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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*$^{lpr/lpr}$ (MRL/lpr) and MRL-*Fas*$^{+/+}$ mouse models. We found a significant increase in CD4$^+$CD25$^+$Foxp3$^+$ Treg cells, albeit with an altered phenotype (CD62L$^-$CD69$^+$) 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-23b, 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. ***The Journal of Immunology*****, 2011, 186: 924–930.**

Normal animals possess subsets of CD4$^+$ and CD8$^+$ T cells that limit the activation of pathogenic autoreactive T cells and control the development of autoimmune disease, particularly under conditions of autoimmune stimuli (1). The development and/or function of such regulatory, inhibitory, or suppressor cells are generally impaired in autoimmune-prone mice (1). Regulatory CD4$^+$ T (Treg) cells expressing CD25 and Foxp3 are one such subset that controls self-reactive T cells and maintains tolerance (2, 3). Systemic lupus erythematous (SLE) is an autoimmune disease characterized by loss of immune tolerance, resulting in activation and expansion of autoreactive CD4$^+$ T helper cells (4). Several studies have found numerical and/or functional insufficiency of Treg cells in humans and mice with SLE (5–11), although a few reports contradict these findings. Mechanisms underlying Treg cell defects in SLE are poorly understood.

RNase III family endoribonucleases Dicer and Drosha play an important role in generation of microRNAs (miRNA, miR). Drosha cleaves primary miRNA into 70-nt precursor miRNA (12) and Dicer generates small noncoding 20- to 22-nt miRNA (13), which are further stabilized by Argonaute (14). These miRNAs bind the 3′-untranslated region of a target mRNA and inhibit its translation (15). Recent studies suggest a role for Dicer and Drosha-controlled miRNA pathways in Treg cell-mediated immune tolerance (16–19) and of miRNAs in the development and function of Treg cells (19, 20). In fact, Treg cells from normal animals display a specific miRNA profile (16). It is unclear whether Dicer and miRNAs play a role in eliciting Treg cell defects in humans and mice with autoimmune diseases such as lupus.

In this study, we investigated the phenotype, including Dicer and miRNA profile, and function of Treg cells in the spleen of lupus-prone MRL-*Fas*$^{lpr/lpr}$ mice (hereafter referred to as MRL/lpr) and congenic *Fas*-intact MRL-*Fas*$^{+/+}$ (MRL$^{+/+}$) mice (21) and in non-autoimmune C3H/HeOuj (C3H) mice. Contrary to our initial expectations, we found that lymphoid organs of MRL/lpr mice have increased numbers of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells that exhibit an altered phenotype, reduced suppressive capacity, and reduced *Dicer* expression, along with a distinct miRNA profile. Unexpectedly, autoimmune disease development in MRL/lpr mice was associated with significantly increased miR-155, despite a profound reduction in *Dicer* expression. Using computational and experimental approaches, we further identified miR-155 to regulate altered phenotype of Treg cells in SLE. These data suggest

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The PCR array data in this article have been submitted to Array Express under accession number A-MEXP-1924.

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Abbreviations used in this article: C3H, C3H/HeOuj; miRNA, microRNA; MRL$^{+/+}$, MRL-*Fas*$^{+/+}$; MRL/lpr, MRL-*Fas*$^{lpr/lpr}$; qPCR, quantitative real-time PCR; SLE, systemic lupus erythematous; Treg, regulatory T.

a role of Dicer and miR-155 in conferring Treg cell defect in lupus.

Materials and Methods

Mice

Breeding pairs of MRL/lpr (lupus-prone mice with *lpr* mutation in their *Fas* 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 (1×10^6) 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 TY/11.8), CD69 (clone H1.2F3), CD127 (clone A7R34), Foxp3 (clone FKJ-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-FITC, CD25-allophycocyanin, CD4 (clone CT-CD4)-Pacific Orange, CD127-PECy7, 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

Splenocyte cells (50×10^6) 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 MinElute 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 OD_{260} 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-ACGGTCCAAAG-3′) and DicerR1 (5′-CATGACTCTTCAACTCAAACT-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^2 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% CO_2 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. 1*A*) or CD4$^+$CD25$^+$Foxp3$^+$ T cells (Fig. 1*B*), 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^{hi} 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 per se, 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. 1*A*, 1*B*). Such increase in Treg cells in these mice precedes the onset of clinical disease (Supplemental Fig. 1*A*). 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 splenic CD4$^+$CD25$^+$Foxp3$^+$ T cells from C3H and MRL/lpr mice using eight-color multiparametric flow cytometry (Fig. 1*C*, 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

