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Graded Levels of IRF4 Regulate CD8+ T Cell Differentiation and Expansion, but Not Attrition, in Response to Acute Virus Infection

Ribhu Nayar,* Elizabeth Schutten,* Bianca Bautista,* Keith Daniels,* Amanda L. Prince,* Megan Enos,* Michael A. Brehm,† Susan L. Swain,* Raymond M. Welsh,* and Leslie J. Berg*

In response to acute virus infections, CD8+ T cells differentiate to form a large population of short-lived effectors and a stable pool of long-lived memory cells. The characteristics of the CD8+ T cell response are influenced by TCR affinity, Ag dose, and the inflammatory cytokine milieu dictated by the infection. To address the mechanism by which differences in TCR signal strength could regulate CD8+ T cell differentiation, we investigated the transcription factor, IFN regulatory factor 4 (IRF4). We show that IRF4 is transiently upregulated to differing levels in murine CD8+ T cells, based on the strength of TCR signaling. In turn, IRF4 controls the magnitude of the CD8+ T cell response to acute virus infection in a dose-dependent manner. Modest differences in IRF4 expression dramatically influence the numbers of short-lived effector cells at the peak of the infection, but have no impact on the kinetics of the infection or on the rate of T cell contraction. Furthermore, the expression of key transcription factors such as T cell factor 1 and Eomesodermin are highly sensitive to graded levels of IRF4. In contrast, T-bet expression is less dependent on IRF4 levels and is influenced by the nature of the infection. These data indicate that IRF4 is a key component that translates the strength of TCR signaling into a graded response of virus-specific CD8+ T cells. The Journal of Immunology, 2014, 192: 000–000.

In response to acute infections, CD8+ T cells undergo priming, differentiation, and expansion to generate robust effector responses that are required for Ag clearance (1, 2). At the termination of the response, the majority of these effector CD8+ T cells die by apoptosis, whereas a small population of efficient memory T cells survives. These memory CD8+ T cells are primed for rapid proliferation and effector functions upon reinfection.

The magnitude and quality of the CD8+ T cell response to an infection is influenced by many factors, including the affinity of TCR–peptide/MHC interactions, the Ag load, costimulatory molecule expression, and the inflammatory cytokine environment. Differences in TCR affinity do not affect the initial activation of Ag-specific CD8+ T cells, but at later time points, T cells with the highest affinity for the Ag dominate the response (3). Similarly, Ag load does not influence the numbers of CD8+ T cells at the early expansion phase but does regulates the size of the overall response at the peak of infection (4–6). To date, the molecular mechanisms linking TCR affinity and Ag density to the magnitude of the CD8+ T cell response have not been characterized.

CD8+ T cell responses to acute infections are also regulated by variations in transcription factor expression. High expression of T-bet and Blimp1 drive the differentiation of primed CD8+ T cells into terminal effectors, whereas T cell factor 1 (TCF1) and Eomesodermin (Eomes) are important for the generation and maintenance of memory cells (7–12). Although the cytokine milieu influences the transcription factor profile of activated CD8+ T cells (8, 13), how these different molecular programs are initially established is not known.

The transcription factor IRF4 is upregulated by BCR and TCR signaling (14, 15). In B cells, different levels of IRF4 regulate differentiation to Ab-secreting plasma cells versus germinal center cells (16). In T cells, IRF4 is required for the differentiation of helper CD4+ T cell subsets, functional regulatory T cells, and effector and innate like CD8+ T cells (17–26). Furthermore, IRF4 was initially found to be required for normal T cell responses to acute lymphocytic choriomeningitis virus (LCMV) infection (17). However, the role of IRF4 in CD8+ T cell differentiation to acute infections has not been characterized in detail, and importantly, the regulation of this process by distinct levels of IRF4 has not been investigated. In this study, we show that variations in Ag dose or in the affinity of TCR–peptide/MHC interactions lead to different levels of IRF4 expression in CD8+ T cells. In turn, these differences regulate the magnitude of the CD8+ T cell response to acute virus infection at the peak of the infection without having any substantial effect on CD8+ T cell attrition. Eomes and TCF1 expression are highly sensitive to distinct levels of IRF4, whereas the effects of IRF4 on CD8+ T cell attrition are dependent on the nature of the infection. These data indicate that IRF4 is a key factor that links signals from the TCR to the transcriptional programing of CD8+ T cells.
Materials and Methods

Mice
Mice were housed in specific pathogen-free conditions at the University of Massachusetts Medical School in accordance with Institutional Animal Care and Use Committee guidelines. Irf4+/fl and Irf4fl/fl mice were used as wild-type (WT) controls. Alterations in peptide dose also impacted IRF4 expression. As shown, higher doses of GP33 peptide (1 μM and 100 nM) induced strong IRF4 expression at 24 and 48 h poststimulation relative to the 10 nM stimulation condition (Fig. 1B). By 72 h, differences in IRF4 levels were observed between each of the peptide doses, with the highest peptide dose leading to the most sustained IRF4 expression (Fig. 1B). These data indicate that expression of IRF4 is transient and is regulated by the strength of TCR stimulation.

CD8+ T cells, IRF4 negatively regulates the expression of the transcription factor, Eomes, that is required for the maintenance of memory cells postinfection (11, 26). As shown in Fig 1C, stimulation with the lower affinity F6L peptide resulted in higher Eomes expression, correlating with its reduced IRF4 expression. Similar results were seen with diminishing doses of GP33 peptide (Fig. 1D). Eomes expression in CD8+ T cells is positively regulated by the transcription factor, TCF1 (10). As shown in Fig. 1E, stimulation with GP33 or F6L peptide resulted in similar TCF1 expression 24 h postactivation; however, at later time points, TCF1 remained highest in cells stimulated with the lower affinity F6L ligand. A similar pattern was seen with the lowest dose of GP33 peptide (Fig. 1F). Taken together, these data demonstrate that varying TCR signal strength, either by changes in TCR-MHC/peptide affinity or dose, leads to distinct expression patterns of three key transcription factors in CD8+ T cells.

