Silencing of microRNA-21 \textit{in vivo} ameliorates autoimmune splenomegaly in lupus mice

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MicroRNAs (miRNAs) have been implicated in B cell lineage commitment, regulation of T cell differentiation, TCR signalling, regulation of IFN signalling, and numerous other immunological processes. However, their function in autoimmunity, and specifically in systemic lupus erythematosus (SLE), remains poorly understood. \(B.\text{Sle123}\) is a spontaneous genetic mouse model of SLE characterized by autoantibody production, lymphosplenomegaly, and glomerulonephritis. We identified several differentially regulated miRNAs in B and T lymphocytes of \(B.\text{Sle123}\) mice. We found that miR-21 expression in lupus B and T cells is upregulated and that \textit{in vivo} silencing of miR-21 using a tiny seed-targeting LNA reversed splenomegaly, one of the cardinal manifestations of autoimmunity in \(B.\text{Sle123}\) mice, and de-repressed PDCD4 expression \textit{in vivo} and \textit{in vitro}. In addition, treatment with anti-miR-21 altered CD4/CD8 T cell ratios and reduced Fas receptor-expressing lymphocyte populations. Our study shows that tiny LNAs can be used to efficiently antagonize endogenous miRNAs in peripheral lymphocytes \textit{in vivo} and in primary lymphocytes cultured \textit{ex vivo} and can alter the course of a spontaneous genetic disease in mice.

INTRODUCTION

Systemic lupus erythematosus (SLE, lupus) is an autoimmune disease in which a combination of genetic predisposition and possible environmental influences triggers an exaggerated immune reaction against self-antigens and loss of immune tolerance. Antibody production by B cells and aberrant antibody-independent B and T cell functions have placed B and T cells at the centre of development of new therapeutic strategies for treatment of SLE [reviewed in (Crispin et al, 2010; Sanz & Lee, 2010)]. The tri-congenic \(B.\text{Sle1.Sle2.Sle3}\) (\(B.\text{Sle123}\)) mouse model bears three lupus susceptibility loci from the NZM2410 lupus-prone strain, backcrossed onto a C57BL/6 (\(B.\)) background (Morel et al, 2000). \(B.\text{Sle123}\) mice spontaneously develop an autoimmune syndrome that strongly resembles human lupus, characterized by autoantibodies against H2A/H2B/DNA nucleosomes, splenomegaly, lymphadenopathy, and immune complex-mediated glomerulonephritis. Anti-histone autoantibodies are detected early in the life of \(B.\text{Sle123}\) mice; however, splenomegaly and kidney disease are not typically present until the age of 4–6 months (Morel et al, 2000). As in human SLE, autoimmune manifestations in \(B.\text{Sle123}\) mice are associated with lymphocyte signalling defects [reviewed in (La Cava, 2009; Liu & Mohan, 2009)] and perturbation of cell proliferation and apoptosis (Mohan et al, 1997; Mohan et al, 1999).

MicroRNAs (miRNAs) are ~22 nt non-coding RNAs that regulate gene expression post-transcriptionally by mediating translational repression or promoting degradation of their target mRNAs (Filipowicz et al, 2008; Nelson et al, 2003). Animal miRNAs have emerged as key players in diverse immunological processes, such as B cell lineage commitment, regulation of T cell differentiation, T cell receptor (TCR) signalling and regulation of interferon (IFN) signalling (Lu & Liston, 2009; Tang et al, 2009). However, their function in autoimmunity, and specifically in SLE, remains poorly understood. Induction of lymphoproliferative syndromes in mice by failed interaction of...
miR-101 with ICOS (Yu et al, 2007) or by transgenic expression of the miR-17-92 cluster (Xiao et al, 2008) highlights an important role for miRNAs in autoimmunity. Furthermore, aberrant miRNA expression in lymphocytes and peripheral blood mononuclear cells (PBMCs) from patients with SLE (Dai et al, 2007; Te et al, 2010) and miRNA regulation of signalling pathways involved in the induction or maintenance of lupus (Divekar et al, 2011; Tang et al, 2009; Zhao et al, 2011) add another layer of complexity to the molecular pathways that are disordered in lupus.

