 4 F, G) than WT $CD8^+$ T cells. Once again, IRF4 bound directly to the *Bcl2l11* locus (Figure S4 F and Figure 4 H). Thus, IRF4 promotes the expansion of $CD8^+$ T cells by targeting its transcriptional repression activity to genes encoding CDK inhibitors and Bim.

IRF4 is required for $CD8^+$ T cell proliferation and survival *in vivo*

We next examined whether IRF4 is required for the optimal proliferation and survival of $CD8^+$ T cells *in vivo*. To do so, control or $Irf4^{\Delta CD8}$ mice were infected with influenza and $CD8^+$ T cell proliferation was measured on day 7 by BrdU incorporation. Results showed that IRF4-deficient $CD8^+$ T cells incorporated dramatically less BrdU in the lung, indicating that IRF4 expression is vital for the proliferation of $CD8^+$ T cells *in vivo* (Figure 5A). Furthermore, IRF4-deficient $CD8^+$ T cells in the lung expressed higher active caspase 3 and Bim (Figure 5 B, C). These data are consistent with our *in vitro* observations and suggest that IRF4 expression is required for the proliferation and survival of anti-viral $CD8^+$ T cells during influenza infection.

IRF4 sustains CD8$^+$ T cell effector differentiation

In conjunction with rapid expansion, activated CD8$^+$ T cells undergo an effector differentiation process to gain the ability to rapidly produce both the cytotoxic molecules and effector cytokines required for the clearance of intracellular pathogens. IRF4-deficient CD8$^+$ T cells were able to up-regulate *Gzm*b mRNA and protein early after stimulation (day 1 following activation) (Figure 6 A, B), but failed to sustain the expression of this gene (Figure 6 A, B). Likewise, IRF4-deficient CD8$^+$ T cells were able to up-regulate IFN-γ production early following stimulation but failed to sustain the production of IFN-γ at later times during activation (Figure 6 C and Figure S5 A). T cells stimulated with weak TCR signals also failed to sustain Gzmb and IFN-γ (Figure S5 B, C). These data suggest that IRF4 sustains the effector differentiation of CD8$^+$ T cells following *in vitro* activation.

We next wondered whether the decreased maintenance of effector molecules was merely due to the selective death of effector CD8$^+$ T cells since IRF4-deficient CD8$^+$ T cells showed enhanced cellular apoptosis. The survival of effector CD8$^+$ T cells is controlled by the balanced expression of anti-apoptotic Bcl2 and pro-apoptotic Bim (Kurtulus et al., 2011). Therefore, it is possible that enhanced expression of Bcl2 could counter-balance the effects of Bim expression to prevent the death of IRF4-deficient T cells. To test this, WT or IRF4-deficient CD8$^+$ T cells were transduced with a Bcl2 expressing retrovirus. Ectopic expression of Bcl2 decreased cellular apoptosis of IRF4-deficient CD8$^+$ T cells (Figure S5 D). However, ectopic expression of Bcl2 failed to rescue IFN-γ production by IRF4-deficient CD8$^+$ T cells (Figure 5 D), suggesting that the ability of IRF4 to sustain CD8$^+$ T cell effector differentiation is not due to the selective apoptosis of effector molecule-expressing cells.

We next investigated how IRF4 might be functioning to sustain the effector differentiation of CD8$^+$ T cells. Following T cell activation, IRF4 was required for the expression of *Prdm1* (Figure 6E), a critical gene whose product (Blimp1) promotes the expression of cytolytic molecules (Shin et al., 2009). IRF4 was able to bind directly to multiple DNA sites within the *Prdm1* locus in CD8$^+$ T cells (Figure 6 F), suggesting that IRF4 promotes *Prdm1* transcription. The T-box transcription factors T-bet and Eomes play important roles in the effector differentiation of CD8$^+$ T cells (Intlekofer et al., 2008; Kaech and Cui, 2012; Zhang and Bevan, 2011). For these genes, IRF4 was important for the optimal expression of T-bet (*Tbx21*), but not Eomes (*Eomes*) in CD8$^+$ T cells (Figure 6 G, H and Figure S5 E). Given that IRF4 only partially controlled T-bet expression in CD8$^+$ T cells, we next examined whether IRF4 could regulate T-bet function in CD8$^+$ T cells. To this end, we observed diminished binding by T-bet to the *Gzmb* and *Ifng* promoters in IRF4-deficient CD8$^+$ T cells (Figure 6 I), suggesting that IRF4 is required for the function of T-bet. The effector differentiation of CD8$^+$ T cells is accompanied by intensive epigenetic chromatin modifications in the promoter regions of effector molecule genes (Olson et al., 2010). These active chromatin modifications are required for effector molecule expression and lineage specification of effector CD8$^+$ T cells. IRF4-deficient CD8$^+$ T cells exhibited diminished active histone modifications, the trimethylation of H3K4 and the acetylation of H3K27, in both *Gzmb* and *Ifng* promoter regions (Figure 6 J, K). In addition, IRF4-deficient CD8$^+$ T cells exhibited diminished *Hif1a* (Figure S5 F), a transcription factor that was shown to promote the effector differentiation of CD8$^+$ T cells (Finlay et al., 2012). Taken together, these data suggested that IRF4 sustains the effector differentiation of activated CD8$^+$ T cells by controlling multiple checkpoints of effector differentiation including the expression *Prdm1*, *Tbx21* and *Hif1a*, the binding of T-bet to its targets and the active modifications of histones in the promoter regions of effector molecules.

We also transduced control or IRF4-expressing virus into CD8$^+$ T cells and examined CD8$^+$ T cell effector molecule expression. We found that IRF4 modestly promoted *Gzmb*

expression (Figure S5 G), but dramatically enhanced IFN-γ production by activated $CD8^+$ T cells (Figure 6 L). Furthermore, ectopic expression of IRF4 also promoted IFN-γ production by $CD8^+$ T cells stimulated with low affinity peptide ligands (Figure 6 M). Taken together, these data suggested that IRF4 expression in $CD8^+$ T cells is essential for sustaining the effector differentiation of $CD8^+$ T cells following activation.

IRF4 is required for $CD8^+$ T cell effector differentiation and function *in vivo*

We next examined whether IRF4 is required for the effector differentiation of $CD8^+$ T cells *in vivo*. $Irf4^{\Delta CD8}$ mice were infected with influenza and *Gzmb* expression in $CD8^+$ T cells was examined at day 7 and 9 p.i. At both time points, IRF4 deficiency impaired *Gzmb* expression in both total or influenza-specific PA_{224}-tetramer$^+$ $CD8^+$ T cells (Figure 7 A, B and Figure S6 A, B). IRF4 deficiency in $CD8^+$ T cells also diminished the percentages of IFN-γ^+ cells and, importantly, the per cell expression of IFN-γ (Figure 7 C, D and Figure S6 C, D). Additionally, IRF4 deficiency in $CD8^+$ T cells resulted in diminished airway IFN-γ *in vivo* (Figure 7 E). The diminished Gzmb and IFN-γ production in lung IRF4-deficient $CD8^+$ T cells was associated with their decreased expression of *Tbx21*, *Prdm1* and CDK inhibitors (Figure S6 E). Taken together, these data suggested that IRF4 is required for $CD8^+$ T cell effector differentiation *in vivo*.

Given that IRF4 is both important for sustaining the expansion and the effector differentiation of $CD8^+$ T cells, we sought to investigate if IRF4 is required for host anti-viral responses *in vivo*. We found that $Irf4^{\Delta CD8}$ mice exhibited enhanced infectious virus titers in the airway and increased viral gene expression in the infected lungs at day 9 post infection (Figure 7 F, G), suggesting that IRF4 deficiency in $CD8^+$ T cells impaired host antiviral responses. During influenza infection, $CD4^+$ T cells, $CD8^+$ T cells and B cells play compensatory and redundant roles to eliminate virus and promote host recovery (Braciale et al., 2012; Brown et al., 2004). To examine the effects of IRF4 expression in $CD8^+$ T cells in host recovery from influenza infection, we infected control and $Irf4^{\Delta CD8}$ mice with influenza and then depleted $CD4^+$ T cells. We then monitored host survival following influenza infection. We found that IRF4 deficiency in $CD8^+$ T cells significantly enhanced host mortality following influenza virus infection (Figure 7 H). Taken together, these data have established that IRF4 expression in $CD8^+$ T cells is critical for the antiviral activities of $CD8^+$ T cells during acute respiratory viral infection.