To determine whether IRF4 regulated the expression of Eomes and/or TCF1, we used P14 T cells with one or two alleles of Irf4 deleted (Irf4+/fl × CD4-Cre and Irf4+fl/fl × CD4-Cre, referred to as Irf4fl/fl and Irf4fl/fl, respectively). For these studies, P14 WT, P14 Irf4+/fl, and P14 Irf4fl/fl T cells were stimulated in vitro with GP33 peptide. As expected, WT P14 T cells expressed the highest levels of IRF4, whereas Irf4fl/fl cells expressed intermediate levels of IRF4 relative to Irf4+fl/fl and WT cells 24 and 48 h time points (Fig 2A); furthermore, this pattern of expression showed a striking similarity to that seen following stimulation of WT P14 T cells with the lower affinity F6L ligand or with lower doses of GP33 peptide (compare Fig. 2A with Fig. 1A, 1B). Eomes expression inversely correlated with IRF4 levels; P14 Irf4fl/fl cells expressed the highest levels of Eomes, with Irf4fl/fl T cells expressing intermediate levels of Eomes compared with WT cells (Fig. 2B). TCF1 expression was elevated in Irf4fl/fl and Irf4fl/fl cells at the 72 h time point relative to the WT samples with Irf4fl/fl cells expressing the highest levels of TCF1 (Fig. 2C). These data indicate that a complete or heterozygous deficiency in Irf4 leads to lower expression of IRF4, and in turn, this alteration changes the expression patterns of Eomes and TCF1 in stimulated CD8+ T cells.

**A heterozygous deficiency in Irf4 reduces virus-specific CD8+ T cell clonal expansion**

To elucidate the role of graded IRF4 expression during polyclonal CD8+ T cell differentiation in vivo, WT, Irf4+fl/fl, and Irf4fl/fl recognize the GP33 of LCMV bound to H2-D3. A single amino acid substitution from phenylalanine to leucine at position six generates a lower affinity peptide ligand, F6L (30). F6L–H2-D3 complexes display ~5-fold reduction in the equilibrium dissociation constant (Kd) for binding to the P14 TCR, and 100- to 1,000-fold reduction in functional avidity.

P14 T cells were stimulated with the high-affinity GP33 peptide and the lower affinity F6L variant. At 24 h, both populations of cells expressed similar amounts of IRF4. However, high IRF4 expression was sustained at 48 and 72 h poststimulation in cells stimulated with GP33 peptide, whereas cells stimulated with F6L peptide showed declining IRF4 as early as 48 h postactivation (Fig. 1A). Histograms showing IRF4 staining on IRF4-deficient P14 T cells (Irf4fl/fl) stimulated with the GP33 peptide are included as negative controls. Alterations in peptide dose also impacted IRF4 expression. As shown, higher doses of GP33 peptide (1 μM and 100 nM) induced strong IRF4 expression at 24 and 48 h poststimulation relative to the 10 nM stimulation condition (Fig. 1B). By 72 h, differences in IRF4 levels were observed between each of the peptide doses, with the highest peptide dose leading to the most sustained IRF4 expression (Fig. 1B). These data indicate that expression of IRF4 is transient and is regulated by the strength of TCR stimulation.

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Results
The strength of TCR signaling regulates the levels and duration of transcription factor expression
The expression of IRF4 is upregulated in naïve T cells by TCR signaling (14). This response is dependent on the activation of the Tec kinase Itk (26). To determine whether the levels of IRF4 were affected by the strength of TCR signaling to stimulation by natural ligands, P14 TCR transgenic TCRα- (hereafter referred to as P14 WT) CD8+ T cells (29) were stimulated in vitro, and IRF4 levels were examined by intracellular staining. The P14 TCR...
mice were infected with LCMV-Armstrong. Responses to three LCMV epitopes (i.e., H2-D\textsubscript{b}/GP33–41, H2-Db/NP396–404, and H2-Db/GP276–396 [hereafter referred to as GP33, NP396, and GP276]) were examined using MHC–peptide tetramers. At the peak of the response (i.e., day 8 p.i., the magnitude of the CD8+ T cell response depended on the gene dosage of \textit{Irf4}). WT CD8+ T cells mounted the most robust response, followed by \textit{Irf4}/fl, and then \textit{Irf4}fl/fl cells (Fig. 3A, Supplemental Fig. 1A, 1B). Enumeration of viral titers by plaque assay indicated that all WT (5 of 5) and \textit{Irf4}/fl (3 of 3) mice had cleared the virus, whereas only 30% of \textit{Irf4}fl/fl (3 of 10) mice had cleared LCMV by day 8 p.i. These data indicated that modest reductions in IRF4 expression did not interfere with viral clearance but that a minimal level of IRF4 was required for sterilizing immunity to LCMV (Fig. 3B). As our in vitro studies showed reduced IRF4 expression levels following activation of WT versus \textit{Irf4}/fl P14 T cells, these initial infection experiments indicated that even modest differences in the magnitude of IRF4 expression had a profound effect on the clonal expansion of virus-specific CD8+ T cells.

