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The Transcription Factor Runx2 Is Required for Long-Term Persistence of Antiviral CD8⁺ Memory T Cells

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ABSTRACT
During acute lymphocytic choriomeningitis virus infection, pathogen-specific CD8⁺ cytotoxic T lymphocytes undergo clonal expansion leading to viral clearance. Following this, the majority of pathogen-specific CD8⁺ T cells undergo apoptosis, leaving a small number of memory CD8⁺ T cells that persist long-term and provide rapid protection upon secondary infection. Whereas much is known about the cytokines and transcription factors that regulate the early effector phase of the antiviral CD8⁺ T cell response, the factors regulating memory T cell homeostasis and survival are not well understood. In this article, we show that the Runt-related transcription factor Runx2 is important for long-term memory CD8⁺ T cell persistence following acute lymphocytic choriomeningitis virus–Armstrong infection in mice. Loss of Runx2 in T cells led to a reduction in KLRG1lo CD127hi memory precursor cell numbers with no effect on KLRG1hi CD127lo terminal effector cell populations. Runx2 expression levels were transcriptionally regulated by TCR signal strength via IRF4, TLR4/7, and selected cytokines. These data demonstrate a CD8⁺ T cell–intrinsic role for Runx2 in the long-term maintenance of antiviral memory CD8⁺ T cell populations. ImmunoHorizons, 2018, 2: 251–261.

INTRODUCTION
The T cell response to acute viral infections has been well characterized at the cellular level. Following infection, a robust pathogen-specific CD8⁺ T cell response is observed and within 1–2 wk postinfection, the pathogen is cleared from the infected host. This early effector phase includes the proliferation and differentiation of cytotoxic effector T cells, a process that is dependent on inflammatory cytokines produced by innate immune cells and on the presentation of viral peptides on host APCs (1–3). After viral clearance, the majority of the effector CD8⁺ T cell population will undergo apoptosis, a process that continues for many weeks post–pathogen clearance (4). Ultimately, the host retains a small pool of pathogen-specific memory T cells that provide rapid protection upon secondary infection (5).
and Runx3 (31) are all required for CD8+ T cell memory formation and homeostasis.

In this study, we show that a member of the Runt-related transcription factor family (RUNX), Runx2, is also important for regulating the long-term persistence of CD8+ memory T cells following acute lymphocytic choriomeningitis virus (LCMV)–Armstrong infection. Runx2, like the other RUNX factors, contains a Runt DNA binding domain and pairs with CBFβ to bind to DNA (32). Runx2 functions primarily in bone development in which it is required for osteoblast generation (33) and bone formation (34).

Runx1 and Runx3 have well-characterized roles in T cells, including important functions during regulatory T cell development (35), Tc1 skewing (36), and CD8+ T cell differentiation (31, 37). In contrast, no clear function for Runx2 in T cells has been identified, although an earlier study showed that ectopic overexpression of Runx2 in thymocytes perturbed T cell development at the CD4−CD8− stage (38). A genome-wide regulatory network generated by Hu and Chen (39) also suggested that Runx2 may play a role in CD8+ T cell memory. Using mice carrying floxed alleles of Runx2 crossed to CD4-cre, we find no apparent defects in T cell development or T cell homeostasis under steady-state conditions. However, following infection with LCMV–Armstrong, we identify a CD8+ T cell–intrinsic defect in the development and persistence of virus-specific MPCs. This correlates with our findings that Runx2 expression levels in activated CD8+ T cells are enhanced by TLR and memory cytokine stimulation but inhibited by IFN-γ expression. Together, these data identify Runx2 as an important mediator of virus-specific memory T cells following resolution of infection by LCMV–Armstrong.

MATERIALS AND METHODS

Mice

Mice were bred and housed in specific pathogen–free conditions at the University of Massachusetts Medical School (UMMS) in accordance with Institutional Animal Care and Use Committee guidelines. C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in house. OT-I TCR transgenic mice were a gift from Dr. J. Kang (UMMS). For coadoptive transfers, splenocytes from P14 WT LCMV–Armstrong infection. Runx2, like the other RUNX factors, contains a Runt DNA binding domain and pairs with CBFβ to bind to DNA (32). Runx2 functions primarily in bone development in which it is required for osteoblast generation (33) and bone formation (34).

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Virus and infections

Adult male mice (7–11 wk) were infected with LCMV–Armstrong at 5 × 10^4 PFU i.p. For rechallenge, mice were infected with LCMV–clone 13 at 2 × 10^6 PFU i.v. LCMM–Armstrong and LCMV–clone 13 were graciously provided by Dr. R. Welsh (UMMS). For coadoptive transfers, splenocytes from P14 WT LCMV–clone 13 were transferred i.v. into CD90.2− host mice 1 d prior to infection.

