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Role of microRNA-15a in Autoantibody Production in Interferon-Augmented Murine Model of Lupus

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Abstract

MicroRNAs (miRNAs) are involved in the regulation of immunity via targeting of mRNA encoding immune response elements. In this report, alterations in the expression of microRNAs as autoantibody levels increase was investigated. The (NZB X NZW) F1 or B/W mouse model of systemic lupus erythematosus (SLE) naturally has increased autoantibodies with aging. IFN α (type I IFN) accelerates B/W disease, however, the effects of a related IFN, IFN λ , which is a type III IFN, is largely unknown. The purpose of the study was to investigate the relationship between IFN-accelerated disease, microRNAs, immunoregulatory B cell subsets and autoantibody production in the autoimmune-prone environment *in vivo*. B/W mice received osmotic pumps to chronically deliver IFN α and IFN λ for up to 16 weeks. Urine protein level was monitored weekly by urine strips and proteinuria was used as the disease marker. Splenic cells were taken for flow analysis of B cell subsets and levels of microRNAs determined. Plasma were analyzed for autoantibodies and microRNA levels. As a result of treatment, IFN α accelerated proteinuria in a dose dependent manner, while IFN λ single treatment did not show a significant effect, but greatly enhanced low dose IFN α effects in the combination treatment. Both the splenic cellular and plasma miR-15a were elevated in diseased compared to pre-diseased mice as well as autoantibody levels. Autoantibodies and miR-15a levels were significantly correlated. The immunosuppressive B subpopulation, B-10, was reduced following IFN α treatment. In addition in diseased mice, B-10 versus B-2 ratios were reduced in IFN-treated B/W compared to the control PBS treated group. In all B/W the miR-15a was highly expressed in the B-10 subset and this increased with disease development, suggesting that miR-15a has a specific negative effect on the B-10 subpopulation. In conclusion, our data support the involvement of elevated miR-15a in autoimmune disease development in B/W mice and suggest that decreasing this microRNA might be beneficial in B/W mice.

Keywords

Interferons; Autoantibodies; microRNA; NZB/NZW F1

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1. Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease and the specific cause remains unknown, yet environmental factors, sex hormones and multiple susceptibility alleles are involved in the pathogenesis [1]. A pathological hallmark of SLE is high-titer antibodies against double stranded DNA (dsDNA), chromatin and ribonucleoproteins occurring before development of severe disease symptoms [2, 3]. These autoreactive antibodies are the result of abnormalities in the regulation of B and T cells in SLE, with dysregulation in cytokine levels contributing to the immune imbalance [4]. Treatments such as cell-depleting strategies targeting B cells by anti-CD20, blockade of co-stimulatory interaction between cells by anti-CD40 ligand antibody and anti-cytokines have been employed [5].

Two inbred strains, the NZB and NZW strains, when crossed result in an F1 with a much more severe autoimmune disease than do either parental strain [6]. Female B/W mice die of lupus disease between the age of 8–12 mo, whereas male B/W mice die at 15–20 mo [7], which is similar to the human case where SLE is much more prevalent in women. B/W mice spontaneously develop high affinity anti-DNA antibodies and IgG immune complex deposition in the kidney causing severe glomerulonephritis, which ultimately leads to renal failure and death [8]. It was shown earlier that the subset of female B/W autoimmune mice surviving beyond 1 year develop B-1 cell clonal expansions similar to the NZB chronic lymphocytic leukemia (CLL) model, suggesting the underlying reversibility between autoimmunity and B-1 clonal expansion [9].

High serum IFN α has been considered as a hallmark for disease severity in the lupus patient and anti-IFN α therapy has been applied in phase I clinical trials [10] and phase II trials are currently ongoing [11]. IFN α is believed to have a strong effect in initiating autoantibody production and clinical features in both human SLE and B/W mice [12–15]. IFN λ , a type III interferon, was newly discovered and shown to have similar roles as IFN α in many biological aspects, especially in anti-viral activities [16]. However, these two interferons also have unique non-redundant activities since they bind to different receptors and the cellular distribution of these receptors and their functional kinetics are also different [17, 18]. Based on the similarities and differences between IFN α and IFN λ , it is of interest to investigate the effects of IFN λ in B/W autoimmunity.