\begin{figure}[h]
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\caption{Variations in TCR affinity and Ag dose upregulate IRF4, Eomes, and TCF1 to different levels. P14 WT and P14 \textit{Irf4}fl/fl were stimulated in vitro. At 0, 24, 48, and 72 h, cells were stained and analyzed for IRF4, Eomes, and TCF1 expression. Histograms show gated live CD8\textsuperscript{+}CD45.2\textsuperscript{+}CD44\textsuperscript{hi} T cells. Gray histograms show staining on direct ex vivo CD8\textsuperscript{+}CD45.2\textsuperscript{+} P14 WT cells. P14 \textit{Irf4}fl/fl cells stimulated with 1 \mu M GP33 peptide are included as negative staining controls for IRF4 expression. Data are representative of four independent experiments. Graphs are compilations of raw MFI of gated live CD8\textsuperscript{+}CD45.2\textsuperscript{+}CD44\textsuperscript{hi} T cells. (A, C, and E) P14 WT T cells were stimulated with 1 \mu M GP33 or F6L peptide. *, significant differences in MFI of WT cells stimulated with GP33 versus F6L ligands. (B, D, and F) P14 WT cells were stimulated with the indicated doses of GP33 peptide. (B) 1 \mu M and 100 nM stimulation conditions were significantly different for IRF4 expression at 72 h, 10 nM stimulation was significantly different from 1 \mu M and 100 nM at all time points. (D) 10 nM stimulation was significantly different from 1 \mu M and 100 nM at 24 h. (F) 10 nM stimulation was significantly different from 1 \mu M and 100 nM at 72 h. *p < 0.05, **p < 0.01, ***p < 0.001.}
\end{figure}

\begin{figure}[h]
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\caption{\textit{Irf4} regulates Eomes and TCF1 expression in a dose-dependent manner. (A–C) P14 WT, P14 \textit{Irf4}+/fl, and P14 \textit{Irf4}fl/fl cells were stimulated with 1 \mu M GP33 peptide for the indicated time points, and cells were stained and analyzed for IRF4, Eomes, and TCF1 expression. Histograms show gated live CD8\textsuperscript{+}CD45.2\textsuperscript{+}CD44\textsuperscript{hi} T cells. Gray histograms show staining on direct ex vivo CD8\textsuperscript{+}CD45.2\textsuperscript{+} P14 WT cells. Data are representative of four independent experiments. Graphs are compilations of raw MFI of gated live CD8\textsuperscript{+}CD45.2\textsuperscript{+}CD44\textsuperscript{hi} T cells. (A) IRF4 expression was significantly different between all genotypes at 24 and 48 h and between P14 WT and P14 \textit{Irf4}+/fl cells and P14 WT and P14 \textit{Irf4}fl/fl cells at 72 h. (B) P14 WT and P14 \textit{Irf4}+/fl cells were significantly different at all time points, whereas P14 \textit{Irf4}fl/fl and P14 \textit{Irf4}fl/fl cells were significantly different at 48 and 72 h. (C) TCF1 expression was significantly different between P14 WT and P14 \textit{Irf4}fl/fl cells at 72 h. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.}
\end{figure}
Reduced levels of IRF4 do not affect the kinetics of CD8+ T cell expansion or attrition

The reduced numbers of virus specific Irf4+/fl cells at day 8 p.i. could be attributed to delayed kinetics of Irf4+/fl CD8+ T cell expansion relative to the WT cells. On the basis of previous data indicating a regulatory T cell defect in Irf4/fl/fl mice that disrupts normal T cell homeostasis (24, 26), studies of Irf4/fl/fl mice were not included in the subsequent analyses. Instead, we focused on a comparison of WT versus Irf4+/fl T cell responses to understand the effect of partial loss of IRF4 expression on CD8+ T cell differentiation. Examination of WT and Irf4+/fl CD8+ T cell populations at later time points following LCMV-Armstrong infection indicated that the peak response for both WT and Irf4+/fl CD8+ T cells was at day 8 p.i.; by day 10 p.i., both populations had started to contract (Fig. 3C, Supplemental Fig. 1C, 1D). Following Ag clearance, the majority of CD8+ T cells undergo attrition by apoptosis and form a small but stable pool of memory cells (1, 2). Examination of virus-specific CD8+ T cells at days 14 and 28 p.i. confirmed that this pattern was observed for both WT and Irf4+/fl CD8+ T cells. However, by day 28 p.i., the differences in the numbers of WT and the Irf4+/fl CD8+ T cells were quite modest, and no longer significant for one of the three epitopes examined (Fig. 3C, Supplemental Fig. 1C, 1D). Normalization of virus-specific CD8+ T cell numbers to the peak of the response indicated that loss of one allele of Irf4 did not change the kinetics of the CD8+ T cell response nor did it affect the rate of CD8+ T cell contraction. Thus, in comparison with the numbers of virus-specific T cells present at the peak of the response, greater numbers of WT...
CD8+ T cells were lost between days 8 and 28 p.i. relative to Irf4+/+/Irf4+/−/Irf4−/− CD8+ T cells (Fig. 3C, Supplemental Fig. 1C, 1D). Therefore, not only did WT cells undergo more robust expansion than the Irf4−/− CD8+ T cells, the WT cells also underwent more extensive contraction.

We also examined the CD4+ T cell response to LCMV-Armstrong in infected WT and Irf4+/− mice. Similar to our findings for CD8+ T cells, analysis of GP61 epitope−specific CD4+ T cells at days 14 and 28 p.i. indicated a defect in Irf4+/− CD4+ T cell expansion relative to the WT cells (Fig. 3D). Furthermore, consistent with our analysis of the CD8+ T cell response, we found that greater numbers of WT GP61-epitope−specific CD4+ T cells were lost between days 14 and 28 p.i. relative to Irf4+/− CD4+ T cells (Fig. 3E).