In this study, we identified miRNAs differentially regulated in peripheral lymphocytes in the mouse lupus model B6.Sle123. We identified miR-21 as a constitutively overexpressed miRNA in mouse SLE lymphocytes and showed that in vivo silencing of miR-21 reversed cardinal manifestations of autoimmunity, de-repressed PDCD4 expression and altered lymphocyte populations in B6.Sle123 mice.

RESULTS AND DISCUSSION

Differential miRNA expression in B6.Sle123 peripheral lymphocytes

We investigated whether miRNAs implicated in hematopoiesis (Xiao et al, 2007), in activation of innate immune responses (Taganov et al, 2006), and in apoptosis or cell proliferation (He et al, 2007; Rokhlin et al, 2008; Yamakuchi & Lowenstein, 2009) are differentially regulated in SLE and whether these miRNAs play a role in SLE pathogenesis or course of the disease. To this end, we isolated total RNA from fluorescence-activated cell sorting (FACS)-sorted splenic B and T lymphocytes from individual B6.Sle123 mice and compared their miRNA expression profiles to those of control age-matched B6 mice. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis showed that expression of several miRNAs is differentially regulated in B6.Sle123 splenic B cells, naïve (CD44LO CD62LHI) and memory (CD44HI CD62LLO) T cells (Table 1), particularly from older mice (12 months of age), which typically have advanced splenomegaly and kidney disease (Fairhurst et al, 2008; Morel et al, 2000). For up-regulated miRNAs, expression differences ranged from 1.8- to 13-fold. Specifically, in B6.Sle123 splenic B cells, miRs-21 and 222 were constitutively up-regulated relative to wild-type controls and their expression increased with disease severity. miR-181a was down-regulated in young and also in old mice with severe disease. Expression of microRNAs miR-34a, 146a, 221, 223, 142-5p and miR-155 was up-regulated in old B6.Sle123 mice with severe disease (p < 0.05, Table 1A). In naïve T cells, miR-21 was also constitutively up-regulated relative to wild-type controls and its expression increased with disease severity. miRs-221 and 155 were down-regulated in young B6.Sle123 mice but up-regulated in mice with severe disease. Expression of miR-34a, 146a and 223 increased significantly relative to wild-type controls in mice with severe disease, while expression of miR-222, 150 and 181a decreased (p < 0.05, Table 1B). In memory T cells, miR-21 and miR-146a were up-regulated relative to wild-type controls in young and older mice. miR-221 expression increased in mice with severe disease. miR-155 was down-regulated in young and old mice while miRs-34a, 221 and 222 were up-regulated and miR-181a was down-regulated in older mice with severe disease (p < 0.05, Table 1C).

In summary, interestingly, miR-21 expression was up-regulated in lupus B cells as well as in naïve and memory T cells compared to B6 controls, regardless of the age of mice (Table 1 and Fig 1A). In contrast, miR-181a and miR-150 expression was down-regulated in most lymphocyte subsets examined in lupus mice compared to B6 controls (Table 1A-C).

The Sle2 locus in B6.Sle123 mice confers expansion of the peritoneal and splenic B1-a B cell compartment (Mohan et al, 1997), even though the B2 subset remains the most abundant. To evaluate the relative contribution of each cell subset to individual miRNA abundance in the total pool of splenic CD19⁺ B cells from older SLE mice, we quantified the expression of miR-21, miR-146a and miR-155 in B1-a and B2 subsets. We detected the same trend for miR-21 and miR-146a expression in both lupus B cell subgroups whereas miR-155 was up-regulated only in B2 cells (Table 2). When lupus T cell subsets were examined in older mice, a 10- and 24-fold up-regulation of miR-21 was detected in CD4⁺ and CD8⁺ T cells, respectively, as well as a significant up-regulation of miR-146a and miR-155 compared to controls (Table 2).