Plaque assay

Spleens and fat pads were harvested 9 d after LCMV–Armstrong infection. For rechallenge, kidneys and livers were harvested 4 d after LCMV–clone 13 infection. Organs were homogenized in 1 mL RPMI 1640 medium and stored at −80°C. Plaque assays were performed as described previously (42).

Cell culture

Splenocytes from OT-I TCR transgenic mice were stimulated with OVA, T4, or G4 peptide with indicated doses for 72 h. Cells were harvested and analyzed for Runx2, Eomes, and CD44 expression by intracellular staining. For cytokine experiments, IFN-γ and IL-12 were purchased from R&D Systems (Minneapolis, MN). IL-7 and IL-15 were purchased from PeproTech (Rocky Hill, NJ). OVA, T4, and G4 peptides were purchased from 21st Century Biochemicals (Marlborough, MA). Imiquimod was purchased from InvivoGen (San Diego, CA). LPS was purchased from Sigma-Aldrich (St. Louis, MO). Splenocytes from Irf4−/− CD4–cre−, Irf4+/− CD4–cre−, and Irf4fl/fl CD4–cre− mice were isolated and plated with plate-bound anti-CD3/CD28 for 72 h. Cells were harvested and analyzed for Runx2, Eomes, and CD44 expression by intracellular staining. For cytokine production, splenocytes from infected mice were stimulated with gp3–41, gp276–286, and nucleoprotein (np)396–404 peptide for 4 h in the presence of 1 μg/ml GolgiStop and 1 μg/ml GolgiPlug and Abs to CD107a and CD107b, gp3–41, gp276–286, and np396–404 peptides were generously provided by Dr. R. Welsh (UMMS) and generated by K. Daniels.

Ab and H2-Db tetramer staining

CD127 (FITC), Bcl2 (PE), Bcl6 (PE), IRF4 (PE), CD8α (PE–eFluor 610), TNF-α (PerCP–eFluor 710), Eomes (PerCP–eFluor 710), CD122 (PerCP–eFluor 710), T-bet (PE–Cy7), CD44 (PE–Cy7 and Alex Fluor 700), KLRG1 (PE–Cy7 and eFluor 450), IFN-γ (eFluor 450), IL-2 (allophycocyanin), Vα2 (allophycocyanin), CD27 (allophycocyanin–eFluor 780), and CD90.2 (allophycocyanin–eFluor 780) Abs were purchased from eBioscience (San Diego, CA). CD107a (FITC), CD107b (FITC), CD62L (FITC), 7-AAD, annexin V (PE), and CD90.1 (V500) were purchased from BD Biosciences (Billerica, MA). Abs to granzyme B (PE), LIVE/DEAD Violet, LIVE/DEAD Aqua, and goat anti-rabbit (Alexa Fluor 647) were purchased from Life Technologies (Grand Island, NY). H2-D1 (gp3–41), H2-Db (gp276–286), and H2-Db (np396–404) monomers were obtained from the National Institutes of Health Tetramer Core Facility (Atlanta, GA). Runx2 and TCF-1 Abs were purchased from Cell Signaling Technology (Danvers, MA). Single-cell suspensions from spleens, bone marrow, lymph nodes, lung, and liver were prepared; RBCs were lysed; and Fc receptors were blocked using supernatant from 2.4G2 hybridomas. Lymphocytes were isolated from lung and liver using lympholyte-M (Cedarlane...
Results

To circumvent the neonatal lethality of a germline deficiency in Runx2, we generated mice that lacked Runx2 only in T cells. To this end, Runx2
def mice (40) were crossed to the CD4-cre+ transgenic line (8, 12) (hereafter referred to as Runx2+/mice). Runx2
def mice showed no apparent defect in thymic T cell development or in peripheral T cells compared with Runx2+/CD4-cre+ controls (hereafter called WT mice) (Supplemental Fig. 1A–C). Additionally, Runx2
def mice exhibited no abnormalities in their numbers or proportions of CD8+CD44hi and CD8+CD44lo T cells within the spleen (Supplemental Fig. 1D).

To assess the function of Runx2 in antiviral T cell responses, we infused WT and Runx2
def mice with 5 × 104 PFU LCMV–Armstrong i.p. and harvested spleens at day 9 (the peak of the CD8+ T cell response), day 14 (the attrition phase), and day 28 (the memory phase) postinfection (Fig. 1). Compared with WT mice, Runx2
def mice had no alterations in the numbers of H2-Db
gp33–41 tetramer–specific cells (GP33) at days 9 and 14 postinfection but did show reduced numbers of virus-specific cells at day 28 postinfection (Fig. 1A, IB). Similar results were observed for H2-Db
gp276–286–specific cells (GP276) and H2-Db
np396–404–specific cells (NP396), indicating the defect at day 28 postinfection in the CD8+ T cells was not an epitope-specific phenotype (Supplemental Fig. 1E).