The pathogenic role of B cells, in both lupus patients and B/W mice is known and depletion of all B cells (ie by Rituximab) eliminates autoantibodies and other B cell associated pathogenic factors beyond autoantibodies [19–21]. However, B-10 cells, which have preferential production of IL-10, share phenotypic markers with B-1 cells (B220+CD5+) but also high expression of CD1d. B-10 cells have been shown to suppress autoimmune disease in the B/W lupus model and other inflammatory diseases via IL-10 [22–25]. To reverse B-cell mediated autoimmunity, an alternative to the depletion of all B cells may be to increase selectively the B-10 subpopulation.

Previous reports of microRNA abnormalities in lupus patients point to these small (20 to 23nt) non-coding RNAs as a potential new avenue to target in autoimmune diseases [26]. Because miRNAs have regulatory capabilities with the ability to alter levels of several different proteins via targeting the 3'UTR of their mRNA, a relatively small number of miRNAs may alter the diverse cellular genes responsible for autoimmunity [27, 28]. B cell-specific Dicer deficiency resulted in autoimmune phenotypes in female mice with elevated autoantibodies due to impaired expression of miRNAs in B cells [29]. miR-155 has also been shown to prevent rheumatoid arthritis in the collagen-induced arthritis mouse model [30]. A recent study on miRNAs in mouse models of autoimmunity showed a number of

miRNAs upregulated or downregulated as signatures of lupus disease in MRL/lpr and B/W mouse strains as compared to non-autoimmune mice [31]. In human SLE, analysis of miRNA expression has also revealed abnormal expression [32, 33]. Altering miRNAs have been exploited as potential therapy for cancer [34] and miRNAs may provide an alternative approach in the treatment of SLE [35, 36]. To date, most of the miRNAs studies in SLE and models of SLE are limited to *ex vivo* study of both human and mouse cells. In recent years, miRNAs have been discovered in serum as well as other body fluids, and several studies have reported the value for analysis of plasma levels of miRNAs as useful novel biomarker in clinical research [37–39]. Since miRNAs originate from cells and tissues and stay stable in serum and plasma, the changes in serum miRNAs may reflect the physiological or pathological conditions in various cells, tissues or organs [40, 41]. In the B/W lupus model, abnormal lymphocytes in the peripheral blood and secondary lymphoid organs could be a major source for plasma miRNAs.

In the present study, the relationship between expression of miR-15a, autoantibodies and splenic B cell subsets was investigated. The hypothesis to be tested was that miR-15a expression in lymphocytes increases as the autoimmunity progresses in B/W mice and exogenous delivery of IFNs triggers miR-15a overexpression and the accelerated onset of autoimmunity. This may be the result of a loss of balance between regulatory B cells (B-10) and the pathogenic B cells (B-2), attributed to the differential expression of miR-15a in these cell subsets. To test this hypothesis, IFN α and/or IFN λ were exogenously infused *in vivo* in B/W mice for up to 16 weeks at most, and pre-disease groups and diseased groups analyzed.

