miR-155 Promotes T Follicular Helper Cell Accumulation during Chronic, Low-Grade Inflammation

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SUMMARY

Chronic inflammation is a contributing factor to most life-shortening human diseases. However, the molecular and cellular mechanisms that sustain chronic inflammatory responses remain poorly understood, making it difficult to treat this deleterious condition. Using a mouse model of age-dependent inflammation that results from a deficiency in miR-146a, we demonstrate that miR-155 contributed to the progressive inflammatory disease that emerged as Mir146a−/− mice grew older. Upon analyzing lymphocytes from inflamed versus healthy middle-aged mice, we found elevated numbers of T follicular helper (Tfh) cells, germinal center (GC) B cells, and autoantibodies, all occurring in a miR-155-dependent manner. Further, Cd4-cre Mir155fl/fl mice were generated and demonstrated that miR-155 functions in T cells, in addition to its established role in B cells, to promote humoral immunity in a variety of contexts. Taken together, our study discovers that miR-146a and miR-155 counterregulate Tfh cell development that drives aberrant GC reactions during chronic inflammation.

INTRODUCTION

Chronic, low-grade inflammation is driven by sustained production of inflammatory factors such as cytokines, reactive oxygen species, and autoantibodies and is associated with seven of the top ten causes of mortality—such as heart disease, neurodegeneration, metabolic disorders, autoimmune, and cancer (Akbaraly et al., 2013; Howcroft et al., 2013; Michaud et al., 2013)—in the United States. This deleterious condition, which typically lacks clinical symptoms, can cause damage to important organ systems over time and serve as a primer for many age-related disorders. Despite our growing knowledge of immune regulation during acute inflammatory responses, our understanding of molecular and cellular mechanisms governing chronic, low-grade inflammation remains tenuous. This deficiency has prevented the development of targeted therapies to reduce chronic inflammation and the plethora of disorders associated with one’s inflammatory status during the aging process. By the year 2050, it is projected that those 60 years and older will make up 22% of the world’s population, compared to only 10% in the year 2000 (Dorshkind et al., 2009). Thus, finding therapeutic solutions to chronic inflammatory conditions has become more important than ever before.

It is now appreciated that most of the human genome is transcribed into noncoding RNAs that carry out diverse cellular functions including differentiation, survival, and proliferation (Esteller, 2011). Among noncoding RNAs, microRNAs (miRNAs) regulate gene expression posttranscriptionally and have been shown to modulate a wide range of biological systems (Mendell and Olson, 2012). Further, several miRNAs have been shown to control inflammation in young mice subjected to infection by pathogens or during antigen-induced autoimmunity (Baumjohann et al., 2013; Kang et al., 2013; O'Connell et al., 2010b; Oertli et al., 2011; Rodriguez et al., 2007). Despite their emerging connection to acute inflammation, little is known about the functions of miRNAs during chronic inflammation and diseases associated with aging. Recently, the anti-inflammatory miR-146a has emerged as a molecular safeguard against age-dependent inflammatory disease (Boldin et al., 2011; Zhao et al., 2011; Zhao et al., 2013). Mice deficient in miR-146a have increased serum concentrations of interleukin-6 (IL-6) and autoantibodies and display splenomegaly, myeloproliferation, and inflammatory damage to several tissues as they reach middle age. When Mir146a−/− mice grow even older, they succumb to different types of cancers and hematopoietic neoplasms that reduce their lifespans compared to wild-type (WT) controls. These findings clearly demonstrate that specific miRNAs have evolved to regulate chronic, low-grade inflammation and establish Mir146a−/− mice as an excellent model with which to study this clinically relevant condition. Whereas miR-146a functions to prevent chronic inflammation, we hypothesized that other miRNAs act to promote this deleterious process.

miR-155 has emerged as a multifaceted regulator of immunity that impacts different types of inflammatory responses in young mice (Hu et al., 2013; Huffaker et al., 2012; O'Connell et al., 2010b; Rodriguez et al., 2007; Thai et al., 2007). Further, previous studies find that constitutive overexpression of miR-155 in the hematopoietic compartment causes a chronic...
Figure 1. miR-155 Is Required for Expansion of CD4+ T Cells with a Tfh Cell Gene-Expression Profile in Middle-Aged Mir146a−/− Mice

(A) Spleen weights from 2 (young)- and 10 (middle-age)-month-old age- and sex-matched WT, Mir155−/−, Mir146a−/−, and Mir155−/− Mir146a−/− (DKO) mice.

(B) Representative FACS analysis of activated (CD69+CD62Llo) CD4+ T cells in the spleens and lymph nodes of 10-month-old mice.

