

The lncRNA Malat1 Inhibits miR-15/16 to Enhance Cytotoxic T Cell Activation and Memory Cell Formation

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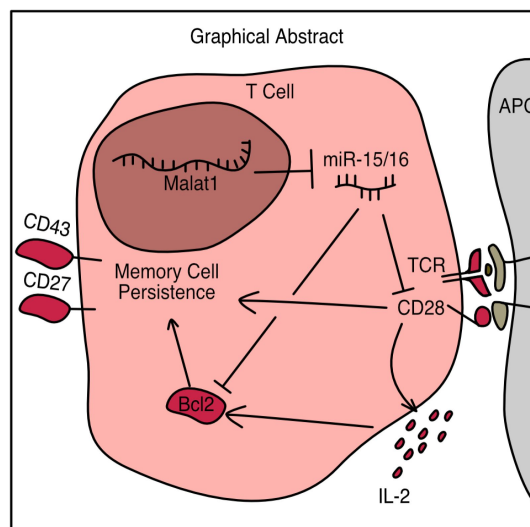
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Abstract

Proper activation of cytotoxic T cells via the T cell receptor and the costimulatory receptor CD28 is essential for adaptive immunity against viruses, many intracellular bacteria and cancers. Through biochemical analysis of RNA:protein interactions, we uncovered a non-coding RNA circuit regulating activation and differentiation of cytotoxic T cells composed of the long non-coding RNA Malat1 (Metastasis Associated Lung Adenocarcinoma Transcript 1) and the microRNA family miR-15/16. miR-15/16 is a widely and highly expressed tumor suppressor miRNA family important for cell proliferation and survival. miR-15/16 also play important roles in T cell responses to viral infection, including the regulation of antigen-specific T cell expansion and T cell memory. Comparative Argonaute-2 high throughput sequencing of crosslinking immunoprecipitation (Ago2 HITS-CLIP, or AHC) combined with gene expression profiling in normal and miR-15/16-deficient T cells revealed a large network of several hundred direct miR-15/16 target mRNAs, many with functional relevance for T cell activation, survival and memory formation. Among these targets, the long non-coding RNA Malat1 contained the largest absolute magnitude miR-15/16-dependent AHC peak in T cells. This binding site was also among the strongest lncRNA:miRNA interactions detected in the T cell transcriptome. We used CRISPR targeting with homology directed repair to generate mice with a 5-nucleotide mutation in the miR-15/16 binding site in Malat1. This mutation interrupted Malat1:miR-15/16 interaction, and enhanced the repression of other miR-15/16 target genes, including CD28. Interrupting Malat1 interaction with miR-15/16 decreased cytotoxic T cell activation, including the expression of IL-2 and a broader CD28-responsive gene program. Accordingly, Malat1 mutation diminished memory cell persistence following LCMV Armstrong and *Listeria monocytogenes* infection. This study marks a significant advance in the study of long noncoding RNAs in the immune system by ascribing cell-intrinsic, sequence-specific *in vivo* function to Malat1. These findings have implications for T cell-mediated autoimmune diseases, antiviral and anti-tumor immunity, as well as lung adenocarcinoma and other malignancies where Malat1 is overexpressed.



eLife assessment

This is an **important** study that revealed a new noncoding RNA regulatory circuit involved in T cell function. The authors provide **compelling** evidence, that is more rigorous than the state-of-the-art, using genetically engineered mice and cell-based experiments. The interpretation of the results should be tempered due to the small effect size observed.

Introduction

Cytotoxic T cells are indispensable for mounting an adaptive immune response against intracellular pathogens and the clearance of mutated cells such as cancer. Over the course of a viral infection, cytotoxic T cells are primed by antigen presenting cells and undergo extensive rounds of intense proliferation (Murali-Krishna et al., 1998 [\[1\]](#)). As they clonally expand, these cells differentiate into effector and memory cells and acquire effector functions, including the production of cytotoxins and critical cytokines such as IL-2, TNF α , and IFN γ (Bachmann et al., 1999 [\[2\]](#)). As the infection is cleared, the cytotoxic T cell response contracts as many cells die via apoptosis, yielding a long lived pool of memory cells poised for secondary expansion and protection against reinfection with the same pathogen (Badovinac et al., 2002 [\[3\]](#); Wherry and Ahmed, 2004 [\[4\]](#)). The factors that control this expansion, differentiation, and contraction have been intensely researched in the past decades, focusing in large part on proteins such as transcription factors, signaling enzymes, and cytokines (Chen et al., 2018 [\[5\]](#)). However, it is of recent interest to understand how non-protein coding regions of the genome contribute to the regulation of these cells as well. These non-protein coding elements can be regulatory in nature such as enhancers (Roychoudhuri et al., 2016 [\[6\]](#); Shapiro et al., 1997-7), or RNA species that are transcribed but not translated. Two such species of interest to our present study are microRNAs (miRNAs) and long non-coding RNAs (lncRNAs).

MicroRNAs are short (21 nucleotide) RNAs which, when loaded into Argonaute (Ago) proteins, can target mRNAs that contain complementary seed sequences in their 3' UTRs for translation inhibition and degradation (Bartel, 2018 [\[7\]](#); Djuranovic et al., 2012 [\[8\]](#); Eichhorn et al., 2014 [\[9\]](#)). In particular, the miR-15/16 family are potent regulators of cell cycle and survival (Liu et al., 2008 [\[10\]](#)). Previous work from our group utilized conditional deletion of the four major miR-15/16 family

members (miR-15a, miR-15b, miR-16-1, miR-16-2) driven by CD4-cre transgene (hereafter referred to as miR-15/16^{ΔΔ} mice or T cells) to demonstrate that this miRNA family has important effects on cytotoxic T cells (Gagnon et al., 2019). In response to viral infection, miR-15/16^{ΔΔ} mice generate more viral-antigen specific T cells, and these cells preferentially differentiate into memory cells that express CD127 and CD27 (Gagnon et al., 2019).

In contrast to the well defined roles of miRNAs, lncRNAs as a class do not have a single defined function. They are broadly defined as RNAs transcribed by polymerase II, over 200 nucleotides (nt) in length, that lack a translated open reading frame (Su et al., 2021; Wilusz et al., 2009). Some lncRNAs bind to chromatin and regulate nearby or distant genes, and others scaffold transcription factors and other protein complexes (Kopp and Mendell, 2018). Most relevant to the present study, some lncRNAs act as competing endogenous RNAs (ceRNAs) that bind to miRNAs, preventing their binding to and subsequent repression of mRNA targets (Poliseno et al., 2010; Su et al., 2021). However, rigorous connections between lncRNA physiological functions and molecular mechanisms of action has been hampered by the lack of precise tools to facilitate their study (Ponting and Haerty, 2022). As lncRNAs lack an open reading frame, insertion or deletion (indel) mutations do not reliably create null alleles. Instead, investigators have frequently deleted large genomic regions around the promoter or excised the genomic locus of the transcript altogether. These approaches risk disrupting cis-regulatory elements or topologically associated domains that control other nearby genes. Other studies that identified physiological roles for lncRNAs rely on RNAi-mediated lncRNA degradation. Both this approach and genetic manipulations that block lncRNA transcription leave a mechanistic gap between sequence features and downstream function. Studies demonstrating sequence dependent function of lncRNAs are comparatively rare (Carrieri et al., 2012; Elguindy and Mendell, 2021; Faghihi et al., 2008; Gong and Maquat, 2011; Kleaveland et al., 2018; Lee et al., 1999; Yoon et al., 2012).

One lncRNA that has garnered much attention is the Metastasis Associated Lung Adenocarcinoma Transcript 1 (Malat1). Malat1 was first identified as highly expressed in both malignant tumors and healthy lung and pancreas with high interspecies conservation (Ji et al., 2003). Malat1 is mostly localized to the nucleus, where it is found within nuclear speckles, although it is not necessary for their formation (Nakagawa et al., 2012). These characteristics have generated numerous hypotheses about the function of Malat1 including that it scaffolds epigenetic and splicing factors and acts as a ceRNA to inhibit a variety of miRNAs (Su et al., 2021; Tripathi et al., 2010).

In the immune system, Malat1 has been studied in dendritic cells, macrophages, T helper cells, T regulatory cells, and cytotoxic T cells (Hewitson et al., 2020; Kanbar et al., 2022; Masoumi et al., 2019; Wu et al., 2018). Malat1 is actively regulated during cytotoxic T cell differentiation with higher expression in memory precursors and lower expression in short-lived effector cells during LCMV infection (Kakaradov et al., 2017). Similarly, T cells stimulated *in vitro* express decreased levels of Malat1 over time (Masoumi et al., 2019). Early reports using large genetic deletions in the mouse Malat1 locus detected widespread gene expression changes but failed to identify a role for Malat1 in mouse development or cytotoxic T cell responses (Nakagawa et al., 2012; Yao et al., 2018; Zhang et al., 2012), though a recent report using RNAi detected altered T cell responses (Kanbar et al., 2022). Clearly there remains much to learn about the sequence-specific mechanisms by which Malat1 tunes pathways and functions essential to cytotoxic T cells.

In the present study, we present a non-coding RNA circuit that regulates the activating signals from CD28 and IL-2, leading to low Bcl-2 expression and loss of memory cells following LCMV infection. We do so by identifying candidate interactions between miRNAs and lncRNAs via a targeted biochemical approach, and by creating CRISPR-targeted transgenic mice with precise

mutation of Malat1 to interrogate the physiological function of the interaction between miR-15/16 and Malat1. We use this novel mouse to identify CD28 responsive gene programs affected by this circuit, and provide a new sequence-specific function of Malat1 *in vivo*.

Results

Malat1 is highly Bound by miR-15/16

To identify candidate noncoding ceRNAs in cytotoxic T cells, we performed Argonaute-2 high throughput sequencing of crosslinking immunoprecipitation (Ago2 HITS-CLIP, AHC). Integrating sequence reads across different classes of transcribed genomic annotations revealed that lncRNAs are bound extensively by Ago2, but the median lncRNA had 7.8 times fewer aligned AHC sequence reads compared to 3' untranslated regions (UTRs) where miRNAs canonically bind to mRNAs (**Fig. 1A**). This difference likely reflects the comparatively low expression of many lncRNAs, and it highlights the relatively low occupancy of Ago2 on most of these transcripts. Nevertheless, there were individual lncRNAs that stood out as highly bound across the transcript. To prioritize lncRNAs for further investigation, we manually curated the transcripts with the largest number of aligned AHC sequence reads. Among the top 10 most highly bound transcripts, 3 overlapped with protein coding genes, 2 were on the mitochondrial chromosome, and 3 were repetitive annotations. Strikingly, all of the top 10 transcripts were either annotated as rRNA or contained an rRNA repeat element, except the 8th most bound transcript, Malat1 (**Table 1**). When the same analysis was repeated with rRNA repeats masked, Malat1 was the second most highly bound transcript (**Table 2**).

Malat1 has been proposed to inhibit miRNAs as a ceRNA (Chen et al., 2017; Luan et al., 2016; Qiao et al., n.d.; Wang et al., 2019; Wu et al., 2018; Xiao et al., 2015; Xie et al., 2017). We used the Piranha peak calling algorithm to identify sites with the highest degree of miRNA binding, as indicated by AHC sequence read number and density (**Fig. 1B**). Within cytotoxic T cells, the algorithm identified 55 AHC peaks in Malat1, the largest of which was extremely pronounced (**Fig. 1C**). Compared with other lncRNA binding peaks, this peak had the 36th most total aligned reads and the 142nd highest read density (15th and 16th respectively when rRNA reads are masked). Even when compared to peaks in 3' UTRs of mRNAs, the largest peak in Malat1 was the 100th most (98th percentile) bound peak in terms of read density (**Fig. 1B**).

miRTarget, the custom miRNA binding prediction algorithm (Liu and Wang, 2019), identified an 8-mer seed binding sequence of the miR-15/16 family centered within the most densely bound region of the called peak (**Fig. 1C**). miR-15/16 has not been previously shown to interact with Malat1 in mice, so to determine whether this peak is miR-15/16 dependent, we performed AHC with cultured CD8⁺ miR-15/16^{Δ/Δ} T cells. In the absence of miR-15/16, Ago2 binding to Malat1 was preserved throughout the whole transcript except for the peak containing the predicted miR-15/16 8-mer seed binding sequence (**Fig. 1C**). We conclude that miRNAs of the miR-15/16 family bind abundantly to Malat1. Further, miR-15/16 binding occurred in a region of high evolutionary conservation (**Fig. 1D**). The corresponding region containing the miR-15/16 binding site in human MALAT1 was highly enriched in two publicly available AHC datasets obtained using the 293 human embryonic kidney (HEK) cell line, consistent with Malat1 regulating miR-15/16 in a colorectal cancer cell line (Karginov and Hannon 2013; Ji et al. 2019; Li et al. 2018) (**Fig. 1E**). We conclude that miR-15/16 bind abundantly to Malat1 in mouse and human cells.

We hypothesized that Malat1 may inhibit the function of miR-15/16 in cytotoxic T cells. To directly address this question in mice, we used CRISPR-Cas9 with homology directed repair to generate mice in which five nucleotides of the miR-15/16 seed binding sequence within Malat1 were scrambled (**Fig. 1F**). Mice homozygous for this mutation are subsequently referred to as *Malat1*^{scr/scr}. To confirm the targeted functional outcome of this mutation, AHC was performed on

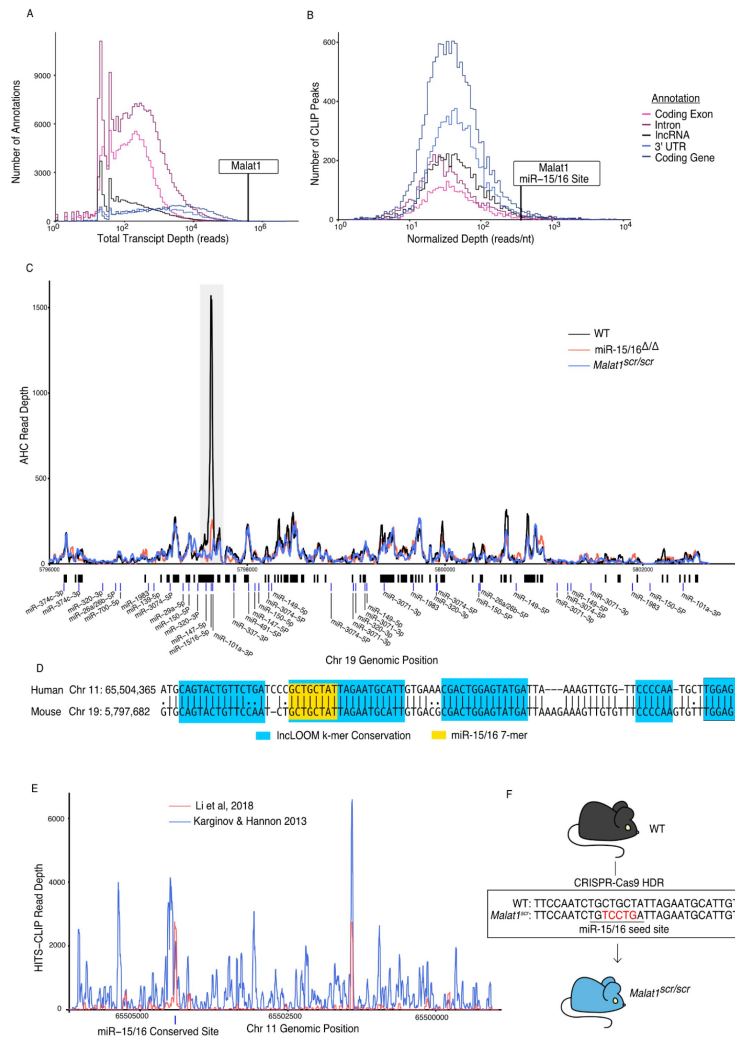


Figure 1.

Malat1 is highly bound by miR-15/16

CD8+ T cells were isolated from spleens, grown *in vitro* for 5 days, then Ago2 transcriptomic occupancy was assayed via Ago2 HITS-CLIP.

(A-B) Transcriptome wide analysis of Ago2 HITS-CLIP libraries prepared from WT cells (combined libraries $n = 2$).

(A) Summed reads across entire annotations. Line indicates total reads across the Malat1 transcript. Malat1 was #8 most highly bound lncRNA annotation, which was in the top 0.0091% of all lncRNA annotations analyzed with >0 HITS-CLIP reads.

(B) Ago2 HITS-CLIP peaks were identified and reads were summed within those called peaks that intersected with the given annotation. Peaks were of variable length so summed reads were normalized by peak length. Line indicates HITS-CLIP reads per nucleotide in the called peak containing the miR-15/16 binding site in Malat1. This peak was the #121 most bound HITS-CLIP peak in lncRNA peaks analyzed, which was in the top 2.3% of all evaluated peaks in lncRNAs.

