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Micro-RNA 155 Is Required for Optimal CD8+ T Cell Responses to Acute Viral and Intracellular Bacterial Challenges

Evan F. Lind,* Alisha R. Elford,* and Pamela S. Ohashi*†,‡

Recent studies have begun to define the role of micro-RNAs in regulating the immune response. Micro-RNA155 (mir-155) has been shown to play a role in germinal center formation, T cell inflammation, and regulatory T cell development. In this study, we evaluated the role of mir-155 in cytotoxic T cell function. We report in this study that mice lacking mir-155 have impaired CD8+ T cell responses to infections with lymphocytic choriomeningitis virus and the intracellular bacteria Listeria monocytogenes. We show by a series of adoptive transfer studies that the impaired CD8+ T cell response to L. monocytogenes is T cell intrinsic. In addition, we observed that CD8+ T cells lacking mir-155 have impaired activation of the prosurvival Akt pathway after TCR cross-linking. These data suggest that mir-155 may be a good target for therapies aimed at modulating immune responses.

M icro-RNAs (miRNA) are short (22–24 nt) RNA species encoded within the mammalian genome that do not code for proteins but rather regulate translation of mRNA. Complementary target mRNA binding is followed by suppression of translation or mRNA degradation. Exact complementation is not required for recognition of the miRNA, and therefore each miRNA has the potential to bind many target mRNAs, allowing any single miRNA to regulate dozens or hundreds of target genes (1).

Mir-155 was identified as the functional sequence in the noncoding BIC gene, which had been identified as the integration site for the avian leukosis virus (2). This miRNA is associated with cellular activation, proliferation, and survival. Transgenic expression of mir-155 under the control of the Eμ promoter in mice results in B cell leukemia (3), implying that this miRNA has the ability to dysregulate cellular pathways involved in survival and proliferation. In addition, overexpression of mir-155 in stem cells results in myeloid hyperplasia (4). Studies have since identified deregulation of mir-155 as a feature in lymphomas (5), breast cancer (6, 7), and liver cancers (8), as well as in autoimmune disorders such as rheumatoid arthritis (9).

Studies of mir-155 in the immune system have revealed a striking role in the proper function of B cells, macrophages, and CD4+ T cells (10). Mice lacking mir-155 have a multitude of immune defects, including impaired germinal center formation (11, 12), CD4+ T cell function, regulatory T (Treg) cell development, and homeostatic expansion (13–15). The defects observed in the CD4+ T cell population include a Th2-skewing bias (11, 12, 16) as well as an inability to produce functional Th17 cells leading to resistance to the induction of experimental autoimmune encephalomyelitis (17, 18) and experimental rheumatoid arthritis (9, 19).

Despite the amount of research aimed at identifying a role for mir-155 in CD4+ T cells, little is known about the function of mir-155 in CD8+ T cells. Modulating mir-155 levels in vitro has shown that mir-155 may regulate the balance between CD8+ T cell effector and central memory cell phenotypes (20). The experiments described in this work are aimed at measuring the potential role of mir-155 in CD8+ T cell responses to the following two types of pathogens in vivo: an intracellular bacteria Listeria monocytogenes and lymphocytic choriomeningitis virus.

Materials and Methods

Mice

C57BL/6 (CD45.2) mice were purchased from Taconic. B6.SJL-Ptpa Pep3b/BoyJ (CD45.1) mice were from The Jackson Laboratory. Mir-155–deficient (155KO) mice (B6.Cg-Mirn155−/−J) were from The Jackson Laboratory and bred as homozygous knockouts in the animal facility at Ontario Cancer Institute. P14t5−/− mice were created in our facility by crossing P14 (21) mice to 155KO mice. Mice used in experiments were 6–10 wk old. Experiments were conducted under approved animal protocols in the animal facility at Ontario Cancer Institute.

Flow cytometry

All Abs were purchased from eBiosciences. The MHC/peptide tetramers were obtained from the Baylor University tetramer core facility. For intracellular cytokine detection, 2 × 106 splenocytes were cultured in IMDM containing 10% FCS plus 10−4 M gp33 peptide and GolgiPlug (BD Biosciences) for 5 h at 37°C. Cells were then stained with Abs to CD8 and congenic markers, fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences), and stained with Abs to IFN-γ. Irrelevant adenoviral peptide control was included in all experiments. Granzyme B was detected by first staining splenocytes with Abs to CD8, followed by fixation and permeabilization using Cytofix/Cytoperm (BD Biosciences). Cell suspensions were stained with anti-human granzyme B PE (BD Biosciences) with appropriate isotype control. All measurements were made using a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star). For phosphorylation analysis, CD8 T cells were purified using CD8-negative selection bead kit (Miltenyi Biotec). Phospho-Akt was measured after activating T cells with plate-bound αCD3 cross-linked by anti-
hamster Abs for 30 min in IMDM with 10% FBS. Cells were fixed and permeabilized using Phosphoflow perm wash kit buffer III (BD Biosciences). Cells were stained for 1 h at room temperature with anti-pAkt serine 473 conjugated to Alexa647 (Cell Signaling Technologies). STAT activation was measured after 30-min activation with dilutions of either IL-2 (pSTAT5) or IFN-α A (PBL IFN Source) (pSTAT1).