(C) Spleen data from multiple young and middle-aged mice in (B).

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inflammatory disease (O’Connell et al., 2008) or leukemia (Costinean et al., 2006), shortening the animal’s lifespan. In the present study, we investigated the role of endogenous miR-155 during chronic, low-grade inflammation that develops in Mir146a−/− mice.

RESULTS

miR-155-Dependent Accumulation of Activated T Cells in Mir146a−/− Mice

To determine whether endogenous miR-155 plays a role in promoting age-dependent disease in Mir146a−/− mice, we aged Mir155−/− Mir146a−/− (DKO) and control mice for 7–10 months (middle-age). As previously reported (Boldin et al., 2011; Zhao et al., 2011; Zhao et al., 2013), middle-aged but not young Mir146a−/− mice had enlarged spleens (Figures 1A). Elevated amounts of activated T cells (CD4+CD69+CD62Llo) were also evident in middle-aged Mir146a−/− mice, both in the spleen and lymph nodes, and this activated T cell phenotype did begin to emerge in young mice (Figures 1B and 1C; Figure S1 available online). In contrast, middle-aged Mir155−/− Mir146a−/− mice had spleen weights and activated CD4+ T cell levels that were similar to middle-aged WT mice, indicating that miR-155 promotes these phenotypes in Mir146a−/− animals (Figures 1A–1C and S1).

The Mir146a−/− mouse phenotype is largely dependent upon lymphocytes (Zhao et al., 2013), and consistent with previous work (Yang et al., 2012), we found that an increase in activated CD4+ T cells precedes other disease manifestations in this model (Figures 1C and S1). Using bone-marrow reconstitution experiments, we also determined that this phenotype was primarily hematopoietic in nature (Figure S1). On the basis of these findings, we next assessed miR-155 expression in CD4+ T cells. Results showed that miR-155 expression trended toward being higher in CD4+ T cells from young Mir146a−/− mice, compared to WT controls, and reached even higher expression in CD4+ T cells taken from middle-aged Mir146a−/− mice (Figure 1D). This correlated with an increased proportion of activated T cells in the bulk CD4+ T cell populations from Mir146a−/− versus WT mice. Upon sorting, we confirmed that activated CD4+ T cells expressed higher miR-155 than naive T cells, and this expression was enhanced further in activated T cells lacking miR-146a (Figure 1E). It has been previously demonstrated that activated Mir146a−/− CD4+ T cells have elevated NF-κB activation, a pathway that we found to promote miR-155 expression in activated CD4+ T cells (Figure 1F). In contrast to T cells, enhanced expression of miR-155 was not observed in B220+ B cells from Mir146a−/− mice (Figure S1). On the basis of these results, we focused our subsequent analysis on the CD4+ T lymphocyte compartment to better understand the role of miR-155 during chronic inflammation.

T Follicular Helper Cells, Germinal Center B Cells, and Autoantibodies Accumulate in Mir146a−/− Mice

We examined gene-expression patterns in CD4+ T cells from 10-month-old WT, Mir155−/−, Mir146a−/−, and Mir155−/− Mir146a−/− mice by RNA-seq. Gene-expression profiles in Mir146a−/− CD4+ T cells were distinct from the other three genotypes according to a cluster analysis while Mir155−/− Mir146a−/− profiles clustered closer to Mir155−/− than WT or Mir146a−/− profiles (Figure 1G). IL-21 expression was significantly higher in Mir146a−/− compared to WT middle-age CD4+ T cells, while there was little difference in interferon-γ (IFN-γ) mRNA amounts and undetectable expression of the IL-17A message in both genotypes (Figure 1H). IL-21 is produced by T follicular helper (Tfh) cells, and its elevated expression in Mir146a−/− T cells prompted us to examine additional Tfh genes. We observed increased expression of B cell lymphoma 6 protein (Bcl6), chemokine (C-X-C motif) receptor 5 (Cxcr5), programmed cell death 1 (Pd1), and inducible T cell costimulator (Icos) in Mir146a−/− CD4+ T cells, and decreased or unchanged expression of these genes in Mir155−/− and Mir155−/− Mir146a−/− T cells, compared to WT controls (Figure 1I). This was confirmed by quantitative RT-PCR (qPCR) (Figure 1J). These data suggested that Mir146a−/− CD4+ T cells from middle-aged mice are enriched in Tfh cells and that this occurs through a miR-155-dependent mechanism.