(C) Ago2 HITS-CLIP binding to the mouse Malat1 locus reads from combined libraries shown ($n = 2$ for each genotype). Grey bar indicates the peak containing the miR-15/16 binding site. Black bars indicate regions identified as peaks by piranha. Blue bars indicate predicted binding sites of miRNAs expressed in our data set from the miRTarget custom sequence prediction algorithm. Grey bar indicates miR-15/16 binding peak.

(D) Local alignment of the human and mouse Malat1 sequences near the miR-15/16 conserved binding site. Highlighting indicates the depth of evolutionary conservation of k-mers as predicted by the IncLOOM algorithm (Ross et al., 2021).

(E) Ago2 HITS-CLIP binding to the human MALAT1 locus from publicly available data sets (Karginov and Hannon, 2013; Li et al., 2018). Blue vertical bar indicates the conserved miR-15/16 binding site.

(F) Schematic representing the creation of the *Malat1*^{scr} allele. Bases in red indicate the 5 nucleotides whose sequence was scrambled by CRISPR-Cas9 HDR to prevent miR-15/16 binding.

Noncode Gene ID	Total Reads	Chromosome	Alias	Note
NONMMUG018330.3	4766634	17	ENSMUST00000198477.1	Contains rRNA repeat
NONMMUG044354.2	2291785	M	mt-Rnr2	Overlaps mt-ND1
NONMMUG044353.2	1208170	M	mt-Rnr1	
NONMMUG076321.1	1012732	6		Overlaps and best aligns to NONMMUG034479.2
NONMMUG034478.3	1012015	6		Overlaps and best aligns to NONMMUG034479.2
NONMMUG034479.2	1011610	6		Contains rRNA repeat
NONMMUG007938.3	785970	11		Intronic of Gm36876, contains rRNA repeat(Weirick et al., 2016)
NONMMUG020671.2	383447	19	Malat1	
NONMMUG015781.2	336664	16		Intronic of Zc3h7a, contains rRNA repeat
NONMMUG044321.2	317822	9		Contained in Lars2 3' UTR, contains rRNA repeat

Table 1.

lncRNAs with the most AHC reads

Noncode Gene ID	Total Reads	Chromosome	Alias	Note
NONMMUG018330.3	688875	17	ENSMUST00000198477.1	Partially anti-sense to XR_877120.2, binding extends beyond rRNA repeat
NONMMUG020671.2	383447	19	Malat1	
NONMMUG094408.1	296723	6		Contains B4A/B3 SINE, binding restricted to SINE
NONMMUG005751.2	291393	11		Intronic of Dock2, contains multiple RLTR44-int repeat elements, binding restricted to RLTRs
NONMMUG094727.1	289326	6		Contains B4A/B3 SINE, binding is restricted to repeats
NONMMUG014644.3	241005	15	Pvt1	Many repeats, but binding is not restricted to any definite subset
NONMMUG096664.1	229834	15	PVt1	Splice variant of NONMMUG014644.3
NONMMUG024327.3	222686	2	Oip5os1, Cyrano	Well described in (Han et al., 2020)
NONMMUG026716.2	207631	3		Overlapping Mbnl1
NONMMUG094659.1	196269	6		Overlapping Foxp1

Table 2.

lncRNAs with the most AHC reads that do not align to rRNA repeat elements

cultured CD8⁺ T cells isolated from *Malat1^{scr/scr}* mice. Ago2 binding was preserved across the Malat1 transcript except at the mutated miR-15/16 binding site, where AHC sequence read density was greatly reduced (**Fig. 1C** [↗](#)). This finding confirmed that miR-15/16 binds to Malat1 at this site in a sequence dependent manner, and provided us with a tool for highly specific investigation of the functional consequences of Malat1:miR-15/16 interaction.

Malat1 inhibits miR-15/16 availability and activity

Malat1:miR-15/16 interaction could lead to regulation and/or degradation of the lncRNA, the miRNAs, or both. To assess whether miR-15/16 degrades Malat1 we compared the expression of Malat1 by mRNA sequencing in primary mouse CD8⁺ T cells. Malat1 expression was unchanged in miR-15/16^{Δ/Δ} cells as well as in the *Malat1^{scr/scr}* cells (**Fig. 2A** [↗](#)). Previous studies investigating Malat1 and other lncRNAs as ceRNA inhibitors of miRNAs have suggested that inhibition occurs by either target RNA-directed miRNA degradation (TDMD) or stoichiometric sequestration of the miRNA from protein-coding mRNA targets (Han et al., 2020 [↗](#); Su et al., 2021 [↗](#)). Therefore, we also tested the possibility that Malat1 lowers miR-15/16 abundance by TDMD or a related mechanism. However, in freshly isolated mouse CD8⁺ T cells, miR-15b and miR-16 were unchanged and only miR-15a, a family member with lower expression, was modestly decreased in *Malat1^{scr/scr}* cells (**Fig. 2B** [↗](#)). We conclude that Malat1 and the miR-15/16 family do not influence each other's absolute abundance in this setting.

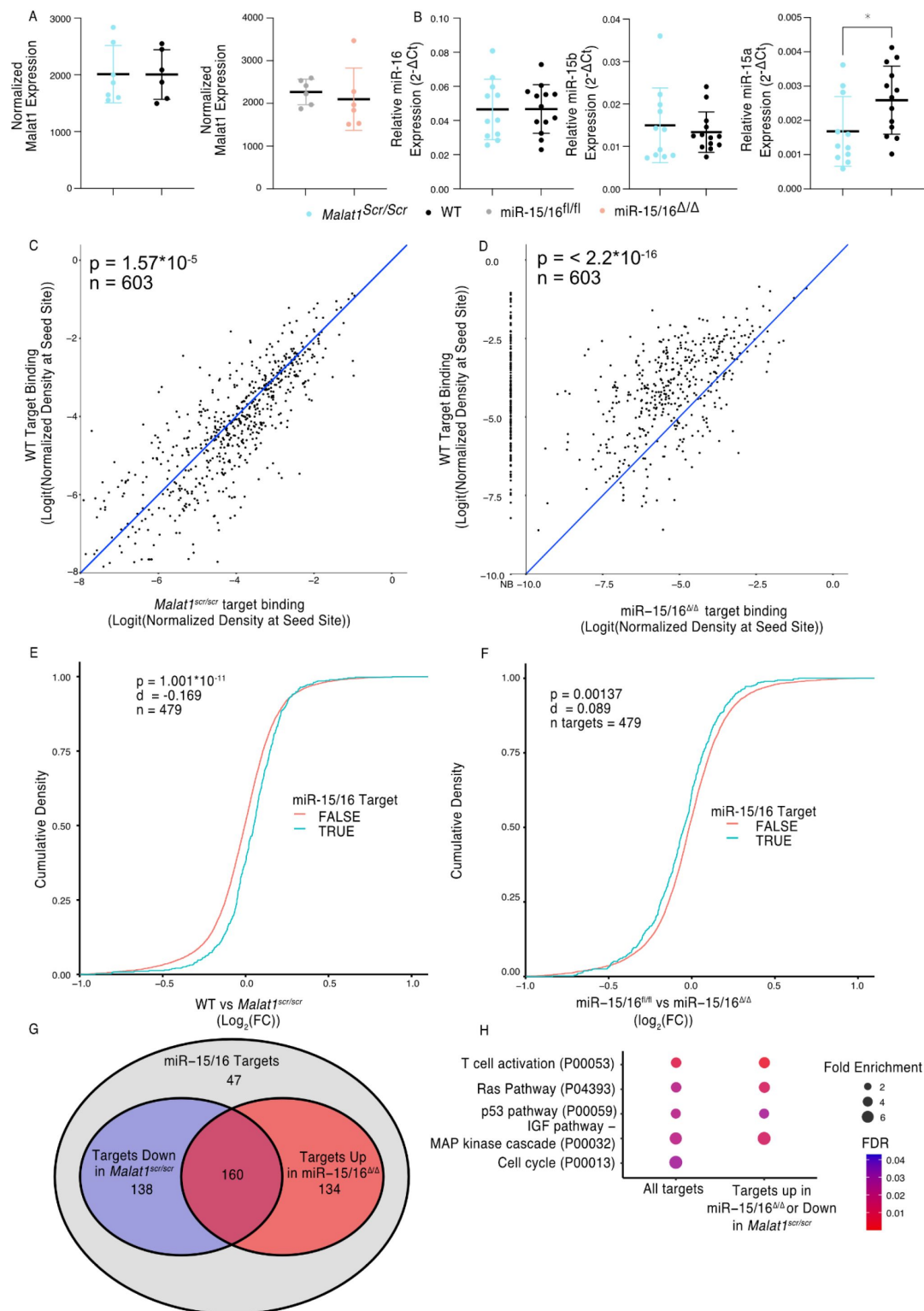


Figure 2.

Malat1 Inhibits miR-15/16 binding and suppressive activity

(A) Malat1 expression measure by RNA-seq from CD8⁺ T cells isolated from spleens and stimulated with αCD3 and αCD28 for 24 hours (WT $n = 6$, *Malat1^{Scr/Scr}* $n = 7$, *miR-15/16^{fl/fl}* $n = 6$, *miR-15/16^{Δ/Δ}* $n = 6$; 1 experiment)

(B) miR-16, miR-15b, and miR-15a expression measured by miRNA qPCR from CD8⁺ T cells freshly isolated from spleens. Expression was determined relative to 5.8s ribosomal RNA expression. Unpaired t-test performed to determine significance.

(C-D) TargetScan predicted miR-15/16 binding sites that contained at least one HITS-CLIP read in both WT and *Malat1^{scr/scr}* CD8⁺ T cells were compared for depth of Ago2 HITS-CLIP reads. First, reads at the predicted seed site were normalized by total Ago2 HITS-CLIP reads in a given 3' UTR. To best visualize all sites, logit transforms of these values are plotted. Paired t-test performed to determine significance. Blue line indicates the identity line. Data for each genotype is from combined libraries of n = 2 biological replicates.

(C) Comparison of WT and *Malat1^{scr/scr}* cells

(D) Comparison of WT and miR-15/16^{Δ/Δ}. Values to the left of the y-axis labeled with NB indicate there was no bind detected at that site in the miR-15/16^{Δ/Δ} cells.

(E-F) Cumulative density plots to determine changes in expression of miR-15/16 targets. Targets determined by TargetScan predicted miR-15/16 mRNA targets that had at least one 3' UTR site with reads in both WT and *Malat1^{scr/scr}* CD8⁺ T cells. Kolmogorov-Smirnov test used to determine significant differences in the distributions of target and non-target genes.

(E) comparison of the Log₂(FC) between WT and *Malat1^{scr/scr}* samples stimulated with αCD3 and αCD28 for 24 hours

(F) comparison of the Log₂(FC) between miR-15/16^{fl/fl} and miR-15/16^{Δ/Δ} samples stimulated with αCD3 and αCD28 for 24 hours.

(G) Venn diagram of miR-15/16 target expression regulated in concordance with the *Malat1*-miR-15/16 circuit. The blue circle indicates genes with WT vs *Malat1^{scr/scr}* log₂(FC) > 0 and the red circle indicates genes with miR-15/16^{fl/fl} vs miR-15/16^{Δ/Δ} log₂(FC) < 0. The purple overlap indicates genes that meet both conditions and the grey indicates genes that do not meet either condition.

(H) Gene ontology analysis of the bound target set used above as well as genes regulated in accordance with the *Malat1*-miR-15/16 circuit (WT vs *Malat1^{scr/scr}* log₂(FC) > 0 or miR-15/16^{fl/fl} vs miR-15/16^{Δ/Δ} log₂(FC) < 0). Enrichment determined within the Panther Pathway annotations. (*, p<0.05)

To test whether *Malat1* affects miR-15/16 function, we examined miR-15/16 target binding in our AHC data. We first defined an experimentally supported list of TargetScan predicted miR-15/16 binding sites with at least 1 AHC read in both WT and *Malat1^{scr/scr}* cells. Using this list, we then examined the read depth at these sites in both *Malat1^{scr/scr}* and WT cells (603 sites contained in 479 genes). In WT cells, these sites constituted on average 3.2% of the binding in each 3' UTR, and this figure increased to 3.4% in *Malat1^{scr/scr}* cells, indicating that Ago2 occupancy preferentially increased at these sites when miR-15/16 binding to *Malat1* was eliminated (**Fig. 2C**). To confirm that binding to these target sites was miR-15/16 dependent, we assessed their AHC read depths in miR-15/16^{Δ/Δ} cells as well. As predicted, binding was greatly reduced in miR-15/16^{Δ/Δ} cells, representing, on average, 1.0% of binding to a given 3' UTR, a 69% reduction in Ago2 binding compared to WT (**Fig 2D**). In contrast, no significant differences were observed for binding at predicted sites for the highly expressed miRNA families of miR-101, Let-7, miR-21, and miR-142, (Fig. S1). Thus, we conclude that the *Malat1^{scr}* allele specifically negatively regulates the first requirement of miR-15/16 function – binding to mRNA targets.

We next sought to investigate whether this increased binding resulted in decreased target mRNA expression. To do so, we analyzed mRNA-sequencing data from primary CD8⁺ T cells 24 hours after stimulation. We generated empirical CDF plots from these data comparing target gene expression in miR-15/16^{fl/fl} to miR-15/16^{Δ/Δ} cells as well as WT to *Malat1^{scr/scr}* cells. For each of these comparisons, we then compared the distribution for miR-15/16 target genes, as defined

above, to the distribution for all other expressed genes. In the case of WT to *Malat1*^{scr/scr} cells, the distribution was shifted in favor of decreased target gene expression in the *Malat1*^{scr/scr} cells (**Fig 2E** [↗](#)). In the case of miR-15/16^{fl/fl} to miR-15/16^{Δ/Δ}, the distribution was shifted in favor of increased target gene expression in the miR-15/16^{Δ/Δ} cells, indicating that this gene set is relieved of miRNA-induced repression (**Fig. 2F** [↗](#)). These data indicate that the increased availability of miR-15/16 leads to increased repression of mRNA target genes when Malat1:miR-15/16 interaction is ablated.

We further investigated genes that displayed reciprocal expression changes in miR-15/16^{Δ/Δ} and *Malat1*^{scr/scr} cells. Of 479 genes in the bound target list, 432 (90%) were either downregulated in *Malat1*^{scr/scr} cells or upregulated in miR-15/16^{Δ/Δ} cells, compared to controls. Among these genes, the expression of 298 (62%) were decreased in *Malat1*^{scr/scr} cells, and 294 (61%) were increased in miR-15/16^{Δ/Δ} cells, with 160 (33%) both increased in miR-15/16^{Δ/Δ} and decreased in *Malat1*^{scr/scr} cells (**Fig 2G** [↗](#)). Gene ontology enrichment analysis revealed multiple pathways associated with growth factor and antigen receptor signaling affected by the Malat1:miR-15/16 circuit in cytotoxic T cells (**Fig. 2H** [↗](#)). Many of these modules, including the T cell activation module, were identified because they share key signaling proteins. For example, gene expression and AHC data support direct miR-15/16 targeting of the costimulatory receptor *Cd28*, the alpha subunit of phosphatidylinositol 3-kinase (*Pik3r1*) and c-Jun N-terminal kinase (JNK1), encoded by *Mapk8* (Fig. S2 and **Fig. 3** [↗](#)). Targeting of these genes by miR-15/16 is consistent with their known tumor suppressor role (Cimmino et al., 2005 [↗](#); Gagnon et al., 2019 [↗](#)) and with Malat1's association with cancer cell proliferation and metastasis (Ji et al., 2003 [↗](#)), providing new mechanistic insight into those observations.