DISCUSSION

The transcriptional programs regulating effector $CD8^+$ T cell responses are incompletely defined. In this report, we have identified a prominent role of IRF4 in regulating robust development of effector $CD8^+$ T cells during acute respiratory virus infection. IRF4 exerts its effects by sustaining both expansion and effector differentiation of activated $CD8^+$ T cells. Previously, several transcription factors have been identified to regulate either the expansion or effector differentiation of primary $CD8^+$ T cell responses. For example, Id2 enhances the expansion of effector $CD8^+$ T cells by maintaining the survival of effector $CD8^+$ T cells (Cannarile et al., 2006), while Runx3, T-bet, Blimp1 and Eomes control the effector differentiation of $CD8^+$ T cells but have little effect on the expansion of effector $CD8^+$ T cells (Kaech and Cui, 2012; Zhang and Bevan, 2011). Thus, the ability of IRF4 to regulate both the expansion and effector differentiation of $CD8^+$ T cells is distinct from previously described factors. We believe that IRF4 regulates the expansion of $CD8^+$ T cells through Id2 independent mechanisms as IRF4 directly represses *Bcl2l11* and CDK inhibitors, thus promoting survival and cell cycle progression. In contrast, IRF4 sustains the effector differentiation of $CD8^+$ T cells indirectly through regulating the expression and

function of *Tbx21* and *Prdm1*. Hence, IRF4-deficient $CD8^+$ T cell show impaired expression of effector molecules such as IFN-γ and Gzmb.

The proper expansion and effector differentiation of $CD8^+$ T cells can be influenced by a variety of signals. However, the strength and quality of TCR signaling appears to play a critical role in the process. Triggering of naive T cells with peptide epitopes with low functional affinity for the TCR often resulted in the early or premature induction of apoptosis in effector cells due to an imbalance in the expression of pro- and anti-apoptotic factors (Hommel and Hodgkin, 2007; Ream et al., 2010). *In vivo*, very weak TCR-ligand interactions are sufficient to activate naive T cells, but resulted in a lower magnitude of expansion and earlier onset of T cell contraction compared to strong TCR-ligand interactions (Denton et al., 2011; Zehn et al., 2009). More recently, the strength of the TCR-ligand was also shown to influence full effector differentiation, tissue infiltration and the pathological activities of $CD8^+$ T cells *in vivo* (King et al., 2012). Currently, the molecular mechanisms underlying the differential responses of $CD8^+$ T cells stimulated with high and low affinity of TCR ligands are unknown. We found that IRF4 expression was highly induced and sustained with strong TCR stimulation, and that IRF4 deficiency in $CD8^+$ T cells resulted in lower magnitude, earlier contraction and diminished effector molecule expression *in vitro* and *in vivo*. Moreover, ectopic expression of IRF4 enhanced $CD8^+$ T cell expansion and effector cytokine production in $CD8^+$ T cells stimulated with low strength of TCR signaling. Thus, we have identified IRF4 as a potential important downstream transcription factor that translates the strength of TCR signaling into the quantity and quality of $CD8^+$ T cell responses. It will be of interest in the future to test whether IRF4 is capable of rescuing the curtailed expansion and effector differentiation of $CD8^+$ T cells in response to low-affinity TCR signaling strength *in vivo* during infection and/or vaccination (Denton et al., 2011; King et al., 2012; Vigano et al., 2012; Zehn et al., 2009). Such information would be useful for designing modalities to manipulate IRF4 expression in T cells to promote effective cellular immunity to immunogens that typically induce weak $CD8^+$ T cell responses.

Our studies have shown that the magnitude of mTOR signaling downstream of the different strength of TCR stimulation regulated IRF4 expression in $CD8^+$ T cells. Interestingly, IRF4 expression in $CD8^+$ T cells also was shown to be dependent on the function of ITK signaling (Nayar et al., 2012). We found here that rapamycin and ITK inhibitors cooperatively inhibited IRF4 expression, suggesting that ITK and mTOR signaling may function in parallel pathways to promote IRF4 expression in $CD8^+$ T cells. It is currently unclear the exact mechanism by which mTOR controls IRF4 expression. mTOR may promote IRF4 expression through its effects on protein translation. Alternatively, mTOR could increase IRF4 transcription by its effects on downstream transcription factors (Laplante and Sabatini, 2013). In this regard, NF-κB activity is subject to mTOR regulation and Rel has been shown to be an important transcription factor for IRF4 expression in lymphocytes (Dan et al., 2008; Grumont and Gerondakis, 2000; Hou et al., 2012). Further studies are needed to clarify these possibilities. Notably, mTOR signaling plays important roles in regulating many aspects of effector $CD8^+$ T cell responses including proliferation, survival and differentiation (Rao et al., 2010a; Rao et al., 2010b). Many of the effects of mTOR in the regulation of $CD8^+$ T cell responses may be mediated through its role in promoting IRF4 expression. For example, mTOR inhibition increases CDK inhibitor function, impairs Blimp1 and T-bet expression, and enhances Eomes expression during primary effector T cell responses (Rao et al., 2010a; Rao et al., 2010b), similar to effects observed in IRF4-deficient $CD8^+$ T cells. Recently, HIF transcription factors have been identified to be downstream of mTOR and regulate the effector differentiation of $CD8^+$ T cells in response to IL-2 treatment (Finlay et al., 2012). We found that *Hif1a* gene expression was diminished in IRF4-deficient $CD8^+$ T cells at day 3 post activation, suggesting that IRF4 may also regulate the HIF pathway to sustain effector

differentiation and/or cellular metabolism (Finlay et al., 2012). However, as HIF-1α protein expression is tightly subject to post-transcriptional regulation by oxygen concentrations in the cells, further studies are warranted to define the exact relationship of IRF4 and HIF transcription factors in $CD8^+$ T cell differentiation. Furthermore, as TCR downstream signaling is often modulated by positive and negative signals derived from various co-stimulatory molecules and cytokines, future studies should examine those signals and their influence on IRF4 expression. Total IRF4 deficiency as well as IRF4 conditional deletion in the double positive stage of thymic development (*Cd4-cre* mediated deletion) lead to the development of innate-like $CD8^+$ T cells with a memory phenotype (Nayar et al., 2012). In contrast, using a mouse model conditionally deleting IRF4 in a late stage of $CD8^+$ T cells, we found little evidence of the development of innate-like T cells, suggesting that IRF4 expression in the double positive stage of thymic T cell development is responsible for the generation of innate-like $CD8^+$ T cells. Alternatively, the intrinsic deletion of IRF4 in $CD8^+$ T cells may be not sufficient to drive the development of innate-like $CD8^+$ T cells, since both $CD8^+$ T cell-intrinsic and extrinsic signals were identified for the development of innate like $CD8^+$ T cells (Nayar et al., 2012).

In conclusion, we have identified here that IRF4 is vital for the development of effective cytotoxic T cell responses during viral infection. IRF4 is also required for the differentiation of multiple $CD4^+$ Th cell lineages including Tfh cells, which promote the formation of germinal centers for the production of high affinity neutralizing Abs (Crotty, 2011). Interestingly, high affinity TCR interaction and high dose antigen stimulation preferentially induce Tfh cell differentiation (Bollig et al., 2012; Fazilleau et al., 2009). It is thus tempting to speculate that high strength of TCR signaling controls IRF4 expression in $CD4^+$ T cells and subsequently facilitates IL-21 and Bcl6 expression for Tfh cell differentiation *in vivo*. Future studies are needed to examine these possibilities. Nevertheless, previous reports of the importance of IRF4 in Tfh and plasma cell differentiation (Bollig et al., 2012; Klein et al., 2006; Kwon et al., 2009; Sciammas et al., 2006) coupled with the data we present here on the role of IRF4 in $CD8^+$ T cell responses highlight the critical function of IRF4 in the development of both humoral and cellular immunity against infection or following immunization. We conclude that selective manipulation of IRF4 expression may serve as a potential strategy to boost both humoral and cellular immunity during vaccination, especially in those (*e.g.*, infants and the elderly) who respond poorly to vaccines.

EXPERIMENTAL PROCEDURE

Mouse and infection

WT C57BL/6 mice were purchased from the Jackson Laboratory. OTI, $Batf^{-/-}$ mice were bred in house. $Irf4^{\Delta T}$ mice were generated by cross $Irf4^{fl/fl}$ mice with mice harboring distal *Lck-cre* transgenic mice (Wang et al., 2001). $Irf4^{\Delta CD8}$mice were generated by cross $Irf4^{fl/fl}$ mice with mice harboring *Cd8α-cre* transgenic mice (Maekawa et al., 2008). All mice were housed in a specific pathogen-free environment and all animal experiments were performed in accordance with protocols approved by the Indiana University Institutional Animal Care and Use Committee. Influenza A/PR8/34 (200 pfu/mouse) and recombinant PR8-OVA (2000 pfu/mouse) infection were performed as described before (Sun et al., 2009). $CD4^+$ T cell depletion was achieved by the i.p. injection of GK1.5 Ab (1 mg/mouse) at day 3 p.i.