These results suggested a potential defect in the virus-specific MPC population in LCMV–Armstrong-infected Runx2/def mice. To assess this possibility, we examined tetramer-positive CD8+ T cells for KLRG1 and CD127 expression at each time point postinfection. This analysis revealed a defect in the total number of GP33, GP276, and NP396 MPCs in Runx2/def mice compared with controls at all three time points tested; in contrast, no differences were found in the virus-specific TEC populations in this comparison (Fig. 1C–E, Supplemental Fig. 1F, 1G). Functional memory cells are able to produce IFN-γ, TNF-α, and IL-2, and this polyfunctionality is an indicator of a robust memory population (43). Comparisons of cytokine production by virus-specific WT and Runx2/def CD8+ T cells revealed a significant reduction in the total numbers of triple cytokine–producing cells when T cells lacked Runx2 expression (Fig. 1F, Supplemental Fig. 1K). These data indicated that the reduced numbers of tetramer-positive CD8+ T cells in LCMV–Armstrong-infected Runx2/def mice was due to a defect in the memory T cell population.

As mentioned above, Runx2/def mice showed no reduction in virus-specific TEC numbers compared with WT mice at days 9, 14, or 28 after LCMV infection. We also observed only modest defects in granzyne B expression and no defects in CD107a+B expression at day 9 postinfection in Runx2/def mice (Supplemental Fig. 1H, 1I). These results suggested there were no defects in antiviral effector cell functions when T cells lacked Runx2. Analysis of viral clearance in LCMV–Armstrong-infected WT versus Runx2/def mice confirmed this supposition (Supplemental Fig. 1J).

Based on these data, we considered whether virus-specific CD8+ T cell subsets might express different levels of Runx2. To test this, we infected WT C57BL/6 mice with LCMV–Armstrong and harvested spleens at day 9 postinfection. Runx2 protein levels were examined in CD8+ MPCs and TECs specific for three viral epitopes. Although Runx2 was upregulated in both subsets of virus-specific cells relative to the levels present in naive CD8+ T cells, we observed increased levels of Runx2 in the MPCs compared with the TECs for each of the epitopes tested (Fig. 1G). These results indicated that Runx2 is upregulated within all activated CD8+ T cells but is higher within the MPC population than in the TEC subset.

To further assess potential molecular differences between WT and Runx2/def antiviral CD8+ T cells, we examined the expression levels of several transcription factors that are important for TEC and MDC differentiation and function. This analysis revealed that at days 14 and 28 postinfection, Runx2/def CD8+ T cells expressed lower levels of Eomes and TCF-1 compared with WT T cells, consistent with the reduced number of MPCS in these mice. In contrast, expression of T-bet, a factor associated with CD8+ effector function, was not altered between WT and Runx2/def cells (Fig. 1H, Supplemental Fig. 1L, 1M), consistent with previous results indicating no defect in CD8+ effector function (Supplemental Fig. 1H, 1I).

We next considered whether the loss of MPCS in LCMV–Armstrong-infected Runx2/def mice were due to Runx2 promoting apoptosis in MPCS. However, examination of splenocytes from LCMV–Armstrong-infected WT or Runx2/def mice at day 10 postinfection failed to show a significant increase in apoptosis of virus-specific CD8+ T cells in the absence of Runx2 (Supplemental Fig. 2). Taken together, these results indicated that Runx2 is regulating an alternative aspect of memory CD8+ T cell persistence, such as homeostatic proliferation, rather than survival.

Loss of virus-specific CD8+ memory T cells is due to a CD8+ T cell–intrinsic deficiency in Runx2

To determine whether the loss of LCMV-specific Runx2-deficient CD8+ memory T cells was caused by an intrinsic loss of Runx2 in
Runx2 IS REQUIRED FOR ANTIVIRAL CD8+ MEMORY T CELLS