To generalize these findings to a distinct infection model, we performed a second series of studies examining CD8+ T cell responses to the PR8-OVA strain of influenza A. At day 8 p.i., both WT and Irf4+/− mice mounted robust OVA257–264-specific polyclonal CD8+ T cell responses. As with LCMV, Irf4+/− CD8+ T cells showed a diminished response relative to WT cells. This reduction was observed in the draining mediastinal lymph node (DLN) as well as in the lungs of infected mice (Fig. 4A, 4B). By day 28 p.i., OVA257–264-specific CD8+ T cell populations had undergone attrition in the DLN and lungs of both groups of mice; furthermore, at this time point, no significant differences in the numbers of cells were observed when comparing WT and Irf4+/− mice. Consistent with this, we found that greater numbers of OVA-specific CD8+ T cells were lost between days 8 and 28 p.i. in both DLN and lungs of WT relative to Irf4−/− mice following infection with PR8-OVA257–264.

Taken together, these data suggest that the effects of reduced IRF4 expression are a general feature of CD8+ T cell responses to viral infections and furthermore are impacting CD4+ T cell responses as well. We conclude that different amounts of IRF4 expression during T cell priming regulate the magnitude of the peak antiviral T cell response without affecting the kinetics of the response or the rate of attrition following Ag clearance.

**Reduced gene dosage of Irf4 regulates effector cytokine expression**

To assess CD8+ T cell effector functions following virus infection, splenocytes from LCMV-Armstrong−infected WT, Irf4+/+, and Irf4−/− mice were examined at days 8 and 28 p.i. for IFN-γ, TNF-α, and IL-2 expression. As expected, the gene dosage of Irf4 strongly correlated with the numbers of IFN-γ-producing CD8+ T cells (Fig. 5, Supplemental Fig. 2A, 2B). No differences in the frequencies of TNF-α–producing CD8+ T cells as a proportion of IFNγ+CD8+ T cells were observed at day 8 p.i. when comparing WT and Irf4+/+ mice; however, Irf4−/− mice showed a substantial reduction in the relative proportion of this double cytokine-producing subset. Furthermore, at day 28 p.i., the median fluorescence intensity (MFI) of TNF-α staining and the frequencies of TNF-α/IFN-γ double-producers and IFN-γ/TNF-α/IL-2 triple-producers were significantly decreased in Irf4−/− mice compared with WT controls. Analyses of granzyme B expression and degranulation as assessed by CD107a and CD107b staining revealed no differences between any of the genotypes at either time point. Overall, these data indicate that reduced expression of IRF4 leads to an impaired ability of virus-specific CD8+ T cells to produce cytokines other than IFN-γ as the cells transition into a long-term memory population.

**Levels of IRF4 expression selectively impact the short-lived CD8+ effector cell population**

In response to acute infections, CD8+ T cells undergo clonal expansion and differentiation to short-lived effector cells (SLEC; KLRG1hiCD127lo) and memory-precursor effector cells (MPEC; KLRG1loCD127hi). Examination of these populations revealed that reduced IRF4 expression had a more substantial impact on the numbers of virus-specific SLEC compared with MPEC for each genotypes at either time point. Overall, these data indicate that reduced expression of IRF4 leads to an impaired ability of virus-specific CD8+ T cells to produce cytokines other than IFN-γ as the cells transition into a long-term memory population.
Lower expression of IRF4 impairs production of effector cytokines at day 28 postinfection. Splenocytes from LCMV-GP33–infected WT, *Irf4*+/fl, and *Irf4*fl/fl mice from days 8 and 28 p.i. were stimulated for 5 h with GP33 peptide and analyzed for IFN-γ, TNF-α, IL-2, granzyme B, and CD107a +CD107b. Dot plots show IFN-γ versus TNF-α staining on gated live CD8+CD44hi T cells, and for day 28 p.i., IFN-γ cells were analyzed for TNF-α versus IL-2 staining. Graphs show a compilation of numbers of IFNγ+ cells from days 8 and 28 p.i., and percentage of TNFα+IL-2+ on gated IFNγ+ cells at day 28 p.i. On the right, histograms show granzyme B, CD107a+CD107b, and TNF-α staining; gray histograms show staining on naive CD8+ T cells from uninfected WT mice; the graph shows a compilation of MFIs of TNF-α staining at day 28 p.i. normalized to WT samples in each experiment. Data are representative of three independent experiments with at least five mice per group per time point. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

**FIGURE 5.** Lower expression of IRF4 impairs production of effector cytokines at day 28 postinfection. Splenocytes from LCMV-GP33–infected WT, *Irf4*+/fl, and *Irf4*fl/fl mice from days 8 and 28 p.i. were stimulated for 5 h with GP33 peptide and analyzed for IFN-γ, TNF-α, IL-2, granzyme B, and CD107a +CD107b. Dot plots show IFN-γ versus TNF-α staining on gated live CD8+CD44hi T cells, and for day 28 p.i., IFN-γ cells were analyzed for TNF-α versus IL-2 staining. Graphs show a compilation of numbers of IFNγ+ cells from days 8 and 28 p.i., and percentage of TNFα+IL-2+ and percentage of TNFα+IL-2+ on gated IFNγ+ cells at day 28 p.i. On the right, histograms show granzyme B, CD107a+CD107b, and TNF-α staining; gray histograms show staining on naive CD8+ T cells from uninfected WT mice; the graph shows a compilation of MFIs of TNF-α staining at day 28 p.i. normalized to WT samples in each experiment. Data are representative of three independent experiments with at least five mice per group per time point. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