Quantitative RT-PCR

mRNA from cultured cells, *in vivo* purified $CD8^+$ T cells or lung homogenates as indicated in the text was isolated with RNeasy kit (Qiagen) and treated with DNase I (Invitrogen). Random primers (Invitrogen) and Superscript II (Invitrogen) were used to synthesize first-strand cDNAs from equivalent amounts of RNA from each sample. RT-PCR was performed

with SYBR Green PCR Master Mix (Applied Biosystems). Data were generated with the comparative threshold cycle (Delta CT) method by normalizing to hypoxanthine phosphoribosyltransferase (HPRT). Sequences of primers used in the studies are available on request.

DC and T cell co-culture

BMDC were generated as described (Sun et al., 2011). $CD8^+$ T cells were isolated from spleen and lymph nodes of indicated mice through MACS-beads (Miltenyi Biotech). Then, we mixed BMDC with $CD8^+$ T cells at the ratio of 1 DC: 10 T cells in round-bottom 96 wells (5×10^4 T cells/well) in the presence of 0.1μg/ml soluble α-CD3. $CD8^+$ T cells from OT-I mice were isolated and cultured with N4, T4 or Q4H7 peptide (4 ng/ml or indicated concentration in the text). In some experiments, hIL-2 (used as indicated U/ml in the text), anti-mIL-2 (JES6-1A12, 20 μg/ml) and IL-21 (20 ng/ml) were used in the culture. Rapamycin (100 nM) and BMS-509744 (ITK inhibitor, 1μM) (Millipore) were used to block mTOR and ITK signaling respectively. In some experiments, T cells were labeled with eFluor670 (eBioscience) or CFSE (Invitrogen) according to manufacturer's protocols.

Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed as described (Ahyi et al., 2009). In brief, 10×10^6 activated wild type or IRF4-deficient $CD8^+$ T cells were cross-linked for 10 min with 1% formaldehyde and lysed by sonication. Cross-linked cells were pre-cleared with salmon sperm DNA, bovine serum albumin, and Protein A agarose (T-bet ChIP) or Protein G agarose (IRF4 ChIP) bead slurry (50%). Cell extracts were incubated with antibodies to rabbit T-bet H-210, goat polyclonal IRF4 M-17 (Santa Cruz), H3K27ac (Millipore), H3K4me3 (Abcam), normal goat IgG (Santa Cruz) or normal rabbit IgG (Millipore) overnight at 4°C. The immunocomplexes were precipitated with either Protein A agarose (T-bet, H3K27ac and H3K4me3 ChIP) or Protein G agarose (IRF4 ChIP) beads at 4°C for 2 h, washed, eluted and cross-links reversed at 65°C overnight. DNA was purified, resuspended in H_20 and analyzed by quantitative PCR as previously described (Pham et al., 2012). Primers for T-bet binding to *Gzmb* and *Ifng* loci as well as IRF4 binding to *Prdm1* locus were described previously (Geng et al., 2010; Sciammas et al., 2006). Additional primers for IFR-4 binding are as follows: *Bim* promoter Forward 5′-GGCTCAACTACCGCAGAGTC-3′, Reverse 5′-GGAGGTGGTGTGAATCCAAG-3′; *Cdkn2a* promoter Forward 5′-GACCGGTAAGTGTGTCCCG-3′, Reverse 5′-GGATGCTCGCGCTTAAAACC-3′. The IRF4 binding sites For *Bim* and *Cdkn2a* were determined using TRANSFAC Transcription factor binding site database.

Retroviral transduction

$CD8^+$ T cells were stimulated with BMDC plus α-CD3 or indicated peptides. At day 1 of the culture, cells were transduced with bicistronic retroviruses through spin infection (2500 rpm, 90min). After transduction, cells were analyzed daily following transduction using flow cytometry.

BrdU incorporation

Control or $Irf4^{\Delta CD8}$ mice were infected with influenza. At day 7 p.i., BrdU (Sigma, 1 mg/mouse) was injected through i.p. Ninety minutes later, mice were sacrificed, and lung and LN cells were collected as described (Sun et al., 2009). Cells were surface stained with CD8 and influenza-specific tetramer (PA_{224}) and intracellular BrdU staining was performed as described in manufacturer's manual (BD Biosciences).

T cell restimulation

For BMDC stimulation, BMDC were harvested and infected with influenza virus at approximate 100 M.O.I. for 6 h. Then BMDC were counted and mixed with total lung cells at a 1.5 to 1 ratio in the presence of Golgi-Stop (BD Biosciences, 1μl/ml) and hIL-2 (40U/ml) for additional 6 h. The surface staining of cell surface markers, intracellular staining of cytokines was performed according to previous report (Sun et al., 2011). For PMA and Ionomycin stimulation of polyclonal cells, cells were restimulated with PMA (100 ng/ml) and Ionomycin (1 μg/ml) for 4 h in the presence of Golgi-Stop (BD Biosciences) as described and then the surface staining of cell surface markers and intracellular staining of IFN-γ were performed as previously described (Sun et al., 2011).

Broncho-alveolar lavage (BAL) cytokine determination

BAL was obtained by flushing the airway multiple times with a single use of 600 μl sterile PBS. Cells in BAL were spun down and supernatants were collected for ELISA analysis (Biolegend) according to the manufacturer's manuals.

Plaque Assay

Influenza plaque assay were performed as described before (Huprikar and Rabinowitz, 1980). Briefly, MDCK cells were grown in 6-well plates and incubated with series dilution of BAL for 1 h. The plates were then overlaid with low melting temperature agarose (0.6 %) in MEM with BSA and tripsin and cultured for 3 days in 37°C incubator. Plates were then fixed with formaldehyde and virus plaques were visualized with the staining of neutral red.

Flow cytometry analysis

Fluorescence-conjugated FACS Abs were purchased from Biolegend, BD Biosciences or eBioscience. Bim and p-S6 Abs were purchased from Cell-Signaling. Bim, Bcl2 and IRF4 staining was performed using Foxp3 staining buffer set (eBioscience). For IRF4 staining, IRF4-deficient $CD8^+$ T cells (from either $Irf4^{\Delta T}$ or $Irf4^{\Delta CD8}$ mice, termed as $Irf4^{-/-}$) were used as negative staining control. Cells were acquired through FACS-Calibur or LSR II (BD Biosciences). Data were analyzed by FlowJo software (Treestar).

Statistical analysis

Data are mean ± SEM of values from individual mice (*in vivo* experiments) or mean ± SD of values from triplicate analysis of the same sample (*in vitro* cell numbers and ChIP analysis). Paired or unpaired two-tailed Student's t-test was used. We consider P values < 0.05 as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Ahyi AN, Chang HC, Dent AL, Nutt SL, Kaplan MH. IFN regulatory factor 4 regulates the expression of a subset of Th2 cytokines. Journal of immunology. 2009; 183:1598–1606.

Belz GT, Smith CM, Kleinert L, Reading P, Brooks A, Shortman K, Carbone FR, Heath WR. Distinct migrating and nonmigrating dendritic cell populations are involved in MHC class I-restricted antigen presentation after lung infection with virus. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101:8670–8675. [PubMed: 15163797]

Biswas PS, Bhagat G, Pernis AB. IRF4 and its regulators: evolving insights into the pathogenesis of inflammatory arthritis? Immunological reviews. 2010; 233:79–96. [PubMed: 20192994]

Bollig N, Brustle A, Kellner K, Ackermann W, Abass E, Raifer H, Camara B, Brendel C, Giel G, Bothur E, et al. Transcription factor IRF4 determines germinal center formation through follicular T-helper cell differentiation. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109:8664–8669. [PubMed: 22552227]

Braciale TJ, Sun J, Kim TS. Regulating the adaptive immune response to respiratory virus infection. Nature reviews Immunology. 2012; 12:295–305.

Brown DM, Roman E, Swain SL. CD4 T cell responses to influenza infection. Semin Immunol. 2004; 16:171–177. [PubMed: 15130501]

Cannarile MA, Lind NA, Rivera R, Sheridan AD, Camfield KA, Wu BB, Cheung KP, Ding Z, Goldrath AW. Transcriptional regulator Id2 mediates CD8+ T cell immunity. Nature immunology. 2006; 7:1317–1325. [PubMed: 17086188]

Cicenas J, Valius M. The CDK inhibitors in cancer research and therapy. J Cancer Res Clin Oncol. 2011; 137:1409–1418. [PubMed: 21877198]

Ciofani M, Madar A, Galan C, Sellars M, Mace K, Pauli F, Agarwal A, Huang W, Parkurst CN, Muratet M, et al. A validated regulatory network for Th17 cell specification. Cell. 2012; 151:289–303. [PubMed: 23021777]

Crotty S. Follicular helper CD4 T cells (TFH). Annual review of immunology. 2011; 29:621–663.