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FIGURE 1. Loss of Runx2 in the T cell compartment leads to a defect in the number of CD8+ memory precursor T cells during LCMV–Armstrong infection. WT and Runx2fl/fl mice were infected with LCMV–Armstrong and analyzed on days 9, 14, and 28 postinfection. (A) Gated CD8a+ splenocytes were stained for H2-D^b gp33–41 tetramer and CD44. (B) Compilation of data shows total number of H2-D^b gp33–41-specific splenocytes in WT versus Runx2fl/fl mice at days 9, 14, and 28 postinfection. (C) KLRG1 versus CD127 staining of WT and Runx2fl/fl CD8a+ CD44hi and H2-D^b gp33–41 tetramer+ splenocytes at days 9, 14, and 28 postinfection. (D and E) Total number of H2-D^b gp33–41–specific TECs (D) and MPCs (E) in WT versus Runx2fl/fl mice at days 9, 14, and 28 postinfection. (F) Cells were restimulated with gp33–41 peptide for 4 h in vitro. Total number of H2-D^b gp33–41–specific IFN-γ, TNF-α, and IL-2+ splenocytes (triple producers) in WT versus Runx2fl/fl CD8a+ and CD44hi cells at days 9, 14, and 28 after LCMV–Armstrong infection. (G) WT C57BL/6 mice were infected with LCMV–Armstrong and analyzed on day 9 postinfection for Runx2 expression levels in CD8a+, CD44hi H2-Db gp33–41, Gp276–286, and Np396–404–specific MPCs versus TECs. (H) Normalized mean fluorescence intensity (MFI) of Eomes, TCF-1, and T-bet in H2-D^b gp33–41 tetramer–specific WT versus Runx2fl/fl splenocytes were analyzed at days 9, 14, and 28 after LCMV–Armstrong infection by staining for Eomes, TCF-1, or T-bet. Data show normalized MFI for transcription factor staining in gated CD8a+ and CD44hi H2-D^b gp33–41 tetramer+ cells. Samples were normalized to average MFI value for each protein in the WT samples in each experiment. Data are from three independent experiments with a total of 9–12 mice per group per time point. Data from (F) are from two independent experiments with a total of 12 mice. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. ns, p > 0.05.
Runx2-dependent CD8+ memory T cell loss does not impair the recall response to LCMV

To determine whether the defect in numbers of Runx2<sup>fl/fl</sup> CD8<sup>+</sup> memory T cells would impact the recall response, we rechallenged LCMV–Armstrong-infected WT and Runx2<sup>fl/fl</sup> mice with LCMV–clone 13. Initially, WT and Runx2<sup>fl/fl</sup> mice were infected with LCMV–Armstrong, and 90 d postinfection, mice were rechallenged with a high dose of LCMV–clone 13. Prior to rechallenge, we examined the surviving populations of virus-specific CD8<sup>+</sup> T cells and found reduced numbers of tetramer-positive cells in Runx2<sup>fl/fl</sup> mice compared with controls; furthermore, Runx2<sup>fl/fl</sup> mice had a more substantial deficit in the CD62L<sup>hi</sup> than in the CD62L<sup>lo</sup> subset of each epitope-specific memory T cell population (Fig. 3A, Supplemental Fig. 3A–C). Four days after rechallenge, we observed substantial expansion of both the WT and Runx2<sup>fl/fl</sup> virus-specific memory cells. This robust recall response was observed for all three viral epitopes tested (Fig. 3B, Supplemental Fig. 3D). A careful examination of TECs and MPCs showed no significant differences in either population when comparing WT to Runx2<sup>fl/fl</sup> mice (Fig. 3C). We also found no differences in viral clearance between WT and Runx2<sup>fl/fl</sup> at day 4 post-rechallenge (Fig. 3D). Phenotypic and functional analysis of the cells after rechallenge showed few significant differences between virus-specific WT and Runx2<sup>fl/fl</sup> cells (Fig. 3E, Supplemental Fig. 3E–J). These results indicate that the loss of memory T cells seen in Runx2<sup>fl/fl</sup> mice does not impair their protective response to secondary challenge.

Based on these data, we considered whether the reduced numbers of virus-specific memory CD8<sup>+</sup> T cells seen in the spleens of Runx2<sup>fl/fl</sup> mice might be due to altered migration of these cells to other tissues rather than to an absolute reduction in the total memory cell population. This hypothesis was suggested by a previous study that showed loss of Runx2 in the plasmacytoid dendritic cell compartment led to a retention of the cells in the bone marrow (44). To test this, we examined several organs for pathogen-specific CD8<sup>+</sup> T cells 14 d after primary infection with LCMV–Armstrong. As shown, we found significantly fewer LCMV-specific memory CD8<sup>+</sup> T cells in nearly all organs examined and for each of the viral epitopes assessed (Fig. 3F, Supplemental Fig. 3K, 3L). These data indicate that the reduced...
numbers of splenic Runx2-deficient LCMV-specific memory CD8+ T cells is not due to enhanced homing or retention of these cells to or in other organs.