population (Fig. 6A, Supplemental Figs. 3A, 4A). Specifically, at day 8 p.i., *Irf4*+/fl mice had a 2.5- to 4.4-fold reduction in numbers of SLEC versus a 1.4- to 2.0-fold reduction in MPEC numbers over the three epitopes examined. Furthermore, despite the increase in MPEC percentages among virus-specific CD8+ T cells in *Irf4*+/fl mice at early times postinfection (days 8, 10, and 14 p.i.), the absolute numbers of MPECs in these mice were decreased. Because the numbers of SLEC are much greater than the numbers of MPEC, these data indicate that diminished SLEC populations are largely responsible for the decrease in the total magnitude of the CD8+ T cell effector response in *Irf4*+/fl mice. Interestingly, by day 28 p.i., WT and *Irf4*+/fl mice had comparable numbers of virus-specific CD8+ T cells, and no significant differences in the numbers of MPEC were observed for two of the three viral epitopes examined. Consistent with these data, examination of virus-specific effector (TEM) and central (TCM) memory populations (Fig. 6B, Supplemental Figs. 3B, 4B). These data indicate that reduced IFR4 expression is regulating the expansion phase of the T cell response but not impacting the generation of long-lived virus-specific CD8+ T cells.

**Differential role for IRF4 in regulating TCF1, Eomes, and T-bet expression**

Virus-specific CD8+ T cell differentiation is regulated by the expression of transcription factors such as TCF1, Eomes, and T-bet at day 8 p.i. (8, 10–12, 31). Consistent with our in vitro stimulation data, LCMV-specific *Irf4*+/fl cells expressed the highest levels of TCF1 and Eomes, *Irf4*fl/fl cells had intermediate levels, and WT cells had the lowest levels of both factors. In contrast, the expression of T-bet was reduced in *Irf4*fl/fl cells compared with WT, but no differences were observed in T-bet levels when comparing WT and *Irf4*+/fl cells (Fig. 6C, Supplemental Figs. 3C, 4C). These data indicated that TCF1 and Eomes expression were more sensitive to modest changes in IRF4 levels than was T-bet expression, indicating an IRF4 dose-dependent variation in the regulation of these key transcription factors. At later time points postinfection, differences in Eomes and TCF1 levels in virus-specific *Irf4*+/fl versus WT cells were less uniform across the three epitope-specific populations, although in general, *Irf4*+/fl cells tended to express higher levels of these factors than WT cells at days 10 and 14 p.i.; however, by day 28 p.i., no further differences were observed between *Irf4*+/fl and WT cells. Consistent with the transient nature of IRF4 expression and the data presented above, these results confirm that variations in IRF4 expression levels have the greatest impact at the peak of the virus-specific CD8+ T cell response and are not generally altering the long-lived population of virus-specific CD8+ T cells found at day 28 p.i.

**Cell-intrinsic role for IRF4 in regulating the magnitude of the CD8+ effector T cell response**

To assess whether the altered virus-specific CD8+ T cell response seen in *Irf4*fl/fl versus WT mice was due to differences intrinsic to the CD8+ T cells, we performed adoptive transfer experiments. This approach also allowed us to examine whether activation of P14 T cells in vivo with an LCMV variant expressing the lower affinity F6L ligand would phenocopy the results of reducing IRF4 expression by a heterozygous deficiency in the *Irf4* gene. We first established that activation of P14 cells with LCMV expressing the GP33 epitope results in higher IRF4 expression relative to P14...
FIGURE 6. Differences in IRF4 expression regulate the nature of CD8+ T cell differentiation. Splenocytes from LCMV-GP33–infected WT, Irf4+/fl and Irf4fl/fl mice were analyzed at days 8, 10, 14, and 28 p.i. (A) Dot plots show KLRG1 versus CD127 staining on CD44hiH2Db-GP33 tetramer–positive live CD8+ T cells. Graphs show compilations of the percentages and numbers of KLRG1hiCD127lo (SLEC) and KLRG1loCD127hi (MPEC) populations. Numbers on time-course graphs indicate the relative difference in SLEC or MPEC numbers between WT and Irf4+/fl mice at day 8 p.i. (B) Dot plots show CD44 versus CD62L staining on CD44hiH2Db-GP33 tetramer–positive live CD8+ T cells at day 28 p.i. Graphs show compilations of the percentages and numbers of Tem and Tcm populations. (C) Dot plots show TCF1 and Eomes staining on CD44hiH2Db-GP33 tetramer–positive live CD8+ T cells at day 28 p.i. Graphs show compilations of the normalized MFI for TCF1 and Eomes. (Figure legend continues)
cells activated in response to LCMV-F6L infection. One million congenerically marked WT P14 (CD45.1^+CD45.2^+) cells were transferred into naive WT (CD45.2^+) hosts. One day later, mice were infected with 1 × 10^5 PFU LCMV-Armstrong expressing either the WT GP33 epitope (LCMV-GP33) or the mutant F6L epitope (LCMV-F6L). Previous studies showed that the single amino acid substitution in the LCMV-F6L virus has no impact on viral replication or viral clearance when compared with LCMV-GP33 (30). As expected, at day 3 p.i., P14 cells activated with LCMV-GP33 expressed higher levels of IRF4 relative to P14 cells activated in response to the F6L epitope (Fig. 7A). These data were consistent with the results seen upon in vitro stimulation of WT P14 cells with the GP33 and F6L ligands (Fig. 1A).