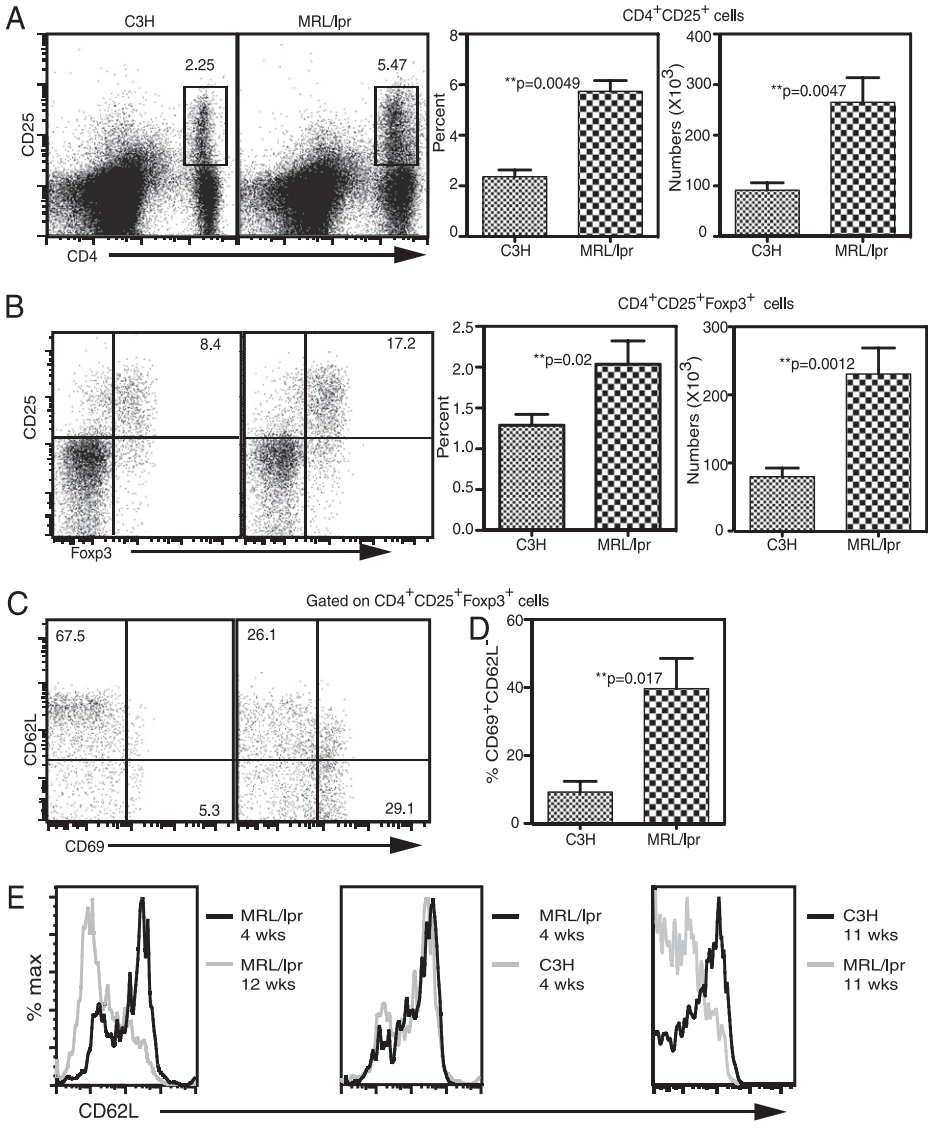


FIGURE 1. $CD4^+CD25^+Foxp3^+$ Treg cells increase and exhibit altered phenotype in MRL/lpr mice. Splenocytes from MRL/lpr and C3H mice were analyzed for Treg cells, as indicated below. *A*, $CD4^+CD25^+$ cells are indicated on dot plots as the proportion of gated small lymphocytes (*left*). Frequency 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 $TCR\beta^+CD4^+CD25^+Foxp3^+$ 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 autoimmune, 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.

showed reduced CD62L expression compared with C3H mice (Fig. 1*C*). As shown in Fig. 1*D*, MRL/lpr mice had significantly increased proportions of $CD4^+CD25^+Foxp3^+CD62L^-CD69^+$ 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. 1*E*). $MRL^{+/+}$ mice also exhibited a similar phenotype (Supplemental Fig. 1*C*), 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. 2*A*, 2*C*). 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. 2*B*). A similar defect in suppressive function was detected in Treg cells from ≥12-wk-old $MRL^{+/+}$ mice (Supplemental Fig. 1*D*). 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. 2*A*, 2*B*), 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. 2*D*), 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. 1*C*.

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

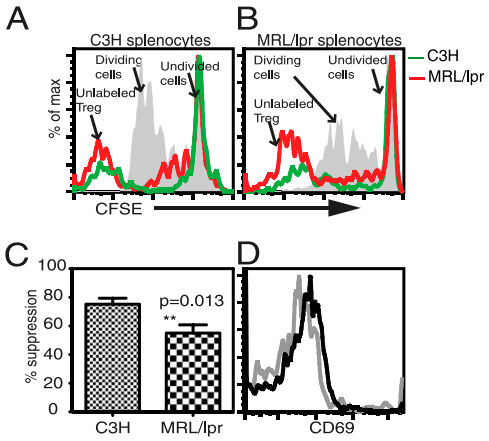


FIGURE 2. Reduced suppression by Treg cells from MRL/lpr mice. Treg cells were purified from spleens of C3H and MRL/lpr mice and mixed with CFSE-labeled responder splenocytes from C3H (*A*) or MRL/lpr mice (*B*) in triplicates. On day 3, triplicate wells were pooled and stained with 7-aminoactinomycin 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. 3*A*). 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 Treg cell-mediated tolerance in knockout animals (17, 19). We asked whether spontaneous Dicer insufficiency in MRL/lpr Treg cells (Fig. 3*A*) 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. 3*B*. 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. 3*C* and 3*D*. 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. 3*B*, 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. 3*A*, 3*C*). 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. 3*A*, 3*C*). Thus, while Dicer normally regulates the generation of miR-155 in Treg 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 ($p = 9.2 \times 10^{-3}$; 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