Runx2 expression is regulated by TLR4/TLR7 signals and cytokine signaling pathways in vitro

Previous work has shown a role for TLR signaling in CD8+ T cell memory formation (45, 46). We next wanted to determine if Runx2 expression in activated CD8+ T cells was regulated by TLR signaling in vitro. To determine the optimal day poststimulation to look at Runx2 expression, we isolated splenocytes from P14 TCR transgenic mice and looked for Runx2 expression up to 5 d after stimulation with GP33 peptide. We found that Runx2 was upregulated by day 3 poststimulation and remained elevated through day 5 (Supplemental Fig. 4A); however, cell death increased dramatically starting at day 4 after rechallenge (Supplemental Fig. 4B). We therefore chose 72 h poststimulation as the optimal time point for assessing Runx2 expression accompanied by minimal cell death in vitro. To test the role of TLR agonists on Runx2 expression, we isolated splenocytes from OT-I TCR transgenic RAG2−/− mice, stimulated OT-I T cells with high or low concentrations of the lower-affinity OVA peptide variant SIITFEKL (T4), and incubated with or without TLR agonists LPS (Fig. 4A) or imiquimod (Fig. 4B). We found that Runx2 was upregulated in cells treated with either TLR agonist at both high and low T4 peptide doses, but this upregulation was more dramatic when cells were stimulated with the low peptide dose.

Previous work from M. Mescher and colleagues (47, 48) has shown that type I IFN and IL-12 regulate memory CD8+ T cell development in several viral and intracellular bacterial infection systems. These findings prompted us to assess whether type I IFN or IL-12 was contributing to the high expression of Runx2 in CD8+ T cells stimulated in the presence of TLR agonists. We stimulated OT-I T cells with either a high or low concentration of

[FIGURE 3. Runx2 deficiency in T cells does not impair the recall response to LCMV.](https://doi.org/10.4049/immunohorizons.1800046)

WT and Runx2−/− mice were infected with LCMV–Armstrong and were rechallenged on day 90 postinfection with a high dose of LCMV–clone 13. (A) Splenocytes were analyzed on day 90 prior to secondary challenge for CD44hi CD62Lhi staining (gated H2-Db gp33–41 tetramer+ CD8+ cells) (left) as well as total numbers of CD62Lhi cells (center) and CD62Llo cells (right). (B) H2-Db gp33–41+ tetramer+ CD8+ CD44hi staining is shown on day 4 after rechallenge (left). Time course of virus-specific cells including day 4 after LCMV–clone 13 rechallenge is shown (right). (C) H2-Db gp33–41–specific CD8+ cells were stained for KLRG1 and CD127 at day 4 after LCMV–clone 13 rechallenge (left), and total cell numbers of H2-Db gp33–41–specific TECs (center) and MPCs (right) were quantified. (D) Plaque assay of LCMV titers in kidney and liver on day 4 after LCMV–clone 13 rechallenge. (E) Mean fluorescence intensity (MFI) of Eomes, T-bet, IRF4, granzyme B, CD107a+b, and total number of triple producers on day 4 after LCMV–clone 13 rechallenge. Intracellular cytokine staining was performed after a 4-h gp33–41 peptide stimulation in vitro. (F) Spleen, lymph node, lung, liver, and bone marrow lymphocytes were isolated on day 14 after primary infection with LCMV–Armstrong. Frequencies of H2-Db gp33–41–specific MPCs in tissues from infected WT versus Runx2−/− mice are shown. Data from day 90 postinfection are from three independent experiments with a total of 12 mice per group. Data from LCMV–clone 13 rechallenge are from two independent experiments with a total of five mice per group. Data in (F) are from three independent experiments with a total of 9–10 mice per group. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
T4 peptide and incubated cells with or without IFN-β (Fig. 4C) or IL-12 (Fig. 4D). We found treatment with IFN-β had no significant effect on Runx2 expression, whereas treatment with IL-12 led to increased Runx2 expression at the lower peptide dose. These results suggest a direct effect of IL-12 on T cells. Furthermore, the fact that IFN-β had no direct effect on T cells suggests that the enhanced Runx2 upregulation observed when stimulation cultures were treated with TLR agonists might be acting via upregulation of costimulatory molecules on splenic APCs.

We also tested additional cytokines known to be important in homeostatic proliferation and survival of memory cells for their effects on Runx2 expression. Splenocytes from OT-I TCR transgenic mice were isolated and stimulated with high or low doses of the T4 peptide. Cultures were supplemented with or without IL-7 (Fig. 4E) or IL-15 (Fig. 4F) and analyzed at day 3 poststimulation. These studies demonstrated that both IL-7 and IL-15 promoted increased expression of Runx2 when T cells were activated with weak TCR stimulation.

Together, these data show that TLR signaling, most likely in APCs, and stimulation of T cells with cytokines known to be important in CD8+ T cell memory development and homeostasis enhance Runx2 upregulation; interestingly, these cytokines and TLR agonist signals have the greatest impact in T cells activated with weaker strength of TCR signaling.