LCMV and L. monocytogenes infections

For LCMV experiments, mice were injected i.v. with 2000 PFU of the Armstrong strain of LCMV. Eight days later, mice were euthanized, and tetrramer and cytokine levels were analyzed. For L. monocytogenes experiments, mice were injected i.v. with 2000 CPU L. monocytogenes expressing LCMV gp33 epitope (22). Tetrramer and cytokine levels were analyzed 7 d later. CTL assays were performed, as previously described (23).

Adoptive transfer experiments

For experiments transferring P14 or P14155−/− cells, CD8+ T cells were purified from spleens and lymph nodes of mice using a CD8+ T cell magnetic bead isolation kit (Miltenyi Biotec). Two thousand cells were infused into recipients i.v., followed by injection with 5 × 10^3 M CFSE (Invitrogen). For CFSE experiments, 1 × 10^5 cells per recipient were injected. Expansion was measured by CFSE dilution 72 h postinfection with L. monocytogenes.

Results

Mice lacking mir-155 have impaired primary CD8+ T cell responses to LCMV infection

To test the potential requirements for mir-155 in CD8+ T cell responses to pathogens in vivo, we infected wild-type (WT) and mir-155KO (155KO) mice with LCMV. The first parameter we measured was the response to a series of viral Ags by CD8+ T cells at day 8 postinfection. We stained splenocytes with tetramers to the LCMV MHC class I epitopes corresponding to NP396, gp33, and gp276. We observed a reduction in the percentage of total CD8+ tetramer+ cells for each epitope when comparing the 155KO splenocytes with WT T cell frequencies (Fig. 1A, 1B, 1D, 1E, 1G, 1I). The total number of CD8+ tetramer+ cells from the spleen for every tetramer measured was drastically reduced (Fig. 1C, 1F, 1H). To test the function of the CD8+ T cells in response to LCMV, we measured the production of cytokines in an Ag-specific recall response to the gp33 epitope. We found that postinfection, CD8+ T cells had the ability to produce IFN-γ (Fig. 1J), but the total number of cells producing IFN-γ in response to gp33 was clearly reduced in the 155KO spleens (Fig. 1K). Reduced functional capacity of the 155KO mice to respond to LCMV was also observed when we assessed the ability of splenocytes to lyse targets pulsed with either NP396, gp33, or gp276 in a CTL assay (Fig. 1L). It is important to note that there was no difference observed in the resting number or activation status of CD8+ T cells in the spleen of naive WT versus 155KO mice (12) (Supplemental Fig. 1). These data clearly show that anti-LCMV CD8+ T cell responses are defective in mice lacking mir-155.

Reduced CD8+ T cell responses are observed in mir-155KO mice in response to L. monocytogenes infection

We challenged WT and 155KO mice with L. monocytogenes to test whether the defect we observed with LCMV is also evident in immune responses against other pathogens. L. monocytogenes is
an intracellular bacterial pathogen that elicits a strong CD8\(^+\) T cell response postinfection in mice. We chose to use a strain of \textit{L. monocytogenes} that had been modified to contain the LCMV gp33 epitope for ease in measuring Ag-specific responses using gp33 tetramers. We measured the response to \textit{L. monocytogenes} \({7 \text{ d postinfection in 155KO and WT mice}}\). We observed that the spleens from 155KO mice were smaller and contained fewer total CD8\(^+\) T cells than their WT counterparts after \textit{L. monocytogenes} infection (Fig. 2A). When we measured the Ag-specific response to \textit{L. monocytogenes}, we found that the total number of CD8\(^+\) T cells specific for the gp33 epitope was \({\sim 4\text{-fold lower in the 155KO than in the WT mice}}\) (Fig. 2B, 2C). To test the functional status of the CD8\(^+\) T cells in 155KO mice postinfection with \textit{L. monocytogenes}, we measured Ag-induced production of IFN-\(\gamma\), a hallmark of CD8\(^+\) T cell activation (24). We observed that the percentage of cells producing IFN-\(\gamma\) in response to gp33 peptide was reduced in the 155KO mice (Fig. 2D). When the total number of CD8\(^+\) T cells producing IFN-\(\gamma\) in response to \textit{L. monocytogenes} was calculated, we observed a clear reduction in the 155KO mouse compared with the WT (Fig. 2E). Similarly, we detected lower percentages and total numbers of CD8\(^+\) T cells producing granzyme B from the spleens of 155KO mice compared with WT (Fig. 2F, 2G). The reduction in Ag-specific CD8\(^+\) T cell response was also observed in the blood at every time point measured in a series of time course experiments (Supplemental Fig. 2). These results mirror those from our experiments challenging mice with LCMV, namely that the overall magnitude of the Ag-specific CD8\(^+\) T cell response is significantly reduced in mice lacking mir-155, but that those cells that do respond maintained the ability to make effector cytokine responses.