Using flow cytometry, we next detected increases in CD44+ CD4+CXCR5+PD1+ Tfh cell numbers in the spleens and LNs of middle-aged Mir146a−/− mice compared to controls (Figures 2A and 2B and S2). Further, these cells also expressed ICOS and BCL6 consistent with their Tfh cell identity (Figures 2C–2E). miR-155 was expressed at higher amounts in WT Tfh compared to non-Tfh cells and further enhanced in Mir146a−/− Tfh cells (Figure 2F). This Tfh cell phenotype began to emerge in young Mir146a−/− mice, suggesting that it might be an early step in disease progression. We also observed an overall increase in Tfh cells in the CD4+ T cell compartment as a function of age in all groups (Figure 2G).

Tfh cells promote antibody class-switching and production by germinal center (GC) B cells (Johnston et al., 2009; Nurieva et al., 2008) and play established roles in driving autoimmunity when their development becomes dysregulated (Linterman et al., 2009). Consistent with this, increases in B220+IgD+IgM−GL7m

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Figure 2. miR-155-Dependent Accumulation of Tfh Cells, GC B Cells, and Autoantibodies in Mir146a−/− Mice

(A) Flow cytometry plots showing CD4+CXCR5+PD1+ Tfh cells in the spleens of middle-aged mice. (B) Average total number of Tfh cells in the spleens of middle-aged mice 7 to 10 months old. (C) Flow cytometry plots showing CD4+ICOS+PD1+ Tfh cells in the spleens of middle-aged mice.

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miR-155 Promotion of GC Formation in Mir146a−/− Mice Precedes the Onset of Systemic Tissue Inflammation

To further assess the miR-155-dependent inflammatory disease that develops in Mir146a−/− mice, H&E stained splenic sections from WT, Mir155−/−, Mir146a−/−, and Mir155−/− Mir146a−/− mice at 1.5, 4, and 11 months of age were prepared (Figures 3A and S3). Immunohistochemistry (IHC) was also performed on splenic sections from 1.5-month-old mice to detect B (B220) and T (CD3) cells, and serial sections were also stained for BCL6 (Figures 3B and S3). Peanut agglutinin (PNA) staining was also performed (Figure 3C). Histological analysis demonstrated the highest number GCs in Mir146a−/− spleens, reduced amounts in WT mice, and the lowest numbers in Mir155−/− and Mir155−/− Mir146a−/− mice at 1.5 and 4 months of age (Figures 3A-3D). The number of GCs increased with age in WT and Mir146a−/− mice, but remained low in Mir155−/− and Mir155−/− Mir146a−/− mice. Consistent with this, there were increased numbers of BCL6+ and PNA+ cells in lymphoid follicles in Mir146a−/− spleens compared to spleens from the other genotypes (Figures 3B and 3C and S3). All of these observations are concordant with our flow cytometry data and further demonstrate that the aberrant GC phenotype in Mir146a−/− mice begins in young mice and becomes more severe with age.

By 11 months of age, Mir146a−/− spleens had disrupted lymphoid follicles that were replaced by increased myeloid cells and other hematopoietic elements, and this was generally not observed in the other age-matched genotypes (Figures 3A and 3E and S3). Further, inflammatory infiltrates into the liver and kidneys of Mir146a−/− mice were found by 12 months of age (Figure 3F), indicating that a multiorgan inflammatory condition was emerging. In contrast, these features were not observed in the Mir155−/− Mir146a−/− mice that were analyzed, suggesting that miR-155 promotes chronic inflammation in multiple tissues when miR-146a is absent.

miR-155 Regulates Tfh Cell Development through a T Cell Intrinsic Mechanism following Immunization

Tfh cell formation involves both cell-intrinsic and -extrinsic mechanisms (Tangye et al., 2013). To determine whether miR-155 plays a cell-autonomous role in Tfh cell development, we created T cell-specific miR-155 deficient mice (Cd4-cre Mir155fl/fl) where the miR-155 gene is floxed and Cre is driven by the CD4 locus (Figures 4A and 4B, Figure S4, and Table S1). In addition to confirming specific deletion of miR-155 in T cells (Figures 4C and S4), we also determined that Cd4-cre Mir155fl/fl mice and controls had similar percentages of CD4+, CD8+, and B220+ cells in the spleens under steady-state conditions (Figure S4). Next, we immunized Cd4-cre Mir155fl/fl mice and relevant controls with Ovalbumin (Ova) in complete Freund’s adjuvant (CFA). After 8 days, we measured the development of Tfh and GC B cells and found that miR-155 expression was specifically required by T cells for proper development of these cell lineages in the spleen and lymph nodes (Figures 4D-4J and S5). Further, reduced anti-Ova immunoglobulin G1 (IgG1) serum antibodies were detected in Cd4-cre Mir155fl/fl mice at multiple time points postimmunization (Figures 4K and 4L and S5), albeit amounts were not as low as in whole-body Mir155−/− mice because miR-155 also functions in B cells (Rodriguez et al., 2007; Thai et al., 2007; Vigorito et al., 2013). Despite substantial reductions in GC phenotype B cells in the spleens of Cd4-cre Mir155fl/fl mice, only partial decreases in antigen-specific antibodies were observed, perhaps reflecting contributions or compensation by extra follicular B cells. Together, our data demonstrate that miR-155 has a T cell-intrinsic role during Tfh cell development and the GC response.