Transcriptional activation of miR-21 in B6.Sle123

We found miR-21 expression to be consistently up-regulated in all SLE cell subsets that we investigated. Notably, the fold change of miR-21 expression in B6.Sle123 B lymphocytes, compared to that from age- and gender-matched B6 mice, positively correlated with the age of mice and thus with severity of their disease (Fig 1A–C). To further dissect the role of miR-21 in mouse SLE, we asked whether transcription of miR-21 gene is activated in lupus by comparing the expression of pri-miR-21 in CD19⁺ B cells isolated from 9 months old B6.Sle123 and age/ gender-matched B6. qRT-PCR analysis revealed up-regulation of pri-miR-21 transcript in B cells from lupus mice compared to controls (Fig 1D). This indicates that, at least in part, miR-21 up-regulation in B6.Sle123 B lymphocytes results from transcriptional activation of miR-21 gene.

In vivo inhibition of miR-21 de-represses PDCD4 expression in B6.Sle123 T cells

Since miR-21 has been shown to regulate apoptosis and cell proliferation pathways (Chan et al, 2005; Cheng et al, 2005; Lu et al, 2008; Sayed et al, 2010; Yang et al, 2004) and was overexpressed in all subsets of lupus lymphocytes, we decided to test whether in vivo inhibition of miR-21 in B6.Sle123 mice could affect the course of their disease. The use of LNA-modified anti-miR oligonucleotides for miRNA silencing in vivo has been previously demonstrated in rodents and non-human primates (Elmen et al, 2008; Worm et al, 2009). Furthermore, recent studies have described an approach that enables efficient antagonism of miR-21 function in vivo using a short LNA-modified seed-targeting anti-miR-21 oligonucleotide (Obad et al, 2011; Patrick et al, 2010). Thus, we designed a short-term in vivo study in which three B6.Sle123 mice were injected...
intravenously at a dose of 25 mg/kg of saline-formulated anti-miR-21 compound on three consecutive days and sacrificed within 24 h after the last dose. In control experiments, three age- and gender-matched B6.Sle123 mice were treated with a LNA scramble compound. Interestingly, our short-term study showed that silencing of miR-21 in vivo results in approximately 20% de-repression of PDCD4 in naïve CD4+ T cells from anti-miR-21 treated mice, as compared to the PDCD4 expression in naïve CD4+ T cells from mice treated with control compound (Fig 2B). PDCD4 is an inhibitor of translation initiation (Zakowicz et al, 2005) and a tumour suppressor (Cmarik et al, 1999; Jansen et al, 2005). Expression of PDCD4 is post-transcriptionally regulated by miR-21 (Asangani et al, 2008; Frankel et al, 2008). The function of PDCD4 in lymphocytes is not yet understood but it is likely involved in cell proliferation and/or apoptosis (Lankat-Buttergiet & Goke, 2009).