\textit{The APC compartment of the mir-155KO mouse is capable of supporting CD8\(^+\) T cell responses to \textit{L. monocytogenes}}

The defects we observed in CD8\(^+\) T cell responses to both LCMV and \textit{L. monocytogenes} could be explained either by abnormalities in the microenvironment and/or the APC compartment in 155KO mice or as a consequence of an intrinsic requirement for mir-155 in CD8\(^+\) T cells. To assess whether mir-155 is critical for APC function or secondary lymphoid organ structure, we transferred congenically marked WT LCMV gp33 Ag-specific CD8\(^+\) T cells (P14) (21) into either WT or 155KO mice and measured their expansion postinfection with \textit{L. monocytogenes}. We labeled the P14 cells with CFSE and measured their proliferation 3 d postinfection with \textit{L. monocytogenes} to measure initial priming effects in the WT or 155KO mice. We observed cell division of transferred P14 cells 3 d postinfection of either WT or 155KO mice with \textit{L. monocytogenes}, as determined by gating on CD8\(^+\) cells and congenic marker (Fig. 3A, left). The transferred P14 cells upregulated CD44 to the same extent in both host mouse genotypes (Fig. 3A, right). The number of rounds of division of P14 cells and the total number of P14 cells at day 3 were the same regardless of whether the host was WT or 155KO (Fig. 3B). There was no difference in total P14 cell numbers detected in WT or 155KO hosts (Fig. 3C). We also measured the accumulation of P14 T cells 7 d after \textit{L. monocytogenes} infection. By using congenic markers we were able to identify the WT transferred P14 cells (Thy1.1\(^+\)) from the endogenous responding cells (Thy1.1\(^-\)). Seven days postinfection of WT or 155KO mice that received the same number of WT P14 cells, we observed a similar percentage of CD8\(^+\) gp33-specific cells by tetramer stain in the spleen (Fig. 3D, left). This population includes both the WT transferred cells (Thy1.1\(^+\)) and the endogenous responding polyclonal CD8\(^+\) gp33-specific cells in the infected mice (Thy1.1\(^-\)). When we gated on the gp33 tetramer-binding cells and calculated the host response versus transferred P14 response in WT hosts, we observed a similar ratio of endogenous to P14 cells (Fig. 3D, top right). In the 155KO hosts, however, we observed a drastic skewing in the proportion of gp33-specific cells in favor of the transferred WT P14 cells (Fig. 3D, lower right). In this case, the great majority of

\textbf{FIGURE 2.} Mice lacking mir-155 have reduced CD8\(^+\) T cell responses to \textit{L. monocytogenes}. WT or 155KO mice were infected with \textit{L. monocytogenes} expressing the LCMV gp33 epitope, and 7 d later splenocytes were stained for gp33 tetramer and CD8. \textbf{(A)} The graphs summarize the data from three mice in one experiment and show the total number of CD8\(^+\) T cells per spleen. \textbf{(B)} Dot plots show CD8 and gp33 staining with the percentage of total CD8\(^+\) splenocytes that bind gp33 tetramer. \textbf{(C)} The total number of gp33-specific CD8\(^+\) T cells per spleen is shown. \textbf{(D)} Dot plots show splenocytes stained for CD8 and IFN-\(\gamma\) with the percentage of total CD8\(^+\) T cells that produce IFN-\(\gamma\) in response to gp33. \textbf{(E)} Graph shows total number of IFN-\(\gamma\)-producing CD8\(^+\) T cells per spleen. \textbf{(F)} Plots show representative stains of CD8 versus granzyme B. Numbers in \textit{upper right} of plots represent percentage of total cells that are CD8\(^+\) and producing granzyme B. \textbf{(G)} Quantitation of total numbers of CD8\(^+\) granzyme B-producing cells in the spleens of animals infected with \textit{L. monocytogenes}. Statistics are Student \(t\) test, \(n = 3\) per group. Experiments were all repeated three times with \(n = 3\) per group per experiment.
gp33-specific CD8+ T cells were WT P14 adoptively transferred cells; only 13% of the cells were endogenous CD8+ T cells, whereas the remaining cells were transferred WT P14. The average percentage of host versus donor (P14) for the three mice in the experiment is displayed in Fig. 3E. Therefore, the defect in the CD8+ T cell response that we observed in 155KO mice is not due to defects in the stromal or APC compartment.