Dan HC, Cooper MJ, Cogswell PC, Duncan JA, Ting JP, Baldwin AS. Akt-dependent regulation of NF-{kappa}B is controlled by mTOR and Raptor in association with IKK. Genes & development. 2008; 22:1490–1500. [PubMed: 18519641]

Denton AE, Wesselingh R, Gras S, Guillonneau C, Olson MR, Mintern JD, Zeng W, Jackson DC, Rossjohn J, Hodgkin PD, et al. Affinity thresholds for naive CD8+ CTL activation by peptides and engineered influenza A viruses. J Immunol. 2011; 187:5733–5744. [PubMed: 22039305]

Fazilleau N, McHeyzer-Williams LJ, Rosen H, McHeyzer-Williams MG. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. Nature immunology. 2009; 10:375–384. [PubMed: 19252493]

Finlay DK, Rosenzweig E, Sinclair LV, Feijoo-Carnero C, Hukelmann JL, Rolf J, Panteleyev AA, Okkenhaug K, Cantrell DA. PDK1 regulation of mTOR and hypoxia-inducible factor 1 integrate metabolism and migration of CD8+ T cells. The Journal of experimental medicine. 2012; 209:2441–2453. [PubMed: 23183047]

Geng D, Zheng L, Srivastava R, Asprodites N, Velasco-Gonzalez C, Davila E. When Toll-like receptor and T-cell receptor signals collide: a mechanism for enhanced CD8 T-cell effector function. Blood. 2010; 116:3494–3504. [PubMed: 20696947]

Grumont RJ, Gerondakis S. Rel induces interferon regulatory factor 4 (IRF-4) expression in lymphocytes: modulation of interferon-regulated gene expression by rel/nuclear factor kappaB. The Journal of experimental medicine. 2000; 191:1281–1292. [PubMed: 10770796]

Guy CS, Vignali KM, Temirov J, Bettini ML, Overacre AE, Smeltzer M, Zhang H, Huppa JB, Tsai YH, Lobry C, et al. Distinct TCR signaling pathways drive proliferation and cytokine production in T cells. Nature immunology. 2013; 14:262–270. [PubMed: 23377202]

Hommel M, Hodgkin PD. TCR affinity promotes CD8+ T cell expansion by regulating survival. Journal of immunology. 2007; 179:2250–2260.

Hou YC, Chiu WC, Yeh CL, Yeh SL. Glutamine modulates lipopolysaccharide-induced activation of NF-kappaB via the Akt/mTOR pathway in lung epithelial cells. American journal of physiology Lung cellular and molecular physiology. 2012; 302:L174–183. [PubMed: 22003094]

Huber M, Brustle A, Reinhard K, Guralnik A, Walter G, Mahiny A, von Low E, Lohoff M. IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. Proceedings of the National Academy of Sciences of the United States of America. 2008; 105:20846–20851. [PubMed: 19088203]

Huber M, Heink S, Pagenstecher A, Reinhard K, Ritter J, Visekruna A, Guralnik A, Bollig N, Jeltsch K, Heinemann C, et al. IL-17A secretion by CD8+ T cells supports Th17-mediated autoimmune encephalomyelitis. The Journal of clinical investigation. 2013; 123:247–260. [PubMed: 23221338]

Huprikar J, Rabinowitz S. A simplified plaque assay for influenza viruses in Madin-Darby kidney (MDCK) cells. J Virol Methods. 1980; 1:117–120. [PubMed: 7228969]

Intlekofer AM, Banerjee A, Takemoto N, Gordon SM, Dejong CS, Shin H, Hunter CA, Wherry EJ, Lindsten T, Reiner SL. Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. Science. 2008; 321:408–411. [PubMed: 18635804]

Kaech SM, Cui W. Transcriptional control of effector and memory CD8+ T cell differentiation. Nature reviews Immunology. 2012; 12:749–761.

King CG, Koehli S, Hausmann B, Schmaler M, Zehn D, Palmer E. T cell affinity regulates asymmetric division, effector cell differentiation, and tissue pathology. Immunity. 2012; 37:709–720. [PubMed: 23084359]

Klein U, Casola S, Cattoretti G, Shen Q, Lia M, Mo T, Ludwig T, Rajewsky K, Dalla-Favera R. Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. Nature immunology. 2006; 7:773–782. [PubMed: 16767092]

Kurtulus S, Tripathi P, Moreno-Fernandez ME, Sholl A, Katz JD, Grimes HL, Hildeman DA. Bcl-2 allows effector and memory CD8+ T cells to tolerate higher expression of Bim. Journal of immunology. 2011; 186:5729–5737.

Kwon H, Thierry-Mieg D, Thierry-Mieg J, Kim HP, Oh J, Tunyaplin C, Carotta S, Donovan CE, Goldman ML, Tailor P, et al. Analysis of interleukin-21-induced Prdm1 gene regulation reveals functional cooperation of STAT3 and IRF4 transcription factors. Immunity. 2009; 31:941–952. [PubMed: 20064451]

Laplante M, Sabatini DM. Regulation of mTORC1 and its impact on gene expression at a glance. Journal of cell science. 2013; 126:1713–1719. [PubMed: 23641065]

Li P, Spolski R, Liao W, Wang L, Murphy TL, Murphy KM, Leonard WJ. BATF-JUN is critical for IRF4-mediated transcription in T cells. Nature. 2012; 490:543–546. [PubMed: 22992523]

Maekawa Y, Minato Y, Ishifune C, Kurihara T, Kitamura A, Kojima H, Yagita H, Sakata-Yanagimoto M, Saito T, Taniuchi I, et al. Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. Nature immunology. 2008; 9:1140–1147. [PubMed: 18724371]

Nayar R, Enos M, Prince A, Shin H, Hemmers S, Jiang JK, Klein U, Thomas CJ, Berg LJ. TCR signaling via Tec kinase ITK and interferon regulatory factor 4 (IRF4) regulates CD8+ T-cell differentiation. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109:E2794–2802. [PubMed: 23011795]

Olson MR, Russ BE, Doherty PC, Turner SJ. The role of epigenetics in the acquisition and maintenance of effector function in virus-specific CD8 T cells. IUBMB Life. 2010; 62:519–526. [PubMed: 20552633]

Pham D, Vincentz JW, Firulli AB, Kaplan MH. Twist1 regulates Ifng expression in Th1 cells by interfering with Runx3 function. J Immunol. 2012; 189:832–840. [PubMed: 22685315]

Prlic M, Bevan MJ. Cutting edge: beta-catenin is dispensable for T cell effector differentiation, memory formation, and recall responses. Journal of immunology. 2011; 187:1542–1546.

Rao RR, Li Q, Odunsi K, Shrikant PA. The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin. Immunity. 2010a; 32:67–78. [PubMed: 20060330]

Rao RR, Li Q, Shrikant PA. Fine-tuning CD8(+) T cell functional responses: mTOR acts as a rheostat for regulating CD8(+) T cell proliferation, survival and differentiation? Cell Cycle. 2010b; 9:2996–3001. [PubMed: 20699660]

Ream RM, Sun J, Braciale TJ. Stimulation of naive CD8+ T cells by a variant viral epitope induces activation and enhanced apoptosis. Journal of immunology. 2010; 184:2401–2409.

Sciammas R, Shaffer AL, Schatz JH, Zhao H, Staudt LM, Singh H. Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. Immunity. 2006; 25:225–236. [PubMed: 16919487]

Shin H, Blackburn SD, Intlekofer AM, Kao C, Angelosanto JM, Reiner SL, Wherry EJ. A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. Immunity. 2009; 31:309–320. [PubMed: 19664943]

Sun J, Dodd H, Moser EK, Sharma R, Braciale TJ. CD4+ T cell help and innate-derived IL-27 induce Blimp-1-dependent IL-10 production by antiviral CTLs. Nature immunology. 2011; 12:327–334. [PubMed: 21297642]

Sun J, Madan R, Karp CL, Braciale TJ. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. Nature medicine. 2009; 15:277–284.

