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Tec Kinases I tk and Rlk Are Required for CD8<sup>+</sup> T Cell Responses to Virus Infection Independent of Their Role in CD4<sup>+</sup> T Cell Help<sup>1</sup>

Luana O. Atherly, Michael A. Brehm, Raymond M. Welsh, and Leslie J. Berg<sup>2</sup>

I tk and Rlk are members of the Tec kinase family of nonreceptor protein tyrosine kinases that are expressed in T cells, NK cells, and mast cells. These proteins are involved in the regulation of signaling processes downstream of the TCR in CD4<sup>+</sup> T cells, particularly in the phosphorylation of phospholipase C-γ1 after TCR activation; furthermore, both I tk and Rlk are important in CD4<sup>+</sup> T cell development, differentiation, function, and homeostasis. However, few studies have addressed the roles of these kinases in CD8<sup>+</sup> T cell signaling and function. Using I tk<sup>−/−</sup> and I tk<sup>−/−</sup>/Rlk<sup>−/−</sup> mice, we examined the roles of these Tec family kinases in CD8<sup>+</sup> T cells, both in vitro and in vivo. These studies demonstrate that the loss of I tk and Rlk impairs TCR-dependent signaling, causing defects in phospholipase C-γ1, p38, and ERK activation as well as defects in calcium flux and cytokine production in vitro and expansion and effector cytokine production by CD8<sup>+</sup> T cells in response to viral infection. These defects cannot be rescued by providing virus-specific CD4<sup>+</sup> T cell help, thereby substantiating the important role of Tec kinases in CD8<sup>+</sup> T cell signaling. The Journal of Immunology, 2006, 176: 1571–1581.

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Abbreviations used in this paper: PLCγ1, phospholipase C-γ1; FasL, Fas ligand; LCMV, lymphocytic choriomeningitis virus; np, nuclear protein.
aspect of the T cell response that was defective could not be determined.

In contrast to the studies described above, only a single study to date has examined CD8+ T cell responses in Itk−/− mice (11), and none has examined CD8+ T cell function in Itk−/− Rlk−/− mice. In addition, no studies have yet directly addressed the potential biochemical defects in purified CD8+ T cells lacking Itk or Itk and Rlk. In the one study that did examine the function of Itk−/−Rlk−/− T cells, mice were infected with three different viruses, lymphocytic choriomeningitis virus (LCMV), vaccinia virus, and vesicular stomatitis virus (11). This study showed that Itk−/− mice were mildly impaired in their ability to generate functional CTL responses to LCMV infection and, although able to clear a vaccinia virus infection, did so with delayed kinetics. Although this study provides the only evidence to date that CD8+ T cell function is also affected by the loss of Itk, no evidence was provided to address the mechanism(s) that might have contributed to the impaired CD8+ T cell responses seen in Itk−/− mice. Furthermore, these data did not address the role of Itk in other aspects of the CD8+ T cell-mediated antiviral response, such as CD8+ T cell expansion and attrition or the ability to generate an efficient and protective recall response, responses in which the efficacy of the TCR-derived signal is critically important. Finally, as mentioned above, the additional role of Rlk and the potential differential effect of loss of both Itk and Rlk on CD8+ T cell-mediated antiviral immune responses have never been explored.

To address these issues, we first examined both the biochemical and functional responses of Itk−/− and Itk−/−Rlk−/− CD8+ T cells in vitro. We then took advantage of the well-characterized model of LCMV infection, in which the Ag-specific immune response can be followed at the single-cell level. These studies demonstrate that Itk and Rlk are critically important to CD8+ T cell expansion and effector cytokine production. We also show that the impaired expansion of virus-specific CD8+ T cells lacking Itk or Itk and Rlk cannot be rescued by providing LCMV-specific CD4+ T cell help, thereby substantiating the important role of Tec kinases in CD8+ T cell signaling.

Materials and Methods

Mice

Itk−/− mice (5) have been backcrossed more than nine generations to the C57BL/10 strain. Itk−/− Rlk−/− mice (7) were a gift from P. Schwartzberg (National Human Genome Research Institute, National Institutes of Health, Bethesda, MD) and were backcrossed more than eight generations to C57BL/10 mice. C57BL/10 mice were used as controls. OT-1 TCR (H-2d) transgenic mice (12) were provided by K. Rock (University of Massachusetts Medical School, Boston, MA) and were crossed to Itk−/− mice. All mice used were between 6 and 12 wk of age and were maintained at the University of Massachusetts Medical School specific-pathogen-free animal facility after review and approval by the institutional animal care and use committee.

Abs and flow cytometric analysis

Anti-CD8-FITC, anti-CD4-PE, anti-CD44-Cy, anti-TNF-α-allophycocyanin, anti-IFN-γ-PE, anti-BrDU-FITC, and anti-CD3-biotin Abs were all purchased from BD Pharmingen. Immobilin-P membrane was purchased from Millipore. Abs to phospho-PLCγ1-1,4 phospho-ERK, phospho-p38, and PI3K p85 protein were all purchased from Cell Signaling Technologies. The anti-Fas ligand (anti-FasL) Ab, MFL3, was purchased from eBiosciences. BrDU was purchased from Sigma-Aldrich. CFSE was purchased from Molecular Probes. The recombinant vaccinia-OVA virus was obtained from K. Rock (University of Massachusetts Medical School). For flow cytometric analysis, cells were analyzed on a FACSCalibur cytometer (BD Biosciences), and data were analyzed using FlowJo software (Treestar).

CD8+ T cell isolation and expansion

For in vitro biochemical and functional experiments, cells were prepared from spleens and lymph nodes of wild-type, Itk−/−, and Itk−/−Rlk−/− mice. After RBC lysis and washing, the cells were incubated with anti-CD8-conjugated magnetic beads and purified on an AutoMacs machine. Cells were then analyzed directly as primary ex vivo CD8+ T cells or were stimulated with PMA (2.5 ng/ml) and ionomycin (375 ng/ml) for 36–48 h and then expanded in medium containing 20% IL-2 supernatant to generate cultured CD8+ T cells. After this in vitro expansion, cultured CD8+ T cells from wild-type, Itk−/−, and Itk−/−Rlk−/− mice had comparable levels of expression of CD44 and CD62L (data not shown).