To assess the cell-intrinsic role of reduced TCR stimulation, either alone or in combination with reduced IRF4 expression, in regulating the CD8^+ T cell response, congenerically marked WT P14 (CD45.1^+CD45.2^+) and Irf4^+/fl P14 (CD45.2^+) cells were mixed 1:1 and transferred into naive WT (CD45.1^+) hosts (Fig. 7B). When analyzed at day 8 p.i., we observed a substantially greater proportion of WT P14 cells relative to the Irf4^+/fl P14 population, indicating differential expansion of the two populations (Fig. 7C, 7D). This trend was observed when a total of 2,000 or 20,000 P14 cells were transferred as a 1:1 mixture of the two genotypes. Strikingly, infection with the LCMV-F6L virus reduced the overall expansion of both WT and Irf4^+/fl P14 cells but maintained the competitive advantage of the WT over the Irf4^+/fl cells (Fig. 7D). These findings indicate that the decreased expansion of P14 cells in response to LCMV-F6L relative to LCMV-GP33 is highly correlated with decreased IRF4 expression. Thus, these data suggest that variable upregulation of IRF4 in CD8^+ T cells is responsible for the effect of TCR signal strength on the magnitude of the peak effector response, as reported previously (3).

More detailed analyses of the P14 populations were performed with mice receiving 20,000 transferred P14 cells prior to infection, because this provided a greater number of cells for analysis. Examination of SLEC versus MPEC subsets among the P14 populations indicated that the graded magnitude of the response seen among the four experimental groups could be largely attributed to differences in the expansion of P14 SLEC (Fig. 8A). This was affected by changes in IRF4 expression because of deletion of one Irf4 allele or by infecting mice with an LCMV variant expressing only a lower affinity ligand for the P14 TCR, or both. In contrast, the MPEC populations were largely unaffected by loss of one functional Irf4 allele. Regardless of the virus used, Irf4^+/fl P14 cells expressed higher total levels TCF1 because of the increased proportions of TCF1^+ cells among the Irf4^+/fl P14 populations (Fig. 8B). However, similar to our observations regarding the MPEC population, the absolute numbers of TCF1^+ cells were not affected by a heterozygous deficiency in Irf4 (Fig. 8B). This is in contrast to a recent report showing a ~2-fold decrease in tcf7 mRNA in vitro–activated Irf4-deficient OT-I CD8^+ T cells (32). This discrepancy could arise from differential priming of CD8^+ T cells in vivo in response to a virus infection versus that occurring when T cells are activated in vitro; alternatively, the differences seen might arise from differences in the time points examined in our ex vivo analysis versus that used for the in vitro stimulation studies. Nonetheless, consistent with previous data (10), we found that higher TCF1 expression in Irf4^+/fl P14 cells correlated with enhanced Eomes expression in these cells (Fig. 8C). In contrast, we observed no differences in the expression of T-bet when comparing WT to Irf4^+/fl P14 cells (Fig. 8C).

The costimulatory molecule CD27 is important for survival of CD8^+ effector and memory cells (33); interestingly, we found increased expression of CD27 on Irf4^+/fl P14 cells than WT P14 cells (Fig. 8C). This finding is consistent with our observation that, following the peak of the antiviral response, Irf4^+/fl cells undergo less cell death than their WT counterparts. To address this possibility further, we examined Bcl2 expression. As shown, the loss of one allele of Irf4 and/or infection with LCMV-F6L resulted in enhanced expression of the prosurvival molecule Bcl2 in P14 cells (Fig. 8D). This increase could be mainly attributed to higher Bcl2 expression in the Irf4^+/fl versus the WT P14 MPEC population (Fig. 8D). Taken together, these data are consistent with the greater survival potential of Irf4^+/fl P14 cells relative to WT and provide an explanation for the reduced numerical attrition of virus-specific Irf4^+/fl CD8^+ T cells observed (Figs. 3C, 4A, 4B, Supplemental Fig. 1C, 1D). Finally, these data also indicate that the differences in the clonal expansion and differentiation of virus specific effector CD8^+ T cells observed upon infection of intact WT and Irf4^+/fl mice were due to a CD8^+ T cell–intrinsic requirement for high levels of IRF4; furthermore, these differences mainly arose from the variable expansion of the short-lived effector cells.

We next confirmed the cell intrinsic role of IRF4 in CD8^+ T cell expansion using the Influenza A infection system. WT and Irf4^+/fl OT-I CD8^+ T cells were mixed 1:1 and transferred into WT hosts, which were then infected with the PR8-OVA strain of virus A that expresses the epitope recognized by OT-I cells. Analysis of OT-I populations at the peak of the response (day 8 p.i.) indicated a defect in the ability of Irf4^+/fl OT-I cells to clonally expand relative to WT OT-I populations in the spleen, DLN, and lung (Fig. 9A). These data are in agreement with the findings from analysis of polyclonal OVA-specific CD8^+ T cell responses in intact WT and Irf4^+/fl mice (Fig. 4A, 4B), indicating the CD8^+ T cell–intrinsic nature of this effect. We also observed that the expression of TCF1 and Eomes were higher in Irf4^+/fl OT-I cells compared with WT OT-I cells in the two lymphoid organs examined, whereas little difference was observed when comparing the two OT-I populations in the lung (Fig. 9B). Interestingly, unlike our findings with acute LCMV infection, influenza A infection revealed a reduction in T-bet levels in Irf4^+/fl OT-I cells compared with WT OT-I cells in the spleen and DLN (Fig. 9B). The differences in T-bet expression seen following influenza A, but not LCMV, infection might be due to a difference in the cytokine milieu between these two virus infections. Whereas high levels of IL-12 are induced during acute Influenza infection, LCMV-Armstrong infection induces little IL-12 (34, 35). Because IL-12 is a po-
Differential T cell expansion is driven by variations in the levels of IRF4 expressed in competing T cell populations

