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Dicer Insufficiency and MicroRNA-155 Overexpression in Lupus Regulatory T Cells: An Apparent Paradox in the Setting of an Inflammatory Milieu

Anagha A. Divekar, Shweta Dubey, Pallavi R. Gangalum, and Ram Raj Singh

Systemic lupus erythematosus is a chronic autoimmune disease characterized by loss of tolerance to self-Ags and activation of autoreactive T cells. Regulatory T (Treg) cells play a critical role in controlling the activation of autoreactive T cells. In this study, we investigated mechanisms of potential Treg cell defects in systemic lupus erythematosus using MRL-Fas<sup>+/−/−</sup> (MRL/lpr) and MRL-Fas<sup>−/−</sup> mouse models. We found a significant increase in CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells, albeit with an altered phenotype (CD62L<sup>−</sup>CD69<sup>+</sup>) and with a reduced suppressive capacity, in the lymphoid organs of MRL strains compared with non-autoimmune C3H/HeOuj mice. A search for mechanisms underlying the altered Treg cell phenotype in MRL/lpr mice led us to find a profound reduction in Dicer expression and an altered microRNA (miRNA, miR) profile in MRL/lpr Treg cells. Despite having a reduced level of Dicer, MRL/lpr Treg cells exhibited a significant overexpression of several miRNAs, including let-7a, let-7f, miR-16, miR-23a, miR-23h, miR-27a, and miR-155. Using computational approaches, we identified one of the upregulated miRNAs, miR-155, that can target CD62L and may thus confer the altered Treg cell phenotype in MRL/lpr mice. In fact, the induced overexpression of miR-155 in otherwise normal (C3H/HeOuj) Treg cells reduced their CD62L expression, which mimics the altered Treg cell phenotype in MRL/lpr mice. These data suggest a role of Dicer and miR-155 in regulating Treg cell phenotype. Furthermore, simultaneous appearance of Dicer insufficiency and miR-155 overexpression in diseased mice suggests a Dicer-independent alternative mechanism of miRNA regulation under inflammatory conditions.

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Abbreviations used in this article: C3H, C3H/HeOuj; miRNA, microRNA; MRL<sup>−/−</sup>, MRL-Fas<sup>−/−</sup>; MRL/lpr; MRL-Fas<sup>+/−/−</sup>; qPCR, quantitative real-time PCR; SLE, systemic lupus erythematosus; Treg, regulatory T

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MicroRNA-155 is a potent miRNA that targets CD62L, a major adhesion molecule involved in T cell migration and homing. Overexpression of miR-155 in Treg cells was associated with reduced CD62L expression, which mimics the altered phenotype of Treg cells in MRL/lpr mice. These data suggest that miR-155 overexpression may contribute to the reduced CD62L expression and altered phenotype of Treg cells in systemic lupus erythematosus.
Materials and Methods

Mice
Breeding pairs of MRL/lpr (lupus-prone mouse with lpr mutation in their Fox gene), congenic MRL-+/+ and MHC-matched C3H mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at the University of California at Los Angeles specific pathogen-free facility. Animal experiments were performed following approved institutional guidelines. To assess the relevance of findings in relation to disease, most experiments were performed using 4-, 12-, and 16-wk-old MRL/lpr mice, which correspond to preautoimmune, autoimmune but preclinical, and early clinical disease stages, respectively. MRL-+/+ mice develop lupus disease by 8–10 mo of age.

Flow cytometry
Spleens were processed into single-cell suspension followed by RBC lysis (Pharm Lyse; BD Biosciences, San Jose, CA). Cells (1 × 10⁷) were incubated on ice for 20 min at 4°C using Ab cocktails for following markers: CD25 (clone PC61.5), CD62L (clone MEL-14), CD73 (clone TY11/8), CD69 (clone H1.2F3), CD127 (clone A7R34), Foxp3 (clone FJK-16s) (all from eBioscience, San Diego, CA); and CD4 and TCRβ (BD Biosciences). Intracellular staining was performed to detect Foxp3, according to the manufacturer’s guidelines. A seven-color panel comprised of Foxp3-PE-TTC, CD25-allophycocyanin, CD4 (clone CT-CD4)-Pacific Orange, CD127-PE-Cy5, CD69-PECy5, CD62L-allophycocyanin-cy7, and CD73 or CD39 (clone A1)-PE was used. Seven-color panels were acquired on a FACSAria or LSRII flow cytometer (BD Biosciences). Four-color panels were acquired on a FACSCalibur (BD Biosciences). For the analysis of Treg cells, the gate was set on small lymphocytes based on forward versus side scatter. Data were analyzed using FlowJo (Tree Star, Ashland, OR).

Purification of Treg cells
Splenocytes (50 × 10⁶) were used to enrich CD4⁺CD25⁺ cells using a Treg cell isolation kit from Miltenyi Biotec (Auburn, CA). Postpurification purity of cells was >95%.