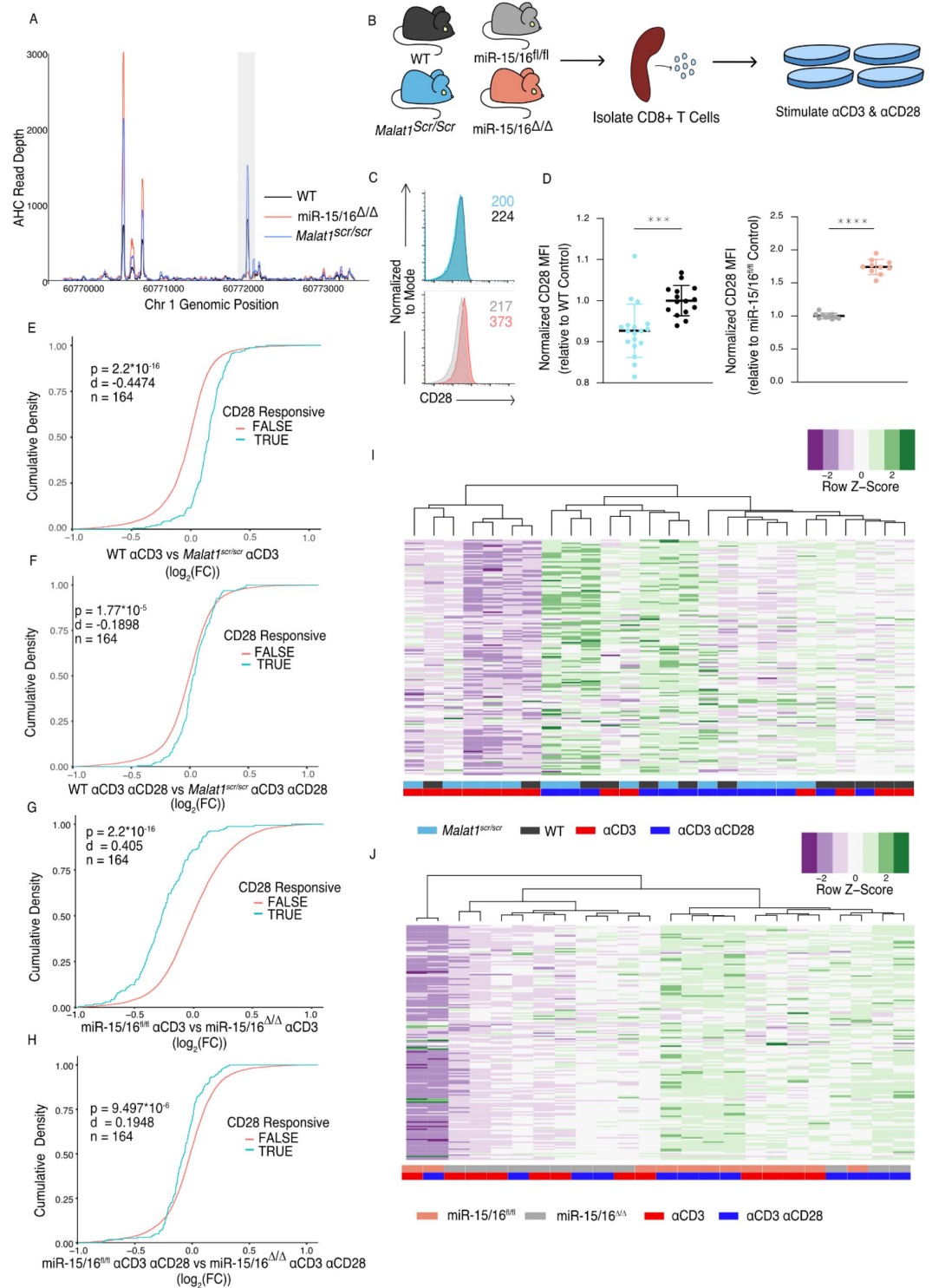


Figure 3.

The Malat1-miR-15/16 Circuit Increase CD28 Expression and Co-stimulation induced Gene Expression

(A) Ago2 HITS-CLIP binding at the Cd28 locus. Sequencing libraries generated from CD8⁺ T cells isolated from spleens and cultured for 5 days (combined libraries from $n = 2$ for each genotype). Grey bar indicates the peak containing the TargetScan predicted miR-15/16 binding site.

- (B) Schematic illustrating the assay scheme to assay acute gene expression downstream of CD28 co-stimulation
- (C) Representative flow cytometry plots of CD28 expression on naive ($CD62L^+ CD44^-$) $CD8^+$ T cells from spleens of unchallenged mice. Mean fluorescence intensity for the sample reported in the upper right of the plot.
- (D) Quantification of CD28 mean fluorescence intensity normalized to the relevant control. (*Malat1^{scr/scr}* compared to WT from 3 independent experiments; *miR-15/16^{fl/fl}* compared to *miR-15/16^{Δ/Δ}* from 2 independent experiments)
- (E-H) Cumulative density plots comparing expression of CD28 responsive gene set defined as genes from (Martínez-Llordella et al., 2013 [DOI](#)) with $\alpha CD3\alpha CD28$ vs $\alpha CD3$ $\log_2(FC) > 1.5$ and adjusted p value < 0.001 .
- Kolmogorov-Smirnov test used to determine significant differences in the distributions of target and non-target genes. $\alpha CD3$ used at 1 $\mu g/mL$, and $\alpha CD28$ used at 1 $\mu g/mL$.
- (E) Comparison of CD28 responsive genes in WT vs *Malat1^{scr/scr}* cells stimulated with $\alpha CD3$ alone
- (F) Comparison of CD28 responsive genes in WT vs *Malat1^{scr/scr}* cells stimulated with $\alpha CD3$ and $\alpha CD28$
- (G) Comparison of CD28 responsive genes in *miR-15/16^{fl/fl}* vs *miR-15/16^{Δ/Δ}* cells stimulated with $\alpha CD3$ alone
- (H) Comparison of CD28 responsive genes in *miR-15/16^{fl/fl}* vs *miR-15/16^{Δ/Δ}* cells stimulated with $\alpha CD3$ and $\alpha CD28$
- (I-J) Heatmaps of CD28 responsive gene set expression by genotype and stimulation condition. Dendrograms represent unbiased hierarchical clustering of the samples.
- (I) *Malat1^{scr/scr}* and WT samples compared with $\alpha CD3 \pm \alpha CD28$
- (J) *miR-15/16^{fl/fl}* vs *miR-15/16^{Δ/Δ}* samples compared with $\alpha CD3 \pm \alpha CD28$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$)

Malat1 enhances CD28 Expression and Downstream CD8 T Cell Activation

Given that the *Malat1:miR-15/16* circuit regulated genes essential to T cell activation, and in particular CD28, we next investigated the functional consequences of perturbing this circuit in cytotoxic T cells. First, we looked directly at CD28. AHC in WT cells detected a prominent peak in the CD28 3'UTR at the TargetScan predicted binding site for *miR-15/16*. This peak was absent in *miR-15/16^{Δ/Δ}* cells, whereas other binding peaks were preserved, empirically verifying that this binding event is *miR-15/16*-dependent. AHC in *Malat1^{scr/scr}* cells indicated a modest increase in Ago2 binding at this site compared to WT cells relative to Ago2 binding in the whole 3' UTR (**Fig 3A** [DOI](#)). These data, along with our previous demonstration that the CD28 3' UTR is *miR-15/16* responsive, indicates that CD28 is part of a module of *miR-15/16* target genes that are highly likely to be affected by the *Malat1^{scr}* allele (Gagnon et al., 2019 [DOI](#)).

Indeed, CD28 expression and T cell activation were regulated by the *Malat1:miR-15/16* circuit. Flow cytometric measurement in primary splenic $CD8^+$ T cells revealed that CD28 protein expression was decreased in *Malat1^{scr/scr}* mice and enhanced in *miR-15/16^{Δ/Δ}* mice (**Fig 3B-C** [DOI](#)). To investigate activation-induced gene expression, we performed RNA-seq on splenic $CD8^+$ T cells stimulated for 24 hours with plate-bound $\alpha CD3$ with or without $\alpha CD28$ cross-linking antibodies diagrammed in (**Figure 3D** [DOI](#)). CD28 stimulation enhances distinct activation-induced gene expression changes in T cells (Martínez-Llordella et al., 2013 [DOI](#)), and these CD28-responsive genes were altered in *miR-15/16^{Δ/Δ}* and *Malat1^{scr/scr}* cells (**Fig 3E-H** [DOI](#)). The previously defined set of 164 genes that are upregulated in WT cells stimulated with $\alpha CD3 + \alpha CD28$ compared to $\alpha CD3$ alone was

also significantly upregulated in the α CD3 + α CD28 condition compared to α CD3 alone for each genotype tested in our experiments (Fig S3A-D). Importantly, the Malat1:miR-15/16 circuit affected this gene set in the α CD3 alone condition, with Malat1^{scr/scr} cells exhibiting decreased expression compared to WT cells, and miR-15/16 ^{Δ/Δ} cells exhibiting increased expression compared to miR-15/16^{fl/fl} cells (Fig 3E,G). This trend was preserved, but to a lesser degree, in the α CD3 and α CD28 condition (Fig 3 F,H). Thus, Malat1:miR-15/16 interaction enhanced expression of a costimulation-responsive gene expression program in activated T cells, and it further enhanced expression of that module even when CD28 costimulation was directly engaged.

Unsupervised hierarchical clustering of these samples based on the expression of the 164 costimulation-responsive gene set further underscored the costimulatory-like effect of the Malat1:miR-15/16 circuit. In comparing gene expression in Malat1^{scr/scr} and WT samples, 3 major groups emerged. The group with the lowest average costimulation-responsive gene expression contained only samples stimulated with α CD3 alone and primarily Malat1^{scr/scr} samples. The group with intermediate expression was the largest group with an even representation of Malat1^{scr/scr} samples and WT samples. While samples in this intermediate group were from both stimulation conditions, the Malat1^{scr/scr} samples tended to be from the α CD3 + α CD28 condition and the WT samples had an even representation from both stimulation conditions. The group with the highest average expression contained predominantly α CD3 + α CD28 stimulated samples with an even representation of Malat1^{scr/scr} and WT samples (Fig 3I). Thus, Malat1:miR-15/16 interaction and engagement of CD28 signaling additively induced costimulation-responsive genes. This observation is further supported when clustering the miR-15/16 ^{Δ/Δ} and miR-15/16^{fl/fl} samples. The 2 largest clusters were divided nearly exclusively by genotype. The group with lower average expression of costimulation-responsive genes contained predominantly miR-15/16^{fl/fl} samples stimulated with α CD3 alone. The group with the higher average expression contained a majority of miR-15/16 ^{Δ/Δ} samples, and the only miR-15/16^{fl/fl} samples within the group received α CD3 + α CD28 stimulation. Within the higher expression group, the miR-15/16^{fl/fl} samples stimulated with α CD3 + α CD28 sub-clustered with miR-15/16 ^{Δ/Δ} samples stimulated with α CD3 alone (Fig 3J). A third, much smaller cluster was composed of both samples from a single outlier biological replicate. Overall, these data show that miR-15/16 restrict costimulation-responsive gene expression, and that Malat1:miR-15/16 interaction limits this effect.

In addition to the proximal changes in gene expression, downstream functional outcomes of CD28 costimulation were also affected. Early activation genes have been well described in T cells, with CD69 responding to many cues including TCR and CD28 ligation, and Nur77 responding very specifically to TCR signals (Ashouri and Weiss, 2017; Vandenberghe et al., 1993). In similar fashion as above, we assessed the expression of these proteins 2 and 4 hours after stimulation with plate bound cross linking antibodies on primary CD8⁺ T cells. CD69 exhibited decreased expression in Malat1^{scr/scr} cells at both 2 and 4 hours post stimulation (2 hr: $p=0.0014$ and 4 hr: $p=0.0598$) and increased expression in miR-15/16 ^{Δ/Δ} cells at 4 hours post stimulation only ($p < 0.0001$) (Fig 4 A-B), in accordance with costimulation-responsive gene module expression (Fig 3). There was no difference, however, in expression of Nur77 across Malat1^{scr/scr}, WT, and miR-15/16 ^{Δ/Δ} cells, indicating that TCR signals were equivalent (Fig 4 A,C). Another key consequence of CD28 ligation is the production of IL-2 (Fraser et al., 1991; Maggirwar et al., 1997; Vandenberghe et al., 1993). Therefore, we assessed cytokine production in the supernatants of these cultures 16 hours after stimulation. In line with previous findings in LCMV-infected mice (Gagnon et al., 2019), miR-15/16 ^{Δ/Δ} cells made significantly more IL-2 and TNF α after α CD3 stimulation both with and without α CD28, with a trend toward increased IFN γ as well (Fig 4D-F). Malat1^{scr/scr} cells stimulated with α CD3+ α CD28 exhibited a reciprocal trend specifically for IL-2 (23% decrease compared to WT, $p=0.26$) (Fig 4D). Together, these data show that the Malat1:miR-15/16 circuit regulates key functional outcomes of CD28-mediated costimulation, from proximal gene expression changes to early activation protein expression and cytokine secretion.

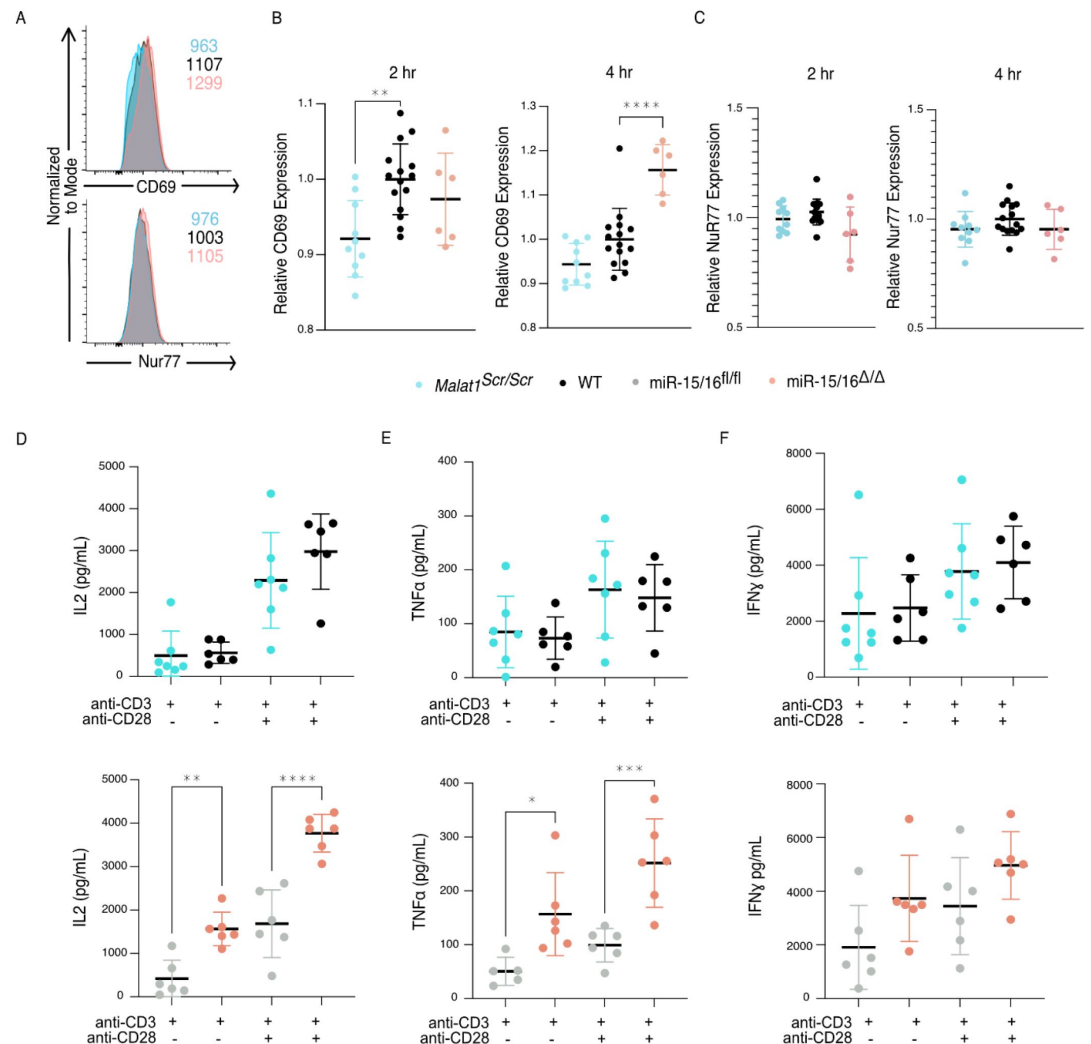


Figure 4.

The Malat1-MiR-15/16 circuit increases functional outcomes of CD28 co-stimulation

(A-C) CD8⁺ T cells were isolated from spleens and stimulated with αCD3 and αCD28 antibodies, results displayed are gated on activated cells (CD69⁺ Nur77⁺)

(A) Representative histograms of CD69 and Nur77 expression 4 hours after stimulation

(B) Quantification of CD69 mean fluorescence intensity 2 and 4 hours after stimulation. Both time points reflect statistically significant changes ($p < 0.01$) by ordinary one-way ANOVA; statistics displayed on graph represent results of post-hoc multiple comparisons of *Malat1^{Scr/Scr}* to WT and *miR-15/16^{Δ/Δ}* to WT. Data from 2 independent experiments, each normalized to WT average value.

(C) Quantification of Nur77 mean fluorescence intensity 2 and 4 hours after stimulation. No significant changes determined by ordinary one-way ANOVA. Data from 2 independent experiments, each normalized to WT average value.

(D-F) Quantification of cytokine secretion into the supernatant by CD8⁺ T cells isolated from spleens, stimulated αCD3 ± αCD28, and cultured 16 hours. Cell free supernatant protein concentration measured by ELISA. Data from a single experiment

(D) Quantification of IL-2 secretion. By 2-way ANOVA, in both experiments there was a significant ($p < 0.0001$) increase in IL-2 with the addition of α CD28 stimulation. But the only significant ($p = 0.0001$) genotypic effect was increased IL-2 secretion in $\text{miR-15/16}^{\Delta/\Delta}$ vs $\text{miR-15/16}^{\text{fl/fl}}$. Comparisons shown on plot are the results of post-hoc multiple comparison tests.

(E) Quantification of TNF α secretion. By 2-way ANOVA, in both experiments there was a significant ($p < 0.0001$) increase in IL-2 with the addition of α CD28 stimulation. But the only significant ($p = 0.003$) genotypic effect was increased IL-2 secretion in $\text{miR-15/16}^{\Delta/\Delta}$ vs $\text{miR-15/16}^{\text{fl/fl}}$. Comparisons shown on plot are the results of post-hoc multiple comparison tests.