Calcium mobilization analysis

Wild-type, Itk−/−, or Itk−/−Rlk−/− cultured CD8+ T cells (1 × 10⁵) were loaded with the calcium-sensitive dyes fluo-3 and fura-red for 1 h at 37°C. Cells (1 × 10⁶) were removed to serve as unstimulated controls. The remaining cells were incubated with 25 µg of biotinylated anti-CD3 Ab for 45 s, then cross-linked with 40 µg of streptavidin for 5 min. As a positive control, the cells were stimulated with ionomycin at 10 µg/ml. Calcium mobilization was determined by assessing the ratios of fluo-3 vs fura-red fluorescence over time using Facs Assistant software (BD Biosciences).

PLCγ and MAPK phosphorylation assays

To assess activation of MAPK signaling pathways, 5 × 10⁴ wild-type, Itk−/−, and Itk−/−Rlk−/− cultured CD8+ T cells were incubated with 25 µg/ml biotinylated anti-CD3 Ab, followed by cross-linking with 1 mg/ml streptavidin for 0, 2, 5, and 10 min. As a positive control, cells were stimulated with PMA (2.5 ng/ml) and ionomycin (375 ng/ml). The reactions were terminated by addition of 1 ml of ice-cold stop solution (1× PBS containing 20 mM sodium fluoride and 1 mM Na3VO4). Cell pellets were then lysed for 15 min on ice using 50 µl of protein lysis buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X, 1 mM PMSF, 1 mM Na3VO4, and 10 µg/ml leupeptin. Total cell lysate was resolved on a 10% SDS-PAGE gel, transferred to an Immobilon-P membrane, blocked, and then blottedted with Abs to phosphorylated-PLCγ1, p42/44 MAPK (ERK1/2), and phospho-p38. Membranes were probed for the P38κ p85 subunit as a protein loading control.

LCMV and vaccinia-OVA infections

To generate acutely infected mice, wild-type, Itk−/−, and Itk−/−Rlk−/− mice were infected i.p. with 100 µl of LCMV Armstrong at 2–5 × 10⁵ PFU/ml. Spleens were harvested on various days after infection (days 2–11 for acute infection and day 63 for analysis of the memory response), and single-cell suspensions were generated. Vaccinia-OVA infection mice were infected with 200 µl of vaccinia-OVA at 5 × 10⁶ PFU/ml, and spleens were harvested on days 3–8 after infection. RBC were lysed by incubation in buffered ammonium chloride for 2–5 min. Cells were then washed and resuspended for additional analysis.

Peptide stimulations and intracellular cytokine staining

For intracellular cytokine staining, 2–4 × 10⁶ total splenocytes from wild-type, Itk−/−, and Itk−/−Rlk−/− mice were infected i.p. with 100 µl of LCMV Armstrong at 2–5 × 10⁵ PFU/ml for 5 h at 37°C in medium containing brefeldin A and monensin. The LCMV-specific peptides used were gp33-41, nuclear protein 396–404 (np396-404), gp276–286, and np205–212 to stimulate CD8+ T cells, and gp86-80 and np309-328 to stimulate CD4+ T cells (13). OT-1 T cells were stimulated with the OVA SIINFEKL peptide. After incubation, cells were stained for surface expression of CD4 and CD8, permeabilized, then stained for IFN-γ, TNF-α, or IL-2 using the Cytotox/Cytoperm Kit (BD Pharmingen).

Plaque assay

Viral supernatants were generated from infected spleens harvested at various times after LCMV infection. The resulting tissue suspension was spun at 1500 rpm for 15 min, and supernatants were removed and frozen in 500 µl aliquots. An aliquot of viral supernatant was thawed and then serially diluted. Briefly, 100 µl of each serial dilution was added to one well of a six-well plate containing an ~70% confluent monolayer of Vero cells. The plates were incubated at 37°C for 90 min, then each well was overlaid with a 1:1 mixture of agarose and EMEM complete (1 part 0.8% Seakem agarose:1 part EMEM complete-2× EMEM, 6 ml of FCS, 5 ml of penicillin-streptomycin/glutamine). Four days later, the plaques were visualized by overlaying the initial agarose layer with 2 ml of agarose mix containing 1% neutral red. Titers were calculated as (number of plaques × dilution factor)×volume plated) × total volume of spleen supernatant.

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**CTL assay**

RMA target cells were incubated with 10 μM gp33 or np396 peptide and 400 μCi of 51Cr for 1 h at 37°C. After extensive washing, 1 × 10⁶ targets were incubated with splenocytes from days 7, 8, 9, and 11 LCMV-infected mice at various E:T cell ratios for 5 h at 37°C. Where indicated, EGTA was added at 2.5 mM, and MgCl₂ was added to a final concentration of 4 mM, respectively. The two populations of labeled cells were then counted, representative of three independent experiments. In all experiments, spontaneous release was calculated as (percent loss of the peptide-labeled population relative to the control population as determined by flow cytometric analysis.

**BrdU labeling**

LCMV-infected mice were given injections i.p. of 100 μl of BrdU (15 mg/ml in PBS) 12 h before harvest. Splenocytes (4 × 10⁶) from these mice were then stained for surface Ags CD4, CD8, and CD44. The cells were then fixed in Cytofix/Cytoperm (BD Pharmingen) for 20 min at 4°C, washed, and then fixed again with a freshly made solution of 1% formaldehyde containing 1% Tween 20. Cells were then washed twice in PBS at room temperature, treated with DNase, and stained with the anti-BrdU Ab.