Proliferation assays
Splenocytes from C3H or MRL/lpr mice were used as responder cells, which were labeled with CFSE (Invitrogen, Carlsbad, CA), and stimulated with 1 μg/ml anti-CD3 (clone 145-2C11; BD Biosciences). Purified Treg cells were mixed with CFSE-labeled splenocytes at responder/Treg cell ratios of 1:2, 1:4, 1:8, and 1:16. After 72 h, cells were harvested, triplicates were pooled, and cells were stained with anti-CD4 allophycocyanin to detect proliferating CD4⁺ T cells. Dead cells were gated out by size and 7-aminoactinomycin D staining.

Isolation of large and small (micro) RNA
Spleens were processed into a single-cell suspension for purifying Treg cells, as described above. The enriched Treg cells were washed three times with PBS. RNA extraction was performed using TRIzol (Invitrogen) and chloroform (Sigma-Aldrich, St. Louis, MO), followed by DNAse treatment and clean-up using the RNeasy MiniElute cleanup kit (Qiagen, Valencia, CA). Large RNA and miRNA were separately purified, as per the manufacturer’s guidelines (Qiagen). RNA was estimated on a spectrophotometer, and OD260 was used for quantification of RNA samples.

Quantitative real-time PCR for Dicer
Large RNA from purified Treg cells was used for quantitative real-time PCR (qPCR) assay. Total RNA was converted to cDNA using the QuantiTect reverse transcription kit (Qiagen). Ten nanograms of total RNA was used per well of the qPCR reaction and each sample was run in triplicates. SYBR Green was purchased from Qiagen. A separate qPCR assay was performed using eight different housekeeping genes. Data were analyzed using geNorm software to choose the most stable housekeeping gene for comparison between C3H and MRL strains of mice (22). The primers for Dicer were as follows: DicerF1 (5’-CCTGACAGTG-ACGGTCCAAGG-3’) and DicerR1 (5’-CATGACTCTCAAATCACAAT-3’) (19).

miRNA PCR arrays
Sixty to 100 ng of miRNA from purified Treg cells was converted to cDNA using the miRNA first-strand kit. miRNAs were detected using the miFinder RT⁺ miRNA PCR arrays (MAM-001A; SABiosciences, Frederick, MD) that profile the expression of the 88 most abundantly expressed and best characterized miRNA sequences. Plates were run on a Bio-Rad iCycler (Bio-Rad, Hercules, CA) and data analyzed using the SABiosciences Web site portal. The PCR array data were submitted to Minimum Information about a Microarray Experiment-compliant Array Express database (http://www.ebi.ac.uk/arrayexpress/) with the accession no. A-MEXP-1924.

qPCR to detect miRNAs
Primers for qPCR for miR-23a, -27a, -141, -140, and -155 were purchased from Qiagen. miRNA was converted to cDNA using the miScript reverse transcription kit (Qiagen). Real-time PCR reactions were performed, according to manufacturer’s directions, and the reactions were run on a Bio-Rad iCycler.

miRNA overexpression studies
Purified Treg cells were transfected with 20 or 40 nM mimics of miR-155 and -23a and HiPerfect transfection reagent (Qiagen), according to the manufacturer’s directions. Cells were incubated at 37°C in 5% CO2 for 24 h, harvested, and stained for CD4 and CD62L.

Statistical analysis
Groups were compared using the nonparametric Mann–Whitney U test or Student t test. miRNA array data were analyzed using the SABiosciences Web site analysis portal.

Results

CD4⁺CD25⁺Foxp3⁺ Treg cells accumulate in the spleen of lupus-prone MRL strains
Previous studies on the frequency of Treg cells in humans and animals with SLE have reported variable results (5–8, 19). To first reconcile these discrepancies, we undertook a comprehensive analysis of Treg cells in MRL mice. Results show that frequencies and total numbers of Treg cells, defined as CD4⁺CD25⁺ (Fig. 1A) or CD4⁺CD25⁺Foxp3⁺ T cells (Fig. 1B), were not reduced in the spleens of MRL/lpr compared with C3H mice. In fact, numbers of these cells were significantly higher in MRL/lpr than in C3H mice. Frequencies of CD4⁺CD25⁺ cells that are identified as bona fide Treg cells in humans (23) were also higher in MRL/lpr mice than in C3H mice (not shown in figures). Treg cell expansion in MRL/lpr mice is related to autoimmunity and not to the lpr mutation in their lpr gene, since the congenic MRL-+/+ mice that develop a similar but delayed disease (21) also exhibit increased frequencies of Treg cells in their spleen compared with C3H mice (Supplemental Fig. 1A, 1B). Such increase in Treg cells in these mice precedes the onset of clinical disease (Supplemental Fig. 1A). Thus, accumulation of Treg cells in lymphoid organs is a feature of lupus-prone MRL strains.