**Table 1. miRNA expression in B6.Sle123 B and T lymphocytes**

| miRNA | 2 mo. (mild disease) | 6 mo. (moderate disease) | 12 mo. (severe disease) |
|-------|-----------------------|--------------------------|------------------------|
|       | RQ ± SEM | p-value | RQ ± SEM | p-value | RQ ± SEM | p-value |
| miR-21 | 1.7 ± 0.15 | 0.001 | 2.2 ± 0.42 | 0.036 | 5.8 ± 0.64 | 0.016 |
| miR-34a | 0.9 ± 0.38 | 0.670 | 1.2 ± 1.33 | 0.821 | 3.2 ± 0.65 | 0.000 |
| miR-146a | 1.0 ± 0.51 | 0.996 | 0.6 ± 0.25 | 0.044 | 5.3 ± 0.75 | 0.032 |
| miR-221 | 1.2 ± 0.29 | 0.504 | 1.2 ± 0.28 | 0.398 | 13.3 ± 1.05 | 0.023 |
| miR-222 | 1.4 ± 0.22 | 0.040 | 2.1 ± 0.26 | 0.015 | 5.8 ± 0.96 | 0.037 |
| miR-223 | 0.9 ± 0.53 | 0.720 | 2.3 ± 1.61 | 0.507 | 11.4 ± 0.68 | 0.006 |
| miR-142-5p | 1.1 ± 0.21 | 0.374 | 0.4 ± 0.34 | 0.017 | 2.7 ± 0.45 | 0.032 |
| miR-155 | 1.3 ± 0.12 | 0.026 | 1.2 ± 0.39 | 0.504 | 2.8 ± 0.53 | 0.050 |
| miR-150 | 0.7 ± 0.45 | 0.314 | 0.5 ± 0.27 | 0.098 | 0.5 ± 0.18 | 0.006 |
| miR-181a | 0.5 ± 0.20 | 0.007 | 0.3 ± 0.78 | 0.069 | 0.4 ± 0.36 | 0.020 |

**A. Differential miRNA expression in B6.Sle123 splenic B cells**

**B. Differential miRNA expression in B6.Sle123 splenic naive T cells**

**C. Differential miRNA expression in B6.Sle123 splenic memory T cells**

In *vivo* inhibition of miR-21 de-represses PDCD4 expression in B6.Sle123 CD4+ T cells

We studied the expression of miR-21 and PDCD4 in primary CD4+ T cells purified from 3 months old B6.Sle123 mice cultured in the presence of bead targeting LNA anti-miR-21 or controls. Primary CD4+ T cells were purified and then mixed with culture media containing LNA anti-miR-21, control compound or no LNA. Cells were subsequently plated and harvested either immediately (time 0) or at the indicated time points (Fig 2D). Interestingly, miR-21 inhibition was observed at time 0, indicating efficient uptake of LNA compounds in the time required to mix, plate and harvest the cells. This unassisted uptake of LNA anti-miR-21 by primary CD4+ T cells cultured *ex vivo* resulted in a functional inhibition of miR-21 as shown by de-repression of PDCD4 (Fig 2C and D). As expected, although efficient LNA uptake by the primary T cells occurs within a short
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Figure 1. miR-21 expression in B6.Sle123 is regulated transcriptionally and correlates with age and disease severity.

A. pre-miR-21 and mature miR-21 expression in B6.Sle123 splenic B cells. Twenty micrograms of total splenic B cell RNA from individual age-matched 2, 6 and 12 months old B6.Sle123 and B6 mice were analysed under denaturing conditions, transferred and hybridized with LNA probe complementary to miR-21. For mass-normalization, the same membrane was stripped and re-probed with mouse snoRNA-429 radiolabelled DNA probe. The data were normalized to snoRNA-429 expression and relative fold-expression values calculated by taking the B6.Sle123/B6 ratio. A shorter exposure of the same membrane was used for the detection of the miR-21.

B. B6.Sle123 disease severity correlates with age: BUN of 2, 6 and 12 months old female B6 and B6.Sle123 mice used as a measure of disease severity.

C. Comparison of spleens extracted from 6 months old B6 and B6.Sle123 mice showing correlation of BUN and splenomegaly.

D. qRT-PCR amplification of primary miR-21 (pri-miR-21) using splenic B cell RNA from individual, age-matched 9 months old B6.Sle123 and B6 mice. The average of three individual experiments is shown. Error bars represent SEM.