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**FIGURE 1.** Loss of Itk and Rlk impairs in vitro CD8⁺ T cell signaling and function. A, Cultured CD8⁺ T cells from wild-type (WT), Itk⁻/⁻, and Itk⁻/⁻ Rlk⁻/⁻ mice were stimulated by anti-CD3 Ab cross-linking for the indicated times or with PMA and ionomycin for 15 min. Whole-cell lysates were then assessed for phosphorylation of PLCγ1, ERK1/2, and p38 MAPK by immunoblotting. Filters were stripped and reprobed with an Ab to PI3K p85 as a loading control. Data are representative of five independent experiments. B, Cultured CD8⁺ T cells before TCR cross-linking. B, Cultured CD8⁺ T cells from WT, Itk⁻/⁻, and Itk⁻/⁻ Rlk⁻/⁻ mice were loaded with fluo-3 and fura-red, then stimulated by anti-CD3 Ab cross-linking. Calcium mobilization is indicated as the ratio of fluo-3 to fura-red fluorescence over time. Data are representative of five independent experiments. CD3bn, Biotin-conjugated anti-CD3 Ab; SA, streptavidin; Iono, ionomycin. C, Primary CD8⁺ T cells from WT, Itk⁻/⁻, and Itk⁻/⁻ Rlk⁻/⁻ mice were stimulated with various concentrations of plate-bound anti-CD3 Ab. Cells were then permeabilized and stained with Abs to IFN-γ and TNF-α. Graphs show the percentage of CD8⁺ CD44high cells in each population producing detectable levels of cytokine. Error bars represent duplicate wells. Data are representative of at least three independent experiments. D, Cultured CD8⁺ T cells from WT, Itk⁻/⁻, and Itk⁻/⁻ Rlk⁻/⁻ mice were labeled with 1 μM CFSE, then stimulated in the presence or the absence of 10 μg/ml plate-bound anti-CD3 Ab for 48 h. Proliferation was assessed by measuring CFSE fluorescence on the CD8⁺ CD44high T cells in each population. Numbers on the graph represent the percentage of dividing CD8⁺ T cells. Data are representative of two independent experiments.
Adoptive transfer of LCMV-immune CD4+ T cells

C57BL/6 CD45.1+ congenic mice were infected with LCMV and rested for 2 mo. CD4+ T cells were isolated from these mice using anti-CD4 Ab-coated magnetic beads and the Auto-Macs. Donor CD45.1+ CD4+ T cells (1 × 10⁷) were injected into wild-type, Itk−/−, and Itk−/−Rlk−/− CD45.2+ host mice. Host mice were then infected with LCMV. Uninfected recipient mice receiving CD4+ T cells and mice infected with LCMV but not receiving CD4+ T cells were used as controls. Mice were harvested 8 days after infection, and the magnitude of the wild-type, Itk−/−, or Itk−/−Rlk−/− host CD8+ T cell response to LCMV was determined.

Adoptive transfer of OT-1+ CD8+ T cells

CD8+ T cells were isolated from pooled single-cell suspensions of OT-1 TCR transgenic wild-type and Itk−/− spleens and lymph nodes. The cells were then labeled with CFSE, and 5 × 10⁶ cells were injected i.v. into C57BL/6 CD45.1+ congenic hosts. Twenty-four hours later, the host mice were infected with 1 × 10⁶ PFU of vaccinia-OVA. Responding cells were identified by staining for Vα2, Vβ5, CD45.2, and CD8. The magnitude of the Ag-specific response was determined by assessing IFN-γ production in response to stimulation with the OVA SIINFEKL peptide.

Statistical analyses

One-tailed Student’s t test was performed using InStat software (GraphPad). Statistical significance is conferred by a value of p < 0.05.

Results

Itk−/− and Itk−/−Rlk−/− CD8+ T cells have impaired responses to TCR stimulation

As a first step in addressing the roles of Itk and Rlk in CD8+ T cell function, we examined several biochemical responses of CD8+ T cells from Itk−/− and Itk−/−Rlk−/− mice to in vitro stimulation. Because Itk−/− and Itk−/−Rlk−/− CD8+ T cells have an activated/memory phenotype (CD44hi, CD62Lhi, Ly6Chi, and CD122hi, data not shown), we first stimulated the isolated Itk−/−, Itk−/−Rlk−/−, and wild-type CD8+ T cells in vitro with PMA and ionomycin, then cultured them in medium containing IL-2 for 5–7 days before analysis. The rationale for this was to create more equivalent populations of T cells for the biochemical studies. Although this protocol yielded CD8+ T cell populations with comparable levels of CD44 expression from wild-type, Itk−/−, and Itk−/−Rlk−/− mice (data not shown), we cannot exclude the possibility that signaling pathways may be altered in these cells compared with those in freshly isolated ex vivo CD8+ T cells. These cells were then assessed for PLCγ1 tyrosine phosphorylation, ERK and p38 MAPK activation, and calcium mobilization after TCR cross-linking. Cultured CD8+ T cells lacking Itk or Itk and Rlk showed impaired phosphorylation of PLCγ1 and the MAPKs, p38 and ERK1/2, in response to TCR stimulation (Fig. 1A). Consistent with these defects, CD8+ T cells lacking Itk, and both Itk and Rlk also failed to generate a sustained calcium response after TCR cross-linking with anti-CD3 Ab (Fig. 1B).

To determine whether these signaling deficiencies translated into functional defects, we assessed the ability of CD8+ T cells from Itk−/− or Itk−/−Rlk−/− mice to proliferate and to produce effector cytokines in response to TCR stimulation. Fig. 1C shows that primary CD8+ T cells from mice lacking Itk or Itk and Rlk were virtually unable to produce IFN-γ or TNF-α in response to TCR cross-linking. Analysis of the proliferative ability of cultured wild-type, Itk−/−, and Itk−/−Rlk−/− CD8+ T cells by CFSE labeling showed that CD8+CD44hi T cells from Itk−/− and Itk−/−Rlk−/− mice were impaired in their ability to proliferate compared with CD8+CD44hi T cells from wild-type mice (Fig. 1D). This deficiency also held true for freshly isolated Itk−/− and
Itk−/−Rlk−/− CD8+ T cells, as assessed by [3H]thymidine incorporation (data not shown). These data demonstrate that Itk and Rlk are critically important for CD8+ T cell signaling and function in vitro.