(F) Quantification of IFN γ secretion. By 2-way ANOVA, in both experiments there was a significant ($p < 0.0001$) increase in IL-2 with the addition of α CD28 stimulation. But no genotypic effect was observed.

(*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$)

Malat1 enhances Cytotoxic Memory T Cell Differentiation

miR-15/16 restrict memory T cell differentiation, cell cycle, and cell survival during the response to LCMV Armstrong infection (Gagnon et al., 2019 [\[1\]](#)). CD28 costimulation is essential for IL-2 production, memory cell formation, and memory recall responses *in vivo* (Borowski et al., 2007 [\[2\]](#); Eberlein et al., 2012-2; Fuse et al., 2008 [\[3\]](#); Grujic et al., 2010 [\[4\]](#); Suresh et al., 2001 [\[5\]](#)). IL-2 is also essential for these same processes and in particular is required in a CD8^+ T cell intrinsic nature for the formation of CD127^+ KLRG1^- memory cells (Blattman et al., 2003 [\[6\]](#); Kahan et al., 2022 [\[7\]](#); Pipkin et al., 2010 [\[8\]](#); Toumi et al., 2022 [\[9\]](#); Whyte et al., 2022 [\[10\]](#)). Since Malat1 inhibits miR-15/16 activity and this circuit impacts proper T cell activation and IL-2 production after CD28 costimulation, we hypothesized that $\text{Malat1}^{\text{scr/scr}}$ cells would exhibit poor memory formation and survival.

We first examined steady state memory populations of polyclonal T cells in unchallenged, young mice. $\text{Malat1}^{\text{scr/scr}}$ mice had normal naive and central memory T cell populations, but a reduced percentage and number of effector memory (CD44^+ CD62L^-) cells in the spleen (Fig S4). Therefore, we investigated the cell intrinsic nature of the $\text{Malat1}^{\text{scr/scr}}$ memory cell impairment during a viral challenge known to induce a large memory response. We bred the $\text{Malat1}^{\text{scr/scr}}$ mice with P14 TCR transgenic mice that express an antigen receptor specific for the immunodominant LCMV GP33 peptide. We then transferred $\text{Malat1}^{\text{scr/scr}}$ and WT P14 T cells into congenic CD45.1 hosts followed by LCMV Armstrong infection and tracked the acute and memory responses in the spleen and liver, the sites of primary LCMV infection (Figure 5A [\[11\]](#)) (Matloubian et al., 1993 [\[12\]](#)).

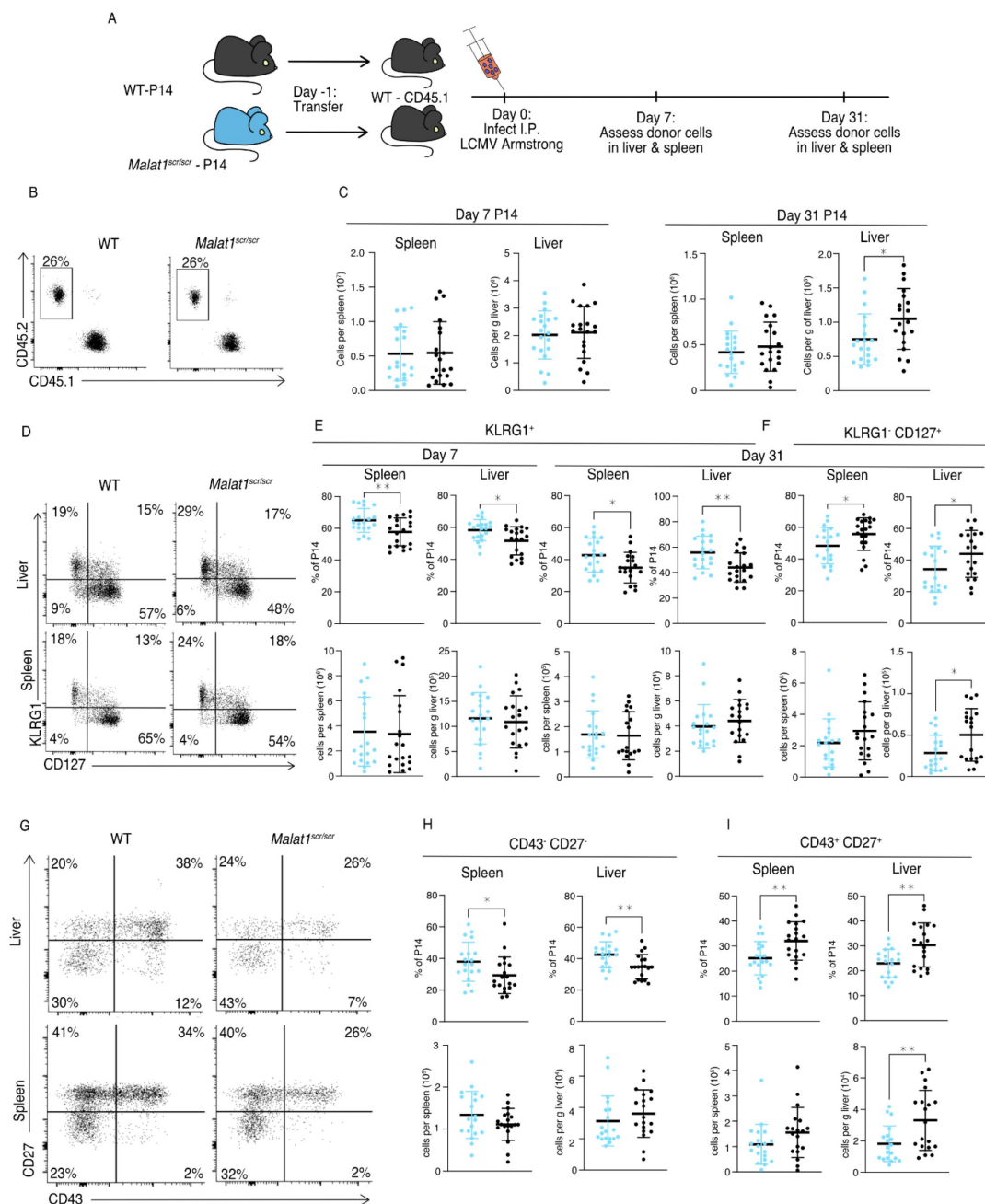


Figure 5.

Malat1 enhances memory T cell persistence following LCMV infection

$Malat1^{scr/scr}$ and WT cells containing the GP33 specific TCR transgene (P14) on the CD45.2 background were transferred separately into congenic CD45.1 WT hosts. One day later the recipient mice were infected with 5×10^5 p.f.u. I.p. lcmv armstrong. LCMV specific responses were assayed by monitoring the transferred cells by flow cytometry in the blood, spleen, and liver over time (data from two independent experiments per time point)

(A) Schematic of experimental design

(B) Representative flow plots to identify and quantify transferred cells

(C) Quantification of transferred P14 cell numbers at day 7 and day 31

(D) Representative flow plots of KLRG1 and CD127 expression on P14 cells at day 31 post infection

(E) Quantification of P14 KLRG1⁺ cells by percent of P14 and total numbers in spleen and liver at day 7 and day 31 post infection

(F) Quantification of P14 KLRG1⁺ CD127⁺ by percent of P14 and total numbers in spleen and liver at day 31 post infection

(G) Representative flow plots of CD43 and CD27 expression on P14 cells at day 31 post infection

(H) Quantification of P14 CD43⁺ CD27⁺ t-Tem cells by percent of P14 and total numbers in spleen and liver at day 31 post infection

(I) Quantification of P14 CD43⁺ CD27⁺ memory cells by percent of P14 and total numbers in spleen and liver at day 31 post infection

Statistics displayed determined by unpaired t-test between *Malat1*^{scr/scr} and WT transferred cells (*, p<0.05; **, p<0.01)

The total numbers of P14 cells were similar in recipients of transferred WT or *Malat1*^{scr/scr} P14 cells in both the spleen and liver at day 7, and in the spleen at day 31. However, there was a reduction in *Malat1*^{scr/scr} P14 cells in the liver at day 31 (Fig 5 B-C). Despite having no effect on cell expansion at the peak of infection and mixed cell number results in the memory phase, the *Malat1*^{scr} allele had a distinct effect on the cellular phenotype across organs and time points. *Malat1*^{scr/scr} P14 cells preferentially displayed a phenotype associated with terminally differentiated effector memory cells (t-TEM), defined by KLRG1 expression and lack of CD127 expression as shown by (Milner et al., 2020), and a corresponding reduction in the percentage of KLRG1⁺ CD127⁺ memory cells (Fig 5 D-F). Previous work has shown that substantial heterogeneity exists in the memory pool and that CD27 and CD43 can be useful in delineating functional differences such as recall potential between different memory cells. For instance, CD27⁺ memory cells tend to produce more IL-2 than CD27⁺ cells (Milner et al., 2020), and CD43⁺ CD27⁺ cells are more effective at clearing *Listeria* upon re-challenge (Hikono et al., 2007; Olson et al., 2013). We therefore assessed these markers on the transferred *Malat1*^{scr/scr} and WT P14 cells. Consistent with increased KLRG1⁺ CD127⁺ t-TEM cells there were proportionally more CD27⁺ CD43⁺ cells in both the spleen and liver at day 31 among the *Malat1*^{scr/scr} transferred cells compared to WT (Fig 5 G,I). CD27⁺ CD43⁺ cells were unchanged. The proportional reduction in CD127⁺ KLRG1⁺ cells corresponded with a decreased proportion of CD43⁺ CD27⁺ cells. Notably, the total numbers of all CD27⁺ subsets were unchanged. The proportional differences we observed were primarily driven by a reduction in the number of CD43⁺ CD27⁺ *Malat1*^{scr/scr} P14 cells compared with wild type P14 cells (45% reduction in liver (p = 0.0058), 30% reduction in spleen (p = 0.113)) (Fig 5 G-I). This phenotype is exactly reciprocal to the increase in CD27⁺ memory cells previously documented in miR-15/16^{Δ/Δ} mice (Gagnon et al., 2019). To test whether the effect of the *Malat1*^{scr} allele was epistatic to miR-15/16, we bred *Malat1*^{scr/scr} mice to miR-15/16^{Δ/Δ} mice to generate triple mutant *Malat1*^{scr/scr} miR-15/16^{Δ/Δ} mice. The poly-clonal LCMV response in these mice phenocopied that of miR-15/16^{Δ/Δ} with a WT allele of *Malat1*, indicating that the observed effects of the *Malat1*^{scr} allele are epistatic to miR-15/16 (Fig S5). We conclude that *Malat1* inhibits miR-15/16 in responding CD8⁺ T cells during LCMV infection, leading to fewer CD43⁺ CD27⁺ memory cells.

Compared to LCMV, *Listeria monocytogenes* (LM) infection induces lower expression of multiple co-stimulatory ligands so the antigen specific response is more sensitive to the ablation of CD28 co-stimulation (Welten et al., 2015). Therefore, we hypothesized that cytotoxic T cell response may be more impaired by the *Malat1*^{scr} allele during LM infection. We first sought to understand the effect of miR-15/16 on cytotoxic T cells during primary LM infection. To do so, we directly infected polyclonal miR-15/16^{fl/fl} and miR-15/16^{Δ/Δ} mice with LM expressing the LCMV GP33 peptide (LM-GP33) (Fig 6A). In contrast to the increased antigen-specific CD8⁺ T cell numbers in LCMV-

infected miR-15/16^{Δ/Δ} mice (Gagnon et al., 2019 [↗](#)), we observed similar cell numbers in LM-GP33 infected miR-15/16^{Δ/Δ} and miR-15/16^{fl/fl} mice (**Fig 6B** [↗](#)). However, the effect on memory cell differentiation was still present with decreased percentages of CD127⁺ KLRG1⁺ t-TEMs and increased percentages of CD127⁺ KLRG1⁺ memory cells (**Fig 6** [↗](#) C,D). Again, miR-15/16^{Δ/Δ} antigen specific cells had a significantly lower proportion of CD43⁺ CD27⁺ cells and a trend toward proportionally more CD43⁺ CD27⁺ cells (p = 0.127) (**Fig 6** [↗](#) E,F).

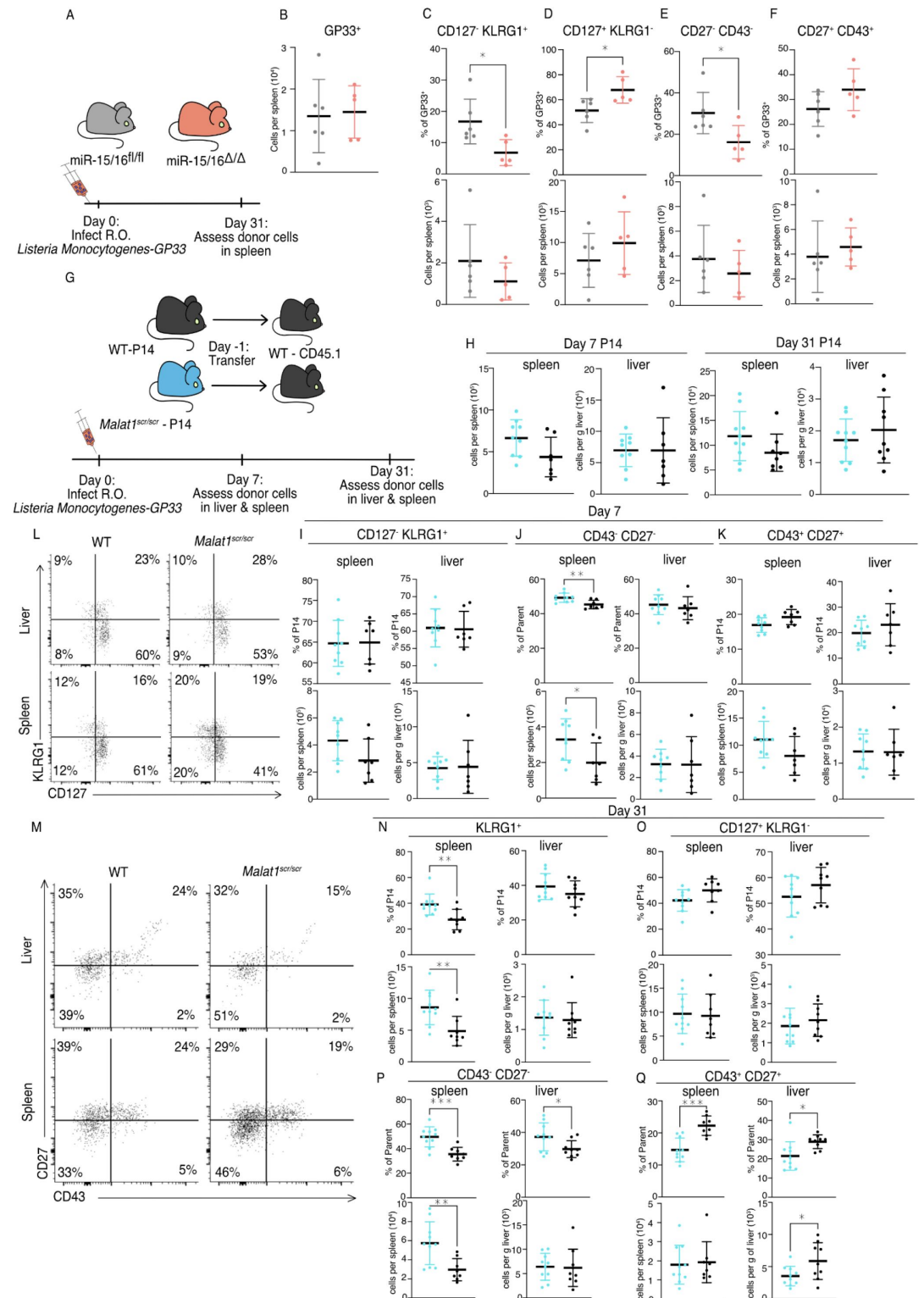


Figure 6.

Malat1 and miR-15/16 alter memory T cell differentiation following *Listeria Monocytogenes* infection

miR-15/16^{fl/fl} and *miR-15/16^{Δ/Δ}* with a polyclonal TCR repertoire were directly infected with 2×10^4 colony forming units (c.f.u.) r.o. *Listeria monocytogenes*-gp33 (LM-GP33). LM-GP33 specific responses were then assayed in the spleen 31 days post infection (*miR-15/16^{Δ/Δ}* n = 5 and *miR-15/16^{fl/fl}* n = 6 from a single experiment).

(A) schematic of experimental design

(B) Quantification of tetramer specific CD8 T cells in the spleen

(C-F) Quantification of tetramer specific subpopulations by percent of GP33+ and numbers for (C) CD127⁻ KLRG1⁺, (D) CD127⁺ KLRG1⁺, (E) CD27⁻ CD43⁺, and (F) CD27⁺ CD43⁺ populations *Malat1*^{scr/scr} and WT cells containing the GP33 specific TCR transgene (P14) on the CD45.2 background were transferred separately into congenic CD45.1 WT hosts. One day later the recipient mice were infected with 2*10⁴ c.f.u. r.o. LM-GP33. LM-GP33 specific responses were assayed by monitoring the transferred cells by flow cytometry in the spleen and liver at discrete time points (data from a single experiment per time point).