Mir-155 is critical for CD8+ T cell responses

To directly measure requirements for mir-155 in Ag-specific CD8+ T cell responses, we crossed the 155KO mice with P14 TCR transgenic mice, resulting in mice with Ag-specific CD8+ T cells lacking mir-155 (P14155−/−). We transferred congenically marked (CD45.2) P14 or P14155−/− into WT CD45.1+ mice and infected them with L. monocytogenes. Postinfection, the percentage of cells expressing the CD45.2 marker was much lower in the mice that received P14155−/− when compared with mice that received WT P14 CD8+ T cells (Fig. 4A). The total number of P14155−/− T cells recovered from the mice on day 7 postinfection was ~7-fold lower than the number of WT P14 cells (Fig. 4B). When we measured the percentages of transferred P14 versus endogenous CD8+ T cells, we observed that ~60% of the glycoprotein-specific
cells were endogenous and 40% were transferred P14 cells (Fig. 4D, left). In contrast, when we transferred CD45.2 P14155−/− into WT hosts, the composition of Ag-specific T cells was skewed away from the P14155−/−, favoring the endogenous T cells, indicating directly that there is a defect in the ability of CD8+ T cells lacking mir-155 to respond to L. monocytogenes infection (Fig. 4D, right). These data demonstrate that mir-155 is important for promoting optimal T cell responses in vivo.

**Discussion**

CD8+ T cell expansion to viruses and intracellular bacteria has been studied extensively for decades. The recent discovery of miRNA-mediated regulation of protein translation requires an entirely new way of looking at cellular processes such as division and survival. We have shown in this study that a single miRNA can have dramatic effects in regulating CD8+ T cell responses. We have found that mice lacking mir-155 have greatly reduced Ag-specific CD8+ T cell responses to both L. monocytogenes and LCMV infection. This reduction in accumulation of CD8+ T cells is T cell intrinsic, as seen by our adoptive transfer studies. This is an important observation, as several recent reports have implicated mir-155 in macrophage and dendritic cell function (25, 27–30). Our data indicate that the innate response to L. monocytogenes was intact because bacterial counts in 155KO mice were similar to WT mice 3 d postinfection (Supplemental Fig. 3). Clearly, in the case of acute L. monocytogenes infection, the potential defects in APC function did not play a major role. Interestingly, although reduced in number, the Ag-specific CD8+ T cells that were activated were functional, as seen by cytotoxicity (Fig. 1L), IFN-γ (Figs. 1J, 1K, 2D, 2E), and granzyme B expression (Fig. 2F, 2G). Importantly, mir-155-deficient T cells were able to clear LCMV as measured by plaque assay (data not shown), demonstrating that the functional ability of the cells remains intact. Therefore, despite the limited numbers of pathogen-specific T cells, the fact that they were functional resulted in the clearance of the pathogens.

Computational and experimental data implicate >4500 possible mRNA targets for mir-155 (www.microrna.org). One such confirmed target is the inhibitory protein SOCS1 (31, 32). It has been published that SOCS1 is regulated by mir-155 in Treg cells and confirmed target is the inhibitory protein SOCS1 (31, 32). It has been published that SOCS1 is regulated by mir-155 in Treg cells and LCMV infection. This reduction in accumulation of CD8+ T cells is T cell intrinsic, as seen by our adoptive transfer studies. This is an important observation, as several recent reports have implicated mir-155 in macrophage and dendritic cell function (25, 27–30). Our data indicate that the innate response to L. monocytogenes was intact because bacterial counts in 155KO mice were similar to WT mice 3 d postinfection (Supplemental Fig. 3). Clearly, in the case of acute L. monocytogenes infection, the potential defects in APC function did not play a major role. Interestingly, although reduced in number, the Ag-specific CD8+ T cells that were activated were functional, as seen by cytotoxicity (Fig. 1L), IFN-γ (Figs. 1J, 1K, 2D, 2E), and granzyme B expression (Fig. 2F, 2G). Importantly, mir-155-deficient T cells were able to clear LCMV as measured by plaque assay (data not shown), demonstrating that the functional ability of the cells remains intact. Therefore, despite the limited numbers of pathogen-specific T cells, the fact that they were functional resulted in the clearance of the pathogens.
pared with WT CD8+ cells. These suggest that mir-155 alters the PI3K/Akt signaling pathway downstream of TCR in CD8+ T cells.