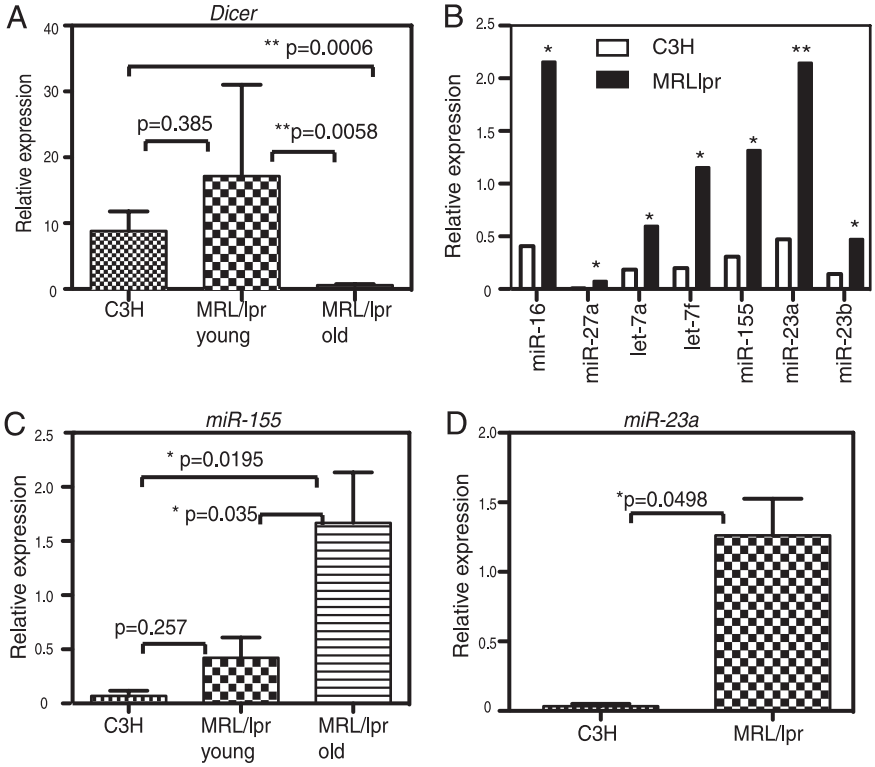


FIGURE 3. Expression of *Dicer* and miRNAs in MRL/lpr mice. *A*, RNA was extracted from purified splenic Treg cells, as described in *Materials and Methods*, and qPCR was performed for *Dicer* expression. Relative *Dicer* expression normalized to *Gapdh* is shown as the mean ± SE in 12- to 16-wk-old 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 ($n = 6$), and 12- to 20-wk-old female C3H ($n = 4$) 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.

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 Treg 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 F_1 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. 1*A*). 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. 1*D*). 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

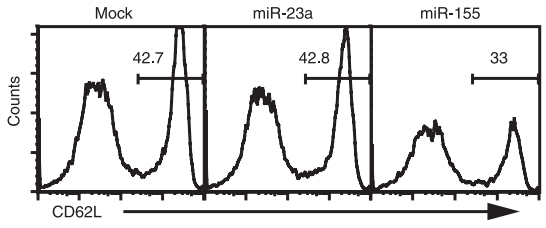


FIGURE 4. Overexpression of miR-155 in otherwise normal Treg cells alters their phenotype. Treg cells enriched from C3H mice were transfected with 40 nM miR-155, miR-23a, or with HiPerfect reagent alone, as described in *Materials and Methods*. Representative histogram shows reduced CD62L expression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

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. 1*C–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 defect 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 $CD4^+CD25^-$ 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 $CD62L^{lo}$ versus $CD62L^{hi}$ 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 $CD62L^{lo}$ and $CD62L^{hi}$ 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 $CD62L^{lo}$ 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 transfected 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