2. Materials and Methods

2.1. Mice and in vivo interferons treatment

Female (NZB X NZW) F1 or B/W mice were obtained from Jackson Labs (Bar Harbor, ME) and housed in pathogen-free environment in the NJMS animal facilities. All mouse procedures were approved by the NJMS IACUC. 13 week-old female B/W were treated with IFN α (PBL InterferonSource) and/or IFN λ (PBL InterferonSource) for a total period of up to 16 weeks. Briefly, IFNs were delivered by micro-osmotic pump (model 2006; ALZET) implanted subcutaneously at different doses diluted in 0.1% BSA-PBS at a maximal pump volume of 250 μ l: IFN α low dose (1 μ g/pump), IFN α high dose (10 μ g/pump), IFN λ (10 μ g/pump), combination treatment with 1 μ g IFN α + 10 μ g IFN λ per pump. Each pump delivered at the rate of 3.6 μ l/day. Nomenclature for each treatment group was designated as the initial IFN dose/pump for convenience, considering that the total amount of IFN delivered was dependent upon the total time the pump was present. Control group received only 250 μ l 0.1% BSA-PBS per pump and age-matched untreated B/W mice were also included. Pumps were replaced at the end of the first 8-week period. Urine was measured weekly to monitor proteinuria using dipsticks (Cole-Taylor urine analysis test strips, Fisher Scientific) and proteinuria >300mg/dl (pro-3+) was considered as a disease marker. Mice were sacrificed within 2 weeks of the development of proteinuria.

2.2. Flow analysis and Sorting

Spleens were taken from sacrificed mice and meshed through 70 μ m cell-strainer (BD Falcon) to make single-cell suspensions. Single-cell suspensions were incubated for 20min in 4°C with the following antibodies: B220 PE-Cy7 (BD Pharmingen), CD5 APC (BD Pharmingen), CD1d PerCP-Cy5.5 (Biolegend), IL-10 FITC (Biolegend), IL-10 PE (Biolegend), IgM FITC (BD Pharmingen). Samples were washed with PBS/2%FBS after staining and fixed in Fixation/Permeabilization solution (BD Biosciences) at 4°C for 20min. For intracellular IL-10 detection, fixed cells were washed twice with perm/wash buffer (BD Biosciences) and stained with IL-10 for 20min in 4°C, followed by another wash with perm/

wash buffer. Then cells were resuspended in 2% paraformaldehyde and acquired by FACS LSRII (BD Biosciences). For sorting subpopulations, 10×10^6 cells from each sample were stained with IgM FITC, CD1d PerCP-Cy5.5 and CD5 APC, followed by washing and resuspended in sorting buffer containing 1X PBS, 0.5% BSA and 2mM EDTA, and sorted using FACSDiVa high-speed cell sorter (BD Biosciences). Data was analyzed using FlowJo (Tree Star, Inc).

2.3. Ex vivo B cell stimulation for IL-10

Splenocytes were resuspended in complete media: 1X RPMI 1640 media (Mediatech Inc, Manassas, VA) supplemented with 10% heat-inactivated Fetal bovine serum optima (Atlanta Biologicals, Lawrenceville, GA), 8mM L-glutamine, 1X penicillin-streptomycin (Mediatech Inc.), 10mM HEPES buffer (Mediatech Inc.), 2.5g/L D-glucose (Gibco, Invitrogen), and 1mM Sodium pyruvate (Gibco, Invitrogen). For IL10 stimulation, 2×10^6 splenocytes were treated with LPS (10ug/ml, Sigma), PMA (50ng/ml, Sigma), Ionomycin (500ng/ml, Sigma) and brefeldin (Biolegend) for 5hr in 37°C incubator.

2.4. ELISA for plasma anti-dsDNA IgG

96 flat bottom well microplates were coated with 50ul of poly-L-lysine (Sigma, 100 ug/ml), and incubated for 45 min at room temperature. After washing plates (3X with 0.1% PBS Tween), wells were coated with 100ul of lambda phage dsDNA (5 ug/ml) (New England Boilable, 500ug/ml 1:100 dilution) and incubated overnight at 4°C. Plates were then blocked with 10% FCS/PBS for 30 min in room temperature (RT), washed 3X and then 100ul of diluted plasma (1:1500) was added to each well and incubated at RT for 2 hours. Plates were washed and 100ul HPR-labeled goat anti-mouse IgG was added to each well followed with 90 min RT incubation, washed and TMB substrate added. Plates were read at 450nm by Tencan GENios microplate reader (Tecan USA, Inc. Durham, NC). Data are reported as mean optical density (OD).