miR-155 is Required for Early Tfh Cell Differentiation by Antigen-Specific, Naive CD4+ T Cells

To gain further insight into the role of miR-155 during Tfh cell development, we crossed Mir155−/− mice with Smarta (SM) TCR Tg mice. Equal numbers of naive splenic WT or Mir155−/− SM cells were injected into WT B6 recipients and mice were infected with Vaccinia virus expressing the lymphocytic choriomeningitis virus (LCMV) glycoprotein (VACV-gp) recognized by SM T cells. Following 3 or 5 days of infection, Mir155−/− SM cells were defective in their production of CXCR5+PD1+ Tfh cells compared to WT controls at both time points (Figures 5A-5C). We also observed defective expression of BCL6 by Mir155−/− SM cells at the 3-day time point (Figure 5D, top). Mir155−/− SM cells demonstrated modest yet significant reductions in the...
proliferation marker Ki67 (Figure 5D, bottom), suggesting that reduced proliferation could be contributing to their diminished numbers compared to WT SM cells. Further, Mir155−/− CXCR5+ PD1+ SM cells also displayed subtle defects in proliferation as assayed by loss of CFSE, as fewer cells had entered the 6th cell division by 3 days postinfection when compared to WT controls (Figures 5E and 5F). No difference in SM cell death was observed between the genotypes according to 7-AAD staining, and homeostatic amounts of SM cells were not reduced by a loss in miR-155 in the absence of infection (Figure S5). These data demonstrate that naive, antigen-specific CD4+ T cells reduced proliferation could be contributing to their diminished homeostatic amounts of SM cells were not reduced by a loss in miR-155 in the absence of infection (Figure S5). These data demonstrate that naive, antigen-specific CD4+ T cells reduced proliferation could be contributing to their diminished

Identification of mir-155 Targets in Tfh Cells
To identify mir-155 target genes involved in Tfh cell development in the context of chronic inflammation, we utilized an integrated approach (Figure S6). RNA was extracted from sorted Tfh cells isolated 8 days after immunizing WT and Mir155−/− mice with Ova, and expression of Bcl6 was confirmed in sorted Tfh cells compared to non-Tfh cells by qPCR (Figure 6A). The RNA was next subjected to RNA-seq to profile gene expression in WT and Mir155−/− Tfh cells, and cluster analysis revealed that the two genotypes had disparate profiles (Figure S6). However, expression of several Tfh-related genes was not significantly changed between WT and Mir155−/− Tfh cells on a per-cell basis, suggesting that miR-155 regulates the quantity and not quality of Tfh cells (Figure S6). Among miR-155 target mRNAs predicted bioinformatically by Targetscape and determined experimentally using Ago HITS-CLIP (Loeb et al., 2012), we observed a bias toward higher expression of these genes in Mir155−/− versus WT Tfh cells, consistent with miR-155 target genes being derepressed (Figures 6B and Table S2).

To identify candidate miR-155 target genes involved in Tfh cell formation during chronic inflammation, we determined which miR-155 targets were (1) elevated in sorted Mir155−/− Tfh cells from immunized mice (Table S2), (2) elevated in middle-aged Mir155−/− and Mir146a−/− Mir146a−/− CD4+ T cells (Table S3), and (3) unchanged or repressed in aged Mir146a−/−/− CD4+ T cells. Using this stringent approach, we identified 21 candidate miR-155 targets putatively involved in the development of Tfh cells in Mir146a−/− mice (Figures 6C and 6D). We confirmed some of these by qPCR (Figure S6), and further validated higher expression of Pel1, Ikbkbe, and Fosl2 at the protein level in Mir155−/− CD4+ T cells compared to WT controls (Figure 6E). We also found that expression of Pel1, Fosl2, and Ikbkbe was lower in WT Tfh compared to non-Tfh cells taken from immunized mice, whereas their expression values in Mir155−/− Tfh cells were similar to WT non-Tfh cells and well above amounts in WT Tfh cells (Figure 6F). To confirm direct targeting of Pel1 and Fosl2, both genes that regulate T cell differentiation, we cloned their 3’ UTRs downstream from luciferase. Luciferase assays confirmed that miR-155 directly repressed protein expression through its binding sites in these 3’ UTRs, because repression was observed with WT 3’ UTRs, but not when miR-155 binding sites were mutated (Figures 6G and 6H).