MRL/lpr CD4⁺CD25⁺Foxp3⁺ Treg cells exhibit an altered phenotype
Expression of CTLA-4, CD39, CD73, and Foxp3 and low levels of CD127 has been used to define Treg cells (24–27). To determine the phenotype of Treg cells in lupus-prone mice, we analyzed expression of these markers on splenich CD4⁺CD25⁺Foxp3⁺ T cells from C3H and MRL/lpr mice using eight-color multiparametric flow cytometry (Fig. 1C, Supplemental Fig. 2). Expression of CD39, CD73, CTLA-4, CD127, and Foxp3 was not significantly different between control C3H and lupus-prone MRL/lpr Treg cells (Supplemental Fig. 2). However, CD69 expression that has been associated with reduced Treg cell function in patients with SLE (28) was increased in CD4⁺CD25⁺Foxp3⁺ Treg cells from MRL/lpr compared with C3H mice. MRL/lpr Treg cells also...
showed reduced CD62L expression compared with C3H mice (Fig. 1C). As shown in Fig. 1D, MRL/lpr mice had significantly increased proportions of CD4+CD25+Foxp3+CD62L−CD69+ T cells as compared with C3H mice. MRL/lpr mice, however, are not born with this altered Treg cell phenotype (i.e., CD69+CD62L−), as CD4+CD25+Foxp3+ Treg cells from 4-wk-old MRL/lpr mice had a phenotype similar to C3H mice (Fig. 1E). MRL+/+ mice also exhibited a similar phenotype (Supplemental Fig. 1C), thus linking Treg cell phenotype abnormality to autoimmunity and not to the lpr mutation per se. Thus, an altered Treg cell phenotype, characterized by increased CD69 and reduced CD62L expression, arises prior to the onset of inflammatory disease in lupus-prone MRL strains.

Reduced suppressive capacity of MRL Treg cells correlates with reduced CD62L and increased CD69 expression

Our data are intriguing, as MRL mice develop a fatal autoimmune disease despite a significant increase in the Treg cell population. This led us to posit that either MRL Treg cells are dysfunctional or that CD4+CD25+Foxp3+ cells are not the bona fide Treg cells in the context of autoimmune disease. We found that while C3H Treg cells potently inhibit the proliferation of anti-CD3–stimulated syngeneic splenocytes, Treg cells from most MRL/lpr mice had a reduced capacity to suppress C3H splenocytes (Fig. 2A, 2C). Splenocytes from adult MRL/lpr mice could not be used as responder cells, as they exhibit a profound defect in proliferation upon anti-CD3 stimulation (S. Dubey and R.R. Singh, unpublished data). However, when we used splenocytes from young MRL/lpr mice as responder cells, MRL/lpr Treg cells continued to exhibit a similarly diminished suppressive capacity compared with C3H Treg cells (Fig. 2B). A similar defect in suppressive function was detected in Treg cells from 12-wk-old MRL+/+ mice (Supplemental Fig. 1D). Thus, Treg cells from MRL strains exhibit a defect in suppressing the proliferation of syngeneic or MHC-matched splenocytes.

MRL/lpr Treg cells displayed a similar inability to suppress splenocytes from both C3H and MRL/lpr mice (Fig. 2A, 2B), suggesting an intrinsic defect in MRL/lpr Treg cells. Interestingly, MRL/lpr mice that did not exhibit a defect in Treg-mediated suppression showed a similar CD69 expression as C3H mice (Fig. 2D), whereas MRL/lpr mice with reduced Treg cell function had increased CD69 and reduced CD62L expression. Thus, the defective function of MRL/lpr Treg cells may be related to their altered phenotype shown in Fig. 1C.

Profound reduction in Dicer expression in MRL/lpr Treg cells

Deletion of Dicer1 in Treg cells results in fatal autoimmunity and altered Treg phenotype (17, 19), akin to that observed in MRL/lpr

FIGURE 1. CD4+CD25+Foxp3+ Treg cells increase and exhibit altered phenotype in MRL/lpr mice. Splenocytes from MRL/lpr and C3H mice were analyzed for Treg cells, as indicated below. A, CD4+CD25+ cells are indicated on dot plots as the proportion of gated small lymphocytes (left). Frequency and total numbers of CD4+CD25+ T cells are shown as the mean ± SE from 10 mice per group, all ≥12 wk old (right). B, CD4+CD25+Foxp3+ T cells are indicated in right upper quadrants as the proportion of gated CD4+ cells (left). Frequency (as a proportion of splenocytes) and total numbers of CD4+CD25+Foxp3+ T cells are shown as the mean ± SE from nine mice per group (right). C, CD69 and CD62L expression on TCRβ+CD4+CD25+Foxp3+ T cells. D, CD69+CD62L− Treg cells are shown as the proportion of CD4+CD25+Foxp3+ cells from six mice per group. E, CD62L expression on gated CD4+CD25+Foxp3+ T cells in 4-wk-old (preautoimmune) and 12-wk-old (early autoimmunity, preclinical) MRL/lpr mice (left), 4-wk-old C3H and MRL/lpr mice (middle), and 11-wk-old C3H and MRL/lpr mice (right). Results represent two to five independent experiments.
A