1. Singh, R. R., F. M. Ebling, D. A. Albuquerque, V. Saxena, V. Kumar, E. H. Giannini, T. N. Marion, F. D. Finkelman, and B. H. Hahn. 2002. Induction of autoantibody production is limited in nonautoimmune mice. *J. Immunol.* 169: 587–594.
2. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
3. Brunkow, M. E., E. W. Jeffery, K. A. Hjerrild, B. Paeper, L. B. Clark, S. A. Yasayko, J. E. Wilkinson, D. Galas, S. F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* 27: 68–73.
4. Singh, R. R., and S. Dubey. 2007. Autoantigens and defects in immune tolerance in lupus. In *Dubois' Lupus Erythematosus*, 7th Ed. D. J. Wallace, and B. H. Hahn, eds. Lippincott, Williams and Wilkins, Philadelphia, p. 370–406.
5. Scalapino, K. J., Q. Tang, J. A. Bluestone, M. L. Bonyhadi, and D. I. Daikh. 2006. Suppression of disease in New Zealand Black/New Zealand White lupus-prone mice by adoptive transfer of ex vivo expanded regulatory T cells. *J. Immunol.* 177: 1451–1459.
6. Parietti, V., F. Monneaux, M. Décossas, and S. Muller. 2008. Function of $CD4^+$, $CD25^+$ Treg cells in MRL/*lpr* mice is compromised by intrinsic defects in antigen-presenting cells and effector T cells. *Arthritis Rheum.* 58: 1751–1761.
7. Valencia, X., C. Yarboro, G. Illei, and P. E. Lipsky. 2007. Deficient $CD4^+$ $CD25^{high}$ T regulatory cell function in patients with active systemic lupus erythematosus. *J. Immunol.* 178: 2579–2588.
8. Bagavant, H., and K. S. Tung. 2005. Failure of $CD25^+$ T cells from lupus-prone mice to suppress lupus glomerulonephritis and sialoadenitis. *J. Immunol.* 175: 944–950.
9. Hahn, B. H., F. Ebling, R. R. Singh, R. P. Singh, G. Karpouzas, and A. La Cava. 2005. Cellular and molecular mechanisms of regulation of autoantibody production in lupus. *Ann. N. Y. Acad. Sci.* 1051: 433–441.
10. Scalapino, K. J., and D. I. Daikh. 2009. Suppression of glomerulonephritis in NZB/NZW lupus prone mice by adoptive transfer of ex vivo expanded regulatory T cells. *PLoS ONE* 4: e6031.
11. Bonelli, M., A. Savitskaya, C. W. Steiner, E. Rath, J. S. Smolen, and C. Scheinecker. 2009. Phenotypic and functional analysis of $CD4^+CD25^-$ $Foxp3^+$ T cells in patients with systemic lupus erythematosus. *J. Immunol.* 182: 1689–1695.
12. Lee, Y., C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Rådmark, S. Kim, and V. N. Kim. 2003. The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425: 415–419.
13. Kim, V. N., J. Han, and M. C. Siomi. 2009. Biogenesis of small RNAs in animals. *Nat. Rev. Mol. Cell Biol.* 10: 126–139.
14. Diederichs, S., and D. A. Haber. 2007. Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. *Cell* 131: 1097–1108.
15. Bartel, D. P. 2009. MicroRNAs: target recognition and regulatory functions. *Cell* 136: 215–233.
16. Cobb, B. S., A. Hertweck, J. Smith, E. O'Connor, D. Graf, T. Cook, S. T. Smale, S. Sakaguchi, F. J. Livesey, A. G. Fisher, and M. Merkenschlager. 2006. A role for Dicer in immune regulation. *J. Exp. Med.* 203: 2519–2527.
17. Liston, A., L. F. Lu, D. O'Carroll, A. Tarakhovsky, and A. Y. Rudensky. 2008. Dicer-dependent microRNA pathway safeguards regulatory T cell function. *J. Exp. Med.* 205: 1993–2004.
18. Chong, M. M., J. P. Rasmussen, A. Y. Rudensky, A. Y. Rudensky, and D. R. Littman. 2008. The RNAseIII enzyme Drosha is critical in T cells for preventing lethal inflammatory disease. *J. Exp. Med.* 205: 2005–2017.
19. Zhou, X., L. T. Jeker, B. T. Fife, S. Zhu, M. S. Anderson, M. T. McManus, and J. A. Bluestone. 2008. Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J. Exp. Med.* 205: 1983–1991.
20. Kohlhaas, S., O. A. Garden, C. Scudamore, M. Turner, K. Okkenhaug, and E. Vigorito. 2009. Cutting edge: the Foxp3 target miR-155 contributes to the development of regulatory T cells. *J. Immunol.* 182: 2578–2582.
21. Hahn, B. H., and R. R. Singh. 2007. Animal models of systemic lupus erythematosus. In *Dubois' Lupus Erythematosus*, 7th Ed. D. J. Wallace, and B. H. Hahn, eds. Lippincott, Williams and Wilkins, Philadelphia, p. 299–355.
22. Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3: RESEARCH0034.
23. Baecher-Allan, C., J. A. Brown, G. J. Freeman, and D. A. Hafler. 2001. $CD4^+$ $CD25^{high}$ regulatory cells in human peripheral blood. *J. Immunol.* 167: 1245–1253.
24. Deaglio, S., K. M. Dwyer, W. Gao, D. Friedman, A. Usheva, A. Erat, J. F. Chen, K. Enjyoji, J. Linden, M. Oukka, et al. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* 204: 1257–1265.
25. Kobie, J. J., P. R. Shah, L. Yang, J. A. Rebhahn, D. J. Fowell, and T. R. Mosmann. 2006. T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5′-adenosine monophosphate to adenosine. *J. Immunol.* 177: 6780–6786.
26. Read, S., V. Malmström, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $Cd25^+Cd4^+$ regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192: 295–302.
27. Seddiki, N., B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay, M. Solomon, W. Selby, S. I. Alexander, R. Nanan, et al. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* 203: 1693–1700.
28. Bonelli, M., A. Savitskaya, K. von Dalwigk, C. W. Steiner, D. Aletaha, J. S. Smolen, and C. Scheinecker. 2008. Quantitative and qualitative deficiencies of regulatory T cells in patients with systemic lupus erythematosus (SLE). *Int. Immunol.* 20: 861–868.
29. Rouas, R., H. Fayyad-Kazan, N. El Zein, P. Lewalle, F. Rothé, A. Simion, H. Akl, M. Mourtada, M. El Rifai, A. Burny, et al. 2009. Human natural Treg microRNA signature: role of microRNA-31 and microRNA-21 in FOXP3 expression. *Eur. J. Immunol.* 39: 1608–1618.
30. Bennett, C. L., J. Christie, F. Ramsdell, M. E. Brunkow, P. J. Ferguson, L. Whitesell, T. E. Kelly, F. T. Saulsbury, P. F. Chance, and H. D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 27: 20–21.
31. Kim, J. M., J. P. Rasmussen, and A. Y. Rudensky. 2007. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat. Immunol.* 8: 191–197.
32. Monk, C. R., M. Spachidou, F. Rovis, E. Leung, M. Botto, R. I. Lechler, and O. A. Garden. 2005. MRL/Mp $CD4^+$,$CD25^-$ T cells show reduced sensitivity to suppression by $CD4^+$,$CD25^+$ regulatory T cells in vitro: a novel defect of T cell regulation in systemic lupus erythematosus. *Arthritis Rheum.* 52: 1180–1184.
33. Kuehbacher, A., C. Urbich, A. M. Zeiher, and S. Dimmeler. 2007. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. *Circ. Res.* 101: 59–68.
34. Cifuentes, D., H. Xue, D. W. Taylor, H. Patnode, Y. Mishima, S. Cheloufi, E. Ma, S. Mane, G. J. Hannon, N. Lawson, et al. 2010. A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science* 328: 1694–1698.
35. Ermann, J., P. Hoffmann, M. Edinger, S. Dutt, F. G. Blankenberg, J. P. Higgins, R. S. Negrin, C. G. Fathman, and S. Strober. 2005. Only the $CD62L^+$ subpopulation of $CD4^+CD25^+$ regulatory T cells protects from lethal acute GVHD. *Blood* 105: 2220–2226.
36. Szanya, V., J. Ermann, C. Taylor, C. Holness, and C. G. Fathman. 2002. The subpopulation of $CD4^+CD25^+$ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. *J. Immunol.* 169: 2461–2465.
37. Fu, S., A. C. Yopp, X. Mao, D. Chen, N. Zhang, D. Chen, M. Mao, Y. Ding, and J. S. Bromberg. 2004. $CD4^+CD25^+CD62^+$ T-regulatory cell subset has optimal suppressive and proliferative potential. *Am. J. Transplant.* 4: 65–78.