Runx2 expression is regulated by TCR signaling pathways in activated CD8+ T cells in vitro

From our TLR and cytokine data, we found that OT-I cells stimulated with low concentrations of T4 peptide expressed higher levels of Runx2 than cells stimulated with a higher concentration of
peptides (Fig. 4). These data suggested an important role for TCR signal strength in controlling Runx2 expression levels. Previous studies have shown an important inverse correlation between TCR signal strength and the development of CD8+ MPCs postinfection (3, 49). To assess more comprehensively a role for TCR signal strength in regulating Runx2 expression, we performed experiments using extensive dose ranges of three peptides recognized by the OT-I TCR. For these studies, OT-I cells were stimulated with APCs plus various concentrations of the highly potent SIINFEKL (OVA) peptide, the medium potency (T4) peptide, or the low potency SIIGFEKL (G4) peptide. These experiments clearly revealed an inverse correlation between peptide dose and Runx2 expression (Fig. 5A). Similar results were also observed for Eomes expression (Fig. 5B). As expected, CD44 upregulation showed a positive correlation with peptide dose (Fig. 5C).

These findings suggested that the transcription factor IRF4 might regulate Runx2 expression. This possibility was suggested by our previous studies showing that IRF4 is upregulated to different levels based on TCR signal strength and, further, that IRF4 is a negative regulator of Eomes expression (8, 12). To test this possibility, we stimulated WT, Irf4<sup>+/fl</sup> or Irf4<sup>fl/fl</sup> mice and plated with αCD3/28 plus IL-2 supernatants. Cells were harvested 72 h after stimulation and stained for Runx2 (D) or Eomes (E). Filled histograms show staining in naive WT OT-I cells. Graphs at right show compilations of data normalized to WT unstimulated control. Data are representative of three to five experiments. ***p ≤ 0.001, ****p ≤ 0.0001.

DISCUSSION

A key aspect of the adaptive immune response is the generation of long-term memory cells that provide rapid and robust protection upon secondary exposure to an infecting pathogen. Improving memory CD8<sup>+</sup> T cell responses is currently a focus of many vaccine efforts, whereas inhibition of memory T cell responses is an ongoing challenge in the development of therapies to treat autoimmune diseases. Thus, there is a need to understand in more detail the molecular mechanisms regulating memory T cell development and persistence.

Our studies identified Runx2 as an important factor in the maintenance of long-term memory CD8<sup>+</sup> T cells. However, we found that Runx2 was not necessary for antiviral effector function or for robust recall responses to secondary challenge. These features of the CD8<sup>+</sup> T cell response to infection in Runx2<sup>fl/fl</sup> mice show a striking resemblance to previous studies examining the consequences of a deficiency in IL-15 or IL-15Ra (50–52). In these earlier studies, IL-15 signaling was found to be essential for long-term memory CD8<sup>+</sup> T cell homeostasis and self-renewal. We observed that IL-15 stimulation of CD8<sup>+</sup> T cells in vitro promoted enhanced Runx2 upregulation only under conditions of weak TCR stimulation. These findings suggest the possibility that in vivo tonic TCR signals plus IL-15 are functioning to maintain memory cells in part through the upregulation of Runx2. Consistent with this...
possibility, IL-15 is thought to promote memory T cell homeostatic proliferation rather than memory cell survival (53–57), a function that aligns well with data demonstrating that Runx2 overexpression in thymocytes promotes uncontrolled cellular proliferation leading to lymphomagenesis (58).

We also found that Runx2 expression levels are enhanced by weak rather than strong TCR signal intensity, likely because of negative regulation by the transcription factor IRF4. This pattern mirrors that of Eomes, another transcription factor associated with long-term memory CD8⁺ T cell maintenance (27). Along with weak TCR signaling, cytokines that promote memory T cell formation and signals that activate APCs also promote enhanced Runx2 upregulation. These data further strengthen an association of Runx2 expression with optimal formation of long-lived memory CD8⁺ T cells (5, 49).