(G) Schematic of experimental design

(H) Quantification of transferred P14 cell numbers at day 7 and day 31

(I-K) Quantification of P14 (I) CD127⁻ KLRG1⁺, (J) CD43⁻ CD27⁻, and (K) CD43⁺ CD27⁺ cells by percent of P14 and total numbers in spleen and liver at day 7

(L) Representative flow plots of KLRG1 and CD127 expression on P14 cells at day 31 post infection

(N) Representative flow plots of CD43 and CD27 expression on P14 cells at day 31 post infection

(N-Q) Quantification of P14 (N) KLRG1⁺, (O) CD127⁺ KLRG1⁺, (P) CD43⁻ CD27⁻, and (Q) CD43⁺ CD27⁺ cells by percent of P14 and total numbers in spleen and liver at day 31 post infection

Statistics displayed determined by unpaired t-test between *Malat1*^{scr/scr} and WT transferred cells (*, p<0.05; **, p<0.01; ***, p<0.001)

Given that antigen-specific miR-15/16^{Δ/Δ} T cells exhibited enhanced memory cell differentiation during LM-GP33 infection, we further tested the role of Malat1:miR-15/16 interaction in this model using the P14 adoptive transfer system (**Fig 6F**). Transferred *Malat1*^{scr/scr} and WT P14 cells accumulated in the spleen and the liver to similar numbers at both day 7 and day 31 following LM-GP33 infection (**Fig 6H**). In contrast to LCMV, there was no change in the proportion or number of CD127⁻ KLRG1⁺ *Malat1*^{scr/scr} P14 cells at day 7 in the spleen or liver (**Fig 6I**). However, in LM-GP33 infection at day 7, CD43 and CD27 expression didn't explicitly mirror the phenotype observed by CD127 and KLRG1 expression. In the spleen, there was a proportional and numerical increase in CD43⁻ CD27⁻ P14 cells with the *Malat1*^{scr/scr} genotype (**Fig 6J**). There was a decreased proportion of CD43⁺ CD27⁺ cells (p = 0.0531), but this was despite a trend towards increased numbers of these cells (p = 0.1056) in the *Malat1*^{scr/scr} P14 cells (**Fig 6K**). In the liver, there were no significant trends in any of these populations at day 7 (**Fig 6J,K**).

Although some of the phenotypes observed with LCMV were attenuated or absent during acute infection with LM-GP33 at day 7, Malat1:miR-15/16 interaction had more pronounced effects on memory cell populations at later times post-infection (**Fig 6L,M**). On day 31, KLRG1⁺ cells were increased in proportion and numbers in the *Malat1*^{scr/scr} P14 cells (**Fig 6N**). In the liver, a similar trend in KLRG1⁺ cell proportion existed (p = 0.233), but the numeric effect was entirely absent (**Fig 6N**). Surprisingly, this effect was restricted to the KLRG1⁺ populations, as CD127⁺ KLRG1⁺ memory cells were unaffected in proportion and number in both the spleen and liver (**Fig 6O**). In both organs CD43⁻ CD27⁻ t-TEMs were proportionally increased and CD43⁺ CD27⁺ memory cells were proportionally decreased in *Malat1*^{scr/scr} P14 cells (**Fig 6P,Q**). The numeric underpinnings of these proportional changes were different in each organ, with a 94% increase in CD43⁻ CD27⁻ cells in the spleen, and a 40% loss of the CD43⁺ CD27⁺ population in the liver (**Fig**

6P,Q [↗](#)). Taken together, these data demonstrate that miR-15/16 restrict memory cell differentiation, and reveal the ability of Malat1:miR-15/16 interaction to enhance memory cell differentiation across infection contexts.

Malat1 Enhances Cytotoxic T Cell IL-2 Production and Survival

Finally, we investigated how the Malat1:miR-15/16 RNA circuit regulates memory cell differentiation and accumulation, giving consideration to the many direct targets of miR-15/16 and the emergent indirect effects on IL-2 and other costimulation-responsive gene expression in CD8 T cells. The pro-survival protein Bcl2 is the first characterized target of miR-15/16 (Cimmino et al., 2005 [↗](#)). Higher abundance of Bcl2 in memory cells (as compared with short-lived effector cells) aids their survival and persistence, counteracting their increased expression of pro-apoptotic factors such as Bim (Kurtulus et al., 2011 [↗](#)). As such, changes in the balance of pro- and anti-apoptotic factors can have selective effects on the accumulation of memory T cells. Bcl2 expression in *Malat1^{scr/scr}* P14 cells was reduced at day 31 in the CD43⁺ CD27⁺ population in both LCMV and LM-GP33 infection, and also in CD43⁺ CD27⁺ cells in LCMV infection (Fig 7A-C [↗](#)). This reduction is consistent with increased direct miR-15/16 action on the Bcl2 3' UTR in the *Malat1^{scr/scr}* cells, but it may also be affected by IL-2 and other co-stimulation responsive factors. Also consistent with reduced Bcl2 expression, increased proportions of dead *Malat1^{scr/scr}* cells were observed in both the KLRG1⁺ and KLRG1[−] cell fractions at day 7 during LM-GP33 infection (Fig 7 [↗](#) D,E). Very few dead cells were detected among P14 cells of either genotype at day 31.

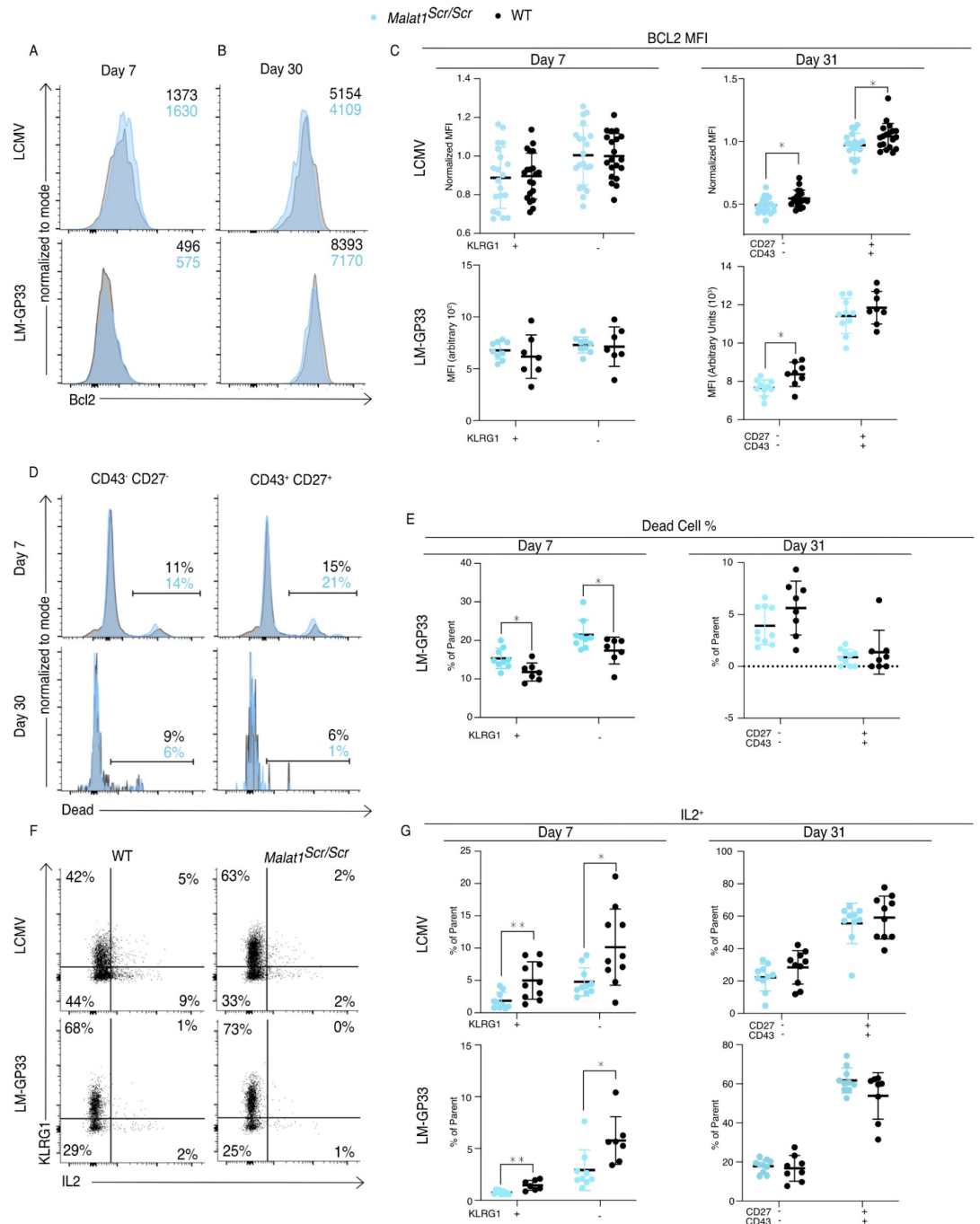


Figure 7.

Malat1 Enhances Pro-survival Cues Downstream of T cell activation

Malat1^{Scr/Scr} and WT cells containing the GP33 specific TCR transgene (P14) on the CD45.2 background were transferred separately into congenic CD45.1 WT hosts. One day later the recipient mice were infected with 2×10⁵ p.f.u. I.p. lcmv armstrong or 2×10⁴ c.f.u. r.o. LM-GP33. Antigen specific responses were assayed by monitoring the transferred cells by flow cytometry in the spleen.

(A-C) Bcl2 expression in transferred P14 cells in the spleen 7 and 31 days post infection for both LCMV and LM-GP33. Data from two independent experiments per LCMV time point and a single experiment per LM-GP33 time point.

(A) Representative flow cytometry plots of P14 KLRG1⁺ CD127⁺ cell Bcl2 expression 7 days post infection. Numbers shown are mean fluorescence intensity.

(B) Representative flow cytometry plots of P14 CD43⁺ CD27⁺ cell Bcl2 expression 31 days post infection. Numbers shown are mean fluorescence intensity.

(C) Quantification of Bcl2 expression producing cells by mean fluorescence intensity within the indicated P14 subpopulation defined by KLRG1 or CD27 and CD43.

(D-E) Analysis of dead cells within splenic P14 CD43 and CD27 subpopulations at days 7 and 31 post LM-GP33 infection, data from a single experiment per time point.

(D) Representative flow cytometry plots of P14 subsets defined by CD43 and CD27. Numbers shown represent percent of dead cells per the parent subpopulation

(E) Quantification of dead cells as a percentage of parent P14 subpopulation

(F-G) Analysis of IL-2 producing P14 subsets in the spleen via IL-2 capture assay at days 7 and 31 post infection for both LCMV and LM-GP33, data from a single experiment per infection per time point.

(F) Representative flow cytometry plots of all P14 cells stained for KLRG1 and captured IL-2 from both infections at day 7. Numbers represent percent of cells in that quadrant of all P14 transferred cells.

(G) Quantification of IL-2 producing cells by percent of parent within the indicated P14 subpopulation defined by KLRG1 or CD27 and CD43.

Statistics displayed determined by unpaired t-test between *Malat1*^{scr/scr} and WT transferred cells, where multiple tests were performed the Holm-Šidák method was used to correct for multiple comparisons (*, p<0.05; **, p<0.01)

IL-2 is critical for the induction of Bcl2 in memory T cells to promote their survival (Toumi et al., 2022 [4](#)). Furthermore, IL-2 production by cytotoxic T cells predisposes them to differentiate into a memory phenotype, likely by an autocrine/paracrine mechanism (Kahan et al., 2022 [4](#); Kurtulus et al., 2011 [4](#); Wojciechowski et al., 2007 [4](#)). Given the enhanced ability of miR-15/16^{Δ/Δ} cells to produce IL-2 and the trend toward impaired IL-2 production in *Malat1*^{scr/scr} cells in vitro (**Fig. 4D** [4](#)), we assessed IL-2 production by *Malat1*^{scr/scr} T cells in LCMV and LM-GP33-infected mice. Corroborating our in vitro observations, a lower proportion of *Malat1*^{scr/scr} P14 cells produced IL-2 when stimulated ex vivo on day 7 in both infection models (**Fig 7F,G** [4](#)). This defect was also shared by KLRG1⁺ and KLRG1⁻ populations and limited to this critical early time point. Higher proportions of memory cells were able to produce IL-2 ex vivo at day 31 after infection with either LCMV or LM-GP33 infection, and *Malat1*^{scr/scr} P14 cells produced equivalent amounts of IL-2 (**Fig 7H** [4](#)).

As CD28 co-stimulation is key to IL-2 production, the observed early defect is consistent with less robust activation while antigen is still present (Shapiro et al., 1997-7). Taken together, these results indicate that *Malat1*^{scr/scr} cells in the context of a viral or bacterial infection receive relatively poor activating cues and subsequently produce less IL-2 early during infection, contributing to a less robust pro-survival and pro-memory state. These findings illustrate how Malat1 regulation of miR-15/16 and its large target gene network can act through multiple connected nodes to coordinate gene expression programs essential to cytotoxic T cell responses.

lncRNAs are a large, diverse class of gene products that perform important physiological functions through a variety of molecular mechanisms. However, functional requirements are typically tested using RNAi to degrade the lncRNA or by disrupting their transcription entirely with no paradigm to dissect sequence specific functions. “Sponging” miRNAs (acting as a ceRNA) is among the most frequently proposed mechanisms of lncRNA function. Networks of noncoding ceRNAs, miRNAs, and target genes likely do shape gene expression programs in many biological contexts. Yet definitive evidence that a lncRNA:miRNA interaction has a physiological effect in a living organism has remained elusive. Guided by a biochemical approach, we investigated the requirements for Malat1 interaction with miR-15/16 by surgically altering just 5 nucleotides within the endogenous 8 kb Malat1 transcript in mice. Using T cells from these animals, we rigorously attribute changes in costimulation-responsive gene expression and in vivo defects in memory T cell formation to this Malat:miR-15/16 circuit.

Malat1 is a pleiotropic lncRNA implicated in a multitude of processes, including scaffolding splicing and epigenetic regulators, binding to chromatin, and interacting with several miRNAs in different cell types (Arun et al., 2020 [↗](#)). Its extremely high expression and interspecies conservation nominated Malat1 as a strong candidate to have an impact on the activity of even highly abundant miRNAs like miR-15/16 that have large effects on gene expression and cell behavior. Less abundant miRNAs can also serve essential functions (Wigton et al., 2021 [↗](#)), and they are likely to be more susceptible to inhibition by Malat1 and other ceRNAs. The same biochemically-driven sequence-specific approach used here could be applied to probe the physiological effects of other lncRNA:miRNA interactions, and it could also be extended to probe the requirement for interaction with Ezh2 and other proteins.

Another important future direction is to better understand how exactly Malat1 inhibits miR-15/16, given that TDMD does not appear to result from this interaction. Most Malat1 resides in the nucleus, whereas miRNAs reside and function in the cytoplasm. Advances in miRNA fluorescent in situ hybridization (miR-FISH) or the implementation of proximity-CLIP (Benhalevy et al., 2018 [↗](#)) may help clarify whether Malat1 nuclear sequestration plays a role in its ceRNA function. Detailed mechanistic understanding of this experimentally tractable circuit may illuminate how ceRNAs may be leveraged by the cell or by cellular engineers to manipulate the miRNA pool and target gene expression.

There is a growing interest in Malat1 function within the immune system, where it is highly expressed in many cell types and often regulated in interesting ways, including differential expression in short lived effector and memory precursor cells produced by asymmetric division of activated T cells during LCMV infection (Kakaradov et al., 2017 [↗](#)). Malat1 overexpression in dendritic cells promotes IL-10 production by T regulatory cells (Wu et al., 2018 [↗](#)), and *Malat1*^{-/-} mice exhibited reduced IL-10 and Maf expression by T helper cells in vivo and in vitro (Hewitson et al., 2020 [↗](#)). Two prior reports addressed Malat1 function in cytotoxic T cell responses. In one report, no significant differences were observed in *Malat1*^{-/-} mice (Yao et al., 2018 [↗](#)). It is difficult to interpret negative data generated from whole-body Malat1 deficiency given the multitude of cell types in which Malat1 may act. A second recent report, using RNAi to suppress Malat1 expression specifically in antigen-specific CD8⁺ T cells, detected enhanced t-Tem differentiation (Kanbar et al., 2022 [↗](#)). This observation, which contrasts with our findings using *Malat1*^{scr/scr} mice, was suggested to occur via an epigenetic mechanism involving Malat1 interaction with Ezh2. This mechanism and the ceRNA function that we describe here are not mutually exclusive, and it is possible to observe divergent phenotypes from the different interventions used to probe Malat1 function in T cells.