Normal viral clearance but impaired accumulation of CD8+ T cells in Itk−/− and Itk−/−Rlk−/− mice after LCMV infection

To assess the importance of Itk and Rlk in CD8+ T cell function in vivo, we used the well-characterized system of LCMV infection, because this response can be followed over time and at the single-cell level. After infection with LCMV (Armstrong strain), CD8+ T cells expand to generate a pool of virus-specific effector cells that rapidly cleared the infection (15–17). The peak of this CD8+ T cell expansion was followed by an equally characteristic and reproducible decline in effector CD8+ T cell number and subsequent generation of LCMV-specific memory CD8+ T cells.

The clearance of LCMV is mediated largely by the perforin-mediated cytolytic mechanisms of virus-specific CD8+ T cells (14, 18, 19). The activation of these cytolytic mechanisms is reportedly influenced by the generation of a sustained calcium flux downstream of TCR signaling in CD8+ T cells (14, 20). Because loss of Itk and Rlk has a substantial impact on the generation of a calcium signal in CD8+ T cells in vitro, we examined whether loss of these proteins would affect the ability of CD8+ T cells from these mice to mediate viral clearance. Wild-type, Itk−/−, and Itk−/−Rlk−/− mice were infected i.p. with 4 × 10^6 PFU of LCMV Armstrong. Viral replication and clearance were assessed by plaque assays of supernatants collected from the spleens of infected mice on various days after infection. These data indicated that the spleens of wild-type, Itk−/−, and Itk−/−Rlk−/− mice were completely clear of LCMV by day 9 after infection (Fig. 2A and data not shown).

Because the clearance of LCMV is largely perforin dependent (19), the fact that wild-type, Itk−/−, and Itk−/−Rlk−/− mice were each able to clear the virus suggested that CD8+ T cells in these mice are capable of cytolytic activity. As shown in Fig. 2B, splenocytes from day 8 infected Itk−/− and Itk−/−Rlk−/− mice exhibited CTL activity against gp33-coated target cells. This killing was dependent on calcium influx, because this was nearly completely blocked by addition of EGTA; furthermore, CTL activity by Itk−/− and Itk−/−Rlk−/− T cells was not mediated by Fas/FasL interactions, because the addition of a blocking anti-FasL Ab had no effect on target cell lysis (data not shown). To confirm these findings, we examined the ability of infected Itk−/− and Itk−/−Rlk−/− mice to clear Ag-specific target cells in vivo. As shown in Fig. 2C, wild-type, Itk−/−, and Itk−/−Rlk−/− mice were each capable of lysing gp33-coated target cells on day 8 after infection. These results suggest that despite the impairment of calcium response seen in in vitro analyses, Itk−/− and Itk−/−Rlk−/− CD8+ T cells are capable of generating effector cytolytic T cells that can lyse viral peptide-coated target cells and clear an LCMV infection.

In contrast to their efficiency in clearing the virus, Itk−/− and Itk−/−Rlk−/− mice failed to show the dramatic increase in CD8+ T cell numbers observed in wild-type mice after LCMV infection (Fig. 3). For instance, the wild-type CD8+ T cell response peaked on day 8 after infection, with 28.7% of the spleen being CD8+ T cells; in contrast, at this time point, CD8+ T cells made up ~18% of the spleen in Itk−/− mice and 22% of the spleen in Itk−/−Rlk−/− mice.

**FIGURE 3.** Impaired CD8+ T cell accumulation in Itk−/− and Itk−/−Rlk−/− mice. A, Wild-type (WT), Itk−/−, and Itk−/−Rlk−/− mice were infected i.p. with 4 × 10^6 PFU of LCMV Armstrong. The kinetics of the CD8+ T cell response was followed by assessing the percentage and total numbers of CD8+ T cells in the spleens of infected mice over the course of the infection. Dot plots show CD4 vs CD8 staining of splenocytes from mice on days 0, 3, 8, 9, and 11 after infection. CD8+ T cell numbers are indicated beneath each dot plot. Data are representative of five independent experiments. B, Graphic representation of the CD8+ T cell response. Results shown are the arithmetic mean ± SEM at each time point of CD8+ T cell numbers per spleen after infection. Data are calculated from groups of eight to 10 mice/time point for days 0–9 and four to six mice for day 11 and are representative of five independent experiments. Differences in CD8+ T cell numbers on day 8 (+) are statistically significant (p < 0.05).
experiments. Despite their ability to lyse LCMV-specific targets and clear the virus, Itk<sup>−/−</sup> and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> CD8<sup>+</sup> T cells are present in greatly reduced numbers compared with wild-type CD8<sup>+</sup> T cells at the peak of infection.

**Ag-specific response to LCMV is impaired in Itk<sup>−/−</sup> and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> mice**

LCMV infection in C57BL/6 mice results in the expansion of CD8<sup>+</sup> T cells specific for the immunodominant epitopes gp33 and np396, in addition to the subdominant epitopes gp276 and np205. LCMV infection of Itk<sup>−/−</sup> and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> mice generated fewer Ag-specific IFN-γ<sup>+</sup> CD8<sup>+</sup> T cells than in wild-type mice (Fig. 4). Specifically, 13.6% of the splenic CD8<sup>+</sup> T cells in wild-type mice responded to np396 stimulation by producing IFN-γ, whereas only ~10 and ~7% of the CD8<sup>+</sup> T cells in Itk<sup>−/−</sup> and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> mice, respectively, responded to np396 (Fig. 4A). Quantitation of the response to the two LCMV immunodominant peptides, np396 and gp33, clearly illustrated the defective CD8<sup>+</sup> T cell response in Itk<sup>−/−</sup> and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> mice (Fig. 4B). A similar trend was seen for the response to the two subdominant peptides, np205 and gp276 (data not shown). In addition to the reduced numbers of LCMV-specific CD8<sup>+</sup> T cells in Itk<sup>−/−</sup> and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> mice, CD8<sup>+</sup> T cells from these mice produced less IFN-γ on a per cell basis, as assessed by the median fluorescent intensity of IFN-γ-staining of IFN-γ-positive cells (Fig. 4A). Together, these data indicate the importance of Tec kinases Itk and Rlk in the generation of an optimal antiviral immune response.