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

cells. 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 $CD4^+CD25^+$ and $CD4^+CD25^+Foxp3^+$ in ≥16-wk-old control C3H, $MRL^{+/+}$ and MRL/lpr mice from 4 different experiments (*$p < 0.05$, **$p < 0.01$; n=9 for C3H and 10 each of MRL+/+ and MRL/lpr).

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

(d) $MRL^{+/+}$ 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 $CD4^+$ 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 $MRL^{+/+}$ Treg cells. Results from a representative of four independent experiments are shown (n = 6 mice per group).

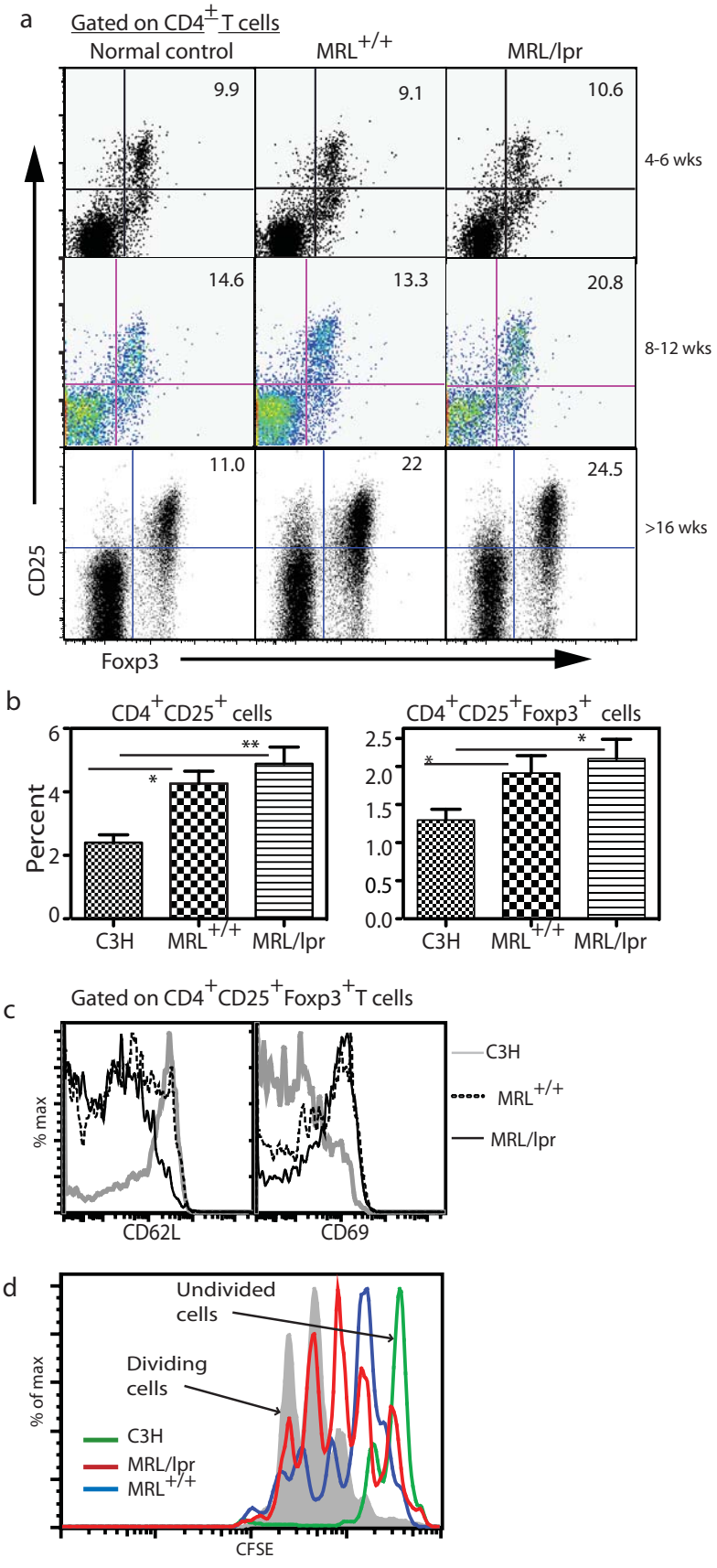
Figure S2. **Detailed phenotypic characterization of Treg cells in MRL mice**. Splenocytes were stained for seven color flow cytometry, as described in Methods, to evaluate markers that have been shown to affect Treg cell functions. Cells were acquired on LSRII, with gates set on

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.