B

C

D

FIGURE 2. Reduced suppression by Treg cells from MRL/lpr mice. Treg cells were purified from spleens of C3H and MRL/lpr mice and mixed with CFSE-labeled responder splenocytes from C3H (A) or MRL/lpr mice (B) in triplicates. On day 3, triplicate wells were pooled and stained with 7-aminoactinomycin D to gate out dead cells, and proliferation of CD4+ T cells was analyzed. CFSE dilution at 1:2 Treg cell/responder cell ratio is shown. Gray shaded area indicates responder C3H (A) or MRL/lpr (B) splenocytes with no Treg cells; red line, responder cells plus MRL/lpr Treg cells; and green line, responder cells plus C3H Treg cells. C. Percentage suppression of proliferation of responder splenocytes in presence of Treg cells from C3H or MRL/lpr mice is shown as the mean ± SE (n = 17 C3H and 21 MRL/lpr mice in eight independent experiments). **p < 0.01. D, Histogram showing CD69 expression on CD4+CD25+Foxp3+ cells from C3H mice (gray line) and MRL/lpr mice that had no defect in Treg suppressive capacity (black line). Representative data from two independent experiments, each using two mice per group, are shown.

mice. This led us to ask whether Dicer expression is reduced in MRL/lpr Treg cells. Indeed, Dicer1 levels, as determined by qPCR, were profoundly lower in MRL/lpr Treg cells than in Treg cells isolated from C3H mice (Fig. 3A). Such spontaneous Dicer insufficiency arose prior to the onset of clinical disease in MRL/lpr mice, but was not seen in these mice at an early age (4 wk). Thus, Dicer insufficiency in MRL/lpr mice may play a role in maintaining normal Treg cell phenotype and function and in preventing systemic autoimmune disease.

Altered miRNA profile in MRL/lpr Treg cells

Dicer controls the generation of miRNAs in T cells, and Dicer-controlled miRNAs appear to play a critical role in T cell-mediated tolerance in knockout animals (17, 19). We asked whether spontaneous Dicer insufficiency in MRL/lpr Treg cells (Fig. 3A) is associated with altered miRNA expression. Purified Treg cells from spleens of MRL/lpr and C3H mice were analyzed for expression of miRNAs using PCR arrays (SABiosciences) that profile 88 most abundantly expressed and best characterized miRNA sequences (Supplemental Fig. 3). Twelve miRNAs were increased and 54 were reduced by $2$-fold in each of the three MRL/lpr mice as compared with the average of three C3H mice. However, only seven miRNAs met these criteria and were statistically significantly different between MRL/lpr and C3H mice ($p < 0.05$), as shown in Fig. 3B. Although recent studies have assigned a miRNA profile to normal Treg cells (16, 29), to our knowledge, our data ascribe for the first time a miRNA signature to lupus Treg cells.

We next performed a qPCR assay to further confirm the expression of miR-155, -23a, -27a, -140*, and -141, which were found to have an altered expression (>5-fold) in MRL/lpr Treg cells in miRNA array experiments and were predicted to confer altered Treg cell phenotype in MRL/lpr mice, as discussed below. Results confirming the overexpression of miR-23a and -155 in Treg cells of diseased MRL/lpr mice are shown in Fig. 3C and 3D. Expression of miR-27a, -140*, and -141 showed significant animal-to-animal variability in these experiments (data not shown). Thus, MRL/lpr Treg cells show increased expression of miR-23a and -155, which may modulate the expression of Dicer and CD62L, respectively.

Inverse correlation between Dicer and miR-155 expression in Treg cells in MRL/lpr mice

Our data showing increased expression of several miRNAs, including miR-155, in MRL/lpr mice (Fig. 3B, Supplemental Fig. 3) are surprising, as we had expected to find reduced miRNAs in these mice owing to markedly reduced Dicer expression (17). Further analyses of Dicer and miR-155 expression showed that Dicer and miR-155 levels were similar between lupus-prone and control mice at a young age prior to onset of autoimmunity (Fig. 3A, 3C). However, $12$-wk-old MRL/lpr mice that begin to exhibit evidence of autoimmunity and inflammatory disease had significantly reduced Dicer and increased miR-155 levels compared with age-matched control mice (Fig. 3A, 3C). Thus, while Dicer normally regulates the generation of miR-155 in T cells (17), alternate mechanism(s) for mature miRNA generation may exist under inflammatory conditions. Alternatively, low levels of Dicer expressed in MRL/lpr Treg cells may be sufficient to generate a few mature miRNAs, including miR-155.