**FIGURE 4.** Loss of Itk and Rlk impairs the Ag-specific CD8<sup>+</sup> T cell response. A. Wild-type (WT), Itk<sup>−/−</sup>, and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> mice were infected i.p. with 4 × 10<sup>6</sup> PFU of LCMV Armstrong. On the indicated days after infection, splenocytes were isolated and incubated with the LCMV peptide np396 for 5 h at 37°C in medium containing protein secretion inhibitors. Cells were then stained for surface expression of CD8, permeabilized, and stained for IFN-γ. Dot plots show CD8 vs IFN-γ staining. In each dot plot, the top number represents the percentage of CD8<sup>+</sup> T cells positive for IFN-γ, and the bottom number indicates the mean fluorescence intensity of IFN-γ staining of the IFN-γ-positive population of cells. B. Graphs show the results of a time course of the responses to np396 and gp33 after LCMV infection. Data points indicate absolute numbers of CD8<sup>+</sup> T cells producing IFN-γ at each time point, as assessed by intracellular cytokine staining after in vitro stimulation with each peptide. The differences between WT and Itk<sup>−/−</sup> as well as between the WT and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> np396 responses on day 8 (+) are statistically significant (p < 0.05). None of the values for the gp33 response was statistically significantly different. Groups of two mice per time point were used. The data shown are representative of nine independent experiments.

**Ik- and Rlk-dependent signals modulate CD8<sup>+</sup> T cell proliferation in response to LCMV infection**

The massive expansion of CD8<sup>+</sup> T cells that occurs during LCMV infection is caused by extensive proliferation of Ag-specific cells during the acute response, a component of which is a programmed response to the initial T cell stimulation (23–25). As shown above, the spleens and CD8<sup>+</sup> T cell compartments of Itk<sup>−/−</sup> and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> mice did not expand to the size seen in wild-type mice in response to LCMV infection. The impaired accumulation of CD8<sup>+</sup> T cells in Itk<sup>−/−</sup> and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> mice could be caused by impaired proliferation or, alternatively, impaired survival of LCMV-specific CD8<sup>+</sup> T cells in these mice. To address this issue, we analyzed CD8<sup>+</sup> T cell turnover in vivo by assessing BrdU incorporation during a time course of LCMV infection. As shown in Fig. 5, early in the response (day 5 after infection), a smaller fraction of Itk<sup>−/−</sup> and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> CD8<sup>+</sup>CD44<sup>high</sup> T cells incorporated BrdU compared with wild-type CD8<sup>+</sup>CD44<sup>high</sup> T cells. Quantitation of five independent experiments demonstrated that there was a statistically significant difference in BrdU incorporation by wild-type, Itk<sup>−/−</sup>, and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> CD8<sup>+</sup> T cells just before the peak of the response (Fig. 5B). This may reflect a reduced rate of proliferation by Itk<sup>−/−</sup> and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> CD8<sup>+</sup> T cells at this stage of the response, or, instead, may be the result of a lower precursor frequency of LCMV-specific CD8<sup>+</sup> T cells in mutant mice.

Itk<sup>−/−</sup> and Itk<sup>−/−</sup>Rlk<sup>−/−</sup> mice generate an LCMV-specific CD8<sup>+</sup> T cell memory pool with an altered cytokine profile

In the weeks following the clearance of an acute virus infection, a memory T cell population develops. Previous data have shown that...
the generation of an efficient memory response depends in part on the magnitude of the acute response (26). To determine whether a deficiency in Itk or in both Itk and Rlk would affect the establishment of effective long-term memory, we examined the CD8\(^+\) T cell population in Itk\(^{-/-}\) and Itk\(^{-/-}\)Rlk\(^{-/-}\) mice 2 mo after the primary infection with LCMV. All three lines of mice analyzed, wild-type, Itk\(^{-/-}\), and Itk\(^{-/-}\)Rlk\(^{-/-}\), had cleared the virus by day 9 after infection; thus, the T cell populations present on day 63 after infection represent a true memory pool. As shown in Fig. 6, wild-type, Itk\(^{-/-}\), and Itk\(^{-/-}\)Rlk\(^{-/-}\) LCMV-immune mice had a similar percentage of gp33-specific IFN-\(\gamma\) memory CD8\(^+\) T cells (3.88 \(\pm\) 0.02% for wild-type mice; 4.44 \(\pm\) 0.06 and 4.64 \(\pm\) 0.08% for Itk\(^{-/-}\) and Itk\(^{-/-}\)Rlk\(^{-/-}\) LCMV-immune mice, respectively). These percentages also corresponded to similar absolute numbers of gp33-specific memory CD8\(^+\) T cells in wild-type, Itk\(^{-/-}\), and Itk\(^{-/-}\)Rlk\(^{-/-}\) LCMV-immune mice. Although the data shown in Fig. 6 assess CD8\(^+\) T cell responses to gp33, similar results were obtained upon analysis of np396-specific CD8\(^+\) T cell responses (data not shown). Thus, unlike the primary response, there was no defect in the maintenance of a memory CD8\(^+\) T cell pool in Itk\(^{-/-}\) and Itk\(^{-/-}\)Rlk\(^{-/-}\) LCMV-immune mice.