Vigano S, Utzschneider DT, Perreau M, Pantaleo G, Zehn D, Harari A. Functional avidity: a measure to predict the efficacy of effector T cells? Clinical & developmental immunology. 2012; 2012:153863. [PubMed: 23227083]

Visekruna A, Ritter J, Scholz T, Campos L, Guralnik A, Poncette L, Raifer H, Hagner S, Garn H, Staudt V, et al. Tc9 cells, a new subset of CD8(+) T cells, support Th2-mediated airway inflammation. European journal of immunology. 2013; 43:606–618. [PubMed: 23254356]

Wang Q, Strong J, Killeen N. Homeostatic competition among T cells revealed by conditional inactivation of the mouse Cd4 gene. The Journal of experimental medicine. 2001; 194:1721–1730. [PubMed: 11748274]

Xu WD, Pan HF, Ye DQ, Xu Y. Targeting IRF4 in autoimmune diseases. Autoimmunity reviews. 2012; 11:918–924. [PubMed: 23010632]

Zehn D, Lee SY, Bevan MJ. Complete but curtailed T-cell response to very low-affinity antigen. Nature. 2009; 458:211–214. [PubMed: 19182777]

Zhang N, Bevan MJ. CD8(+) T cells: foot soldiers of the immune system. Immunity. 2011; 35:161–168. [PubMed: 21867926]

HIGHLIGHTS

- IRF4 expression is determined by TCR signaling strength
- IRF4 sustains $CD8^+$ T cell expansion by repressing Bim and CDK inhibitors
- IRF4 sustains effector differentiation of $CD8^+$ T cells
- Ablation of IRF4 in $CD8^+$ T cells impairs $CD8^+$ T cell responses and function

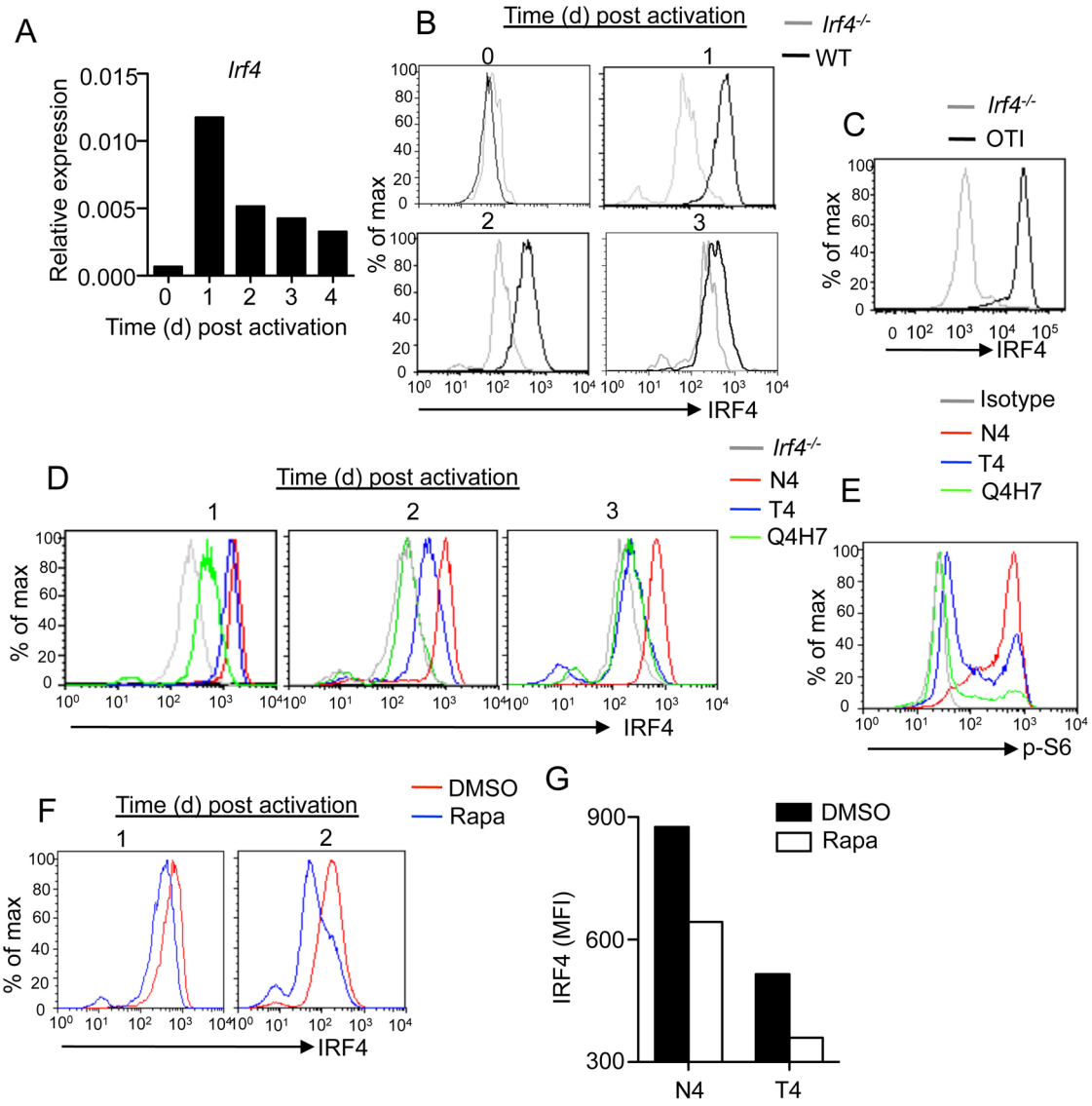

Figure 1. mTOR signaling regulates IRF4 expression in CD8$^+$ T cells

(A, B) IRF4 mRNA (A) and protein expression (B) in polyclonal CD8$^+$ T cells following activation *in vitro*.

(C) OTI T cells were transferred in Thy1 mismatched mice and then the mice were infected with PR8-OVA. IRF4 expression in OTI T cells in draining MLN at day 3.5 p.i.

(D) IRF4 expression in OTI T cells stimulated with N4, T4 or Q4H7 peptide.

(E) mTOR signaling in OTI cells as indicated by S6 phosphorylation following 24 hour N4, T4 or Q4H7 peptide stimulation.

(F) IRF4 expression in polyclonal CD8$^+$ T cells following vehicle (DMSO) or rapamycin (Rapa) treatment.

(G) IRF4 MFI of OTI cells stimulated with N4 or T4 in the presence of DMSO or Rapa at 24 hours post stimulation.

Data are representative of two to four independent experiments. See also Figure S1.

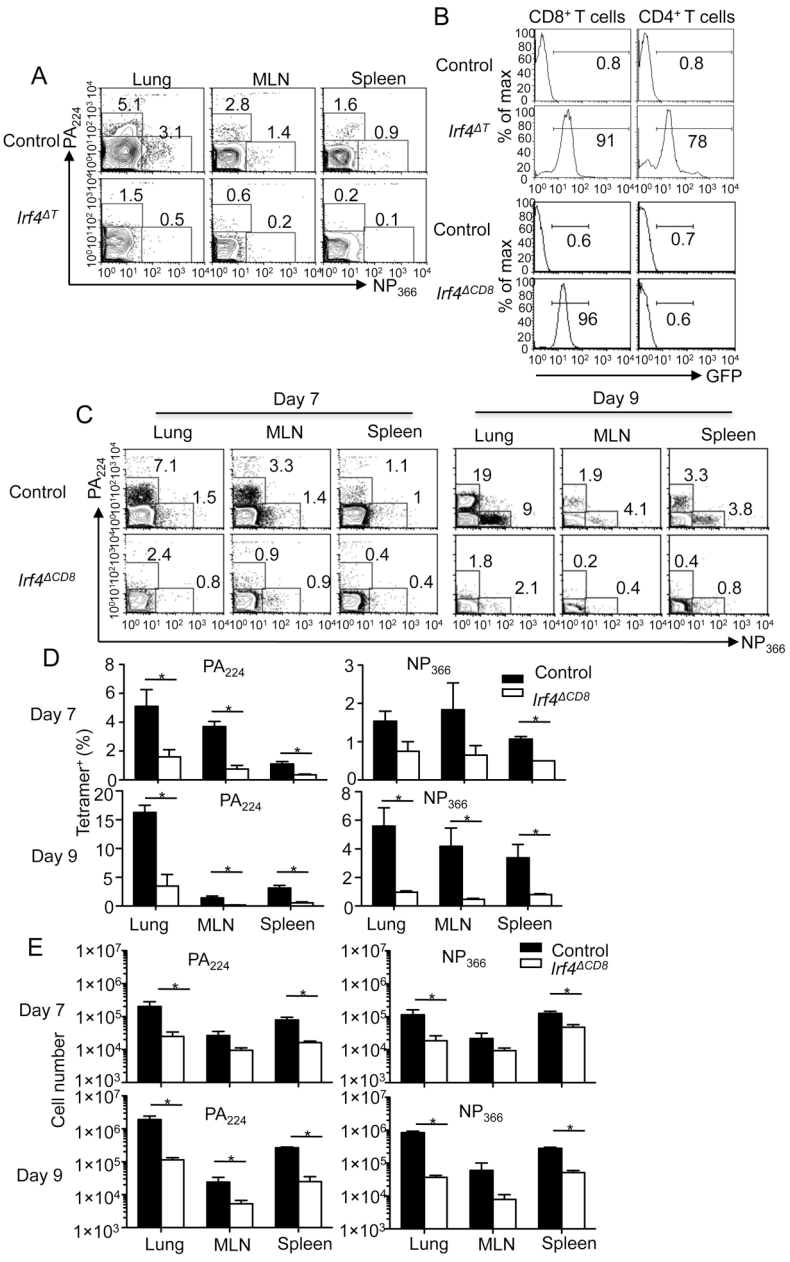

Figure 2. IRF4 ablation in CD8$^+$ T cells impairs the magnitude of CD8$^+$ T cell responses *in vivo*

(A) *Irf4$^{\Delta T}$* mice were infected with influenza. Influenza-specific NP_{366} and PA_{224} tetramer staining in lung, MLN and spleen CD8$^+$ T cells at day 7 p.i.