Pathway analyses indicated that a subset of these targets regulate the NF-κB (Ikbkbe and Pel1) and AP-1 (Fosl2) pathways, both involved in Tfh cell development and autoimmunity (Chang et al., 2011; Clark et al., 2011) (Figure 6D). Another subset of genes repressed by miR-155 has been shown to regulate the mTOR pathway (including Raptor, Rps6ka3, Adrb2, Ikbkbe, and Nf2i2), and there were multiple target genes involved in chromatin modifications (Satb1, Kat2a, Kdm7a, and Nsd1) (Figure 6D). Many of these targets have been shown to influence T helper cell development (Figures 6D and S6).

shRNA silencing of Fosl2 in adoptively transferred Mir155−/− 2D2 TCR-transgenic CD4+ T cells, which are TCRV11+, resulted in improved Tfh cell development following immunization with MOG35–55 (Figures 6I–6K). Silencing of Pel1 also tended toward rescuing the phenotype, supporting our view that multiple miR-155 targets are likely involved in this process (Figure S6). Taken together, our findings suggest that miR-155 facilitates Tfh cell accumulation during chronic, low-grade inflammation through a complex mechanism involving multiple target genes and signaling pathways that instruct early Tfh cell formation.

T Cell-Specific Expression of mir-155 Drives Spontaneous Tfh and GC B Cell Development in Mir146a−/− Mice
To determine the role of T cell-intrinsic miR-155 during the early stages of chronic, low-grade inflammation, when expanded Tfh cell populations are first observed, we crossed Mir146a−/− mice with Cd4-cre Mir155fl/fl animals (Figure 7A). By 4 months of age, Mir146a−/− mice began to exhibit mild splenomegaly, which was not seen in Mir146a−/−/Cd4-cre Mir155fl/fl mice (Figure 7B). Upon further analyzing 1.5- and 4-month-old Mir146a−/−/Cd4-cre Mir155fl/fl mice, we detected reduced numbers of Tfh cells (Figures 7C–7J and S7) and GC B cells (Figures 7K and S7) compared to the numbers found in age-matched Mir146a−/− mice. Further, at the 4-month time point, we also noticed that early signs of the myeloproliferative disease that emerged as Mir146a−/− mice grew older were observed in

Figure 3. Histological Assessment of mir-155-Dependent, Spontaneous GC Formation and Chronic Inflammatory Disease in Mir146a−/− Mice
(A) Representative H&E stained sections of WT, Mir155−/−, Mir146a−/−, and Mir155−/−/Mir146a−/− (DKO) male mice spleens from mice of the indicate ages. Black arrows represent examples of germinal center elements including large centroblasts, mitotic figures, and apoptotic bodies. Red arrows represent myeloid cells. Scale bar represents 40 μm.
(B) Spleen sections from 1.5-month-old mice were also subjected to IHC to detect T cells (CD3), B cells (B220), and BCL6+ cells. Arrows indicate regions with BCL6 positive cells. Scale bar represents 400 μm.
(C) Representative PNA staining of spleens from Mir146a−/− and Mir155−/−/Mir146a−/− mice at 1.5 months of age. Scale bar represents 100 μm.
(D) The number of GCs per splenic lymphoid follicle was determined at 1.5 and 4 months of age.
(E) CD11b+ myeloid cell numbers were measured using flow cytometry.
(F) Representative H&E staining of kidneys and livers from Mir146a−/− and Mir155−/−/Mir146a−/− mice at 12 months of age. Scale bar represents 100 microns. Inflammatory infiltrate is located within the dashed boarder. * denotes a p value of < 0.05 using a Student’s t test. See also Figure S3.
Figure 4. T Cell-Intrinsic Role for miR-155 during Tfh Cell Development and T-Dependent Humoral Responses

(A) Schematic diagram showing the loxP sites flanking the mir-155 hairpin sequence in mir-155<sup>fl/fl</sup> mice, and location of genotyping primers.

(B) Genomic DNAs were isolated from flow-cytometry-sorted CD4<sup>+</sup>CD<sub>3</sub>ε<sup>+</sup> T and B220<sup>+</sup> cells from the indicated mouse spleens and the primers that flank the 5' loxP site were used for PCR. A representative agarose gel is shown to demonstrate T cell specific deletion of mir-155.