Interestingly, analysis of the cytokine profiles produced by LCMV-specific CD8\(^+\) T cells revealed a qualitative defect in the memory response generated in Itk\(^{-/-}\) and Itk\(^{-/-}\)Rlk\(^{-/-}\) mice. Previous studies have demonstrated that memory CD8\(^+\) T cells in LCMV-immune mice are predominantly double producers of IFN-\(\gamma\) and TNF-\(\alpha\) (27). In contrast, Itk\(^{-/-}\) and Itk\(^{-/-}\)Rlk\(^{-/-}\) LCMV-immune mice generated a 50:50 distribution of CD8\(^+\) T cells that produce both IFN-\(\gamma\) and TNF-\(\alpha\) (double producers) and cells that produce only IFN-\(\gamma\) (single producers; Fig. 6). This was observed for responses to gp33 (Fig. 6) as well as np396 (data not shown). The reduced proportion of TNF-\(\alpha\)-producing memory CD8\(^+\) T cells in Itk\(^{-/-}\) and Itk\(^{-/-}\)Rlk\(^{-/-}\) mice may be a consequence of the impaired ability of Itk\(^{-/-}\) and Itk\(^{-/-}\)Rlk\(^{-/-}\) CD8\(^+\) T cells to produce TNF-\(\alpha\) in the primary response to LCMV infection (Fig. 6). These data indicate that although Itk\(^{-/-}\) and Itk\(^{-/-}\)Rlk\(^{-/-}\) mice generate a comparable proportion of LCMV-specific memory CD8\(^+\) T cells as wild-type mice, many of these cells are impaired in their production of an effector cytokine, TNF-\(\alpha\).

**Impaired CD4\(^+\) T cell function in Itk\(^{-/-}\) and Itk\(^{-/-}\)Rlk\(^{-/-}\) is not responsible for the defect in the CD8\(^+\) T cell response to LCMV**

The data presented above indicate that CD8\(^+\) T cells in mice lacking Itk or Itk and Rlk are impaired in their ability to respond to LCMV infection. This defect is particularly evident for the primary response to the virus; Because CD4\(^+\) T cell responses in Itk\(^{-/-}\) and Itk\(^{-/-}\)Rlk\(^{-/-}\) mice are also known to be defective, we considered the possibility that an impaired antiviral CD4\(^+\) T cell response in these mice might contribute to the defective CD8\(^+\) T cell response to LCMV. This concern is based on previous data demonstrating that CD4\(^+\) T cells in Itk\(^{-/-}\) and Itk\(^{-/-}\)Rlk\(^{-/-}\) mice are deficient in IL-2 production (5, 7) and on our own findings that reduced numbers of CD4\(^+\) T cells in the spleens of Itk\(^{-/-}\) and Itk\(^{-/-}\)Rlk\(^{-/-}\) mice produce IL-2 in response to LCMV infection (data not shown). To address this concern, we isolated CD4\(^+\) T cells from mice 2 mo after the primary infection with LCMV. All three lines of mice analyzed, wild-type, Itk\(^{-/-}\), and Itk\(^{-/-}\)Rlk\(^{-/-}\) mice were infected with LCMV-Armstrong, then rest for at least 2 mo to generate LCMV-immune mice. Splenocytes were isolated from mice 63 days after infection (D63). Naive mice (D0) and naive mice infected with LCMV and harvested 8 days after primary infection (D8 1\(^{st}\)) are shown for comparison. Splenocytes were stimulated in vitro with the gp33 and np396 (not shown) peptides, and the Ag-specific response was analyzed by intracellular cytokine staining for IFN-\(\gamma\) and TNF-\(\alpha\).
cells from wild-type congenic (CD45.1+) mice that had been infected with LCMV 2 mo previously. This population of cells, containing LCMV-specific memory CD4+ T cells, was adoptively transferred into wild-type, Itk−/−, and Itk−/−Rlk−/− mice. Mice were then infected with LCMV, and host CD8+ T cell responses were analyzed 8 days after infection. Uninfected mice receiving donor memory CD4+ T cells and mice infected with LCMV, but not receiving donor memory CD4+ T cells, were used as controls.

Transfer of wild-type, LCMV-specific memory CD4+ T cells did not reverse the defect in CD8+ T cell accumulation observed in Itk−/− and Itk−/−Rlk−/− mice after LCMV infection (Fig. 7A). This defect remained despite the substantial expansion of the transferred memory CD4+ T cell population able to produce IL-2 in response to stimulation (Fig. 7B). These data demonstrate that the defect in CD8+ T cell accumulation observed in Itk−/− and Itk−/−Rlk−/− mice is not the result of defects in the CD4+ T cell response in these mice and may, instead, be due to intrinsic defects in the CD8+ T cell population in these Tec kinase-deficient mice.

**Impaired accumulation of TCR transgenic Itk−/− and Itk−/−Rlk−/− CD8+ T cells in response to infection**

These findings suggest that the impaired accumulation of Ag-specific CD8+ T cells in Itk−/− and Itk−/−Rlk−/− mice may be due to intrinsic defects in CD8+ T cell function in the absence of Itk and Rlk. However, given the unique phenotype of CD8+ T cells in Itk−/− and Itk−/−Rlk−/− mice, an alternative explanation is that these T cells express an altered TCR repertoire compared with wild-type CD8+ T cells, resulting in a lower precursor frequency of LCMV-specific cells. In addition, we were concerned that the environment in virus-infected Itk−/− and Itk−/−Rlk−/− mice may be substantially different from that in wild-type mice undergoing a similar immune response due to impaired effector cytokine production by both CD4+ and CD8+ T cell populations in these mice. To address these concerns, we used a second, independent experimental system to address the functional capabilities of Itk−/− CD8+ T cells in response to viral infection.