The data described above establish a CD8+ T cell-intrinsic requirement for IRF4. During polyclonal T cell responses, Ag-specific T cells compete for Ag and inflammatory cytokines. To test whether variations in IRF4 expression levels between competing T cell populations could result in variable T cell expansion, we transferred WT P14 T cells into either WT or Irf4+/− host mice and infected them with LCMV-GP33 or LCMV-F6L virus. Our findings thus far predicted that, in the case where WT P14 cells were responding in a host where all endogenous T cells are Irf4+/−, the WT P14 cells should show an enhanced response relative to their response in a WT host environment. In contrast, we reasoned that the WT P14 cells should show an impaired response following infection with LCMV-F6L in a WT host; however, we predicted that this response should improve if the WT P14 cells are transferred into Irf4+/− hosts, thereby providing the WT P14 cells with an advantage based on two functional alleles of Irf4.

As shown in Fig. 10A, the data from these experiments supported our predictions. A single population of WT P14 T cells was transferred into either WT or Irf4+/− hosts, which were then infected with either LCMV-GP33 or LCMV-F6L. We found that WT P14 cells contributed more dominantly to the response to LCMV-GP33 when the endogenous T cell population was Irf4+/− cells, in contrast to their contribution when responding in a WT host. Alternatively, the WT P14 cells contributed little to the response to LCMV-F6L when present in a WT host, but this response could be greatly improved by transferring these cells into an Irf4+/− host, where the endogenous T cell response was handicapped in Irf4 expression. These data indicate that the contribution of an individual virus-specific CD8+ T cell population to the overall response is not simply regulated by the levels of IRF4 expressed in those cells, but also is determined by the levels of IRF4 expressed in competing T cell populations present in the same individual.

On the basis of these data, we speculated that the CD8+ T cells forming the most robust response to an acute infection would express the highest levels of IRF4, thus accounting for the predominance of these populations at the peak of the response. In acute LCMV infections, CD8+ T cell responses to GP33 and NP396 epitopes are immunodominant whereas those to GP276 are subdominant (37). Because the expression of IRF4 is transient in nature, we were unable to detect differences in IRF4 expression in these populations during the polyclonal response to LCMV-Armstrong. However, we did observe that the magnitude and kinetics of upregulation of TCF1, a target repressed by IRF4, were different between the epitope-specific populations. GP276-specific cells expressed the highest levels of TCF1, followed by GP33-specific cells, and then NP396-specific cells, suggesting lower IRF4 expression in GP276-specific cells and the highest expression in NP396-specific cells (Fig. 9B). Because TCF1 also regulates the differentiation of TEM to TCM (10), these data provide further mechanistic support for the observation that GP276-specific CD8+ T cells are the earliest subset to form TCM in response to LCMV-Armstrong, whereas NP396-specific T cell population are the slowest (38). These results show that variations in IRF4 expression can modulate the relative proportions of different virus-specific T cell populations recognizing the same epitope and suggest that differences observed in the expansion of polyclonal T cell responses to different epitopes may be driven by differential upregulation of IRF4.

Discussion

The adaptive immune system protects us from pathogens by mounting a strong primary response, and then retaining protective cells that form immunological memory. The recognition of pathogens by CD8+ T cells occurs via interactions of the TCR with peptide/MHC complexes. This process not only allows for activation of pathogen-specific T cells, but also for the selection of high affinity CD8+ T cell clones from the pool of responding...
Although even brief Ag exposure is sufficient to induce a programmed proliferative burst of effector CD8+ T cells (39, 40), the ultimate magnitude of the response is nonetheless proportional to the overall Ag load (4–6). These findings indicate that TCR signaling contributes to the programming of the CD8+ T cell response during the short period of initial Ag exposure.

The data presented in this study, along with two recent reports (32, 41), demonstrate that the transcription factor, IRF4, is a central component in translating the strength of TCR signaling into the magnitude of the CD8+ T cell response to infection. Man et al. examined the response of OT-I CD8+ T cells to infections with Influenza A virus expressing different affinity variants of the OVA peptide. This study showed that decreasing the TCR signal strength resulted in lower IRF4 expression in OT-I cells both in vitro and in vivo, and dramatic differences in the numbers of OT-I CD8+ T cells at the peak of the response, a phenotype similar to that observed for IRF4-deficient CD8+ T cells responding to infection. Yao et al. also showed that IRF4 expression was dependent on the strength of TCR signaling in vitro, and that loss of IRF4 expression resulted in a diminished polyclonal CD8+ T cell response at the peak of the infection. Consistent with these two studies, we found that upregulation of IRF4 in P14 TCR transgenic CD8+ T cells was also dependent on the affinity and the dose of the stimulating peptide. Further, reduced expression of IRF4 was also observed following in vitro stimulation of IRF4 haplo-deficient P14 T cells compared with WT. Similar to the findings