Table 2. miRNA expression in B6.Sle123 lymphocyte subsets

| miRNA  | CD5⁻ B2 cells (RQ1 ± SEM (p-value)) | CD5⁺ B1a B cells (RQ1 ± SEM (p-value)) | CD4⁺ T cells (RQ1 ± SEM (p-value)) | CD8⁺ T cells (RQ1 ± SEM (p-value)) |
|--------|-----------------------------------|---------------------------------------|-----------------------------------|-----------------------------------|
| miR-21 | 5.2 ± 0.85 (0.048)                | 2.7 ± 1.30 (0.329)                    | 10.2 ± 0.76 (0.011)               | 24.4 ± 0.97 (0.009)               |
| miR-146a | 1.9 ± 1.09 (0.456)               | 1.9 ± 0.83 (0.319)                    | 5.4 ± 0.48 (0.007)                | 19.4 ± 0.93 (0.010)               |
| miR-155 | 2.1 ± 0.35 (0.038)               | 0.94 ± 1.6 (0.954)                    | 2.9 ± 0.03 (0.0001)               | 7.5 ± 0.69 (0.014)               |

As in Table 1A except CD5⁻ B2 and CD5⁺ B1a B cells and CD4⁺ and CD8⁺ T cells were FACS purified from individual ~12 months old B6.Sle123 and age- and gender matched B6 mice. Standard error and p-values were calculated using an un-paired, two-tailed Student’s t-test.
time period, de-repression of PDCD4 is only observed at subsequent time points (48 and 72 h). Our results indicate that seed-targeting LNA anti-miR-21 can silence endogenous miR-21 efficiently and in a very short period of time in primary B6.Sle123 T lymphocytes cultured ex vivo.

**In vivo inhibition of miR-21 ameliorates splenomegaly in B6.Sle123 mice**

To study the effect of in vivo miR-21 silencing in lupus, we treated 9-week-old female SLE mice by injecting them with anti-miR-21. Control mice were injected with LNA scramble or saline. Twelve 9-weeks-old female B6.Sle123 mice were randomly assigned to four groups. Mice in each group received injections of either LNA anti-miR-21 at a dose of 25 mg/kg, LNA scramble control compound at the same dose, or saline vehicle. All mice received intraperitoneal (IP) injections on three consecutive days and subsequent maintenance IP injections of 25 mg/kg every 3 weeks over a period of 3 months. The last injection was given intravenously. All mice were sacrificed within 24 h after the last dose. Spleens were extracted, weighed and subsequently, splenic lymphocytes were sorted. Northern blot analysis of total RNA isolated from liver or splenic B cells indicated that miR-21 was sequestered in a slower-migrating heteroduplex with the anti-miR-21 in the liver and in B cells (Fig 3A). Notably, silencing of miR-21 in vivo resulted in a significant reduction of splenomegaly in the SLE mice treated with anti-miR-21 compared to the LNA scramble- and vehicle control-treated mice in all four groups (Fig 3B and C). All mice survived the treatment with no observable change in the behaviour to suggest toxicity or intolerance to the treatment. Furthermore, no gross pathologic abnormalities were observed in the spleens (Fig 3B), livers, kidneys or other organs examined macroscopically during the necropsy at the completion of the study.

In addition to the observed reduction in splenomegaly, in vivo miR-21 silencing resulted in a significant decrease in the splenic
Figure 3. Silencing of miR-21 in vivo by tiny LNA-anti-miR ameliorates splenomegaly in B6.Sle123.

A. In vivo sequestration of miR-21 by seed-targeting LNA antimiR-21 in a slower migrating heteroduplex. Four groups of mice were treated with either saline (n = 4), or LNA-scramble control compound (n = 4) or tiny LNA antimiR-21 (n = 4) over a period of 12 weeks. Native Northern blot analysis of total RNA extracted from liver tissue (top panels) or from purified splenic B220+ B cells (lower panels) was performed using a radiolabelled DNA probe complementary to miR-21. The first and second sample in each panel corresponds to in vitro annealed miR-21/LNA anti-miR-21 duplex and synthetic miR-21, respectively, used as controls.