Determining the precise mechanism underlying the loss of memory CD8⁺ T cells in the absence of Runx2 has remained. Early on, studies using microarrays were performed to identify differentially expressed genes between WT and Runx2⁻/⁻ CD8⁺ T cells. Cells isolated from LCMV–Armstrong-infected mice and analyzed directly ex vivo failed to yield informative gene targets of Runx2 regulation; similar uninformative results were obtained when comparing WT and Runx2⁻/⁻ CD8⁺ T cells stimulated in vitro (data not shown). This prompted us to consider alternative mechanisms for Runx2 function, such as a change in rRNA transcription and processing, based on the known role for Runx2 in regulating this pathway in osteoblasts (59); these studies also failed to identify a function for Runx2 in CD8⁺ T cells (data not shown). One additional possibility is that Runx2 is affecting memory formation through regulation of chromatin accessibility, a known function of Runx3 in CD8⁺ T cells (31). RUNX family members have been shown to have redundant roles in CD4⁺ regulatory T cells; thus, the most robust alterations are observed in the absence of Cbfβ, the binding partner for all three Runx proteins (60). Future studies examining CD8⁺ T cell memory using Cbfβ-deficient T cells may be informative in this regard. Alternatively, Runx2 may function intermittently in memory CD8⁺ T cells during short time windows when memory cells associate with stromal cells in lymphoid organs and receive homeostatic TCR and IL-15/IL-7 signals. Capturing the cells during these brief interactions may be required to identify the specific genes and/or pathways regulated by Runx2 in memory CD8⁺ T cells.