(B) GFP expression spleen CD8$^+$ and CD4$^+$ T cells in naïve *Irf4$^{\Delta T}$* and *Irf4$^{\Delta CD8}$* mice.

(C – E) *Irf4$^{\Delta T}$* mice were infected with influenza. % NP_{366} and PA_{224} tetramer$^+$ cells in lung, MLN and spleen CD8$^+$ T cells (C, D) and the numbers of NP_{366} and PA_{224} tetramer$^+$ CD8$^+$ T cells in lung, MLN and spleen (E) at day 7 and 9 p.i. Data are mean ± SEM. Data are representative of at least three independent experiments (n = 3–4 mice per group per experiment). *, $P < 0.05$. See also Figure S2.

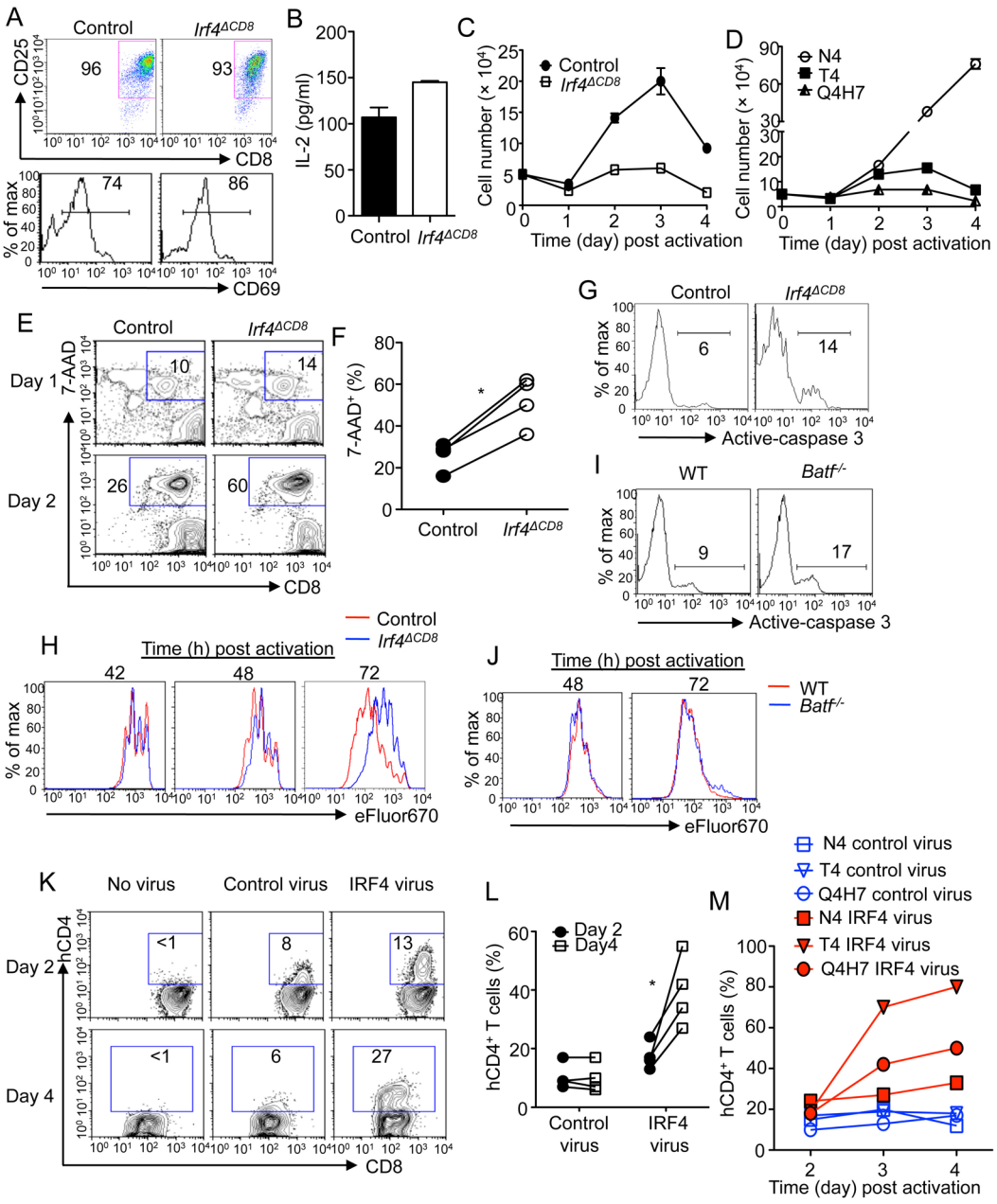

Figure 3. IRF4 is dispensable of early T cell activation but vital for CD8$^+$ T cell expansion

(A) CD25 and CD69 expression of control or IRF4-deficient polycolonal CD8$^+$ T cells (day 1 post activation).

(B) IL-2 in the cultured medium of control or IRF4-deficient polyclonal CD8$^+$ T cells (day 1 post activation). Data are mean ± SD.

(C) Cell numbers of control or IRF4-deficient polyclonal CD8$^+$ T cells following stimulation. Data are mean ± SD.

(D) Cell numbers of OTI T cells stimulated with N4, T4 or Q4H7 peptide. Data are mean ± SD.

(E) 7-AAD staining of control or IRF4-deficient polyclonal CD8$^+$ T cells in the culture.

(F) The percentages of 7-AAD$^+$ cells in control or IRF4-deficient polyclonal CD8$^+$ T cells in the culture (day 2 post activation).

(G) The percentages of active-caspase 3^+ cells in the live control or IRF4-deficient polyclonal $CD8^+$ T cells (day 2 post activation).
(H) Proliferation of control or IRF4-deficient polyclonal $CD8^+$ T cells.
(I) The percentages of active-caspase 3^+ cells in the live WT or BATF -deficient polyclonal $CD8^+$ T cells (day 2 post activation).
(J) Proliferation of WT or BATF-deficient polyclonal $CD8^+$ T cells.
(K, L) WT $CD8^+$ T cells were left untransduced or transduced with control or IRF4-expressing retroviruses. hCD4 staining (K) and the percentages (L) of $hCD4^+$ cells in live $CD8^+$ T cells at day 2 and day 4 following activation.
(M) OTI T cells were stimulated with N4, T4 or Q4H7 peptide and transduced with control or IRF4-expressing retrovirus. The percentages of $hCD4^+$ cells in live OTI T cells were monitored.
Data are representative of two to four independent experiments except in (F, L). (F, L), data are pooled from four independent experiments. *, $P < 0.05$. See also Figure S3.

