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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.

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<sup>fl/fl</sup> and OT-I × CD4-Cre and OT-I × Rag1<sup>−/−</sup> Irf4<sup>fl/fl</sup> CD4-Cre mice have been described previously (26, 27). P14/TcRa<sup>−/−</sup> were purchased from Taconic Farms (Germantown, New York). Ift4<sup>+/+</sup> × CD4-Cre, Ift4<sup>−/−</sup>, and Ift4<sup>−/−</sup> were used as wild-type (WT) controls.

Abs, H2<sup>D</sup>D<sup>2</sup>, and H2K<sup>K</sup>k monomers and staining

CD45.2-V500 and TNF-α-allophycocyanin-cyanine 7 were purchased from BD Biosciences (San Jose, CA). KLRC1–FITC, Eomes–PECy7, CD107a–PE, CD107b–PE, CD27–PE, CD27–PE-Cy5, CD127–PerCP-Cy5.5, Thet-PerCP-Cy5.5, IFN-γ-PerCP-Cy5.5, Eomes-PerCP-efluor710, CD45.1–PECy7, KLRC1–PE-Cy7, Thet–PE-Cy7, Irf4–Alexa Fluor 674, CD44–Alexa Fluor 700, L-selectin (CD62L)-allophycocyanin-efluor780, CD44–fluor450, KLRC1–fluor450, IFNy–fluor450, CD90.2–allophycocyanin-efluor780, CD45.1–allophycocyanin-efluor780, IL-2–PerCP-Cy5.5 were purchased from eBioscience (San Diego, CA). CD8-Pe-TexasRed, granzyme B–PE, granzyme B–allophycocyanin, Live-Dead-Violet, Live-Dead-Aqua and goat-anti-rabbit IgG-Alexa Fluor 674 and -Alexa Fluor 488 were purchased from Life Technologies (Grand Island, NY). H2<sup>D</sup>D<sup>2</sup>-GP33 monomers were prepared at the University of Massachusetts Medical School; LCMV-specific (H2<sup>D</sup>D<sup>2</sup>-NP96 and H2<sup>D</sup>D<sup>2</sup>-GP76) and influenza A PR8-OVA<sub>5</sub>–specific (H2K<sup>K</sup>k-OVA15257) monomers were obtained from the NIH Tetramer Core Facility (Atlanta, GA). Intracellular TCF1 staining was performed using rabbit-anti-mouse TCF1 (Cell Signaling Technology, Danvers, MA), followed by staining with goat-anti-rabbit secondary (Life Technologies). Samples were analyzed on an LSRII flow cytometer (BD Biosciences), and data were analyzed using FlowJo (Tree Star).

Cell culture

Lymph node cells from P14 WT and P14 Irf4<sup>−/−</sup> mice were mixed with equal numbers of WT CD45.1 splenocytes and stimulated with GP33<sub>4-10</sub> epitope (GP33) or F6L peptides for 24, 48, and 72 h. Cells were harvested and analyzed for Irf4, Eomes, and TCF1 expression by intracellular staining. For cytokine production, splenocytes from infected mice were stimulated and analyzed for IRF4, Eomes, and TCF1 expression by intracellular staining. The P14 TCR transgenic TCR<sup>P14</sup> T cells (29) were stimulated in vitro, and IRF4 expression was evaluated by 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<sup>−/−</sup> expression. Similar results were seen with diminishing doses of GP33 peptide (Fig. 1D). Eomes expression in CD8<sup>+</sup> T cells is positively regulated by the transcription factor, TCF1 (10). As shown in Fig. 1E, with diminishing doses of GP33 peptide (Fig. 1F), TCF1 expression was sustained at 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<sup>+</sup> T cells.