Our *Malat1^{scr/scr}* mouse combined with the cell transfer system clearly demonstrates a cytotoxic T cell intrinsic requirement for Malat1 to inhibit miR-15/16 to enhance activation and protect memory cell persistence. This is consistent with our previous work on the miR-15/16 family, and fitting with the increased expression of Malat1 in memory precursors (Gagnon et al., 2019 [DOI](#); Kakaradov et al., 2017 [DOI](#)). The present study builds upon these findings and provides detailed insight into the gene regulatory programs which may drive these phenotypes. Cytotoxic T cells lacking CD28 generate similar numbers of viral specific cells at the height of infection, but fail to maintain equivalent numbers during the memory phase (Borowski et al., 2007 [DOI](#)). Further, a major outcome of CD28 ligation is the production of IL-2, an essential T cell growth factor, and this IL-2 production is required in a cell intrinsic manner for generation of CD127⁺ memory cells (Kahan et al., 2022 [DOI](#)). Here we demonstrate that not only does the Malat1-miR-15/16 circuit regulate CD28 co-stimulation, IL-2 production, and Bcl2 expression, but also the concordant outcome of maintaining CD43⁺ CD27⁺ memory cells. miR-15/16 targets a large number of genes, so it is unlikely that the effect on Bcl2 or CD28 alone is entirely responsible for the observed changes in CD43⁺ CD27⁺ t-TEM and CD43⁺ CD27⁺ memory cells. Our results are consistent with memory cells requiring a heightened level of pro-survival cues to survive the contraction bottleneck after the peak of infection. In this manner elevated Malat1 levels can act as an enhancer of pro-survival and activating cues to prevent excessive cell death in this sensitive memory population. The magnitude of this effect was modest in acute LCMV and Listeria infection, two models that feature robust pathogen clearance, allowing assessment of memory T cells in the absence of chronic antigen persistence. Further work is needed to probe other settings in which Malat1:miR-15/16 interaction may have a bigger impact on the outcome of immune responses.

The importance of cytotoxic T cells is evident in the context of the global COVID-19 pandemic and the advent of CAR-T cell therapies reaching the clinic. Knowledge of the networks that regulate these cells and the critical nodes within these networks have the potential to augment technologies and therapies from vaccines to anti-tumor immunotherapy. Transcription factors and miRNAs have been extensively studied in this fashion as critical nodes in the regulation of gene transcription and translation. In the study presented here, we show Malat1 acts upstream of one such node, the miR-15/16 family. From this one interaction, Malat1 has the potential to combinatorially and synergistically regulate gene networks essential to cytotoxic T cells. This concept is easily extended when Malat1's ability to regulate multiple other miRNA families is considered. If Malat1's sequence specific function is further defined, then editing or expression of specific Malat1 sequences could be used to tune multiple miRNA families in concert while leaving other functions of this enigmatic transcript un-touched.

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A.M. is a cofounder of Arsenal Biosciences, Spotlight Therapeutics, and Survey Genomics, serves on the boards of directors at Spotlight Therapeutics and Survey Genomics, is a board observer (and former member of the board of directors) at Arsenal Biosciences, is a member of the scientific advisory boards of Arsenal Biosciences, Spotlight Therapeutics, Survey Genomics, NewLimit, Amgen and Tenaya, owns stock in Arsenal Biosciences, Spotlight Therapeutics, NewLimit, Survey Genomics, PACT Pharma, and Tenaya and has received fees from Arsenal Biosciences, Spotlight Therapeutics, NewLimit, 23andMe, PACT Pharma, Juno Therapeutics, Trizell, Vertex, Merck, Amgen, Genentech, AlphaSights, Rupert Case Management, Bernstein and ALDA. A.M. is an investor in and informal advisor to Offline Ventures and a client of EPIQ. The Marson laboratory has received research support from Juno Therapeutics, Epinomics, Sanofi, GlaxoSmithKline, Gilead and Anthem. M.H.S. is founder and a board member of Teiko.bio and has received a speaking honorarium from Fluidigm Inc., has served as a consultant for Five Prime, Ono, January, Earli, Astellas, and Indaptus, and has received research funding from Roche/Genentech, Bristol Myers Squibb, Valitor, and Pfizer. J.D.G. is now an employee of Arsenal Biosciences.

Materials and Methods

Mice

WT C57/BL6 mice were bred in our facility. miR-15/16^{Δ/Δ} and miR-15/16^{fl/fl} mice were derived as described in [Gagnon et al 2019](#). *Malat1*^{scr/scr} mice were generated from WT C57/BL6 mouse zygotes electroporated with CRISPR-Cas9 RNPs and HDR template (guide and template sequences below) as described previously ([Chen et al., 2016](#)). B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) strain #002014 were purchased from the Jackson Laboratory. WT P14 mice carrying the TCR transgene specific to the LCMV GP33 peptide were obtained from the Waterfield lab and were backcrossed to C57/BL6 to maintain the line. *Malat1*^{scr/scr} mice were bred to the P14 line to generate *Malat1*^{scr/scr} P14 mice. Male and female age and sex matched mice were used between 5 to 12 weeks of age. All mice were housed and bred in specific pathogen-free conditions in the Animal Barrier Facility at the University of California, San Francisco. Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Ago2 hits clip

CD8⁺ T cells were isolated as below and stimulated for 3 days with αCD3 and αCD28 antibodies and grown with kool aid complete media. Subsequently the cells were rested and expanded in kool aid complete media for 2 days supplemented with 100 U/mL recombinant Human IL-2 (R&D Systems Cat# 202-IL-010/CF). Subsequently, 1*10⁶ cells were used to prepare NGS libraries as described in ([Gagnon et al., 2019](#)) & ([Loeb et al., 2012](#)). Samples were sequenced on HI-Seq 2500 (Illumina). 11 nucleotide adaptors were trimmed from each read and resultant sequences were aligned to the mm10 genome using bowtie2 ([Langmead and Salzberg, 2012](#)). To assure lack of miR-15/16 binding in *Malat1*^{scr/scr} mice was not do to errors in alignment, reads from *Malat1*^{scr/scr} cells were aligned to a mm10 genome that contained a single modification changing Malat1 from the WT allele to the *Malat1*^{scr} allele. To determine maximum binding depth across the genome and to manipulate aligned files the samtools package was used ([Li et al., 2009](#)). To assess Ago2 binding, aligned HITS-CLIP reads were integrated across the follow genomic annotations: lncRNA genes from mouse Noncode v6 (<http://www.noncode.org/download.php>); miRNA target binding sites from TargetScan V7.2 (https://www.targetscan.org/cgi-bin/targetscan/data_download.mmu80.cgi); mm10 introns, exons, 3'UTRs, and coding genes from UCSC genome table browser (<http://genome.ucsc.edu/cgi-bin/hgGateway?db=mm10>). To assess differences in target binding at specific 3' UTR sites between WT and *Malat1*^{scr/scr} cells, reads

within each 3' UTR were normalized to the total reads contained in the given 3' UTR for each genotype. To identify regions of significant Ago2 binding above background, so-called “peaks”, we used the Piranha algorithm (<https://github.com/smithlabcode/piranha>) on our HITS-CLIP. To remove sites from analysis where confounding features were present, such as miRNA expression sites or rRNA repeats, bedtools intersect was used to remove these features from annotation files (Quinlan, 2014). Human AHC data from publicly available datasets were trimmed and aligned to the Hg38 genome as previously described (Karginov and Hannon, 2013; Li et al., 2018).

Cell Transfer and Infections

Spleens were harvested from WT or *Malat1^{scr/scr}* P14 CD45.2 mice into PBS and passed through 70 μ m strainers to generate a single cell suspension. Samples were then stained for live/dead (apc-cy7), Thy1.2 (BV605), CD8 α (apc), and TCRV α 2 (pe). Whole splenocytes were then transferred retro-orbitally (r.o.) into WT BoyJ (CD45.1) 7 week old male recipients such that each recipient received 20,000 Thy1.2⁺ CD8 α ⁺ TCRV α 2⁺ cells in 200 μ L PBS. To initiate LCMV infections, mice were injected intraperitoneally (i.p.) with 2*10⁵ plaque forming units (p.f.u.) LCMV armstrong in 200 μ L plain RPMI. LCMV virus was produced in house as described in (Shehata et al., 2018). To initiate *Listeria Monocytogenes* infection, mice were injected r.o. with 2*10⁵ colony forming units (c.f.u.) *Listeria Monocytogenes*-GP33 in 100 μ L PBS. *Listeria Monocytogenes*-GP33 was prepared in house as described in (Allen et al., 2020). Blood was collected via submandibular bleeds with goldenrod 4 mm lancets collected into sample tubes coated with K2 EDTA (BD Ref# 365974) and 60 μ L of blood was lysed with 500 μ L of ACK lysis buffer. To assay spleens and livers, mice were sacrificed and organs harvested. To assay intracellular cytokine production, splenocytes were plated into 96-well u-bottom plates in complete kool-AID media and stimulated for 6 hours with a final concentration of 0.2 mg/ml GP33-41 (KAVYNFATM) in the presence of Brefeldin A.

Flow Cytometry

Spleens and livers were harvested into 2% FBS PBS with and passed through 70 μ m strainers to generate a single cell suspension. Samples were then spun at 450 r.c.f. for 5 minutes and livers were resuspended in 20% Percoll. Percoll suspensions were spun at 741 r.c.f. for 20 minutes at 25°C and the supernatants discarded. Cell pellets for both spleens and livers were resuspended in 1 mL ACK lysis buffer and incubated at 4°C for 5 minutes. Lysis was stopped with 5 mL 2% FBS PBS and samples again spun at 450 r.c.f. for 5 minutes. Samples were resuspended in 2% FCS PBS and aliquoted into v-bottom 96 well plates. Live dead staining was then performed using the fixable viability dye at 1:2000 in PBS. Subsequently, cells were blocked with mouse Fc block 1:100 in 2% FCS PBS and stained for surface proteins with directly conjugated antibodies diluted 1:100 in 2% FCS PBS. Stains were incubated for 20 minutes at 4°C protected from light. For surface stains alone, LCMV samples were fixed with 4% PFA for 5 minutes at 4°C. For intracellular stains, samples were fixed and permeabilized according to the Transcription Factor Fixation Kit (Invitrogen Cat#00-5521-00). Intracellular antibodies were diluted in 1:100 in permeabilization buffer and incubated at 4°C for 30 minutes. All samples were spun at 821 r.c.f. for 5 minutes prior to being resuspended in 2% FCS PBS with 1:10 AccuCount beads (spherotech Cat #ACBP-100-10) for analysis on either the BD LSR II or the BD LSRFortessa flow cytometry analyzer.

Cell Isolation and In Vitro Functional Assays

CD8⁺ T cells were isolated from spleens using negative selection from the EasySep Mouse CD8⁺ T Cell Isolation Kit (cat# 19853). Cells were counted using trypan blue staining and the nexelcom cellometer spectrum. Cultures were started via stimulation with plate bound α CD3 (1 μ g/mL, clone 2C11) and α CD28 (1 μ g/mL, clone 37.51); plates coated overnight in PBS with Ca⁺⁺ & Mg⁺⁺ at 4°C. Cells were plated in Kool AID complete media (DMEM high glucose media supplemented with 10% FCS, pyruvate, nonessential amino acids, MEM vitamins, L-arginine, L-asparagine, L-glutamine, folic acid, beta mercaptoethanol, penicillin, and streptomycin) and spun at 450 r.c.f. for 5 minutes at 25°C to begin stimulation. For functional assays cells were harvested 2 or 4 hours after

stimulation for flow cytometry, or 24 hours after stimulation for mRNA-seq. For supernatant cytokine expression, plates were spun at 450 r.c.f. for 5 minutes at 4°C 16 hours after stimulation and cell free culture medium was collected and frozen at –80°C. TNFα (Cat #), IFNγ (Cat #), and IL-2 (Cat #) were analyzed by ELISA.

miRNA qPCR

Spleens were harvested and single cell suspensions generated by passing through 70µm strainers. CD8+ T cells were isolated as above, and 2×10^6 cells were pelleted at 450 r.c.f. for 5 minutes at 4°C. Cell pellet was resuspended in 700 µL Trizol reagent (Ambion cat #15596018) and kept at –80°C. RNA was isolated using the Direct-zol-96 RNA Kit (zymogen Cat #R2054). This RNA was used as input to the Mir-X™ miRNA qRT-PCR TB Green® Kit (Takara cat# 638316) to generate miRNA cDNA. Specific primers to miR-15a, miR-15b, and miR-16 were used to quantify those miRNA species on the QPCR MACHINE (eppendorf realplex²). ribosomalRNA 5.8s was used as a housekeeping control for each sample. Each sample was run in duplicate. To quantify miRNA expression technical duplicates were averaged and then normalized to rRNA 5.8s by subtracting the rRNA 5.8s Ct value from miRNA Ct value (Δ Ct). Expression values reported are generated by $2^{-\Delta$ Ct.

mRNA Sequencing

1×10^6 CD8+ T cells were harvested 24 hours after αCD3 ± αCD28 stimulation as described above. Cells were pelleted at 450 r.c.f. for 5 minutes at 4°C. Cell pellet was resuspended in 700 µL Trizol reagent (ambion ref #15596018) and kept at –80°C. RNA was isolated using the Direct-zol-96 RNA Kit (zymogen Cat #R2054). The integrity of total RNA was checked on Fragment Analyzer (Agilent, Cat. No. DNF-472), only RNA with RQN number of above 7 was used for library construction. The starting quantity of 100ng of total RNA was used according to vendor instructions with Universal plus mRNA with Nu Quant (TECAN, Cat. No. 0520), final library PCR amplification was 17 cycles. After library completion, individual libraries were pooled equally by volume, and quantified on Fragment Analyzer (Agilent, Cat. No. DNF-474). Quantified library pool was diluted to 1nM and further diluted as per protocol and sequenced on Illumina MiniSeq (Illumina, Cat. No. FC-420-1001) to check for quality of reads. Finally, individual libraries were normalized according to MiniSeq output reads, specifically by % protein coding genes and were sequenced on one lane of NovaSeq6000 S4 PE100 (Illumina, Cat. No. 20028313). Reads were aligned to the mouse genome GRCm38 and quantified using the STAR aligner software version 2.7.2b. Read normalization and differential expression analysis was performed in the R computing environment version 3.6.1 using the software DESeq2 version 1.26. For RNA sequencing analysis, FDR-corrected p-values were used to evaluate significant differences between experimental groups using a significance threshold of 0.05. Lowly expressed genes that had fewer than 2 reads per million on average across all samples were removed from the analysis. Empirical cumulative density plots were made using the ggplot package for R. Heatmaps and hierarchical clustering was performed via the gplots package for R. Gene ontology analysis was performed using the statistical overrepresentation test for Panther pathways (Mi et al., 2019 [\[19\]](#)).

Statistical and Analytical Software

All flow cytometry data was analyzed using FlowJo (version 10). Statistical analyses and plotting was performed using Graphpad Prism (Version 9.2.0) and R (version 4.2.1).

Data Availability and Software

Ago2 HITS-CLIP data uploaded to NCBI GEO accession #GSE216565. mRNA sequencing data uploaded to NCBI GEO accession #GSE216113.

Code used to analyze HITS-CLIP data summed over annotations and peaks in WT cells can be found at github.com/AnsellLab/WT_HITS_CLIP_Analyses.

Code used to analyze relative binding densities of HITS-CLIP reads can be found at github.com/AnselLab/Malat1_miR1516_AGO2_HITS_CLIP.

Code used to analyze mRNAseq data for both miR-15/16 target expression and CD28 responsive genes can be found at github.com/AnselLab/Malat1_miR1516_CD3CD28_RNAseq.