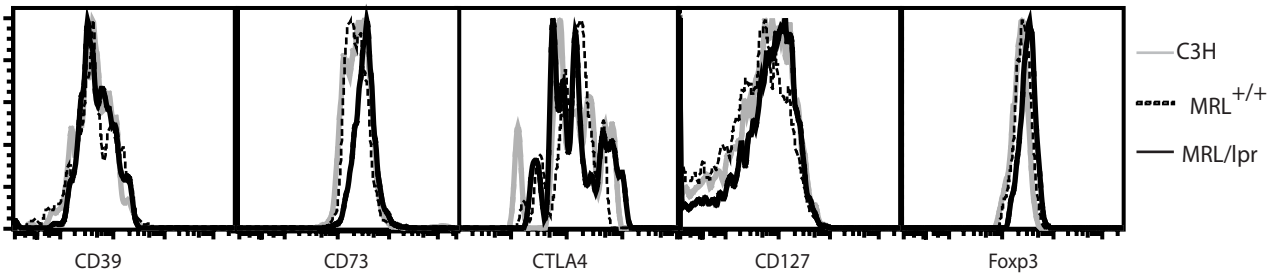
Figure S3. Altered miRNA profile in MRL/lpr Treg cells. Treg cells were purified and their small RNA extracted, as described in Methods. RNA was reverse transcribed to cDNA and applied to the miFinder PCR array plates (SABiosciences). Data were analyzed using the SABiosciences Web Portal. ΔΔCt values 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.

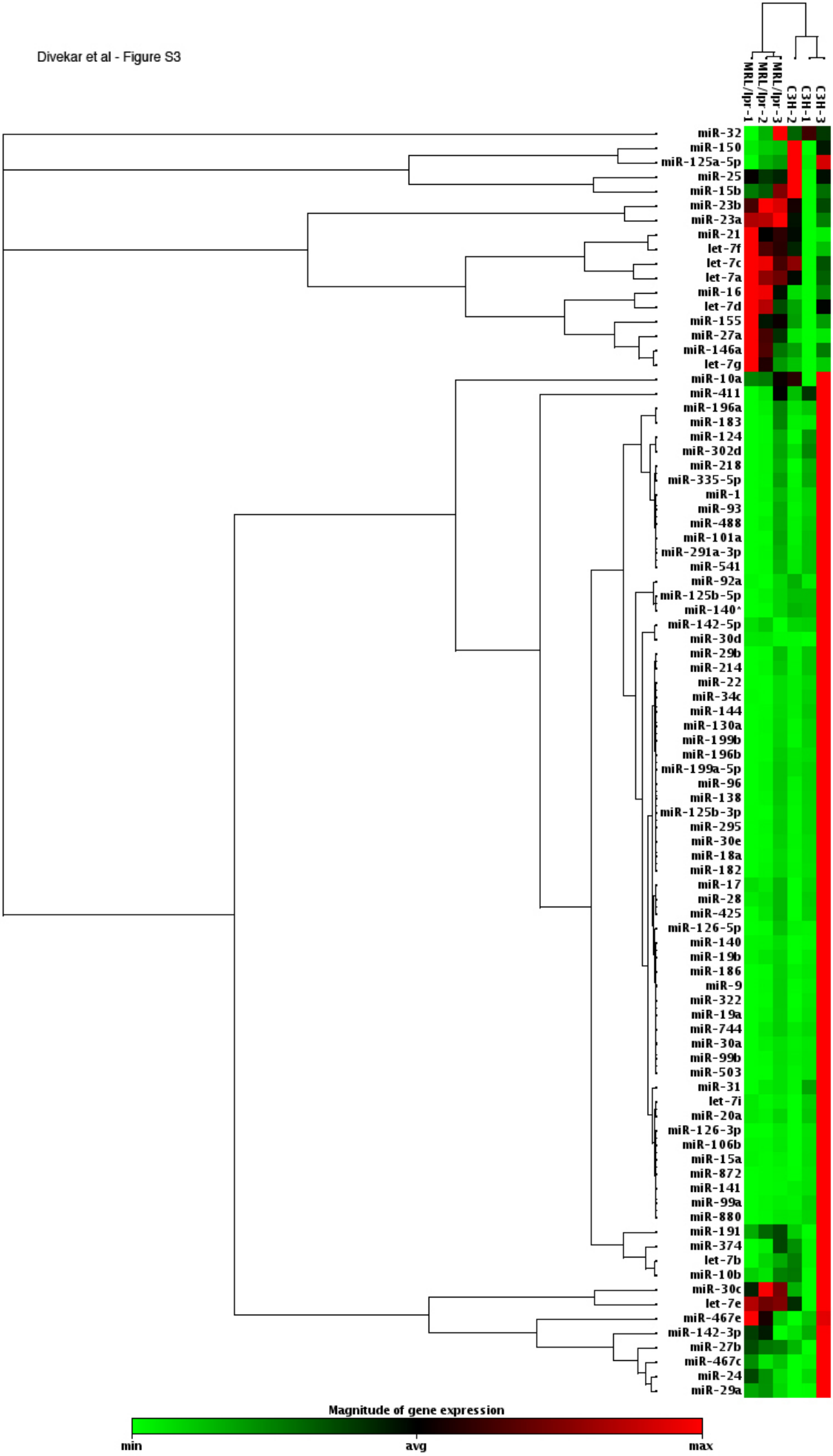
Divekar et al Fig S1

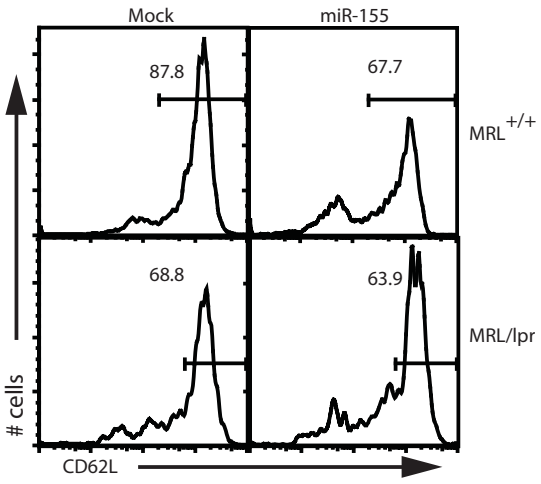


Divekar et al - Figure S2



Divekar et al - Figure S3




Mock
miR-155
87.8
67.7
MRL+/+
68.8
63.9
MRL/lpr
cells
CD62L

In This Issue

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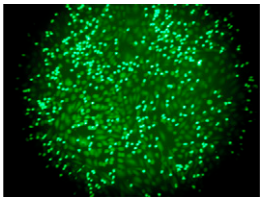
THE JOURNAL OF IMMUNOLOGY