Bioinformatic target prediction analyses for MRL/lpr Treg phenotype

To understand the relevance of altered miRNA profile in lupus Treg cells, we asked which of these altered miRNAs can potentially confer the altered Treg cell phenotype—increased CD69 and reduced Dicer and CD62L—seen in MRL/lpr mice. Using miRBase (Microcosm) and TargetScan prediction databases, we found that CD69 is not predicted to be a target for any of the miRNAs that are significantly differentially expressed in MRL/lpr Treg cells. Two miRNAs (miR-23a and -27a) that are upregulated in MRL/lpr Treg cells and two (miR-141 and -144) that are reduced in MRL/lpr Treg cells can bind Dicer. Of these, miR-23a and -27a are predicted to have lower context scores compared with miR-141 and -144, which indicates a more favorable sequence of the latter miRNAs for binding Dicer. Our ongoing studies will determine whether these miRNAs contribute to reduced Dicer expression in MRL/lpr Treg cells. Finally, CD62L is predicted to be a target for miR-140*, -141 and -144, which are reduced in MRL/lpr Treg cells by $2$-fold ($p = NS$). Importantly, CD62L is predicted to be a target for miR-155 ($p = 9.2 \times 10^{-5}$; Microcosm database), which is significantly increased in MRL/lpr compared with C3H Treg cells ($>4$-fold; $p < 0.05$, in both PCR arrays and qPCR assays). Thus, high levels of miR-155 may be causally related to the reduced CD62L expression in MRL/lpr Treg cells.

Induced expression of miR-155 in otherwise normal Treg cells alters their phenotype

To directly address whether miR-155 regulates CD62L expression, we examined whether the induced overexpression of miR-155 in otherwise normal Treg cells will reduce their CD62L expression. Purified Treg cells from C3H mice were transfected with mimics for miR-155 or for miR-23a that does not target CD62L (control) or with no mimic (mock). Transfected cells were harvested after $24$ h and analyzed for CD62L expression. As shown in Fig. 4, induced
overexpression of miR-155 resulted in reduced CD62L expression as compared with the mock or miR-23a transfected cells. These data suggest a role for miR-155 in regulating CD62L expression.

**Discussion**

To the best of our knowledge, we report the first example of spontaneous *Dicer* deficiency that develops in MRL/lpr Treg cells that exhibit a reduced suppressive capacity and an altered phenotype characterized by increased CD69 and reduced CD62L expression. Despite such a profound reduction in *Dicer*, seven miRNAs are unexpectedly and significantly upregulated in MRL/lpr cells. One of these upregulated miRNAs, miR-155, suppresses CD62L expression. These alterations in Treg cells correlate with the onset of autoimmune disease in lupus mice.

Treg cells play a critical role in maintenance of peripheral tolerance. A reduction in their numbers has been reported to cause autoimmune disease (3, 30, 31). Consistently, many studies have found reduced numbers of Treg cells in humans and NZB/W F1 and NZM.2328 mice with SLE (5–8). Surprisingly, we found that lupus-prone mice had an increase in CD4+CD25+Foxp3+ Treg cells in spleen (Fig. 1), lymph nodes, and thymus (data not shown). These changes are seen prior to the onset of clinical disease, but not at a very young age, as 4-wk-old MRL/lpr mice had no increase in Treg cell numbers compared with C3H mice (Supplemental Fig. 1A). The increase in Treg cells was related to autoimmunity and not necessarily to the *lpr* mutation per se, as the Fas-intact MRL+/- mice also exhibited similar abnormalities, albeit at a later age (Supplemental Fig. 1). Despite such an increase in Treg cells in MRL strains, these mice develop fatal autoimmune disease. Therefore, MRL Treg cells must be defective in their suppressive function. Indeed, the in vitro suppressive capacity was impaired in most MRL mice (Fig. 2, Supplemental Fig. 1D). These data are largely consistent with previous studies showing that while the numbers of Treg cells may be unchanged in the peripheral blood of MRL/lpr mice (6), their suppressive function is reduced.

![FIGURE 3. Expression of Dicer and miRNAs in MRL/lpr mice. A, RNA was extracted from purified splenic Treg cells, as described in Materials and Methods, and qPCR was performed for Dicer expression. Relative Dicer expression normalized to Gapdh is shown as the mean ± SE in 12- to 16-wk-old C3H (n = 13) and MRL/lpr (n = 12, old) mice and in seven young, preautoimmune mice (4 wk old, young). Results represent four independent experiments. B, miRNA was extracted from purified splenic Treg cells and converted to cDNA, as described in Materials and Methods. miFinder PCR arrays were used to profile 88 miRNAs (Supplemental Fig. 3). Raw threshold values for obtained and changes in miRNA expression were calculated using SABiosciences Web analysis platform. Threshold cycle values were calculated from raw threshold cycle data. Threshold cycle values from three independent experiments, each using cells purified from one each of C3H and MRL/lpr mice, were compared. miRNAs that are significantly different between C3H and MRL/lpr mice are shown. *p < 0.05; **p < 0.01; n = 3 each. C and D, qPCR reactions for a few selected miRNAs were set up, as per the manufacturer’s instructions. Results are shown as the mean ± SE for miR-155 in 4- to 5-wk-old MRL/lpr (n = 4) and MRL/lpr (n = 5) mice (C) and for miR-23a in four each of 12- to 20-wk-old female C3H and MRL/lpr mice (D). Results represent three (C) and two (D) independent experiments.](http://www.jimmunol.org/)