Reagent	Source	Detail	Catalog #
IL-2 ELISA	Invitrogen		5018280
TNFα ELISA	Invitrogen		BMS607-3
IFNγ ELISA	R&D Systems		MIF00
IL-2 Secretion Assay APC	Miltenyi Biotec		130-090-987

Malat1 CRISPR Guide 1	Dharmacon	Sequence - GCATTCTAATAGCAGCAGAT	
Malat1 HDRT Ultramer	IDT	Sequence - ACAGACCACACAGAATGCAGGTG TCTTGACTTCAGGTCATGTCTGTT CTTTGGCAAGTAATATGTGCAGTA CTGTTCCAATCTGTCCTGATTAGA ATGCATTGTGACGCGACTGGAGT ATGATTAAAGAAAGTTGTGTTTCC CCAAGTGTTTGGAGTAGTGGTTG TTGGAGGAAAAGCCATGAGTAAC AGGCTGAGTGTT	
anti-CD127 PE	invitrogen	Clone-A7R34	12-1271-83
anti-CD127 FITC	invitrogen	Clone-A7R34	11-1271-82
anti-CD45.2 BV785	BioLegend	Clone-104	109839
anti- CD45.1 Alexa Fluor 700	BioLegend	Clone-A20	110724
anti-CD90.2 BV605	BD Biosciences	Clone-30-H12	740334
anti-CD90.2 eFluor 450	eBiosciences	Clone-53-2.1	48-0902-80
anti-CD44 PE-Cy7	eBiosciences	Clone-IM7	25-0441-82
anti-CD27 APC	eBiosciences	Clone-LG.7F9	17-0271-82
anti-CD27 FITC	eBiosciences	Clone-LG.7F9	11-0271-82
anti-Bim PE	Cell Signaling	Clone-C34C5	12186S
anti-CD43 Percp-Cy5.5	BioLegend	Clone-1B11	121224
anti-CD45.2 PE-Cy7	BD Biosciences	Clone-104	560696
anti-CD45.1 BV785	BioLegend	Clone-A20	110743
anti-Bcl2 Alexa Fluor 647	BioLegend	Clone-BCL/10C4	633510
anti-Bim Alexa Fluor 700	Cell Signaling	Clone-C34C5	28997S
GP33 Tetramer PE	NIH Tetramer Core	Peptide: KAVYNFATM	57624
anti-KLRG1 BV711	BD Bioscience	Clone-2F1	564014
Fixable Viability Dye eFluor 780	eBioscience		65-0865-14

anti-CD8a BV805	BD Bioscience	Clone-53-6.7	612898
anti-CD28 FITC	BioLegend	Clone-E18	122008
anti-CD69 APC	Invitrogen	Clone-H1.2F3	17-0691-82
anti-Nur77 PE	Invitrogen	Clone-12.14	12-5965-80
anti-CD62L BV605	BD Biosciences	Clone-MEL-14	563252

Key Reagent Table

Supplemental Data & Figures

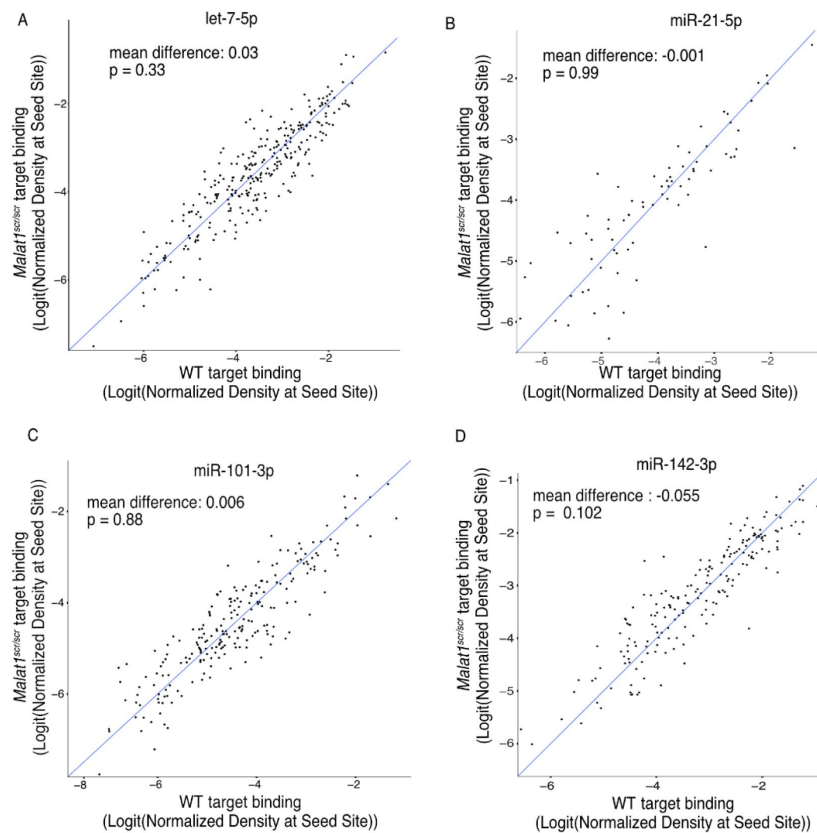


Figure S1.

***Malat1^{scor}* allele does not disrupt other miRNA families**

TargetScan predicted binding sites for highly expressed microRNA families in T cells that contained at least one HITS-CLIP read in both WT and Malat1^{scor/scr} CD8⁺ T cells were compared for depth of Ago2 HITS-CLIP reads. First, reads at the predicted seed site were normalized by total Ago2 HITS-CLIP reads in a given 3' UTR. To best visualize all sites, logit transforms of these values are plotted. Paired t-test performed to determine significance. Blue line indicates the identity line. Data for each genotype is from combined libraries of n = 2 biological replicates.

- (A) Predicted sites for let-7-5p
- (B) Predicted sites for miR-21-5p
- (C) Predicted sites for miR-101-3p
- (D) Predicted sites for miR-142-3p

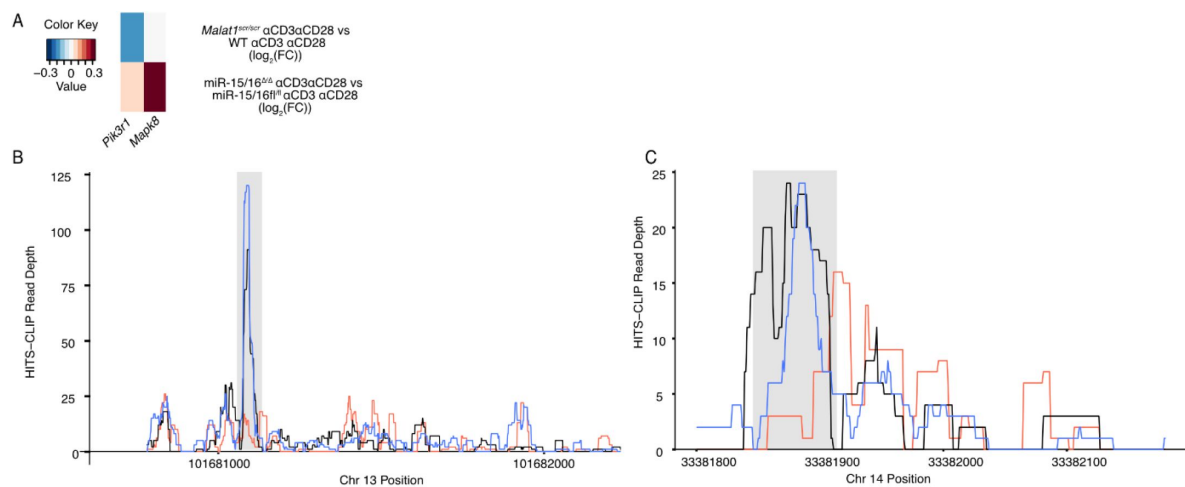


Figure S2.

AHC and gene expression analyses nominate direct miR-15/16 targets involved in growth signaling pathways

(A) Heat map indicating differential gene expression between WT and *Malat1^{scr/scr}* cells, and between miR-15/16^{fl/fl} cells and miR-15/16^{Δ/Δ} cells in RNA sequencing analyses.

(B-C) Histograms of AHC mapping of Ago2 binding in the 3' UTR of *Pik3r1* (B) and *Mapk8* (C) in WT (black), miR-15/16^{Δ/Δ} (red) and *Malat1^{scr/scr}* (blue) cells. As in **Figure 3**, AHC libraries were generated from CD8⁺ T cells isolated from spleens and cultured for 5 days (combined libraries from n = 2 for each genotype). Grey bars indicate the peaks containing TargetScan predicted miR-15/16 binding sites.

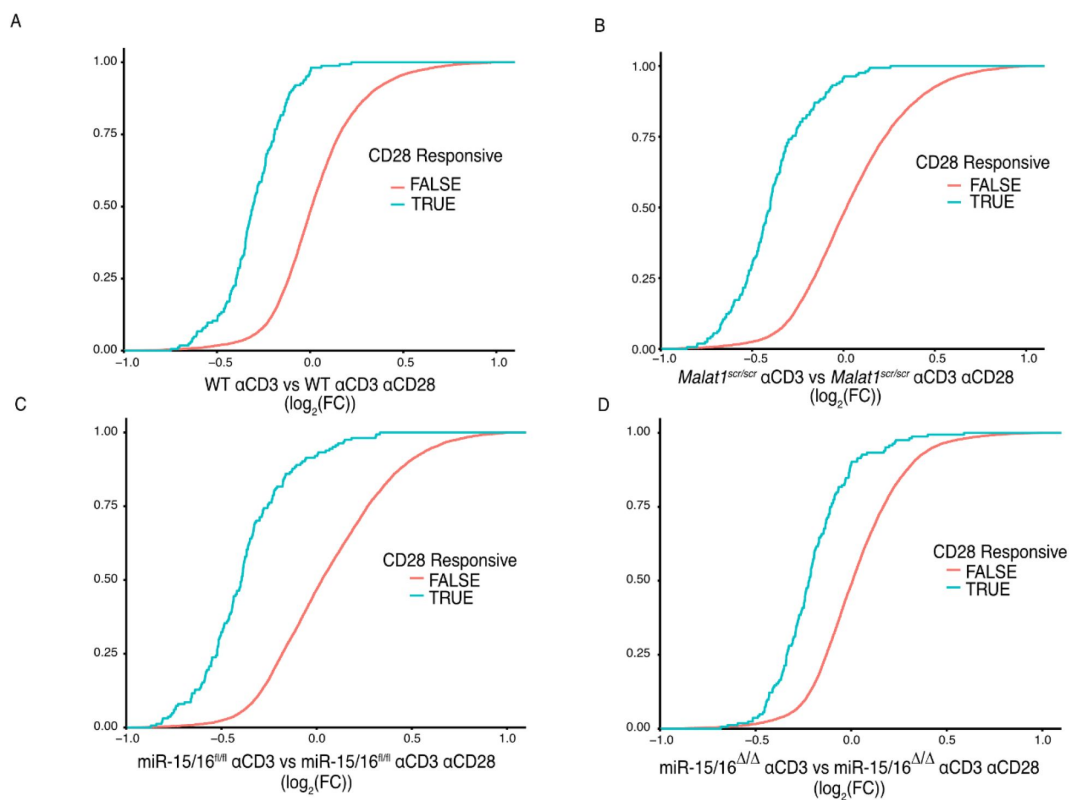


Figure S3.

CD28 responsive genes are induced by α CD28 stimulation in all genotypes tested

Cumulative density plots comparing expression of CD28 responsive gene set defined as genes from (Martínez-Llordella et al., 2013) with α CD3 α CD28 vs α CD3 $\log_2(\text{FC}) > 1.5$ and adjusted p value < 0.001 . Kolmogorov-Smirnov test used to determine significant differences in the distributions of target and non-target genes. α CD3 used at 1 $\mu\text{g}/\text{mL}$, and α CD28 used at 1 $\mu\text{g}/\text{mL}$. Data are from a single experiment with $n=6$ for each genotype and stimulation condition combination.

- (A) Comparison of CD28 responsive genes in WT cells
- (B) Comparison of CD28 responsive genes in *Malat1*^{scr/scr} cells
- (C) Comparison of CD28 responsive genes in miR-15/16^{fl/fl} cells
- (D) Comparison of CD28 responsive genes in miR-15/16 ^{Δ/Δ} cells

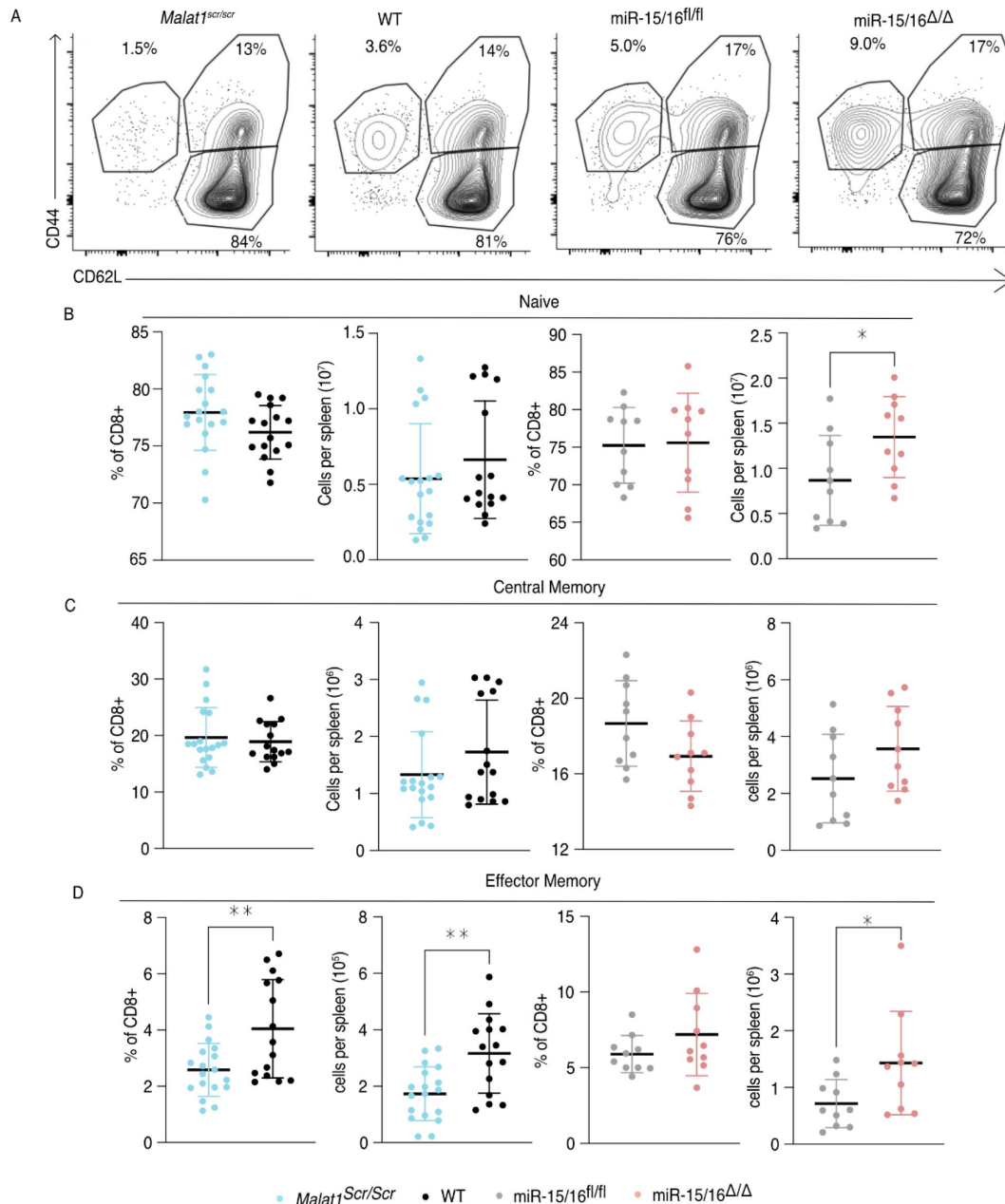


Figure S4.

Malat1 Regulates Memory Formation in Unchallenged Poly Clonal Animals

Cells were isolated from the spleens of young, age-matched, naive mice and analyzed by flow cytometry for CD44 and CD62L to delineate naive, effector memory, and central memory cells. Results shown are gated on CD8⁺ CD5⁺ lymphocytes. Data for *Malat1*^{scr/scr} and WT cells are from 3 independent experiments. Data for miR-15/16^{fl/fl} and miR-15/16^{Δ/Δ} cells are from 2 independent experiments. Statistics displayed determined by unpaired t-test between *Malat1*^{scr/scr} and WT cells or between miR-15/16^{fl/fl} and miR-15/16^{Δ/Δ} cells (*, p<0.05; **, p<0.01)

(A) Representative flow cytometry plots of CD44 and CD62L for each genotype assayed. Percentages shown are of CD8⁺ population.