B. Effect of in vivo miR-21 silencing in autoimmune splenomegaly. Individual spleens harvested from the four groups of mice treated in the study are shown. (i, ii, iii indicate treatment with saline, LNA scramble and LNA antimiR-21, respectively).

C. Mean spleen masses plotted as spleen-mass (mg)/mouse body mass (g) ratios. Error bars represent the SEM of the four independent experiments. p-values were calculated using an un-paired, two-tailed t-test. *p = 0.0149, **p = 0.000127.
CD4$^+$ to CD8$^+$ T cell ratio and a reduction in the number of Fas receptor-expressing splenic B cells (Fig 4 and Table 3). Interestingly, elevated CD4$^+$ to CD8$^+$ ratios are a characteristic phenotypic alteration in Sle3-bearing T lymphocytes (Mohan et al, 1999). Thus, our results suggest that miR-21 inhibition skews the CD4$^+$ to CD8$^+$ ratio towards that of the background non-autoimmune strain (Mohan et al, 1999). In the LNA scramble-treated group, we observed increased CD4$^+$ to CD8$^+$

Figure 4. Silencing of miR-21 in vivo results in altered CD4/CD8 T cell ratios and reduced populations of Fas receptor-expressing B cells. Four groups of B6.Sle123 mice were treated in vivo with LNA antimiR-21 or controls for 12 weeks, as described in the text. Splenocytes from B6.Sle123 mice injected with saline (left panels), LNA scramble control (middle panels) or LNA antimiR-21 (right panels) were stained with appropriate fluorophore-conjugated antibodies and subjected to flow cytometry.

A. Top panels: CD4$^+$ and CD8$^+$ populations from the four treatment groups plotted as the percentage of viable lymphocytes. Horizontal bar indicates the mean value. Top Right: Chart of the mean CD4/CD8 ratios in the four treatment groups. $^*p = 0.0135$, $^{**}p = 0.0025$. Bottom panels: representative flow cytometry plots from one treatment group.

B. Top panels: Fas$^+$ IgD$^-$ populations from the four treatment groups plotted as the percentage of CD19$^+$ B220$^+$ lymphocytes. Horizontal bar indicates the mean value. Bottom panels: representative flow cytometry plots from one treatment group.
miR-21 in SLE

Our study shows that seed-targeting tiny LNAs can be used for in vivo inhibition of miR-21 in rapidly proliferating cells, such as splenic lymphocytes. Efficient, unassisted uptake of tiny LNAs in cultured cell lines has been recently reported (Obad et al, 2011); our study is the first to show unassisted uptake of tiny seed-targeting LNAs by primary T lymphocytes. Combined with our functional results, this new evidence supports a central role for miR-21/PDCD4-controlled pathways in mouse lupus. Further elucidation of these pathways with studies in mouse models and human cells will shed light on the molecular complexity of this prototype autoimmune disease.

In summary, our study shows that tiny seed-targeting LNAs can be used to efficiently antagonize endogenous miRNAs in peripheral lymphocytes in vivo and in vitro and that pharmacological inhibition of a miRNA using such compounds can alter the course of a spontaneous genetic disease in mice. Our work, together with a recent study showing efficient silencing of miRNA families by seed-targeting LNAs (Obad et al, 2011), opens new directions for investigations of tiny LNAs as novel therapeutic tools. In addition, our results in a mouse model of lupus corroborated by recent studies in human SLE, indicate that miR-21 plays a central role in regulating autoimmune responses in lupus and that miR-21 inhibition can favourably alter the course of a systemic autoimmune process, opening new directions for explorations of miRNA therapeutics in SLE.
The paper explained

PROBLEM:
Systemic lupus erythematosus (SLE) is a potentially fatal systemic autoimmune disease. The pathogenesis of SLE is poorly understood, however, aberrant functions of the immune system place B and T lymphocytes at the centre of investigations for novel therapeutic targets. miR-21 is implicated in cancer, however, its function in autoimmunity and specifically in SLE is poorly understood.