For these experiments, we crossed Itk−/− mice to the OT-1 TCR transgenic line, thus creating a homogeneous population of CD8+ T cells specific for the OVA peptide, SIINFEKL, bound to Kb (12). In these OT-1+1 Itk−/− mice, ~12% of the splenic T cells were CD8+ and expressed the OT-1 TCR; importantly, >85% of these Itk−/−OT-1+1 CD8+ T cells had a naive phenotype and had wild-type levels of expression of CD44, CD25, and CD62L. In addition, comparable to wild-type CD8+OT-1+ T cells, ~95% of the CD8+Itk−/−OT-1+ T cells were Vα2/Vβ5+ (data not shown).

CD8+ T cells purified from Itk+/+OT-1+ and Itk−/−OT-1+ TCR transgenic mice were adoptively transferred into C57Bl/6 (CD45.1+) congenic mice, which were then infected with a recombinant strain of vaccinia virus expressing the chicken OVA protein (28). As shown in Fig. 8, Itk−/−OT-1+ CD8+ T cells were impaired in their Ag-specific response to vaccinia-OVA infection. As measured both by quantifying the numbers of cells staining for the congenic marker CD45.2+ as well as by production of IFN-γ in response to in vitro stimulation with the SIINFEKL peptide (Fig. 8), a greatly reduced number of Itk−/−CD8+OT-1+ cells accumulated at the peak of the response compared with wild-type CD8+OT-1+ T cells. These data demonstrate that even in a wild-type environment and with a similar precursor frequency of responding cells, Itk−/−CD8+ T cells are impaired in their ability to mount an efficient Ag-immune response to viral infection.
The Tec kinases Itk and Rlk are known to be important in signaling downstream of the TCR in CD4+ T cells and in CD4+ T cell responses to various pathogens. In this study we demonstrate a role for Itk and Rlk in CD8+ T cell signaling and function both in vitro and in vivo. Stimulation of CD8+ T cells lacking Itk or Itk and Rlk resulted in impaired phosphorylation and activation of PLCγ1, ERK, and p38 MAPK and loss of a sustained calcium response. Surprisingly, our data also showed that resting wild-type CD8+ T cells that had previously been activated with PMA and ionomycin and then cultured in IL-2 have high basal levels of PLCγ1 tyrosine phosphorylation. Although serum starvation for 5 h attenuated the basal levels of PLCγ1 phosphorylation of Itk−/− and Itk−/−Rlk−/− CD8+ T cells, the same did not occur for wild-type CD8+ T cells, perhaps due to the presence of IL-2 in the culture medium. Nonetheless, the impaired phosphorylation of PLCγ1 in Itk−/− and Itk−/−Rlk−/− CD8+ T cells is still apparent in these experiments.

The reduced biochemical signaling downstream of the TCR in Itk−/− and Itk−/−Rlk−/− CD8+ T cells correlates with dramatically impaired proliferation and effector cytokine production after in vitro stimulation of these cells. These data are consistent with previous studies of CD4+ T cells lacking Itk or Itk and Rlk, in which loss of these proteins resulted in defects in the activation of transcription factors such as NFAT and NF-κB and in the diminished production of effector cytokines such as IL-2, IL-4, and IFN-γ (4–8).

Despite the substantial defects in CD8+ T cell responses observed in vitro, we found that antiviral immune responses proceed fairly efficiently in the absence of Itk and Rlk. This obvious disparity between the in vitro capacities of Itk−/− and Itk−/−Rlk−/− CD8+ T cells and their mild impairments in vivo suggest that these T cell defects can be compensated for by the substantial innate immune response accompanying an LCMV infection. One potential aspect of this compensation may be the cytokines produced in response to LCMV, such as IFN-α/β, IL-12, and IFN-γ (29). These cytokines may enable Itk−/− and Itk−/−Rlk−/− CD8+ T cells to overcome their intrinsic defects in TCR signaling, resulting in an improved calcium response in this in vivo setting.

After viral infection of Itk−/− and Itk−/−Rlk−/− mice, we observed a decline in the frequency of Ag-specific cells capable of producing the effector cytokine IFN-γ and a nearly complete loss of CD8+ T cells capable of producing TNF-α (Fig. 6). Surprisingly however, loss of Itk and both Itk and Rlk did not affect the cytolytic capabilities of CD8+ T cells. We hypothesize that the TCR-dependent signal needed for the release of perforin and other cytotoxic granules is lower than that necessary for slower effector functions, such as cytokine production and T cell proliferation. This hypothesis is supported by data showing that the release of perforin and other granules requires a biphasic increase in intracellular calcium levels and a sustained influx of extracellular calcium, whereas effector functions requiring de novo protein synthesis are more dependent, instead, on the sustained influx of extracellular calcium (20, 30), a process that is defective in CD8+ T cells lacking Itk and both Itk and Rlk (5). Furthermore, it has been shown that the generation of T cell cytotoxicity did not require the formation of a stable and mature immunological synapse and thus may be independent of a sustained and strong TCR signal (31).

We also found impaired accumulation of Itk−/− and Itk−/−Rlk−/− CD8+ T cells in response to LCMV infection and of OT-1 Itk−/−CD8+ T cells in response to vaccinia-OLA infection. Thus, in the latter system, even with identical precursor frequencies and TCR specificities of responding cells, CD8+ T cells lacking Itk are greatly impaired in their ability to accumulate and produce effector cytokines after viral infection. This diminished accumulation of CD8+ T cells was not the result of a poor CD4+ T cell response to LCMV in Itk−/− and Itk−/−Rlk−/− mice, because adoptive transfer of wild-type LCMV-specific memory CD4+ T cells did not restore the CD8+ T cell response to LCMV in Tec kinase-deficient mice, a conclusion that is also supported by the adoptive transfer data generated with Itk−/−OT-1 CD8+ T cells responding to vaccinia-OLA in an otherwise wild-type mouse. Together, these findings indicate that the loss of Itk or Itk and Rlk results in an intrinsic defect in CD8+ T cell function. These findings are striking, particularly those observed in the LCMV system, because this viral infection is known to induce a very robust CD8+ T cell response. Thus, we anticipate that even more substantial defects in CD8+ T cell function would be observed in systems in which the inflammatory response and/or Ag burden is less pronounced.