(B) Quantification of naive cells (CD62L⁺ CD44⁺)

(C) Quantification of central memory cells (CD62L⁺ CD44⁺)

(D) Quantification of effector memory cells (CD62L⁺ CD44⁺)

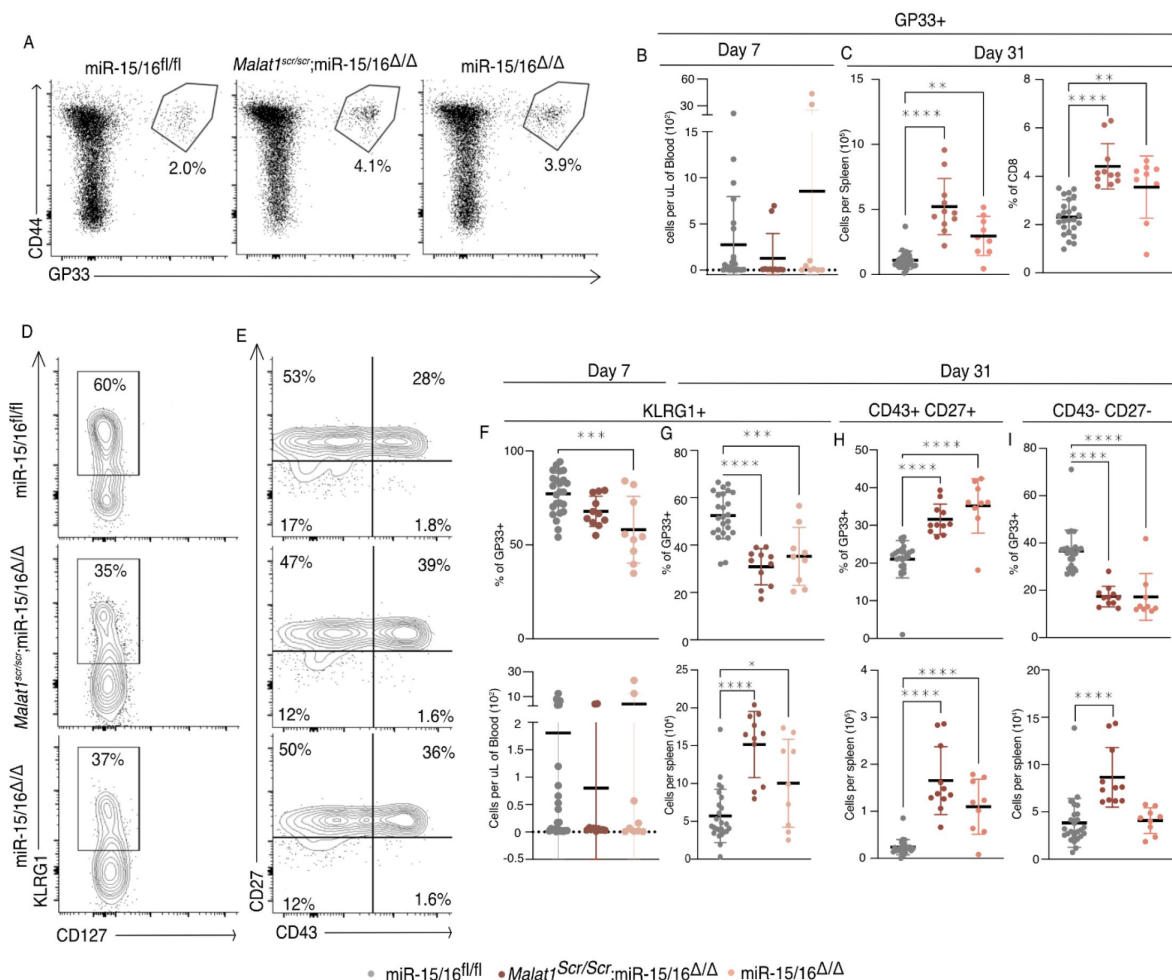


Figure S5.

Malat1 is epistatic to miR-15/16 in the regulation memory cell expansion following LCMV infection

Polyclonal mice of the *miR-15/16^{fl/fl}*, *miR-15/16^{Δ/Δ}*, and *Malat1^{scr/scr};miR-15/16^{Δ/Δ}* genotypes were directly infected i.p. with 5×10^5 p.f.u. I.p. lcmv armstrong. Antigen specific responses were tracked in the blood and spleen using the GP33 tetramer at day 7 and day 31 post infection.

(A) Representative flow cytometry plots illustrating the gating on antigen specific cells using CD44 and GP33

(B) Quantification of GP33+ cells in the blood at day 7

(C) Quantification of GP33+ cells in the spleen at day 31 by absolute numbers and percent of the CD8+ T cell population

(D) Representative flow cytometry plots of KLRG1 and CD127 expression at day 31 in the antigen specific cell population

(E) Representative flow cytometry plots of CD27 and CD43 expression at day 31 in the antigen specific cell population

(F) Quantification of KLRG1+ antigen specific cells in the blood at day 7 by relative percentage and absolute numbers

(G) Quantification of KLRG1+ antigen specific cells in the spleen at day 31 by relative percentage and absolute numbers

(H) Quantification of CD43+ CD27+ memory cells in the spleen at day 31 by relative percentage and absolute numbers

(I) Quantification of CD43- CD27- t-Tem cells in the spleen at day 31 by relative percentage and absolute numbers

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Reviewer #1 (Public Review):

Wheeler et al. have discovered a new RNA circuit that regulates T-cell function. They found that the long non-coding RNA Malat1 sponges miR-15/16, which controls many genes related to T cell activation, survival, and memory. This suggests that Malat1 indirectly regulates T-cell function. They used CRISPR to mutate the miR-15/16 binding site in Malat1 and observed that this disrupted the RNA circuit and impaired cytotoxic T-cell responses. While this study presents a novel molecular mechanism of T-cell regulation by Malat1-miR-15/16, the effects of Malat1 are weaker compared to miR-15/16. This could be due to several reasons, including higher levels of miR-15/16 compared to Malat1 or Malat1 expression being mostly restricted to the nucleus. Although the role of miR15/16 in T-cell activation has been previously published, if the authors can demonstrate that miR15/16 and/or Malat1 affect the clearance of Listeria or LCMV, this will significantly add to the current findings and provide physiological context to the study.

Reviewer #2 (Public Review):

This study connects prior findings on MicroRNA15/16 and Malat1 to demonstrate a functional interaction that is consequential for T cell activation and cell fate.

The study uses mice (Malat1scr/scr) with a precise genetic modification of Malat1 to specifically excise the sites of interaction with the microRNA, but sparing all other sequences, and mice with T-cell specific deletion of miR-15/16. The effects of genetic modification on in vivo T-cell responses are detected using specific mutations and shown to be T-cell intrinsic.

It is not known where in the cell the consequential interactions between MicroRNA15/16 and Malat1 take place. The authors depict in the graphical abstract Malat1 to be a nuclear lncRNA.

Malat 1 is very abundant, but it is unclear if it can shuttle between the nucleus and cytoplasm. As the authors discuss future work defining where in the cell the relevant interactions take place will be important.

In addition to showing physiological phenotypic effects, the mouse models prove to be very helpful when the effects measured are small and sometimes hard to quantitate in the context of considerable variation between biological replicates (for example the results in Figure 4D).

The impact of the genetic modification on the CD28-IL2- Bcl2 axis is quantitatively small at the level of expression of individual proteins and there are likely to be additional components to this circuitry.

Author Response

Review 1:

Major concerns that need to be addressed:

Investigate the effects of Malat1 on the clearance of Listeria or LCMV.

In our prior publication (Gagnon et al, Cell Reports) we showed that miR-15/16 deficiency in T cells does not affect the clearance of LCMV, and that transferred memory T cells formed in these mice can function normally to clear a secondary infection with Listeria expressing the LCMV gp33 peptide. However, the size of the memory pool was clearly changed, as was the programming of memory cells. Here, we show that disrupting miR15/16 binding to MALAT1 induces a reciprocal phenotype, validating a biological function for this RNA:RNA interaction. We employed these systems because they are widely used to reveal key aspects of T cell memory, but both infections are readily cleared by the host. These changes in the memory response likely play a limiting role in some biological context(s), and we agree that further investigation to uncover such situations would further validate the importance of this RNA circuit.

Demonstrate that Malat1 shuttles to the cytosol, this will strengthen the conclusions that Malat1 sponges miR15/16.

The location of miR-15/16 interaction with Malat1 is an interesting area for future study. Many prior studies have shown clearly that Malat1 is primarily located in the nucleus, but since T cells express such a large excess of this lncRNA, even the remaining fraction detected in the cytosol may be sufficient to “sponge” a significant amount of miR-15/16. Alternatively, these molecules may interact in the nucleus, or during mitosis. As the reviewer suggests, Malat1 may shuttle between compartments, raising the intriguing possibility that it could not only “sponge” but “drag” miR-15/16 away from its targets into the nucleus. A proper analysis of the mechanism of ceRNA function is beyond the scope of this paper, but we do believe that this circuit may be an especially good one for further study.

Through flow cytometry or immunoblot analyses, investigate the effects of Malat1-miR15/16 on genes listed in table 3. This would add credence to the sequencing and CLIP data.

We thank the reviewer for bringing to our attention the manuscript’s overemphasis on the former Table 3 gene set, which represented just a few of the hundreds of genes for which our data provide evidence for miR-15/16 binding and inhibition of expression. We have removed this table to avoid the appearance of suggesting an oversimplified model for how miR-15/16

regulate T cell responses, and replaced it with a short description of two targets (Pik3r1 and Mapk8) that link the roles of miR-15/16 in T cell activation and tumor suppression. Like transcription factors, miRNAs function as network regulators of gene expression, gaining biological power through their ability to coregulate many genes with convergent effects on cell behavior. In the case of miR-15/16, our published data, reinforced by the data in this manuscript, indicates that the relevant target network is very large, and that even very small changes in the expression of these targets is sufficient to alter the fate of antigen-responsive T cells in the setting of acute infection.

This comment also raises the important issue of target validation, which is often difficult, since the effect size for each miRNA target is small (typically 10-30%, sometimes reaching 50% reduction). The expected effect of Malat1 inhibition of miR-15/16 is some fraction of that. Nevertheless, in Figure 3 and Figure 7, we validated two direct targets (CD28 and Bcl2) using flow cytometry, a technique that facilitates precise sampling of protein expression on a large number of individual cells.

Minor concerns:

The discussion is too broad and does not address the limitations of the study.

We added a sentence to acknowledge the limitation regarding small effect sizes and the shortcomings of the acute infection models used in this study:

“The magnitude of this effect was modest in acute LCMV and Listeria infection, two models that feature robust pathogen clearance, allowing assessment of memory T cells in the absence of chronic antigen persistence. Further work is needed to assess other settings in which Malat1:miR-15/16 interaction may have a bigger impact on the outcome of immune responses.”

Reviewer 2:

1. Given the lack of an effect on microRNA or Malat1 levels following the genetic modification is it possible that Malat1 is actually not directly bound by the miRNA? Could the knock-out of the miRNA could induce Ago2 loss on Malat1 by indirect mechanisms? If there is any room for doubt about a direct interaction the authors should at least mention discuss.

There is very little room for doubt about the direct interaction between miR-15/16 and Malat1. The AHC data we report indicates that the loss of Ago2 binding to the mutant Malat1 occurs predominantly at the site containing the miR-15/16 binding site of interest. This suggests that the mutation we created does not affect global Ago2 levels or occupancy across the rest of the transcript. Further, the miR-15/16 KO data directly support this result, showing that miR-15/16 is necessary for Ago2 binding at that site. If loss of miR15/16 resulted in a non-specific indirect loss of binding to Malat1, we would expect that other binding events would be affected as well, which we do not observe.

In the Results, the authors write: "miR-15/16 has not been previously shown to interact with Malat1", but they should cite/discuss: MALAT1 regulates the transcriptional and translational levels of proto-oncogene RUNX2 in colorectal cancer metastasis, Qing Ji et al, 2019.

We thank the reviewer for bringing this study to our attention, and we have cited it in our updated version of the manuscript. While the interaction between miR-15/16 and Malat1 has been shown before, our study represents a significant step beyond this study in two

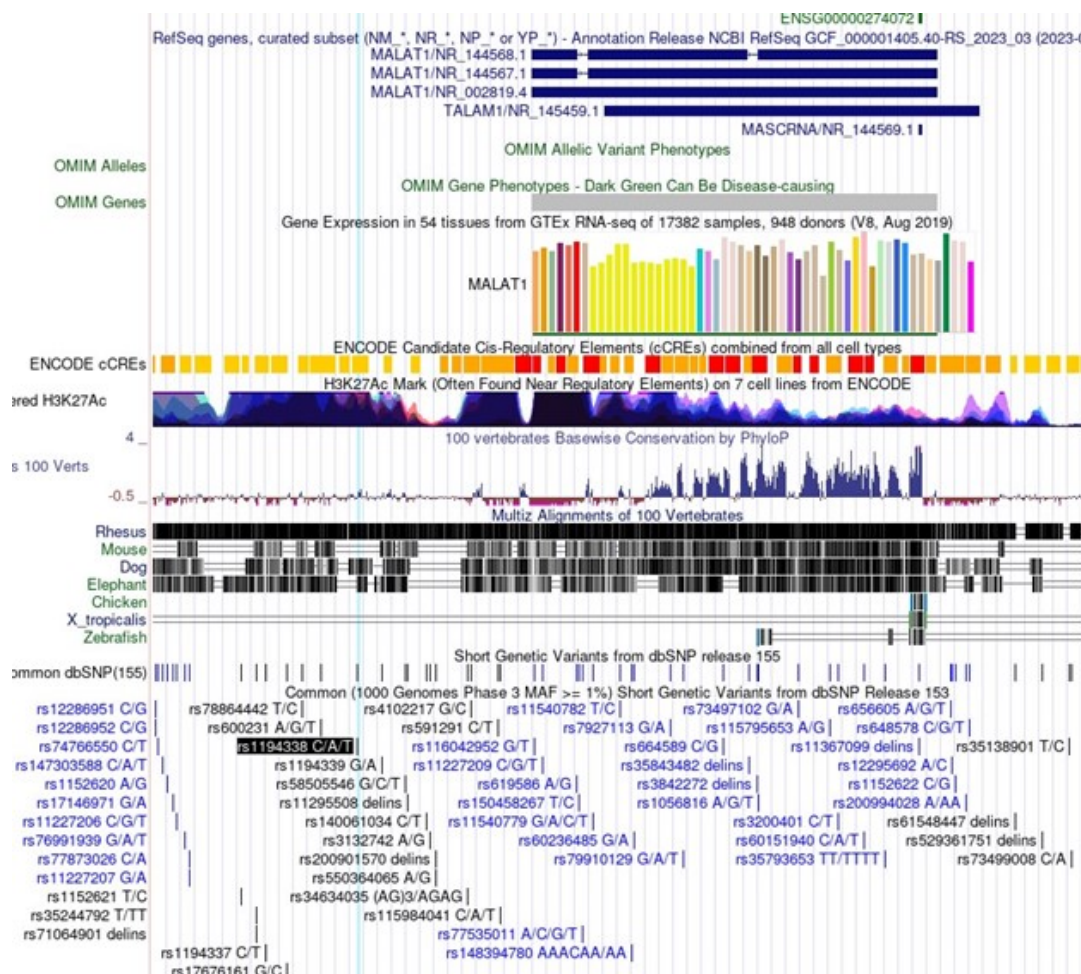
important ways: The rigorous biochemical mapping of the miR-15/16:Malat1 interaction site, and direct evidence for the role of a miR:lncRNA interaction in an in vivo physiological phenotype.

1. The authors write: "Only a few studies demonstrate sequence dependent function of lncRNAs (Elguindy and Mendell, 2021; Kleaveland et al., 2018; Lee et al., 1999)". But this seems more common that the statement implies (see for example this review: <https://www.sciencedirect.com/science/article/pii/S0022283612008960#s0065>). Moreover, SNPs in lncRNAs are associated with pathologies (see for example: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6306726/>, where also SNPs in Malat1 are presented). The authors could acknowledge this and by reformulating their sentence and citing these.

A large number of studies uncovered lncRNA functions without identifying RNA sequences that are responsible for that activity, but evidence for sequence-specific effects remain rare. We thank the reviewer for providing direction to additional sequence-specific studies and we have now cited several of them in the updated version of the introduction:

"Studies demonstrating sequence dependent function of lncRNAs are comparatively rare (Carrieri et al., 2012; Elguindy and Mendell, 2021; Faghihi et al., 2008; Gong and Maquat, 2011; Kleaveland et al., 2018; Lee et al., 1999; Yoon et al., 2012)."

In particular, association of important SNPs with lncRNA loci is an exciting motivator in the study of lncRNAs and can be informative in the dissection of lncRNA function. For Malat1 in the linked Minotti et al publication, we do not believe the SNPs referenced represent indications of sequence-specific transcript function. The SNPs identified for Malat1 are rs1194338, rs4102217, and rs591291. In the UCSC genome browser screenshot in Author response image 1, you can see that all of these SNPs are upstream of Malat1 and in regions of extremely dense H3K27Ac, suggesting enhancer function. These SNPs do not represent sequence specific function of the Malat1 transcript, but rather more likely genomic sequence regulation of Malat1 (or nearby gene) expression.



- Figure 2H: In the figure legend, could the authors clarify what they mean by "same conditions as in F"?

We have updated the figure legend for clarity.

- Figure 3 panel labels B, C, D don't match figure.

We have corrected this and provided an updated figure.

- Figure 4 D, E, F: Can the authors comment more about why in their opinion early activation genes are not significantly decreased in Malat1 scr/scr?

Figure 4A shows that interrupting Malat1 interaction with miR-15/16 does affect the early induction of the immediate early gene CD69. Even miR-15/16 deficiency did not affect Nur77 expression, indicating that Malat1 and miR-15/16 regulate specific cues and signaling pathways involved in T cell activation. In particular, the transcriptomic analysis led us to focus on effects on costimulation-induced genes (Figure 3). Figure panels 4D, E, and F show the production of cytokines, including IL-2, which has been well documented to be responsive to CD28 signaling and clearly did so in our experiments. These data show a consistent increase in miR-15/16-deficient T cells, despite considerable noise in the assay. The

trend toward reduced IL-2 in Malatscr/scr T cells is of smaller magnitude, as expected, and not statistically significant. Repeating this assay to obtain a better p value doesn't seem warranted. However, we did independently observe decreased IL-2 production in Malatscr/scr T cells in an ex vivo cytokine capture assay (Figure 7F-G).