2.5. MicroRNA PCR Array

For miRNA PCR microarraying, pooled RNA (at least 3 individual mice for each group) was obtained from spleens of B/W mice. For comparison, RNA from mice in pre-disease stage (proteinuria-free) and mice with proteinuria (pro-3+) were collected. RNA extraction was performed with miRNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. RNA quantity and quality was measured by NanoDrop 1000 (Thermo Scientific, Wilmington, DE) and A260/280 and A260/230 ratios were all above 2.0. Following this, cDNA synthesis was performed with RT² miRNA first strand kit (SABiosciences, Qiagen, Frederick, MD), and mouse miFinder miRNA arrays (SABiosciences, Qiagen, Frederick, MD) were used. Analysis was performed using RT² Profiler PCR Array Data Analysis version 3.4. (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>)

2.6. Realtime PCR

Quantitative real time PCR (qPCR) was used to quantitate mature expression in RNA samples. Cellular RNA ($\sim 5 \times 10^6$ cells) was extracted by miRNeasy mini kit (Qiagen). Plasma RNA was extracted by diluting 20ul of plasma in RNase-free H₂O to a final volume of 200ul and extracted by TRIzol LS (Invitrogen) according to the manufacturer's instructions. However, for sorted cell subsets, no RNA extraction was performed, instead, heat-disruption at 95°C for 5min was directly applied to small amount of samples (1000 cell/ 50ul/sample) followed by quick-chilling at 4°C. cDNA synthesis and realtime PCR were performed by TaqMan microRNA Reverse Transcription and TaqMan miRNA hsa- Assay Kit (Applied Biosystems, Foster City, CA). The qPCR reaction was run on the Applied

Biosystems 7500 Real-Time PCR Systems for 40 cycles at 60°C. The miR-15a relative quantification (RQ) values were determined using the standard $2^{-\Delta\Delta CT}$ method according to the manufacturer's protocol and normalized to U6 snRNA or miR-30c (Applied Biosystems). For non-miRNA RT-qPCR assays, total RNA extracted as described above were reverse transcribed by multiscribe reverse transcriptase (Applied Biosystems) in the presence of random hexamers and dNTPs (1mM of each dNTPs in reactions) (all components from Applied Biosystems). Realtime PCR was performed based on SYBR Green PCR master mix (Applied Biosystems) according to manufacturer's protocol. Primers were: 5'-ACCGAGACTGCTTTTACC-3' and 5' CACTTCCTTCCTGTCCAA-3' for Syndecan-1, 5'-TCCTCGGACCATCAGGACAG-3' and 5'-CCTGTTGATGGAGCTGACGC-3' for PAX5, and 5'-TGACCACAGTCCATGCCATC-3' and 5'-GACGGACACATTGGGGGTAG-3' for GAPDH.

2.7. Statistical analysis

Values are presented as mean $\pm$ SEM and statistical analysis performed using an unpaired two-tailed Student t test by GraphPad (GraphPad, San Diego, CA) or Excel (Microsoft). The analysis of median survival time of B/W mice from all treatment groups was performed by comparing groups using unpaired, two-tailed t Test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. IFNs accelerated autoimmunity as well as induced microRNA changes in lupus-prone B/W mice