DISCLOSURES

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Supplemental Figure 1: T cell development and steady-state T cell homeostasis are normal in Runx2^β/β^ mice, but CD8^+^ MPCs are reduced in Runx2^0/β^ mice after LCMV-Armstrong infection. (A) Thymocytes were isolated from WT or Runx2^0/β^ and stained with antibodies to CD4 and CD8. Dot-plots show CD4 versus CD8 staining. (B) Percentages of DP thymocytes (left), CD4SP thymocytes (center), and CD8SP thymocytes (right) in WT and Runx2^0/β^ mice are shown. (C) Splenocytes from WT and
Runx2<sup>β/β</sup> mice were stained analyzed for CD8 versus live/dead staining (left); numbers of live CD8<sup>+</sup> cells are shown at right. (D) WT and Runx2<sup>β/β</sup> splenocytes were stained for CD8 versus CD44 (left) and absolute numbers of CD8<sup>+</sup> CD44<sup>lo</sup> (center) and CD8<sup>+</sup> CD44<sup>hi</sup> (left) cells are shown. (E) Total numbers of splenic H2-D<sup>b</sup> GP<sub>276-286</sub> tetramer-specific cells (left) or NP<sub>396-494</sub> tetramer-specific cells (right) at various timepoints post LCMV Armstrong infection. Cells are gated on CD8α<sup>+</sup> CD44<sup>hi</sup> cells. (F) Total numbers of H2-D<sup>b</sup> GP<sub>276-286</sub> specific TECs (left) and MPCs (right) in WT versus Runx2<sup>β/β</sup> mice. Cells are gated on CD8α<sup>+</sup> CD44<sup>hi</sup> H2-D<sup>b</sup> GP<sub>276-286</sub> tetramer specific cells. (G) Total numbers of H2-D<sup>b</sup> NP<sub>396-404</sub> specific TECs (left) and MPCs (right) in WT versus Runx2<sup>β/β</sup> mice. Cells are gated on CD8α<sup>+</sup> CD44<sup>hi</sup> H2-D<sup>b</sup> NP<sub>396-404</sub> tetramer specific cells. (H) MFI of Granzyme B in WT versus Runx2<sup>β/β</sup> cells day 9 post infection. Cells were restimulated <i>in vitro</i> for 4h with GP<sub>33-41</sub> peptide, GP<sub>276-286</sub> peptide, or NP<sub>396-404</sub> peptide and stained for intracellular Granzyme B. (I) MFI of CD107a<sup>+</sup>b in WT versus Runx2<sup>β/β</sup> cells day 9 post infection. Cells were restimulated <i>in vitro</i> for 4h with GP<sub>33-41</sub> peptide, GP<sub>276-286</sub> peptide, or NP<sub>396-404</sub> peptide and stained for CD107a<sup>+</sup>b. (J) PFU/ml LCMV virus in spleen or fat pad of WT versus Runx2<sup>β/β</sup> at day 9 post infection. Positive control shows LCMV-Armstrong stock solution (K) Total numbers of H2-D<sup>b</sup> GP<sub>287-286</sub> specific (left) or H2-D<sup>b</sup> NP<sub>396-404</sub> specific (right) IFNγ<sup>+</sup>, TNFα<sup>+</sup>, IL-2<sup>+</sup> cells (triple producers) in WT versus Runx2<sup>β/β</sup> cells at days 9, 14, and 28 post infection. Cells were restimulated with GP<sub>276-286</sub> or NP<sub>396-404</sub> peptide for 4 hours <i>in vitro</i> prior to intracellular cytokine staining. Samples were gated on CD44<sup>hi</sup> CD8α<sup>+</sup> cells. (L) Normalized MFI of Eomes (left), Tcf-1 (center), and T-bet (right) in H2-D<sup>b</sup> GP<sub>276-286</sub> tetramer-specific WT versus Runx2<sup>β/β</sup>. Cells were gated on CD8α<sup>+</sup> CD44<sup>hi</sup> H2-D<sup>b</sup> GP<sub>276-286</sub> tetramer-specific cells. Samples were
normalized to average MFI values of WT samples in each experiment. (M) Normalized MFI of Eomes (left), Tcf-1 (center), and T-bet (right) in H2-D\textsuperscript{b} NP\textsubscript{396-404} tetramer-specific WT versus \textit{Runx2}\textsuperscript{fl/fl}. Cells were gated on CD8\textsuperscript{α+} CD44\textsuperscript{hi} H2-D\textsuperscript{b} NP\textsubscript{396-404} tetramer-specific cells. Samples were normalized to average MFI values of WT samples in each experiment. Data are from 3 independent experiments with a total of 9-12 mice per group per timepoint.
Supplemental Figure 2: Loss of Runx2 does not increase apoptosis of virus-specific TEC or MPCs following LCMV Armstrong infection. WT and Runx2\textsuperscript{fl/fl} mice were infected with LCMV-Armstrong and splenocytes were isolated 10 days post-infection. (A) Cells were analyzed for Annexin-V and 7-AAD staining as shown. (B) Percentage of AnnexinV\textsuperscript{+} 7-AAD\textsuperscript{−} TECs and MPCs among each virus-specific population, including H2-D\textsuperscript{b} GP\textsubscript{33-41}, H2-D\textsuperscript{b} GP\textsubscript{276-286}, and H2-D\textsuperscript{b} NP\textsubscript{396-404} tetramer specific cells in WT and Runx2\textsuperscript{fl/fl} mice. Samples are gated on CD\textsubscript{8α}\textsuperscript{+}, CD44\textsuperscript{hi}, tetramer\textsuperscript{+} cells. Data are from 2 independent experiments with a total of 8 mice per group.
Supplemental Figure 3: Loss of Runx2 does not impair the recall response to LCMV rechallenge. WT and Runx2<sup>fl/fl</sup> mice were infected with LCMV-Armstrong, and then 90 days later challenged with a high dose of LCMV-clone 13. (A) Dot-plot shows CD62<sup>hi</sup> and CD62<sup>lo</sup> cells from an uninfected control to indicate gating strategy. (B) Total numbers of CD62<sup>hi</sup> (left) and CD62<sup>lo</sup> (right) H2-D<sup>b</sup> GP<sub>276-286</sub> specific cells at day 90 post-primary infection. (C) Total numbers of CD62<sup>hi</sup> (left) and CD62<sup>lo</sup> (right) H2-D<sup>b</sup> NP<sub>396-404</sub> specific cells day 90 post-primary infection. (D) Total numbers of GP<sub>276-286</sub> (left) and NP<sub>396-404</sub> (right) specific cells 4 days after LCMV Clone 13 rechallenge. (E) MFI of Eomes (left), T-bet (center), and IRF4 (right) in GP<sub>276-286</sub> specific cells 4 days post rechallenge. (F) MFI of Granzyme B (left) and CD107a+b (right) in GP<sub>276-286</sub> specific cells 4 days post rechallenge. (G) Total numbers of GP<sub>276-286</sub> specific triple producer cells 4 days post rechallenge. (H) MFI of Eomes (left), T-bet (center), and IRF4
(right) in NP\textsubscript{396-404} specific cells 4 days post rechallenge. (I) MFI of Granzyme B (left) and CD107a+b (right) in NP\textsubscript{396-404} specific cells 4 days post rechallenge. (J) Total numbers of NP\textsubscript{396-404} specific triple producer cells 4 days post rechallenge. (K-L) Percentages of GP\textsubscript{276-286} (K) and NP\textsubscript{396-404} (L) specific MPCs in the spleen, LN, lung, liver, and bone marrow 14 days post-primary LCMV-Armstrong infection. Data from day 90 post-infection are from 3 independent experiments with a total of 12 mice per group. Data from LCMV-clone 13 rechallenge are from 2 independent experiments with a total of 5 mice per group. Data in (K-L) are from 3 independent experiments with a total of 9-10 mice per group.
Supplemental Figure 4: Timecourse of Runx2 upregulation after *in vitro* stimulation.

(A) Runx2 expression and (B) cell death in P14 splenocytes stimulated with GP<sub>33-41</sub> peptide (1uM) and IL-2 supernatants. Each day, cells were analyzed for Runx2 and cell death by flow cytometry. Graphs show MFI of Runx2 staining (A) and percentages of dead cells (B). Data are representative of 3 experiments.