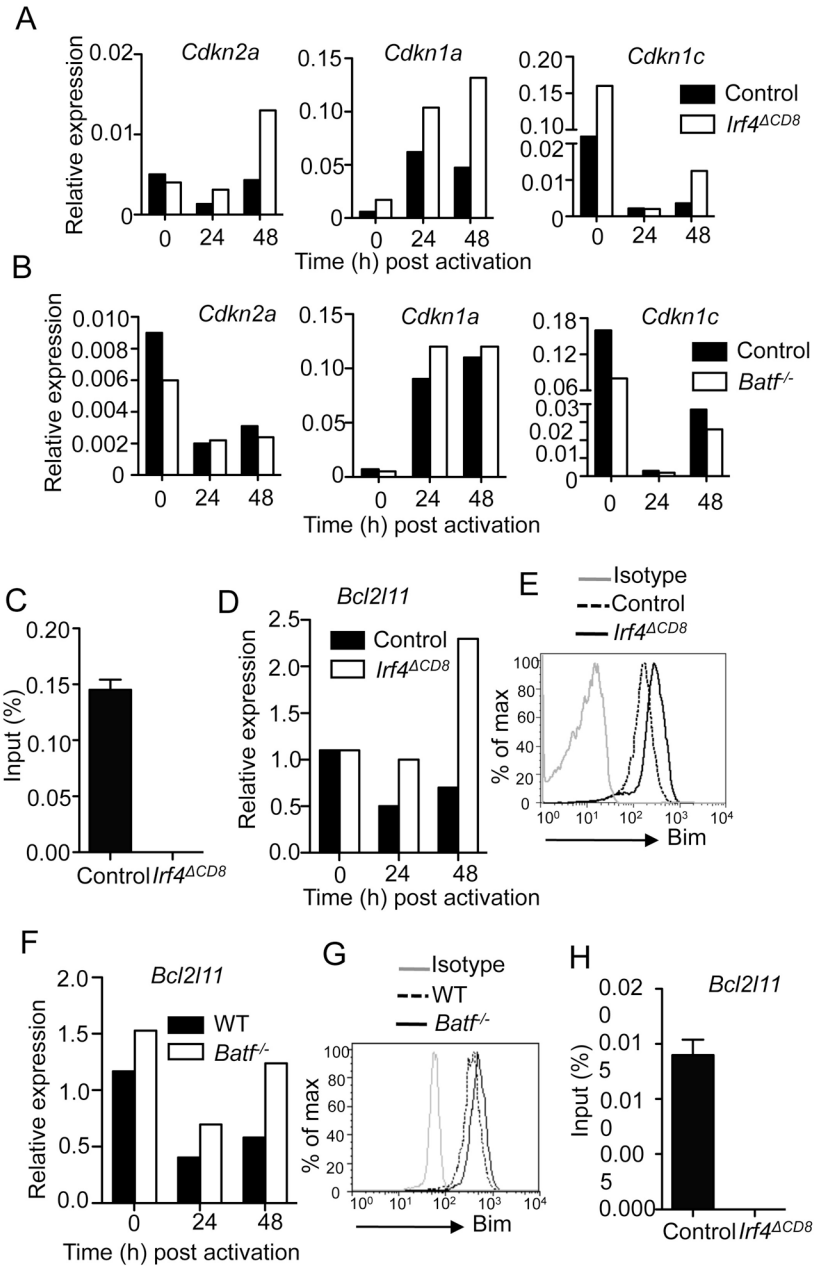

Figure 4. IRF4 regulates CD8$^+$ T cell expansion by repressing CDK inhibitors and Bim

(A) Expression of various CDK inhibitors in control or IRF4-deficient polyclonal CD8$^+$ T cells.

(B) Expression of various CDK inhibitors in WT or BATF-deficient polyclonal CD8$^+$ T cells.

(C) IRF4 binding to *cdkn2a* locus in control or IRF4-deficient polyclonal CD8$^+$ T cells was determined by ChIP assay (day 2 post activation). Data are mean ± SD.

(D, E) Bim mRNA (*Bcl2l11*) (D) and protein (E) expression in control or IRF4-deficient polyclonal CD8$^+$ T cells at indicated times (D) or day 2 (E) post activation.

(F, G) Bim mRNA (*Bcl2l11*) (F) and protein (G) expression in control or BATF-deficient polyclonal CD8$^+$ T cells at indicated times (F) or day 2 (G) post activation.

(H) IRF4 binding to *Bcl2l11* locus in control or IRF4-deficient polyclonal $CD8^+$ T cells was determined by ChIP (day 2 post activation). Data are mean ± SD.
Data are representative of three to four independent experiments. See also Figure S4.

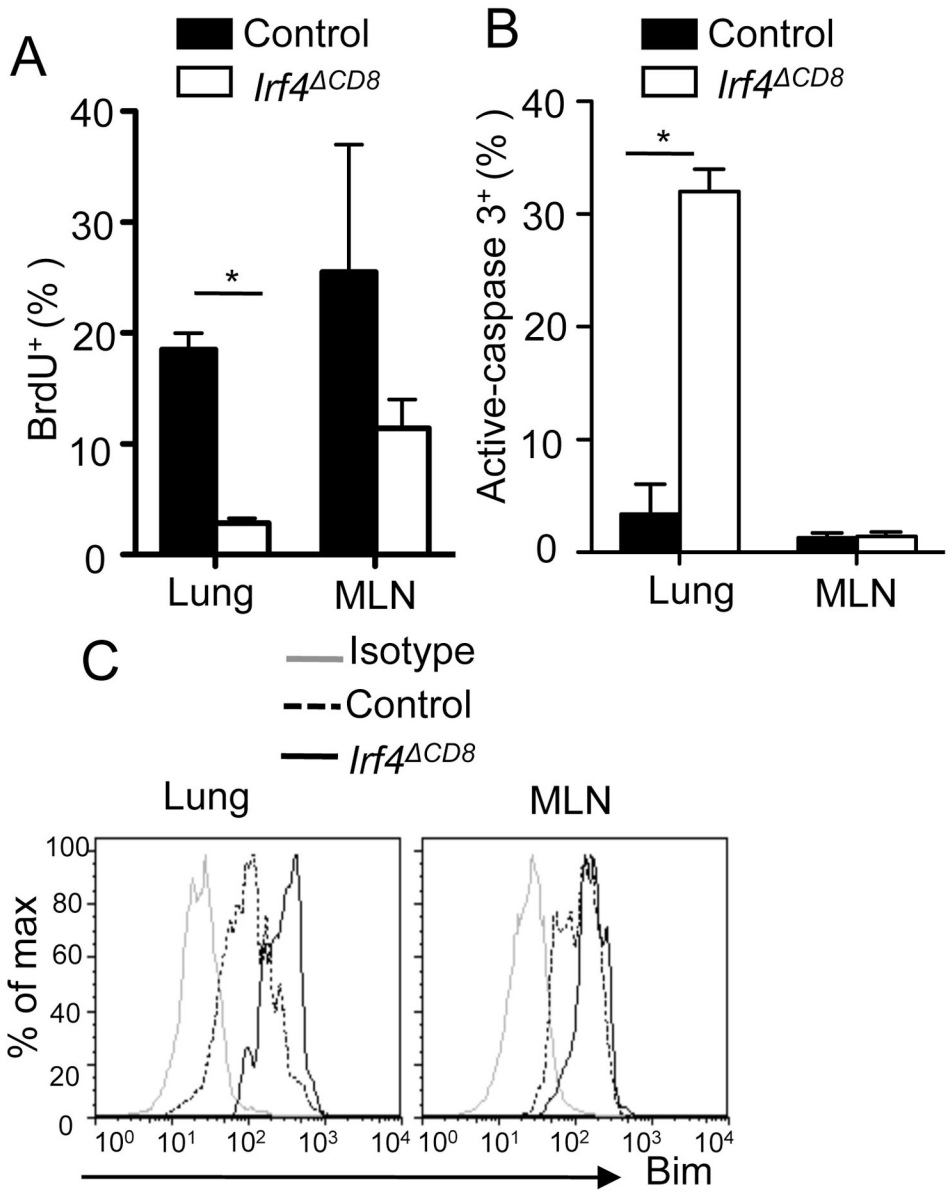

Figure 5. Selective ablation of IRF4 in CD8$^+$ T cells impairs CD8$^+$ T cell proliferation and survival *in vivo*

Control or *Irf4$^{\Delta CD8}$* mice were infected with influenza and injected with BrdU at day 7 p.i.

(A) The percentages of BrdU$^+$ cells in lung or MLN PA_{224} tetramer$^+$ T cells. Data are mean ± SEM.

(B) The percentages of active-caspase 3$^+$ cells in lung or MLN PA_{224} tetramer$^+$ T cells. Data are mean ± SEM.

(C) Bim expression in lung or MLN PA_{224} tetramer$^+$ T cells.

Data are representative of three independent experiments (n = 3–4 mice per group per experiment). *, $P < 0.05$.