One of the disease manifestations of lupus in B/W mice is proteinuria as a result of autoantibody-associated immune complex deposition in kidneys [8]. We used proteinuria as the marker for the development of autoimmunity in these mice following *in vivo* delivery of IFNs. To ensure sustained chronic IFNs treatment, the pumps, which continuously released compound at the rate of 0.15ul/hr for up to 8 weeks, were replaced if mice survived beyond 8 weeks of treatment with new pumps with the same amount of materials for an additional 8 weeks. To verify the bioactivities of these cytokines for the duration of infusion, residue IFNs from the pumps at the end of treatment were tested on IFN α and IFN λ responsive cell lines and demonstrated that the *in vivo* conditions in the pump did not affect bioactivity of both IFNs (data not shown). IFN α high dose (10ug/pump) treatment achieved the greatest effect, with proteinuria onset occurring as early as 4 weeks post implantation (16wk of age), and 50% survival at 19 weeks of age. The next affected group was IFN α low dose (1ug/pump) in combination with IFN λ (10ug/pump) treatment, in which 50% survival was 23 weeks. However, neither IFN α low dose (1ug/pump) or IFN λ (10ug/pump) single treatment groups had an effect when compared to PBS control group, in which more than half of the mice remained proteinuria free beyond the age of 29 weeks (Fig. 1A). IFN λ 10ug single treatment did not achieve significant effects in B/W mice, however, in combination with IFN α 1ug, greatly accelerated disease onset than that seen in either single treatment with the same dose.

Analysis of large-scale microRNA expression in these mice was performed using PCR array analysis. The analysis included 88 most characterized microRNAs selected to be profiled using mouse miFinder PCR array. Pools of splenic RNA for each treatment group were extracted: IFN α 10ug proteinuria-3+ (α 10 pro-3+), IFN α 1ug + IFN λ 10ug pro-3+ (α 1ug + λ 10ug pro-3+), IFN λ 10ug pro-3+ (λ 10ug pro-3+), PBS pro-3+, IFN λ 10ug pro-free (λ 10ug pro-free) and untreated pro-free (UT pro-free) with 2 pools of the last group as controls. The untreated control mice were approximately the same age with α 10ug pro-3+, α 1ug + λ 10ug pro-3+ and λ 10ug pro-free groups (5.5mo on average), the λ 10ug pro-3+ were 6.5mo and

PBS pro-3+ were 7mo of age. A heat map was generated of selected microRNAs altered by the different treatments (Fig. 1B). The complete results for all the microRNAs are included in Supplementary Material. Many of the microRNA levels were similar in RNA obtained from mice with the same disease status (pro-3+ vs. pro-free), in which more microRNAs were upregulated as proteinuria was established and only a few were downregulated. No changes were specific to a particular type of IFN treatment (based on fold changes, data not shown). Thus, IFNs treatment accelerated proteinuria in B/W mice, but all B/W mice with proteinuria induced similar microRNA pattern changes when compared to untreated proteinuria-free B/W mice.