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**MicroRNAs Regulate Tfh Cells during Inflammation**

*Mir146a−/−*, but not *Mir155−/− Mir146a−/−* or *Mir146a−/− Cdx4−/− Cdx4−/−* Mir155+/− mice (Figure S7). These included elevated CD11b+ and decreased Ter119+ cells in the bone marrow. Overall, these findings demonstrate that miR-155 plays a T cell-intrinsic role in promoting spontaneous germinal center reactions in *Mir146a−/−* mice.

**DISCUSSION**

The humoral response gradually loses its potency against novel, exogenous antigens and begins to initiate responses against self-tissues as a function of age (Dorshkind et al., 2009; Linteman, 2014). Our study indicates that cells with a Tfh cell phenotype might be involved in this age-dependent conversion, because both their numbers and downstream effects (e.g., increases in GC B cells) increase before the onset of autoantibody production in *Mir146a−/−* mice. This concept is also in accordance with clinical studies that report increases in the Tfh cell growth factors IL-6 (Akaraly et al., 2013) and IL-21 (Agrawal et al., 2012), memory phenotype T cells (Moro-Garcia et al., 2013), autoantibodies (Nagele et al., 2013), and autoimmune disease (Yung and Julius, 2008) in some older versus younger individuals. During future studies, it will be important to assess both the quality and quantity of Tfh cell populations as a function of age in human tissues and to determine whether different features of chronic, low-grade inflammation in human populations involves aberrations to this cellular population.

Mechanistically, our results identify opposing roles for miRNAs in controlling progressive, spontaneous Tfh cell expansion, where miR-146a restricts and miR-155 promotes this program in mice. This suggests that factors controlling the ratio of miR-155:miR-146a can influence this process. Both of these miRNAs are transcriptionally induced by inflammatory stimuli or T cell receptor (TCR) engagement (Haasch et al., 2002; Yang et al., 2012). Further, genetic variants in the miR-155 gene locus have been linked to autoimmune disease (Paraboschi et al., 2012), while specific polymorphisms in the passenger strand of miR-146a lead to reduced production of mature miR-146a (Jazdzewski et al., 2008). These observations indicate that both genetic and environmental factors are involved in controlling the concentrations of these miRNAs and thus their abilities to regulate humoral responses during the aging process. Furthermore, while we found a variety of age-related inflammatory phenotypes in *Mir146a−/−* mice to involve miR-155 through the use of whole-body *Mir155−/− Mir146a−/−* mice, and focused on miR-155’s T cell-intrinsic role in promoting GC reactions in this setting, future investigation is needed to determine whether miR-155 functions in either Tfh or non-Tfh cell types to promote other aspects of the disease that emerge in this model. It is also plausible that other miR-146a-dependent phenotypes are independent of miR-155.

In addition to its well-established function in B cells during Ig class-switching and affinity hypermutation (Rodriguez et al., 2007; Thai et al., 2007; Vigorito et al., 2007), our data identify a previously unappreciated role for miR-155 in the CD4+ T cells as they provide help to B cells during the germinal center reaction. In particular, we describe a decreased capacity by *Mir155−/−* CD4+ T cells to develop into the Tfh cell lineage following immunization, viral infection, or during age-related inflammatory disease. Because we observe decreased Tfh cell numbers, while our expression analysis indicates that effector function might be intact on a per cell basis, it is possible that miR-155 is involved in Tfh cell differentiation and expansion as opposed to their functions once mature. Our findings also indicate that multiple miRNAs are involved in regulating Tfh cell biology, as recent studies have described roles for the miRNAs 17~92 family (Baumjohann et al., 2013; Kang et al., 2013) and miR-10a (Takahashi et al., 2012) during Tfh cell formation.

We identified 21 direct miR-155 targets in Tfh cells that regulate critical signaling pathways including NF-κB, AP-1, and mTOR, in addition to several genes that regulate chromatin modifications. Consistent with many previous studies (Hu et al., 2013; Huffaker et al., 2012; Loeb et al., 2012), our results continue to support a model whereby miR-155 regulates T cell biology through a multitarget mechanism that enables development of different T effector cell subsets in distinct contexts. However, it remains unclear whether miR-155 targets unique sets of genes to regulate the distinct effector T cell lineages that it has been linked to, including regulatory T (Treg) cells (Lu et al., 2009), Th17 cells (Kurowska-Stolarska et al., 2011; O’Connell et al., 2010b), Th1 cells (Oertli et al., 2011), Th2 cells (Malmhall et al., 2013), and now Tfh cells, or whether there is a core “targetome” that is commonly required to license the formation of these subtypes. This will be an important area of future research that will require target identification in multiple T cell types in parallel using the same technology.