RESULTS:
This study identifies a set of miRNAs differentially expressed in peripheral lymphocytes in a mouse model of SLE. miR-21 is up-regulated in all lymphocyte subsets examined. In vivo and in vitro silencing of miR-21 using small, seed targeting LNA inhibitors de-represses PDCD4, a protein involved in cell proliferation and apoptosis and ameliorates cardinal SLE manifestations in mouse lupus.

IMPACT:
This work provides evidence for the involvement of miR-21 in SLE. Furthermore, this study is the first to show that pharmacological in vivo inhibition of miR-21 using seed-targeting LNAs can alter the course of a spontaneous, genetic disease in mice. Our work opens new directions for the use of small LNA inhibitors as therapeutic tools in autoimmune diseases.

MATERIALS AND METHODS

Mice
Breeder pairs of B6.Sle1Sle2Sle3 (B6.Sle123) (Morel et al, 2000) mice were a kind gift from Dr. Laurence Morel (University of Florida, Gainesville). B6.Sle123 or C57BL/6 (B6) (Jackson Laboratory, Bar Harbor, ME, USA) mice were bred and maintained in pathogen-free, Institutional Animal Care and Use Committee (IACUC)-approved animal facility at the University of Pennsylvania. Care and experimental procedures were approved by the IACUC at the University of Pennsylvania. BUN was measured in freshly drawn cardiac blood (Azostix reagent strips, Siemens Healthcare Diagnostics Inc.). BUN of 5–15 mg/dL was considered consistent with no detectable renal disease, 15–26 mg/dL consistent with mild disease, 30–40 mg/dL consistent with moderate disease and 50–80 mg/dL consistent with severe disease.

Antibodies
Rat anti-mouse CD19 (1D3), CD44 (IM7), CD44 (30-F11), CD62L (MEL-14), CD19 (1D3) and hamster anti-mouse CD3e (145-2C11), CD69 (H1.2F3), CD11c (HL3), were purchased from BD Pharmingen. Rat anti-mouse CD45R (B220; RA3-6B2), CD5 (53-7.3), CD4 (L3T4), CD4 (GK1.5), CD8 (53-6.7), IgD (11-26c), hamster anti-mouse CD3e (145-2C11), and mouse anti-mouse CD95 (APO-1/FasR; 13A7) were from eBioscience.

Splenocyte preparation and lymphocyte purification
All procedures were performed on ice in FACS buffer (1X DPBS (Ca²⁺/Mg²⁺ free); 0.5% BSA; 2 mM EDTA). Single-cell suspensions were prepared by crushing spleens between frosted glass slides. Cells were blocked with rat anti-mouse CD16/32 (2.4G2) monoclonal antibody (mAb) for 15 min at room temperature (RT) and stained for 30 min on ice with appropriate fluorophor-conjugated antibodies. Dead cells were excluded from FACS by staining with DAPI. FACS sorting was performed at 4°C on a FACSAria cytometre (BD Biosciences) to a purity >95%. Alternatively, B cells were purified using autoMACS magnetic cell separation columns. Following purification, cells were either pelleted and snap-frozen or processed immediately.

Flow cytometry
All flow cytometry experiments were performed on a Becton Dickinson FACSCalibur at the University of Pennsylvania and the acquired data analysed with the FloJo software package (Tree Star Inc.).

RNA purification and quantitative real-time PCR
Total RNA was extracted using either Trizol reagent (Invitrogen) or the mirVana miRNA Isolation Kit (Ambion). For miRNA-specific reverse transcription (RT), 20 ng purified total RNA was reverse-transcribed (TaqMan miRNA-specific RT PCR primers, Applied Biosystems). qRT-PCR was performed in triplicates using 1:10 dilution of the RT product (Applied Biosystems 7500 real-time PCR system, according to the manufacturer’s instructions). snoRNA-429 expression was used as control. Relative quantification (RQ) of B6.Sle123 miRNA expression was assessed by calculating the 2−ΔΔCt, where ΔΔCt = ΔCtmiRNA−Dct snoRNA.