To determine whether Irf4<sup>−/−</sup> regulated the expression of Eomes and/or TCF1, we used P14 T cells with one or two alleles of Irf4 deleted (Irf4<sup>−/−</sup> × CD4-Cre and Irf4<sup>−/−</sup> × CD4-Cre, referred to as Irf4<sup>−/−</sup> and Irf4<sup>−/−</sup> respectively). For these studies, P14 WT, P14 Irf4<sup>−/−</sup>, and P14 Irf4<sup>−/−</sup> T cells were stimulated in vitro with GP33 peptide. As expected, WT cells expressed the highest levels of Irf4<sup>−/−</sup>, whereas Irf4<sup>−/−</sup> cells expressed intermediate levels of Irf4<sup>−/−</sup> relative to Irf4<sup>−/−</sup> 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 Irf4<sup>−/−</sup> cells expressed the highest levels of Eomes, with Irf4<sup>−/−</sup>-<sup>+</sup> T cells expressing intermediate levels of Eomes compared with WT cells (Fig. 2B). TCF1 expression was elevated in Irf4<sup>−/−</sup> and Irf4<sup>−/−</sup> cells at the 72 h time point relative to the WT samples with Irf4<sup>−/−</sup> 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<sup>−/−</sup>, and in turn, this alteration changes the expression pattern of Eomes and TCF1 in stimulated CD8<sup>+</sup> T cells.

A heterozygous deficiency in Irf4 reduces virus-specific CD8<sup>+</sup> T cell clonal expansion

To elucidate the role of graded Irf4 expression during polyclonal CD8<sup>+</sup> T cell differentiation in vivo, WT, Irf4<sup>−/−</sup>, and Irf4<sup>−/−</sup>
mice were infected with LCMV-Armstrong. Responses to three LCMV epitopes (i.e., H2-D\textsuperscript{b}/GP33–41, H2-D\textsuperscript{b}/NP396–404, and H2-D\textsuperscript{b}/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\textsuperscript{+} T cell response depended on the gene dosage of \textit{Irf4}). WT CD8\textsuperscript{+} T cells mounted the most robust response, followed by \textit{Irf4+/fl}, and then \textit{Irf4fl/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{Irf4fl/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\textsuperscript{+} T cells.

**FIGURE 1.** Variations in TCR affinity and Ag dose upregulate IRF4, Eomes, and TCF1 to different levels. P14 WT and P14 \textit{Irf4fl/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{Irf4fl/fl} cells stimulated with 1 \textmu 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 \textmu 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 \textmu M and 100 nM stimulation conditions were significantly different for IRF4 expression at 72 h, 10 nM stimulation was significantly different from 1 \textmu M and 100 nM at all time points. (D) 10 nM stimulation was significantly different from 1 \textmu M and 100 nM at 24 h. (F) 10 nM stimulation was significantly different from 1 \textmu M and 100 nM at 72 h. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

**FIGURE 2.** \textit{Irf4} regulates Eomes and TCF1 expression in a dose-dependent manner. (A–C) P14 WT, P14 \textit{Irf4+/fl}, and P14 \textit{Irf4fl/fl} cells were stimulated with 1 \textmu 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{Irf4fl/fl} cells at 72 h. (B) P14 WT and P14 \textit{Irf4fl/fl} cells were significantly different at all time points, whereas P14 \textit{Irf4+/fl} and P14 \textit{Irf4fl/fl} cells were significantly different at 48 and 72 h. (C) TCF1 expression was significantly different between P14 WT and P14 \textit{Irf4+/fl} cells at 72 h. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
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 Irf4fl/fl mice that disrupts normal T cell homeostasis (24, 26), studies of Irf4fl/fl 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$^{+/\beta}$ CD8$^+$ T cells (Fig. 3C, Supplemental Fig. 1C, 1D). Therefore, not only did WT cells undergo more robust expansion than the Irf4$^{+/\beta}$ 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$^{+/\beta}$ 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$^{+/\beta}$ 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$^{+/\beta}$ 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 PR8-OVA strain of influenza A. At day 8 p.i., both WT and Irf4$^{+/\beta}$ mice mounted robust OVA$^{257-264}$-specific polyclonal CD8$^+$ T cell responses. As with LCMV, Irf4$^{+/\beta}$ 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., OVA$^{257-264}$-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$^{+/\beta}$ 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$^{+/\beta}$ mice following infection with PR8-OVA.