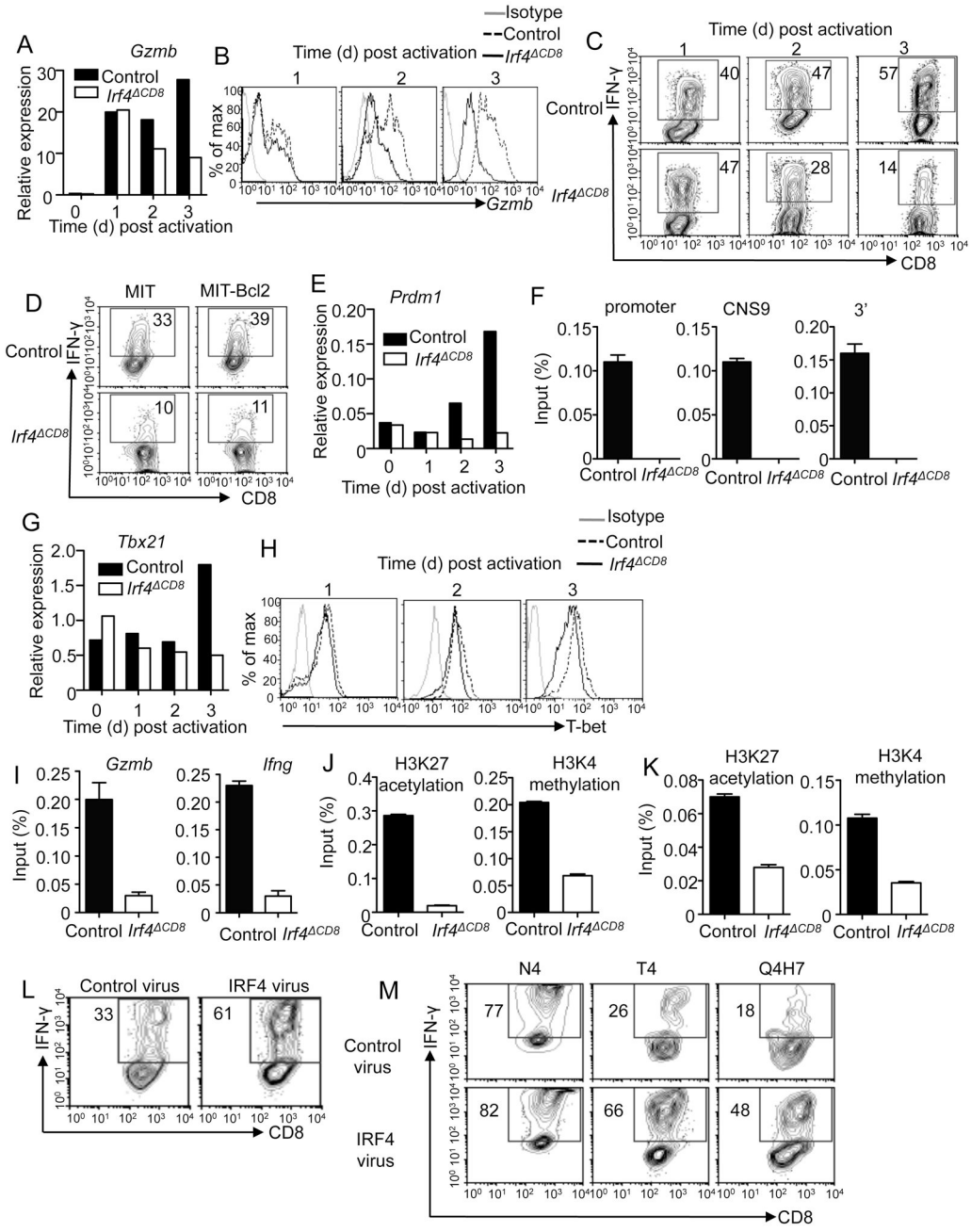

Figure 6. IRF4 sustains CD8$^+$ T cell effector differentiation

(A, B) *Gzmb* mRNA (A) and protein (B) expression in control or IRF4-deficient polyclonal CD8$^+$ T cells.

(C) IFN-γ production in control or IRF4-deficient polyclonal CD8$^+$ T cells following PMA and Ionomycin stimulation.

(D) IFN-γ production in MIT or MIT-Bcl2 retrovirus-transduced control or IRF4-deficient polyclonal CD8$^+$ T cells following PMA and Ionomycin stimulation at day 3 post activation.

(E) Blimp1 mRNA (*Prdm1*) expression in control or IRF4-deficient polyclonal CD8$^+$ T cells.

(F) IRF4 binding to *Prdm1* promoter, CNS9 and 3-prime loci in control or IRF4-deficient polyclonal $CD8^+$ T cells was determined by ChIP (day 2 post activation). Data are mean ± SD.
(G, H) T-bet mRNA (*Tbx21*) (G) and protein (H) expression in control or IRF4-deficient polyclonal $CD8^+$ T cells.
(I) T-bet binding to *Gzmb* and *Ifng* promoters in control or IRF4-deficient polyclonal $CD8^+$ T cells was determined by ChIP assay (day 2 post activation). Data are mean ± SD.
(J, K) H3K27 acetylation and H3K4 methylation in *Ifng* (J) and *Gzmb* (K) promoter regions in control or IRF4-deficient polyclonal $CD8^+$ T cells were determined by ChIP (day 2 post activation). Data are mean ± SD.
(L) IFN-γ production by control or IRF4-expressing retrovirus-transduced WT polyclonal $CD8^+$ T cells following PMA and Ionomycin stimulation at day 4 post activation (3 days post transduction).
(M) OTI T cells were stimulated with N4, T4 or Q4H7 peptide and transduced with control or IRF4-expressing retrovirus. IFN-γ production by transduced T cells following PMA and Ionomycin stimulation at day 4 post activation (3 days post transduction) is depicted.
Data are representative of two to four independent experiments. See also Figure S5.

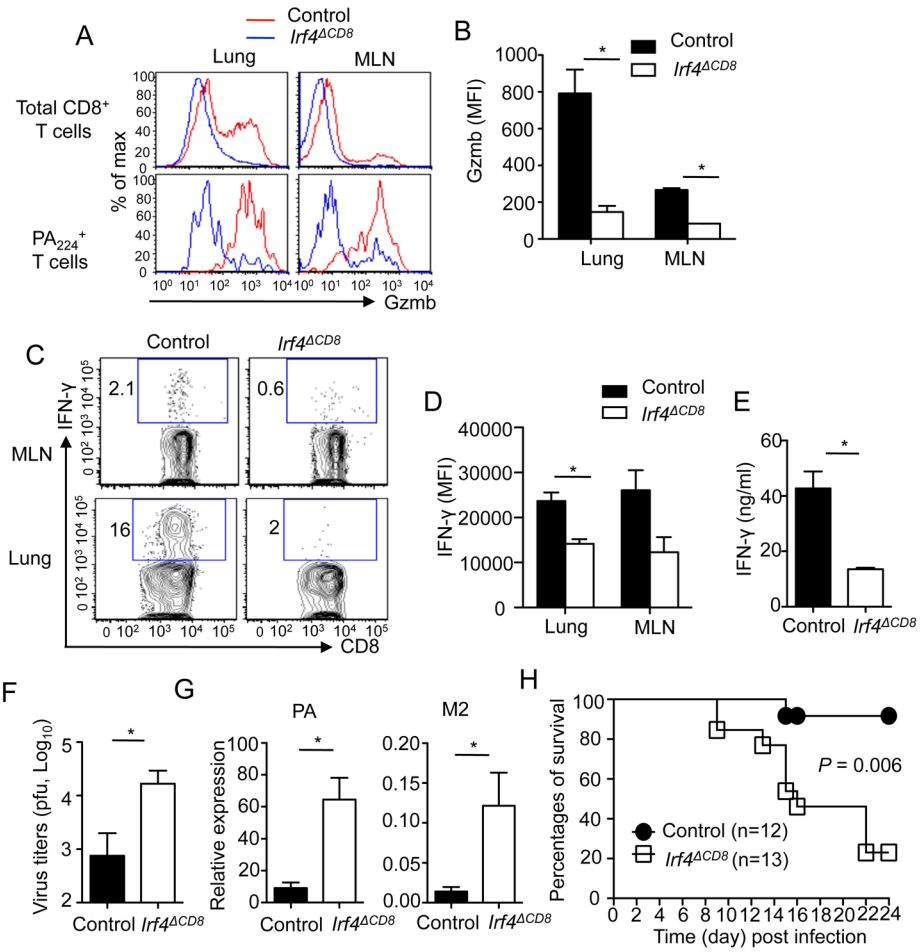

Figure 7. Selective ablation of IRF4 in CD8$^+$ T cells impairs CD8$^+$ T cell effector differentiation and function *in vivo*

Control or *Irf4$^{\Delta CD8}$*mice were infected with influenza.

(A, B) Gzmb expression (A) and MFI (B) in total or PA_{224} tetramer$^+$ T cells at day 7 p.i.

(C, D) IFN-γ production (C) and MFI (D) of day 7 CD8$^+$ T cells following stimulation with influenza-infected BMDCs.

(E) IFN-γ in the BAL at day 7 p.i.

(F) Influenza virus titers in the BAL at day 9 p.i.

(G) Influenza virus gene (PA and M2) expression in the lung at day 9 p.i.

(H) The survival of infected control or *Irf4$^{\Delta CD8}$* mice that were depleted with CD4$^+$ T cells.

(A–E, G). Data are representative of three independent experiments (n = 3–4 mice per group per experiment). (F, H) Data are pooled from total of three independent experiments. Data are mean ± SEM. *, $P < 0.05$. See also Figure S6.