**FIGURE 8.** Irf4 haplodeficiency selectively impairs terminal effector CD8+ T cell numbers and alters transcription factor, CD27 and Bcl2 expression. A total of 20,000 P14 cells comprising a 1:1 mix of P14 WT (CD45.1+CD45.2+) and P14 Irf4+/fl (CD45.2+) were transferred into CD45.1+ congenic hosts 1 d prior to infection with LCMV-GP33 or LCMV-F6L, and splenocytes were analyzed on day 8 p.i. (A) Dot plots show KLRG1 versus CD127 staining on gated live CD8+CD44hi P14 populations. Graphs show compilations of percentages and numbers of SLEC and MPEC populations for each genotype. (B) The histogram shows TCF1 expression on gated live CD8+CD44hi P14+ cells. Open histograms, LCMV-WT responding cells; shaded histograms, LCMV-F6L responding cells. Colors are as indicated in graphs. The graph below shows the compilation of TCF1 MFI normalized to WT cells responding to LCMV-WT virus in each experiment. Dot plots show CD8 versus TCF1 staining on gated live CD8+CD44hi P14 cells. The graphs at the right show compilations of percentages and absolute numbers of CD8+TCF1+ population for each genotype. (C) Histograms show Eomes, T-bet, and CD27 staining on CD8+CD44hi P14 cells of each genotype, and the graphs below show compilations of MFI for each stain normalized to WT cells responding to LCMV-WT virus in each experiment. (D) Left histogram shows Bcl2 staining on gated live CD8+CD44hi P14 populations from LCMV-WT responding cells (open histograms) and LCMV-F6L responding cells (shaded histograms). Colors are as indicated in the graph. The graph below shows the compilation of Bcl2 MFI normalized to WT cells responding to LCMV-WT virus in each experiment. Data are representative of two to three independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
of Man et al. (31), we also found a dose-dependent decrease in CD8+ T cell expansion upon loss of one or both alleles of Irf4, a phenomenon that could be phenocopied using an LCMV variant expressing a lower affinity ligand for the P14 TCR. Taken together, these studies provide strong support for the conclusion that levels of IRF4 are tightly regulated by the strength of TCR signaling and, in turn, regulate the magnitude of the CD8+ T cell response to infection.

Most importantly, we show that modest variations in the levels of IRF4 expression, such as those achieved by a heterozygous

**FIGURE 9.** IRF4 regulates the numbers and differentiation of CD8+ T cells in response to influenza A infection. A total of 6000 OT-I cells comprising a 1:1 mix of OT-I WT (CD45.1) and OTI Irf4+/fl (CD45.2) were transferred into CD90.1 congenic WT hosts and infected with PR8-OVAI. Spleens, DLN, and lungs were harvested at day 8 p.i. (A) Dot plots show CD45.1 versus CD45.2 staining on live CD8+CD44hiCD90.2+ cells. Graphs show the ratios of OT-I WT to OTI Irf4+/fl cells in each organ. (B) Histograms show TCF1, Eomes, and T-bet staining of OT-I WT (red) and OTI Irf4+/fl (blue) cells relative to isotype controls (gray histograms). Graphs show MFI of transcription factor staining normalized to WT samples in each experiment. Data are representative of two independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

**FIGURE 10.** Ability to express higher levels of IRF4 provides a competitive advantage to Ag-specific CD8+ T cells. (A) A total of 1000 P14 WT (CD45.1) or Irf4+/fl hosts (CD45.2) 1 d prior to infection with LCMV-GP33 or LCMV-F6L, and splenocytes were analyzed at day 8 p.i. Dot plots show CD45.1 versus CD45.2 staining on gated live CD8+CD44hi cells. Graphs show a compilation of percentages of P14 WT populations. Data are representative of two independent experiments with more than or equal to four mice per group. (B) Splenocytes from LCMV-GP33-infected WT mice were analyzed at days 8, 14, and 28 p.i. Histograms show TCF1 staining of CD44hiH2D2-NP396, -GP33, and -GP276 tetramer-positive live CD8+ T cells; gray histograms represent isotype control staining. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
deficiency in Irf4, are sufficient to have a dramatic impact on the peak expansion of virus-specific CD8+ T cells. In our studies, reduced Irf4 expression decreased the maximum numbers of Ag-specific CD8+ T cells by 2- to 3-fold and, furthermore, had a greater impact on the numbers of short-lived effector cells compared with the memory precursor effector cells. These data provide a mechanistic explanation for previous studies demonstrating that shortening the duration of Ag exposure decreases the total CD8+ T cell response and in particular the size of the SLEC compartment, without affecting the numbers of MPEC (4, 6, 8).

Our studies also showed that, following virus clearance and the bulk of the T cell attrition, there was virtually no impact on the numbers of Ag-specific T cells remaining, regardless of their ability to express high levels of Irf4. This latter finding is strikingly similar to the observations of Bevan and colleagues (3) in their elegant study examining the response of OT-I T cells to strains of *Listeria monocytogenes* expressing different affinity variants of the OVA peptide. In this study, dramatic differences in the peak expansion of OT-I T cells were seen following activation by *Listeria* strains expressing the different OVA variants; however, following bacterial clearance, few differences were found in the numbers of long-term surviving OT-I memory T cells. A likely explanation for these data are that the higher affinity OVA variants induced higher levels of IRF4 than did the lower affinity variants, thus accounting for the relative response of the OT-I T cells to each bacterial strain. Furthermore, because the expression of IRF4 is transient in nature, the effects of different levels of IRF4 are limited to CD8+ T cell priming and the peak expansion phase but not thereafter. We also found increased expression prosurvival factors, CD27 and Bcl2, in P14 Irf4 haplosufficient cells, consistent with a greater survival potential of these cells relative to WT. These data provide a potential mechanism to account for the findings of Bevan and colleagues, that ligands representing a broad range of TCR affinities generated relatively similar numbers of Ag-specific T cells by 2- to 3-fold and, furthermore, had reduced IRF4 expression decreased the maximum numbers of Ag-specific CD8+ T cells also convert to TCM more rapidly than WT T cells. Thus, differences in the magnitude and/or duration of IRF4 expression may be one factor that could account for observed differences in the responses of CD8+ T cells to distinct viral epitopes. Overall, our findings, along with those of others, demonstrate that variations in the levels of IRF4 expressed during T cell priming fine-tune the size and quality of the pathogen-specific adaptive immune response.

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**Disclosures**

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**References**