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$^{+/\beta}$, and Irf4$^{\beta\beta}$ 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. 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$^{+/\beta}$ mice; however, Irf4$^{\beta\beta}$ 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$^{+/\beta}$ 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; KLRG1hiCD127hi). 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 genotype; the number on the graph indicates the fold difference in the average numbers of WT vs. Irf4$^{+/\beta}$ or Irf4$^{\beta\beta}$ T cells at days 8 and 28 p.i. (i.e., average number day 8 − average number day 28; right) for each genotype; the number on the graph indicates the fold difference in the average numbers of WT cells lost between D8 and D28 relative to the loss of Irf4$^{+/\beta}$ cells. Data are a compilation of two independent experiments with at least five mice per group per time point. *p ≤ 0.05.
population (Fig. 6A, Supplemental Figs. 3A, 4A). Specifically, at day 8 p.i., Irf4+/− 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+/− 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+/− mice. Interestingly, by day 28 p.i., WT and Irf4+/− 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 (T effector memory) and central (T central memory) memory populations (Fig. 6A, Supplemental Figs. 3A, 4A) showed that the percentages of virus-specific CD8+ T cells in WT mice at early times postinfection were less uniform across the three epitope-specific populations, although in general, Irf4+/− 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+/− 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+/− 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 T cells. WT cells had the lowest levels of both factors. In contrast, the expression of T-bet was reduced in Irf4+/− cells compared with WT, but no differences were observed in T-bet levels when comparing WT and Irf4+/− 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+/− versus WT cells were less uniform across the three epitope-specific populations, although in general, Irf4+/− 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+/− 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.

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+/− cells expressed the highest levels of TCF1 and Eomes, Irf4+/− cells had intermediate levels, and
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. Numbers on time-course graphs indicate the relative difference in Tem or Tcm numbers between WT and Irf4+/fl mice at day 28 p.i. (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 of TCF1 and Eomes. Numbers on time-course graphs indicate the relative difference in TCF1 or Eomes expression between WT and Irf4+/fl mice at day 28 p.i. (Figure legend continues)
cells activated in response to LCMV-F6L infection. One million congenically marked WT P14 (CD45.1<sup>+</sup>CD45.2<sup>+</sup>) cells were transferred into naive WT (CD45.2<sup>+</sup>) hosts. One day later, mice were infected with 1 × 10<sup>5</sup> 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<sup>+</sup> T cell response, congenically marked WT P14 (CD45.1<sup>+</sup>CD45.2<sup>+</sup>) and <sup>−</sup>Irf4<sup>−/−</sup> P14 (CD45.2<sup>+</sup>) cells were mixed 1:1 and transferred into naive WT (CD45.1<sup>+</sup>) hosts (Fig. 7B). When analyzed at day 8 p.i., we observed a substantially greater proportion of WT P14 cells relative to the <sup>−</sup>Irf4<sup>−/−</sup> P14 population, indicating differential expansion of the two populations (Fig. 7C). 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 <sup>−</sup>Irf4<sup>−/−</sup> P14 cells but maintained the competitive advantage of the WT over the <sup>−</sup>Irf4<sup>−/−</sup> 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<sup>+</sup> 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 <sup>−</sup>Irf4 allele or by infecting mice with an LCMV variant expressing either the WT GP33 epitope (LCMV-GP33) or the mutant F6L epitope (LCMV-F6L; Supplemental Fig. 1C, 1D). Finally, these data also indicate that the differences in the clonal expansion and differentiation of virus-specific effector CD8<sup>+</sup> T cells observed upon infection of intact WT and <sup>−</sup>Irf4<sup>−/−</sup> mice were due to a CD8<sup>+</sup> T cell–intrinsic requirement for high levels of IRF4; furthermore, these differences largely arose from the variable expansion of the short-lived effector cells.