In This Issue

Sepsis Saps Endothelium



Dysregulation of endothelial cell function during sepsis, which can be triggered by TLR stimulation, contributes to excessive inflammation and organ failure. Shin et al. (p. 1119) now describe a multitude of damaging effects that TLR2 stimulation inflicts on endothelial cells. TLR2 agonists induced IL-6 and IL-8 secretion and E-selectin expression in a variety of primary human endothelial cells, including those from the umbilical vein, lung, and coronary artery. IL-6 responses to TLR2 agonist treatment were TLR2 dependent, as IL-6 secretion increased significantly in endothelial cells from wild-type but not $TLR2^{-/-}$ mice. Human umbilical vein endothelial cells treated with TLR2 agonist showed increased membrane permeability and dysregulation of multiple coagulation factors, as well as upregulation of TLR2 expression. Increased expression of E-selectin, P-selectin, and MCP-1 mRNAs and greater neutrophil infiltration, indicated by increased myeloperoxidase levels, were observed in the lungs of wild-type but not $TLR2^{-/-}$ mice upon systemic TLR2 agonist treatment. Lung fibrin also increased significantly in mice treated intratracheally with a TLR2 agonist compared with carrier-treated mice. These results highlight the extent to which TLR2 agonists affect endothelial cells, supporting the notion that TLR2 activation may be a major cause of endothelial damage during sepsis.

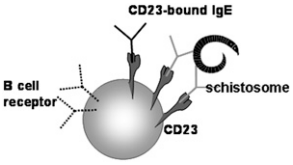
Host Seeking Long-Term IgM

IgM secretion is commonly considered an early immune response to infection, which is typically followed by the development of a longer lasting IgG response. Racine et al. (p. 1011) show that a long-lasting IgM response against the intracellular bacterium *Ehrlichia muris* is sufficient to protect mice against lethal infection. Previous work has shown that *E. muris* infection is associated with a strong IgM response. In this study, a population of $CD138^{high}IgM^{high}$ cells in the bone marrow was linked to the generation of a protective IgM response against a chronic, lethal *E. muris* infection in mice. This unique Ag-specific cell population had phenotypic features of both plasmablasts and plasma cells. Ag-specific $CD138^{high}IgM^{high}$ cells were found in the bone marrow, even in the absence of detectable infection following antibiotic treatment, and these cells could be reactivated to produce a protective IgM response upon reinfection. Moreover, IgM responses were shown to be sufficient to protect activation-induced cytidine deaminase-deficient mice, which produce only IgM and not IgG, against lethal *E. muris* infection. These findings define a unique role for the IgM response in protection against a chronic intracellular bacterial infection, and may offer a new approach for designing antibacterial therapies.

Igniting Tumor Inflammation

IL-12 immunotherapy has shown limited efficacy in anticancer clinical trials, which may be due in part to immunosuppressive mechanisms acting within tumors. Medina-Echeverz et al. (p. 807) successfully eliminated established tumors in a murine colon cancer model by combining IL-12 therapy with depletion of immunosuppressive cells, using anti-CD25 mAb or cyclophosphamide (CPA). Both of these combination therapies reduced the frequency of intratumoral regulatory T cells (Tregs) and myeloid-derived suppressor cells. Nonetheless, only IL-12 plus CPA treatment significantly reduced tumor growth and improved survival. Regressing tumors from IL-12 plus CPA-treated mice were enriched with a heterogeneous population of $Ly6C^{high}$ $Ly6G^{low}$ monocytes and $Ly6C^{+}Ly6G^{high}$ neutrophils that were termed inflammatory myeloid cells (IMCs). IMCs did not induce conversion of $CD4^{+}$ T cells into Tregs but promoted intratumoral infiltration of tumor Ag-specific $CD8^{+}$ effector T cells. Tumor regression halted upon depletion of IMCs via anti-Gr1 mAb during IL-12 plus CPA treatment, which confirmed that IMCs were required for the antitumor effects of this therapy. Taken together, IL-12 plus CPA is a potent combination for shutting down immunosuppressive mechanisms within colon tumors and activating antitumor defenses, and may lead to new strategies for formulating effective cancer therapies.

CD23 and IgE Pulverize Parasites



$BCR\mu^{+}$ B cells express CD23, a low-affinity IgE receptor, and these B cells can present Ag and activate T cells upon endocytosis of CD23 molecules bound by IgE cross-linked with Ag. Protection against infection with *Schistosoma mansoni* is associated with high *S. mansoni*-specific serum IgE levels and an increase in $CD23^{+}$ B cells in humans. Griffith et al. (p. 1060) sought to understand if IgE-mediated resistance to *S. mansoni* involved CD23-bound IgE on B cells. CD23 cross-linking induced signaling events in naïve B cells similar to those caused by BCR stimulation, including Syk and ERK1/2 phosphorylation. $CD23^{+}$ B cells from individuals chronically exposed to *S. mansoni* showed a decrease in basal B cell activation upon ex vivo stimulation with crude *S. mansoni* Ag, compared with stimulation with TLR agonists or anti-CD3/CD28 Abs. The addition of CD23-bound *S. mansoni*-specific IgE restored B cell responses to *S. mansoni* Ag.