Under normal conditions, CD8+ T cells are extremely responsive to antigenic stimulation. Numerous studies demonstrate that CD8+ T cells undergo a massive and autonomous program of proliferation in response to activation signals (16, 17). However, to date, the role of intrinsic vs extrinsic factors in the rate of CD8+ T cell expansion is a matter of substantial debate. Although many mathematical models have been generated to predict the effect of factors extrinsic to the CD8+ T cell (32, 33), an influx of recent data has demonstrated that CD8+ T cells can undergo an autonomous program of expansion and differentiation (23–25), leading to a revision of these earlier models (34). Although the influence of extrinsic factors, such as cytokines and viral load, has been the subject of much investigation, factors that affect the intrinsic program of CD8+ T cell expansion remain largely uncharacterized. Our data indicate that signaling molecules, such as Itk and Rlk, may have a role in establishing the magnitude of that intrinsic program.
Surprisingly, although it has been reported that the magnitude of the primary immune response affects the size of the memory CD8+ T cell pool (26), the impaired accumulation of Ag-specific CD8+ T cells seen during the primary immune response to LCMV in Itk−/− and Itk−/−Rlk−/− mice did not affect the magnitude of the memory response in these mice. It is interesting to note that the LCMV-specific memory response in Itk−/− and Itk−/−Rlk−/− mice was not impaired by the well-documented CD4+ T cell defects in these mice (see Ref. 35 for review), because several recent studies have indicated an important role for CD4+ T cells in the generation and/or maintenance of CD8+ T cell memory (see Ref. 36 for review). Loss of Itk and Rlk did, however, alter the effector cytokine profiles produced by memory CD8+ T cells. Although wild-type memory LCMV-specific CD8+ T cells secrete both IFN-γ and TNF-α (double producers) in response to peptide restimulation (27), Itk−/− and Itk−/−Rlk−/− memory CD8+ T cell pools are comprised of cells producing only IFN-γ as well as cells producing both cytokines.

In the case of TNF-α production, Itk and Rlk play a particularly critical role, because virtually no Itk−/− or Itk−/−Rlk−/− CD8+ T cells are able to produce TNF-α during the primary immune response. TNF-α is a very important effector cytokine, the production of which is regulated both transcriptionally and post-transcriptionally (37). The transcription of TNF-α in activated T cells depends on the generation of a sustained calcium response and the subsequent activation of NFATp (37, 38). There are also putative NF-κB, AP-1, and early growth response gene-1 binding sites in the TNF-α promoter region (39). Given the importance of Itk and Rlk in the signaling pathways leading to efficient activation of NFAT, NF-κB, AP-1, and early growth response gene transcription factors (6, 8, 9, 40), it is likely that these kinases are involved in regulating the transcription of TNF-α. TNF-α is also regulated post-transcriptionally; this pathway is mediated by the activation of p38 MAPK and leads to increased stability of TNF-α mRNA (41). Because p38 MAPK activation is impaired in the absence of Itk and Rlk, a defect in the post-transcriptional regulation of TNF-α may contribute to the overall reduction in TNF-α protein production by Itk−/− and Itk−/−Rlk−/− CD8+ T cells. Nonetheless, despite these defects, a subset of LCMV-specific Itk−/− and Itk−/−Rlk−/− CD8+ T cells acquires the capacity to produce TNF-α in the memory phase of the immune response.

These data demonstrate the importance of signaling molecules, such as Itk and Rlk, in the adaptive immune response. Based on our findings, we conclude that optimal TCR signaling is necessary to generate a robust CD8+ T cell-mediated antiviral response that encompasses maximum T cell expansion with production of the full panoply of effector cytokines. Interestingly, the requirement for maximal strength TCR signaling was independent of TCR specificity and/or affinity for viral peptide/MHC complexes, because we observed similar responses with a polyclonal TCR repertoire as with a single fixed TCR specificity. Overall, these studies indicate that modulating the strength of TCR signaling by inhibiting Itk and Rlk would greatly reduce the magnitude and efficacy of the CD8+ T cell response to viral infections.

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Disclosures
The authors have no financial conflict of interest.

References


CORRECTIONS


Figure 8 is incorrect. The corrected figure is shown below.


The second author’s middle initial was omitted. The correct name is Robert L. Ferris.


The eighth author’s last name was misspelled. The correct name is Leda Q. Vieira.

The ninth author’s last name was misspelled. The correct name is Hideaki Nakajima.


In Figure 2A, the three left hand dot plot panels from Ly9+/+ cells were mistakenly duplicated in the three right hand dot plot panels of Ly9-/- cells. The numbers in each of the quadrants are correct and the error does not change any interpretation in the article. The corrected figure is shown below.


One of the first author’s affiliations was omitted. The corrected list of authors and affiliations is shown below.


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In Figure 1B, the WT Ca flux data line is missing from the Ca flux graph. The corrected figure is shown below.

In Discussion, the last reference in the paper is incorrect. The corrected sentence and reference are shown below.

> It is known that the cytoplasmic domains of several components of the TCR complex tend to homo-oligomerize at high concentrations (41); perhaps ligand-induced clustering of the TCR drives the cytoplasmic domains of proximal receptors to rearrange, exposing the Nck binding epitope and propelling other signaling cascade processes.


In Discussion, in the second sentence of paragraph six, 10S-HDNA should have been 10S-HDHA. The corrected sentence is shown below.

> Recently, classic steric analysis of 10S-HDHA and the formation of 10,20-diHDHA and 17-H(p)DHA were reportedly optimized for the plant LOs (49).


The title of the article is incorrect. The corrected title is shown below. The error has been corrected in the online version, which now differs from the print version as originally published.

Thymocyte Negative Selection Is Mediated by Protein Kinase C- and Ca$^{2+}$-Dependent Transcriptional Induction of Bim