Our data also provide evidence that Fosl2, and to some extent Peli1, are functionally relevant miR-155 targets. Fosl2 is a repressor of CD4+ T cell plasticity (Ciofani et al., 2012) that binds to Jun proteins and is thought to compete with Batf for DNA binding sites. Batf-containing AP-1 complexes bind cooperatively with IRF4 to define DNA elements called AP-1-IRF composite elements (AICEs) (Glasmacher et al., 2012), and both of these factors are necessary for Tfh cell development (Betz et al., 2010; Bollig et al., 2012). However, Fosl2-containing complexes are unable to recruit IRF4 once bound to these sites (Glasmacher et al., 2012), pointing to a mechanistic basis for its repressive role during T cell differentiation. Thus, because Fosl2 is at higher amounts in *Mir155−/−* CD4+ T cells, our data...
suggest that it is able to hinder Tfh cell development by interfering with normal Batf and IRF4 functions. In the case of Pell1, it has been shown to inhibit NF-κB activation, which is also involved in the induction of Tfh cell-associated genes (Chang et al., 2011; Chen et al., 2010). Future work will investigate these connections further and determine their relevance in other Th cell
lineages, including Th17 and Treg cells where miR-155 and Fosl2 have been shown to have opposing functions.

Finally, our observations have many translational implications with relevance to human disease. First, the relative expression of miR-155 and miR-146a in middle-aged individuals might have predictive, diagnostic, or prognostic value in the context of chronic, low-grade inflammation. Next, therapeutic targeting of miR-155 or miR-146a with antisense oligonucleotides in patients with chronic, low-grade inflammation could be an effective strategy to reduce certain disorders that stem from aberrant humoral responses. Conversely, from a vaccine development standpoint, these findings indicate that manipulation of miR-155 in both T and B cells might have a synergetic effect on the production of high affinity, class-switched antibodies that can be induced via immunization to target tumor cells or pathogenic microbes.

**EXPERIMENTAL PROCEDURES**

**Mice**

All experiments were approved by the University of Utah Institutional Animal Care and Use Committee (IACUC). Mir155−/−Miri46a−/− (DKO) mice were generated as previously described (Huflaker et al., 2012). Mir155fl/fl mice were generated at Taconic and crossed with SMARTA TCR Tg+ mice were crossed with Mir155fl/fl mice, and WT SMARTA TCR Tg+ mice were crossed with Mir155−/− mice to generate Mir155−/− SMARTA TCR Tg+. Mir155−/− 2d2 TCR Tg+ mice were described previously (Hu et al., 2013).

**Flow Cytometry**

Fluorophor-conjugated antibodies against the indicated surface markers were used to stain RBC-depleted splenocytes, LN cells, BM cells, and peripheral blood cells. For intracellular staining, cells were first stained for lineage markers and then fixed, permeabilized, and stained with antibodies against Bcl6, IL-17A, IFN-γ, or Ki67 (BioLegend). Stained cells were analyzed with a BD LSR Fortessa flow cytometer, and further data analysis was carried out with FlowJo software.

**Bone-Marrow Reconstitution**

RBC-depleted bone-marrow cells from WT mice expressing the congenic marker CD45.1 were mixed with bone marrow from WT, Mir155−/−, Mir146a−/−, or Mir155−/− Mir146a−/− mice expressing CD45.2 in equal proportions and injected into lethally irradiated (1,050 Rads) WT mice expressing CD45.1, similar to (O’Connell et al., 2010a).

**Adaptive Transfer of SM T Cells and VACV-gpc Infection**

Untouched naive (Thy1.1+CD44lo) CD4+ T cells were isolated from WT or Mir155−/− SMARTA mice (SM T cells) using magnetic beads, per the manufacturer’s instructions (Stem Cell Technology), and injected intravenously into C57BL/6 mice. The next day, mice were infected with recombinant Vaccinia virus that expresses the LCMV glycoprotein (referred to as VACV-gpc). For CFSE labeling, T cells were incubated with 0.625 μM CFSE (final concentration) for 10 min and washed with FBS before adoptive transfer. To determine viability, we used 7-AAD staining.

**Adaptive Transfer of shRNA-Expressing 2d2 TCR Tg CD4+ T Cells and MOG35–55 Immunization**

Untouched naive CD44lo CD4+ T cells were injected intravenously into B6 mice following spin infection with control or shRNA retroviral vectors, as described (Hu et al., 2013). The sequences targeting Fosl2 and Peli1 are shown in Table S4. The next day, the mice were immunized with MOG35-55 (0.5 mg/ml) emulsified in complete Freund’s adjuvant (CFA) at the base of the tail (200 μl each mouse), as described (Hu et al., 2013).