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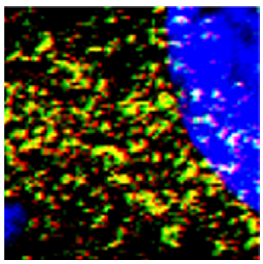
BCR signaling was reduced in naïve B cells cross-linked with both anti-CD23 and anti-BCRμ Abs, suggesting that CD23-bound IgE signaling can overpower BCR signaling. Thus, the antiparasitic attributes of IgE may be due in part to its influence on B cell activation and subsequent Ag-specific T cell activation.

Autophagy Keeps Skin Cool



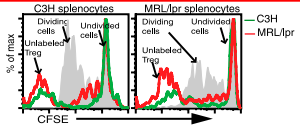
Emerging evidence suggests that intracellular degradation processes defined as autophagy may influence inflammatory responses. The scaffolding adapter protein p62, a putative autophagy receptor, can interact with polyubiquitinated proteins and target them for autophagic degradation. In this issue, Lee et al. (p. 1248) observed that inflammation in human keratinocytes is influenced by autophagy-driven suppression of p62. Treatment of keratinocytes with TLR2/6 or TLR4 agonist induced autophagy and p62 expression by stimulating NADPH oxidase-dependent reactive oxygen species generation. Keratinocytes treated with autophagy inhibitors exhibited a significant increase in p62 expression and inflammatory cytokine secretion compared with untreated or negative control keratinocytes. Conversely, RNA interference-mediated silencing of p62 caused a decrease in NF-κB–associated responses and inflammatory cytokine secretion. These data support other findings indicating that autophagy preserves cellular homeostasis by regulating p62 expression in response to inflammatory stimuli. p62 expression was significantly higher in epidermal samples from psoriatic skin than in atopic dermatitis or healthy control samples. Thus, autophagy appears to be a regulator of keratinocyte inflammation through its effect on p62 expression, and the autophagy pathway may potentially be exploited for the development of novel psoriasis therapies.

Ornery Old Myeloid Cells

Numerous defects in the immune system emerge in aging animals, but the primary causes of these defects remain poorly defined. Enioutina et al. (p. 697) previously observed that immature $Gr1^+CD11b^+$ myeloid cells accumulated in the spleens of aged mice and have now expanded this observation by characterizing attributes of these cells that influence immune senescence. An elevated number of immature $Gr1^+CD11b^+$ myeloid cells bearing phenotypic similarity to myeloid-derived suppressor cells were observed in the peripheral blood, lymph nodes, and bone marrow, as well as the spleens, of aged mice compared with young mice. Relative to comparable cells from young mice, a significantly higher percentage of both $CD4^+$ T cells and $Gr1^+CD11b^+$ myeloid cells from aged mice produced proinflammatory cytokines constitutively or upon stimulation. $Gr1^+CD11b^+$ myeloid cells from aged mice suppressed adaptive immune responses, but this effect was reversed by depletion of $Gr1^+$ cells such that Ag-specific $CD4^+$ T cell proliferation and Ab responses were similar to those of young mice. $Gr1^+CD11b^+$ myeloid cells had a defect in PI3K-Akt signaling, thus leading to uncontrolled GSK3β activity that promoted proinflammatory responses. The aberrant functions of $Gr1^+CD11b^+$ myeloid cells in aged mice suggest that these cells are influential in the events surrounding immune senescence, and further study is needed to better define their impact.

In the Transcription Trenches

Two papers in this issue feature the critical role of transcriptional regulation in T cell function. The first paper explores the influence of the transcription factor Kruppel-like factor 2 (KLF2) on T cell trafficking and cell cycle regulation by using novel knockout mice. KLF2 has been described as a transcriptional regulator in T cells, and its re-expression in T cells following activation is affected in different ways by IL-2 or IL-15 exposure. Using conditional and inducible KLF2 knockout mouse models, Takada et al. (p. 775) determined that KLF2 expression in post-activated $CD8^+$ T cells was required for transcription of the trafficking molecules CD62L and $S1P_1$. Transcription of cell cycle regulatory genes was affected differently by treatment with IL-2 or IL-15, as described previously, but this differential regulation did not require endogenous KLF2 expression. Moreover, KLF2 deficiency altered Ag-specific $CD8^+$ T cell migration in vivo but did not affect their proliferation or quiescence. Overall, these findings do not support a direct effect of KLF2 expression on cell cycle regulation, but do clarify how KLF2 expression affects activated $CD8^+$ T cell trafficking by influencing the transcription of cell surface molecules.



Irregularities in regulatory T cell (Treg) function have been observed in mice and humans with systemic lupus erythematosus, but the mechanisms behind these defects are not well understood. In the second paper, Divekar et al. (p. 924) observed aberrations in the microRNA (miRNA) pathway of Tregs from lupus-prone MRL-*Fas*$^{lpr/lpr}$ (MRL/lpr) mice. MRL/lpr mice with lupus symptoms had significantly more Tregs in their spleens, compared with control C3H mice. These Tregs had altered functional and phenotypic characteristics, including increased CD69 and reduced CD62L expression and a diminished suppressive capability relative to Tregs from C3H mice. Further examination of these abnormal Tregs revealed a significant reduction in the expression of the endoribonuclease Dicer, which is involved in miRNA generation. A number of miRNAs, including miR-155, were overexpressed in these Tregs, in spite of the Dicer deficiency. miR-155 was identified as a target of CD62L, and otherwise normal Tregs from C3H mice transfected with miR-155 showed reduced CD62L expression, compared with controls. These data suggest that abnormalities in the Tregs of lupus-prone MRL/lpr mice may be caused by multiple defects in miRNA regulation that influence transcription of genes essential to Treg function.

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