![FIGURE 4. Overexpression of miR-155 in otherwise normal Treg cells alters their phenotype. Treg cells enriched from C3H mice were transfected with 40 nM miR-155, miR-23a, or with HiPerfect reagent alone, as described in Materials and Methods. Representative histogram shows reduced CD62L expression in C3H cells transfected with miR-155 mimic. Similar results were obtained in five of seven mice tested in three independent experiments.](http://www.jimmunol.org/)

Roetman and colleagues (28) recently reported the first example of a spontaneous *Dicer* deficiency, with the results indicating a role for *Dicer* in the development of SLE. It is possible that the changes in Dicer expression, miRNA expression, and miRNA function in MRL/lpr Treg cells may be a result of the *Dicer* deficiency. However, the changes in miRNA expression and miRNA function in MRL/lpr Treg cells are not solely due to the *Dicer* deficiency, as the Fas-intact MRL+/- mice also exhibit similar abnormalities, albeit at a later age (Supplemental Fig. 1).
reduced in these mice as well as in other mouse models of SLE and in humans with SLE (6, 7, 11, 28).

The reduced functional capacity of MRL Treg cells was associated with a characteristic phenotype (i.e., increased CD69 and reduced CD62L expression) (Fig. 1C–E). None of the other markers tested was significantly different between MRL and control mice (Supplemental Fig. 2). Ongoing studies will investigate whether altered CD62L expression affects the ability of Treg cells to migrate to target organs affected by lupus disease.

Mechanisms underlying Treg defect in SLE remain mostly unclear. Altered CTLA-4 on Treg cells, increased CD80/CD86 expression on APCs, and increased IL-6 production have been implicated in defective function in Treg cells in lupus (6, 7, 32). We did not find reduced CTLA-4 expression on MRL/lpr Treg cells (Supplemental Fig. 2). Previous studies have suggested an altered amenability of MRL/lpr CD4⁺ T effector cells to suppression by Treg cells in MRL/lpr mice (6, 32). However, MRL/lpr Treg cells exhibited a similar inability to suppress splenocytes from MRL/lpr and C3H mice, suggesting an intrinsic defect of MRL/lpr Treg cells and not in the responder population. CD73 and CD39 have been shown to mediate Treg function through adenosine-mediated inhibition (24, 25). We found that although a few MRL/lpr mice had reduced CD73 expression compared with C3H mice (Supplemental Fig. 2), there was no correlation between expression of CD73 or CD39 and Treg suppressive defective in MRL/lpr mice. CD127 expression that inversely correlates with the suppressive function of human Treg cells (27) was also not different between C3H and MRL/lpr mice (Supplemental Fig. 2).

Animals rendered deficient in the endoribonuclease Dicer develop uncontrolled autoimmune disease (19). Interestingly, the Dicer knockout mice exhibit a Treg cell phenotype that is similar in many ways to the Treg cell phenotype in MRL/lpr mice. To the best of our knowledge, our data demonstrates the first example of an acquired deficiency of Dicer, which occurs spontaneously in MRL/lpr mice just prior to the onset of inflammatory disease. Thus, genetic deficiency of Dicer in knockout mice as well as its acquired deficiency in MRL/lpr mice is associated with Treg abnormalities and development of autoimmune disease, suggesting an important role of Dicer in Treg cell function and prevention of autoimmunity. Indeed, the Dicer-controlled miRNA pathway has been shown to be important in the control of autoimmunity (17, 19).

The reduced generation of miRNAs owing to profoundly reduced Dicer may contribute to reduced suppressive function of Treg cells in MRL/lpr mice. Our PCR array analysis did reveal a ≥2-fold reduction in MRL/lpr Treg cells in 54 of 88 miRNAs tested. Unexpectedly, however, seven miRNAs were significantly upregulated in MRL/lpr compared with C3H Treg cells. Thus, low levels of Dicer in MRL/lpr Treg cells may be sufficient for the generation of mature miRNAs. Alternatively, alternate pathways to generate mature miRNAs (33) may arise under inflammatory conditions associated with reduced Dicer expression, such as in MRL/lpr mice. Bolstering this idea is a recent report that identified a novel miRNA processing pathway that is independent of Dicer (34).

Recent studies have reported miRNA signatures in Treg cells from humans and mice (16, 29). Interestingly, three of the five human Treg cell signature miRNAs were differentially expressed in MRL/lpr versus C3H Treg cells. The expression of miR-21, a signature miRNA for both human and mouse Treg cells, was 13-fold increased in MRL/lpr versus C3H Treg cells (p = 0.07). The two miRNAs, miR-31 and -125a, that are underexpressed in human Treg cells were also underexpressed in MRL/lpr Treg cells by 4.6- and 2.4-fold (p > 0.1), respectively, compared with C3H Treg cells. A further analysis of these miRNAs using additional animals at different age groups is needed, because a previous study in human Treg cells has shown that miR-31 negatively regulates FOXP3 expression by binding directly to its potential target site in the 3’-untranslated region of FOXP3 mRNA, whereas miR-21 acts as a positive regulator of FOXP3 expression (29). Thus, overexpression of miR-21 and underexpression of miR-31 in MRL/lpr Treg cells might be related to the increase in Foxp3⁺ Treg cells in these mice.