The activity of Akt has been linked to protein translation, which is critical for proliferation and survival of cells. The observed phenotype of decreased CD8+ T cell accumulation could be explained by either reduced proliferation after priming or reduced survival. Our data demonstrate that P145/5−/− T cells are able to undergo similar rounds of division (Fig. 5D), but do not accumulate in vivo (Fig. 5B, 5D). This suggests that the reduction in 155KO CD8+ T cell numbers is due to impaired Akt signaling resulting in reduced cell survival rather than impaired proliferation. Akt is activated after TCR cross-linking and can promote T cell survival by increasing levels of the anti-apoptotic protein Bcl-xL (37, 38). Although we have found that 155KO CD8+ T cells have impaired Akt signaling, there are several other target molecules that have been identified that could also be important contributors to the phenotype, such as the proapoptotic transcription factor FOXO3a (15, 39), which is downstream of Akt.

In summary, to our knowledge, these data show for the first time that mir-155 is required for acute CD8+ T cell responses to both virus and intracellular bacteria in vivo. Our data clearly show defects in immune responses to LCMV and intracellular bacteria in vivo. Our data demonstrate that P145/5−/− T cells are able to undergo similar rounds of division (Fig. 5D), but do not accumulate in vivo (Fig. 5B, 5D). This suggests that the reduction in 155KO CD8+ T cell numbers is due to impaired Akt signaling resulting in reduced cell survival rather than impaired proliferation. Akt is activated after TCR cross-linking and can promote T cell survival by increasing levels of the anti-apoptotic protein Bcl-xL (37, 38). Although we have found that 155KO CD8+ T cells have impaired Akt signaling, there are several other target molecules that have been identified that could also be important contributors to the phenotype, such as the proapoptotic transcription factor FOXO3a (15, 39), which is downstream of Akt and regulates T cell responses to viral infections (40, 41). We thank Yoko Kosaka (Princess Margaret Hospital, Toronto, ON, Canada) for critically reading the manuscript.

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Disclosures

The authors have no financial conflicts of interest.

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Supplementary Figure 1.

**Naïve WT and 155KO mice have comparable CD8 T cell populations.** Splenocyte suspensions from WT or 155KO mice were stained with antibodies to CD8 and CD44. (a) Plots show CD8 staining (left) or CD8 vs. CD44 (right) profiles for a representative WT (top plots) or 155KO (bottom plots). (b) Total numbers of splenocytes (c) or CD8+ cells. (d) Bar graph shows the mean fluorescence intensity. Experiment representative of 3 experiments. Statistics show Student’s t-test, n=5 mice per group.
Supplementary Figure 2.
Reduced CD8 response to LM in the blood of 155KO compared to WT mice at several times after infection.
Groups of WT or 155KO mice were infected with LM-GP and blood was collected at indicated times for tetramer staining. Plots of CD8 vs. GP33 Tetramer at day 14 from the blood of WT (a) or 155KO mice (b). Numbers in upper right represent percent of CD8+ Tet+ cells. The percent of CD8+ Tet+ cells in the blood at day 14 is quantitated (c). Percent of CD8+ GP33 Tet+ cells at various time points (circle represents WT, squares represent 155KO) (d). Experiment repeated twice, n=4 per group.
Supplementary Figure 3.
The innate response to LM appears intact in 155KO mice.
Spleens and livers from groups of WT or 155KO mice were collected 3 days post infection with 2000 CFU LM-GP. Tissues were homogenized and treated serial dilutions of homogenate were plated on BHI agar plates. Data representative of 3 experiments.
Supplementary Figure 4.

(a) CD8 T cells were isolated from WT (pink) or 155KO spleens (blue) and exposed to indicated amounts of IL-2 for 30 minutes in vitro. Cells were then fixed and stained for phosphorylation of STAT5.

(b) CD8 T cells were isolated from WT (pink) or 155KO spleens (blue) and exposed to indicated amounts of recombinant IFN-α for 30 minutes in vitro. Cells were then fixed and stained for phosphorylation of STAT1. Gray shaded histograms represent irrelevant isotype control staining. Both experiments were performed twice with similar results.