We next confirmed the cell intrinsic role of IRF4 in CD8<sup>+</sup> T cell expansion using the Influenza A infection system. WT and <sup>−</sup>Irf4<sup>−/−</sup> OT-I CD8<sup>+</sup> T cells were mixed 1:1 and transferred into WT hosts, which were then infected with the PR8-OVA<sub>83</sub> strain of influenza 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 <sup>−</sup>Irf4<sup>−/−</sup> 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<sup>+</sup> T cell responses in intact WT and <sup>−</sup>Irf4<sup>−/−</sup> mice (Fig. 4A, 4B), indicating the CD8<sup>+</sup> T cell–intrinsic nature of this effect. We also observed that the expression of TCF1 and Eomes were higher in <sup>−</sup>Irf4<sup>−/−</sup> 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 <sup>−</sup>Irf4<sup>−/−</sup> 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 potent inducer of T-bet expression (36), the presence of IL-12 might reveal a requirement for high levels of IRF4 to achieve the maximum expression of T-bet. In addition, these data confirm the critical role of high IRF4 expression in regulating the magnitude of the effector CD8<sup>+</sup> T cell response in lymphoid organs but indicate a lesser impact on the CD8<sup>+</sup> T cells present at the site of infection in a nonlymphoid peripheral tissue.
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⁺/+ or 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 Tcm 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
T cells (3). Although even brief Ag exposure is sufficient to induce a programmed proliferative burst of effector CD8+ T cell (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 cell 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 haplodeficient P14 T cells compared with WT. Similar to the findings

**FIGURE 8.** *Irff* 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 *Irff+/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+CD44hiP14+ 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+CD44hiP14 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+CD44hiP14+ 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 SLEC samples for 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](image1)

**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 OT-I 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. Graph shows the ratios of OT-I WT to OT-I Irf4+/fl cells in each organ. (B) Histograms show TCF1, Eomes, and T-bet staining of OT-I WT (red) and OT-I 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](image2)

**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+CD45.2+) T cells were transferred into WT 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 CD44hiH2Db-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, despite the dramatic differences in T cell expansion at the peak of the response (3).

Inflammatory cytokines produced during an infection also increase TCR activation by sustaining phosphorylation of ZAP-70 and phospholipase Cγ (42). Because the activation of phospholipase Cγ, as well as the levels of Irf4 expression are Itk dependent (26, 43), it is possible that factors such as cytokines also may help sustain Irf4 expression, which may lead to an overall enhancement in the SLEC response. For instance, IL-12 enhances the expression of T-bet (36), another transcription factor that positively regulates the size of the SLEC compartment (8). Because the expression of T-bet was more dependent on Irf4 following infection with influenza A than LCMV, it is possible that Irf4 also functions to integrate signals from the TCR and cytokines to dictate the magnitude of the CD8+ T cell response.

Another transcription factor, Blimp-1, also plays a central role in terminal effector cell differentiation. Blimp-1-deficient CD8+ T cells have higher expression of Tcf1 and Eomes transcripts and lower expression of T-bet mRNA (7, 9). Three recent studies found a role for Irf4 in Blimp-1 expression in CD8+ T cells (32, 41, 44). In our study, we find a dose-dependent effect of Irf4 levels on the expression of Tcf1 and Eomes in CD8+ T cells responding to infection. These observations not only confirm our previous data that Irf4 is a negative regulator of Eomes expression (26) but also suggest that regulation of Eomes is more complex and possibly involves multiple transcription factors functioning in a temporal manner. Taken together, these data indicate that Irf4 is a central component of this transcriptional program and that the magnitude of Irf4 upregulation during CD8+ T cell priming is a critical determinant of the outcome of the response.

Our data also provide a potential explanation for observations regarding the variable expansion, as well as the varying kinetics, of CD8+ T cell responses to different viral epitopes. For instance, polyclonal CD8+ T cells responses to LCMV epitopes GP33 and NP396 form the dominant response, whereas those to GP276 are subdominant (37), suggesting that Irf4 expression is higher in the former populations. This possibility would fit with our data showing that the response of Irf4−/− P14 T cells in an Irf4−/− host following LCMV infection gives the P14 cells a substantial competitive advantage over the endogenous response, compared with those same cells responding in a WT host. Because of the transient nature of Irf4 expression during T cell priming, we were unable to directly test this hypothesis on the polyclonal T cell response to LCMV. However, we did observe that the levels of Tcf1, a downstream target of Irf4, were different between the different epitope-specific populations. Tcf1 is also important for the conversion of TCM to TEM cells (10).Because LCMV GP276-specific cells convert to TEM earlier than NP396-specific cells following acute infection (38), these data are consistent with a lower level of Irf4 expression in the NP396-specific subset. In further support of this hypothesis, Irf4−/− CD8+ T cells also convert to TEM 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
The authors have no financial conflicts of interest.

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