**ELISAs**

Titters of autoantibodies against dsDNA in the serum of aged mice were measured with a commercial ELISA test (Biovendor) according to the manufacturer’s protocol. Serum from Ova-immunized mice (0.5 mg/ml emulsified in CFA) was also collected, and Ova antigen-specific IgG and IgG1 antibodies were measured by ELISA, as described (O’Connell et al., 2010b).

**qPCR**

Sybrgreen-based quantitative real-time PCR was conducted to assay relative mRNA amounts with the Light Cycler 480 PCR system (Roche) and gene-specific primers (Table S4). For mature miR-155 and miR-146a expression analyses, gene-specific primers were purchased from Exiqon. 5S or L32 specific primers (Table S4). For mature miR-155 and miR-146a expression analyses, gene-specific primers were purchased from Exiqon. 5S or L32 were used to normalize.

**RNA Sequencing**

For both experiments, total RNA was isolated with the miRNeasy kit (QIAGEN). Stranded RNA sequencing (following RiboZero treatment and library preparation) was conducted using Illumina HiSeq 2000 Sequencing and carried out by the University of Utah core facility (https://bioserver.hci.utah.edu/microarrayweb/ordering.html). The analysis approach is described in our Supplemental Experimental Procedures.

**Immunoblotting**

Cell extracts were subjected to gel electrophoresis and transferred onto a nitrocellulose membrane followed by antibody staining and detection of Peli1, Ikbke, Fosl2, Jα-actin, or a-tubulin, as described (Hu et al., 2013).

Figure 6. Identification of Tfh Cell-Relevant miR-155 Target Genes in CD4+ T Cells from Middle-Aged Mice

(A) qPCR analyses of mature miR-155 and BCL6 mRNA expression in sorted Tfh and non-Tfh cells from the indicated mouse spleens following immunization.

(B) Pie charts indicating the percentage of genes that are upregulated (>1.2) or downregulated (<0.8) in Mir155−/− versus WT Tfh cells based on all genes in the data set or specifically miR-155 target genes.

(C) Venn diagram showing the number of common miR-155 targets in purified Tfh cells and CD4+ T cells from middle-aged mice.

(D) Genes targeted by miR-155 according to the overlap in (C). Bars represent relative fold change of gene expression in the mutant cells compared to WT controls based on RNA-seq data. Boxes indicate different pathways in which these genes function, and * denotes that a role for the gene has been described in T cell differentiation.

(E) Representative immunoblot analysis of Peli1, Ikbke, Fosl2, and a-tubulin expression in activated WT and Mir155−/− CD4+ T cells (left). Quantification of Peli1 (n = 9), Ikbke (n = 6), and Fosl2 (n = 4) expression in T cells from multiple mice normalized to a-tubulin (right).

(F) Representative flow cytometry plots showing the percentage of CXCR5+PD1+ Tfh cells among CD3+CD4+Vj111+GFP+ cells in the spleens following 7 days of immunization with MOG35–55.

(G) Average percentage of Tfh cells among CD3+CD4+Vj111+GFP+ cells from (F). Error bars represent a SEM. * denotes a p value of < 0.05 using a Student’s t test. See also Figure S6.
MicroRNAs Regulate Tfh Cells during Inflammation

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Luciferase Assay

The 3′ UTR regions of mouse Fos2 and Peli1 that contain the miR-155 binding sites, or mutant variants, were synthesized by GeneArt Technology (Life Technologies) and cloned into pMiR reporter plasmid. Experiments were performed with 293T cells, as described (Hu et al., 2013).

Histological Analyses

Tissue preparation and H&E staining were performed as described previously (O’Connell et al., 2008). IHC was performed with antibodies against B220, CD3, and BCL6 or PNA.

Statistical Analysis

Statistical significance was determined by performing an unpaired t test with Graphpad Prism. All quantitative data are reported as mean ± SEM or mean ± ns. Significance is denoted as *p ≤ 0.01, **p ≤ 0.05, and ***p ≤ 0.005.

ACCESSION NUMBERS

All RNA-seq data have been deposited into the NCBI GEO database under the accession number GSE58373.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.09.015.

AUTHOR CONTRIBUTIONS

R.H. and R.M.O. designed the study. R.H. carried out all experimental work with assistance from D.A.K., T.B.H., M.C.R., J.L., M.A., and E.B. and guidance from R.M.O., M.A.W., and J.L.R. W.S., T.M., and G.A.G. contributed to the miR-155fl/fl mice. D.S.R. performed histology analyses. R.H. and R.M.O. wrote the manuscript with contributions from all authors.

ACKNOWLEDGMENTS

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