Mouse Treg signature miRNAs (16), including miR-16, -19a, -21, -23a, -23b, -30e, -142-5p, -146, -150, and -155, were further differentially expressed in the same direction by 2- to 13-fold in MRL/lpr Treg cells as compared with C3H Treg cells (p < 0.05 to <0.01 for miR-16, -23a, -23b, and -155, and p > 0.05 for other miRNAs). However, miR-30 that is underexpressed in C57BL/6 lymph node Treg cells compared with syngeneic C4D⁺CD25⁺ T cells was overexpressed in MRL/lpr Treg cells, whereas miR-125a and -214, which are overexpressed in C57BL/6 mouse Treg cells, were underexpressed in MRL/lpr Treg cells compared with C3H Treg cells. Additional miRNAs were also differentially expressed in MRL/lpr versus C3H Treg cells. Among the miRNAs that were underexpressed in MRL/lpr mice, miR-22, -140*, -141, and -214 have predicted target sites for Treg cell-related molecules Dicer1 and CD127. Target prediction algorithms have also identified CD62L and Dicer1 to be targets for miR-155 and miR-23a, respectively. An altered expression of these miRNAs may thus alter the function of their target genes in Treg cells, rendering them defective in function or phenotype.

The Treg signature miRNA miR-155 has been reported to play a role in Treg cell development and function. In this article, we describe a new role of miR-155. We found that the induced overexpression of this miRNA altered the phenotype of otherwise normal (C3H) Treg cells by reducing CD62L expression, which mimicked the Treg phenotype in MRL/lpr mice. Induced overexpression of miR-155 in young, 4-wk-old MRL mice also elicited a similar reduction in CD62L expression (Supplemental Fig. 4). Consistent with previous reports showing a similar in vitro suppressive capacity of CD62L⁺ versus CD62L⁻ Treg cells (35–37), our preliminary studies found no difference in T cell suppression in vitro induced by C3H Treg cells transfected with mock versus miR-155. The same previous studies also showed that although both CD62L⁺ and CD62L⁻ Treg cells can suppress T cell proliferation in vitro, CD62L⁺ subpopulation of CD4⁺CD25⁺ cells protected against type 1 diabetes, acute graft-versus-host disease, and transplant rejection upon in vivo adoptive transfer. They found that the CD62L⁺ subset of Treg cells was less effective in controlling diabetes or transplant rejection in vivo (35–37). Hence, ongoing studies will determine whether miR-155 transfect Treg cells that exhibit reduced CD62L will have reduced ability to suppress autoimmune disease upon transfer in vivo, whereas inhibition of miR-155 in MRL/lpr Treg cells will enhance their in vivo suppressive capacity. Finally, it would be important to determine whether miR-155 mediated downregulation of CD62L reduces the trafficking of Treg cells from lymphoid to target organs, thus contributing to the accumulation of Treg cells in the lymphoid organs of MRL/lpr mice.

In summary, we have made three novel observations in this article. To the best of our knowledge, we report the first example of acquired deficiency of Dicer, which develops spontaneously in an autoimmune disease model. Second, the progression to autoimmune disease is associated with increased miR-155 expression despite a marked reduction in Dicer expression in Treg cells from MRL/lpr mice. Third, miR-155 can regulate CD62L expression in Treg cells. Ongoing studies will investigate how these alterations
in Treg phenotypes, characterized by reduced CD62L and Dicer and increased mir-155 and -23a, correlate with their suppressive functions in vivo. Identifying mechanisms underlying Treg cell impairment in autoimmune diseases will open new avenues of modulating immune tolerance and suppressing disease.

Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Data


dicer insufficiency and miR-155 over expression in lupus Treg cells: an apparent paradox in the setting of an inflammatory milieu

AA Divekar, S Dubey, PR Gangalum, and RR Singh.

Figure S1. Similar to MRL/lpr mice, MRL+/+ mice show abnormalities in phenotype and function of Treg cells, albeit at a later age. (a, b) Splenocytes from MRL/lpr, MRL+/+, and several control strains of mice (BALB/c, B10.BR and C3H) were stained for CD4, CD25, Foxp3 and TCRβ. Cells were acquired on FACSCalibur (upper and middle rows) or LSRII (lower row) flow cytometers, using small lymphocyte gate based on forward and side scatter. (a) Representative dot plots show CD4+CD25+Foxp3+ T cells as a percent of CD4+ T cell gate. Similar Treg cell frequencies were detected among the three normal control mouse strains tested, including BALB/c, B10.BR and C3H mice. Shown are representative plots from non-autoimmune BALB/c (upper panel) or C3H (middle and lower panels) mice. An overall analysis of data from over 80 mice shows that 60% of MRL+/+ mice that develop lupus disease by 8–10-mo of age had increased frequencies of Treg cells by 4-mo of age compared to age-matched normal control strains. Among MRL/lpr mice that begin to exhibit clinical disease by 3-mo of age, occasional animals had increased Treg cells as early as 6-wks of age, and more than 50% animals by 12-wks of age and 90% animals by 16-wks of age had increased frequencies of Treg
Thus, the increase in Treg cell frequencies precedes the onset of clinical disease in these two autoimmune-prone mouse strains.