For qRT-PCR amplification of the primary miR-21 (pri-miR-21), primers (F: 5’-TCA ATG GCT GTA-3’ and R: 5’-GAT GCC GGG TAA TGT TTG AAT G-3’) spanning a 209 nt sequence containing the mouse pre-miR-21, were designed by identifying a mouse genomic sequence with 91% similarity to the human primary miR-21. Splenic B cells (CD19⁺) were isolated from 3 nine months old B6.Sle123 and three age- and gender-matched C57BL/6 and B6.Sle123 mice using anti-CD19 MACS microbeads (Miltenyi Biotec, Germany). Total RNA was extracted with Trizol (Invitrogen) and double treated with TURBO DNase (Ambion, Inc., Austin, Texas, USA) to remove genomic DNA. Six hundred nanograms of total RNA was reverse-transcribed (Random primers, Applied Biosystems and Superscript III Reverse Transcriptase, Invitrogen). qRT-PCR was performed in triplicates using 1/3 of the RT product for pri-miR-21 and 1/3 for β-actin amplification (β-actin primers from Applied Biosystems Mm 00607939_S1). No-RT (-RT) RNA was used as negative control to exclude amplification due to genomic DNA contamination. RQ of B6.Sle123 pri-miR-21 expression was assessed by calculating the 2−ΔΔCt, where ΔΔCt = ΔCtpri-miR-21−ΔCtβ-actin.
Northern blot analysis
Total RNA samples were resolved on 15% urea-polyacrylamide gels (20% polyacrylamide for native Northern blots), transferred to nitrocellulose membranes, hybridized with radiolabelled DNA or LNA probes complementary to miR-21. snoRNA-429 expression was used for normalization. Detection and analysis: Storm 860 (Molecular Dynamics) and ImageQuant (GE).

Protein isolation and Western blot analysis
Purified cell populations were pelleted, washed with PBS then resuspended (10^6 cells/ml) in RPMI 1640 media (supplemented with L-glutamine, 1% non-essential amino acids, 50μM 2-mercaptoethanol and 100 U/ml IL-2) containing either PBS, 50 μM LNA scramble oligonucleotide or 50 μM LNA anti-miR-21 and plated in 96-well round-bottom plates (1 x 10^3 cells/well) and maintained at 37°C ± 5% CO2. At selected time points, cells were harvested and miR-21 expression was assessed by qRT-PCR.

Ex vivo T cell culture and miR-21 inhibition
Spleens from 3 months old B6.Sle123 mice were harvested and single-cell suspensions prepared as described. Splenocytes were pooled and CD4^+ T cells purified by incubation with anti-CD4 MACS microbeads (L4T4) followed by separation on MACS LS columns (Miltenyi Biotec, Auburn, CA) according to the Manufacturer’s instructions. The purity was >90% CD4^+, as determined by flow cytometry. Cells were resuspended (10^5 cells/ml) in RPMI 1640 media (supplemented with 10% FBS, 25 mM HEPES, 100 U/ml penicillin/streptomycin, 2 mM

Author contributions
MK and BGG designed the studies and prepared the manuscript; BGG performed cell purification, miRNA expression, in vivo silencing, protein expression studies and statistical analysis; RC and RAE assisted with the design and analysis of cell sorting and flow cytometry studies; AP assisted with the statistical analysis; PYY performed immunoassays; YTL and OBE cloned and analysed pri-miR-21 expression; MK, BGG and SK designed the in vivo silencing studies and analysed the data; SO and BGG validated the LNA compounds; MK supervised the studies and performed data analysis.

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Conflict of interest statement: SO, AP, and SK are employees of Santaris Pharma, a clinical stage biopharmaceutical company that develops RNA-based therapeutics. The other authors declare that they have no conflict of interest.

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