(b) Bar diagrams depict frequencies (as percentages of total lymphocytes) of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> in ≥16-wk-old control C3H, MRL<sup>+/+</sup> and MRL/lpr mice from 4 different experiments (*p < 0.05, **p < 0.01; n=9 for C3H and 10 each of MRL<sup>+/+</sup> and MRL/lpr).

(c) Splenocytes from ≥16-wk-old mice were stained for CD4, Foxp3, CD25 and CD69 or CD62L. Representative histograms gated on CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells from MRL<sup>+/+</sup> mice show increased CD69 and reduced CD62L expression similar to that observed in MRL/lpr mice, although these abnormalities were less profound in MRL<sup>+/+</sup> mice.

(d) MRL<sup>+/+</sup> Treg cells show a functional defect similar to MRL/lpr mice. Purified splenic Treg cells were incubated with CFSE-labeled responder splenocytes from C3H mice at ratios of 1:2, 1:4, 1:8 and 1:16 in triplicates. On day 3, triplicate wells were pooled and cells stained with 7-AAD to gate out dead cells and acquired using FACSCalibur. T cell proliferation is shown as CFSE dilution on gated CD4<sup>+</sup> T cells at Treg:responder cell ratio of 1:2. Gray shaded area – proliferation of responder C3H splenocytes in absence of Treg cells, green line – suppression of proliferation in presence of C3H Treg cells, red line – suppression of proliferation in presence of MRL/lpr Treg cells, and blue line – suppression of proliferation in presence of MRL<sup>+/+</sup> Treg cells. Results from a representative of four independent experiments are shown (n = 6 mice per group).

**Figure S2. Detailed phenotypic characterization of Treg cells in MRL mice.** Splenocytes were stained for seven color flow cytometry, as described in Methods, to evaluate markers that have been shown to affect Treg cell functions. Cells were acquired on LSRII, with gates set on
small lymphocytes. There were no significant differences in these markers between C3H, MRL \(^{+/+}\), and MRL/lpr mice (n = 5 mice per group). Results represent three independent experiments.

**Figure S3. Altered miRNA profile in MRL/lpr Treg cells.** Treg cells were purified and their small RNA extracted, as described in Methods. RNA was reverse transcribed to cDNA and applied to the miFinder PCR array plates (SABiosciences). Data were analyzed using the SABiosciences Web Portal. ΔΔCt values were calculated from raw threshold cycle data. Each column represents Treg cell miRNA from a mouse. Color legend indicates relative log scale intensity of expression. Hierarchical clustering shows expression of miRNAs in 3 each of female C3H and MRL/lpr mice.

**Figure S4. Effect of induced overexpression of miR-155 on Treg cell phenotype in MRL\(^{+/+}\) and MRL/lpr mice.** Treg cells enriched from 4-wk-old MRL \(^{+/+}\) and MRL/lpr mice were transfected with 40nM of miR-155 or with HiPerfect reagent alone and analyzed for Treg cell phenotype, as described in Materials and Methods. Representative histograms show markedly reduced CD62L expression in MRL \(^{+/+}\) Treg cells transfected with miR-155 mimic, as shown for C3H mice in Fig. 4. Such effect of transfection with miR-155 mimic on CD62L expression was less pronounced in MRL/lpr Treg cells, probably because some MRL-lpr mice begin to have an increase in miR-155 expression as early as 4-wks of age as shown in Fig. 3.
**Figure S1**

**a** Gated on CD4⁺ T cells

<table>
<thead>
<tr>
<th>Normal control</th>
<th>MRL⁺⁺⁺</th>
<th>MRL/lpr</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-6 wks</td>
<td>9.9</td>
<td>9.1</td>
</tr>
<tr>
<td>8-12 wks</td>
<td>14.6</td>
<td>13.3</td>
</tr>
<tr>
<td>&gt;16 wks</td>
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</tr>
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**b**

<table>
<thead>
<tr>
<th>CD4⁺CD25⁺ cells</th>
<th>CD4⁺CD25⁺Foxp3⁺ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percent</strong></td>
<td></td>
</tr>
<tr>
<td>C3H</td>
<td>MRL⁺⁺⁺</td>
</tr>
<tr>
<td>0.0</td>
<td>0.5</td>
</tr>
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<td>1.5</td>
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<tr>
<td>2.0</td>
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**c** Gated on CD4⁺CD25⁺Foxp3⁺ T cells

**d**

Undivided cells

Dividing cells

% of max

CFSE

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- CD39
- CD73
- CTLA4
- CD127
- Foxp3

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C3H
MRL^{+/+}
MRL/lpr
Mock miR-155

MRL+/+

MRL+/+

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# cells

CD62L

87.8

68.8

67.7

63.9