3.2. MicroRNA-15a upregulation induced by IFNs treatment preceded and correlated with autoantibody elevation

One microRNA of particular interest was miR-15a which was found to have elevated expression in diseased mice (Fig. 1B) and have a greater than 2 fold increase in mice with proteinuria compared to proteinuria-free mice based on the PCR array results (data not shown). In order to further investigate miR-15a in B/W disease progression, the findings of the microarray were validated by TaqMan realtime PCR. MiR-15a was measured for individual splenic RNA samples from age-matched (5mo) α 10 pro-free, α 10 pro-3+ and PBS pro-free mice, in parallel with the measurement of plasma anti-dsDNA IgG autoantibodies from the same mice (Fig. 2A). The increase in miR-15a levels seen in α 10 pro-free mice was significant compared to PBS control even before an increase in anti-dsDNA IgG levels, indicating overexpression of miR-15a induced by IFN α might be an initiation factor for autoantibody production (Fig. 2A). It has been shown that SLE patients have increased CD138+ (syndecan-1) plasma cell in the peripheral [42] and syndecan-1+ plasma cells also accumulated in inflamed kidneys in B/W mice [43]. In addition, IFN α triggered human plasma cell differentiation [44] and induced short-lived CD138+ plasma cells in the spleen in B/W mice [45]. PAX5, a paired box transcription factor, is a master transcriptional factor for all stages of B cell development, however, is turned off in plasma cells [46], more importantly, PAX5 has been shown to be a negative regulator of miR-15a expression [47]. Thus, it was expected that plasma cells would be increased in the spleens from IFN α treated diseased mice. As it is shown, the ratio between mRNA level of plasma cell marker (syndecan-1 or CD138) and PAX5 was higher in spleens from IFN α treated diseased mice compared to age-matched disease free controls, indicating more plasma cells (and reduced PAX5 expression) in the IFN α treated group (Fig. 2B). The increased expression of miR-15a was sustained and overexpression was not a transient phenomenon upon IFN α treatment, however, it was maintained and increased as autoimmunity progressed. To verify this, the overall correlation between miR-15a expression and autoantibody levels in samples from α 10 pro-3+, α 1ug + λ 10ug pro-3+, PBS pro-3+ and PBS pro-free mice was determined (Fig. 2C). Results showed that splenic miR-15a expression and autoantibody levels were correlated. Since plasma microRNA analysis has been intensely studied as a potential disease marker [39], it was hypothesized that the cellular level of miR-15a might also be reflected in the plasma levels of this microRNA. The miR-15a levels in the plasma increased as did the plasma autoantibody levels with disease development (PBS pro-3+ mice relative to the UT pro-free mice) (Fig. 2D). Furthermore, we compared all the individual mice in each IFN-treatment group with proteinuria and their age-matched PBS proteinuria-free counterpart for splenic miR-15a and plasma autoantibodies, showing that the increase in miR-15a accompanied the increase in autoantibodies (Fig. 3). This suggests that increased miR-15a may be an early marker of plasma cell development and may serve as an important regulator of autoantibody production.

3.3. Splenic B cell subpopulations and IL10 production in B-10 cells is altered by IFN

B cell abnormalities in lupus autoimmunity may result from either pathogenic B-2 overactivation resulting in excessive production of autoantibodies, and/or a deficiency in regulation by B-10 cells, via an IL-10-dependent pathway [23], resulting in a further enhanced activation of the immune system. Thus, the splenic B cell subset composition changes following IFNs treatment of B/W mice were determined by flow cytometric analysis. Cell surface markers B220, CD5 and CD1d were used to distinguish B-2, B-1 and B-10 cells (Fig. 4A). For measurement of IL-10 production in B-10 cells, spleen cells were stimulated *ex vivo* for 5h with a cocktail of LPS, PMA, ionomycin and brefeldin (abbreviated as LPIB) (Fig. 4B). B-10 absolute numbers were analyzed (Table 1). B-10 cells were significantly inhibited by IFN α high dose (IFN α 10) treatment even before proteinuria development. However, IFN λ did not act similarly, in contrast, treatment with IFN λ transiently induced significant B-10 upregulation before the development of proteinuria compared to all other treatment groups including PBS pro-free mice of the same age. With disease development, all the IFN treatment groups had absolute numbers of B-10 cells that were lower than PBS pro-3+. The ratio between B-10 and B-2 subpopulations, which may represent the ability of the immunoregulatory B-10 subpopulation to control or inhibit B-2 activity, was determined (Fig. 4C). Consistent with the trend we observed in B-10 absolute number analysis of diseased B/W mice, B-10 to B-2 ratios were decreased by IFNs treatment. Because the suppressive capabilities of B-10 cells is mediated by the production of IL-10 by these cells [48], the IL-10 levels in the B-10 subpopulation was determined. IL-10 expression intensity was shown representatively in the B-10 subpopulation (following 5h stimulation). Each treatment had as an internal control of total unstimulated spleen cells (Fig. 4D) and the change in mean fluorescence intensity (delta MFI) was determined. IL-10 expression post *ex vivo* stimulation in B-10 cells was low (similar MFI in B-10 vs. whole spleen) in the IFN α treatment group both before and after proteinuria when compared to either PBS or IFN λ groups. In summary, analysis of B cell subsets and IL-10 expression indicated that IFN α high dose treatment in B/W mice inhibits B-10 levels which results in an early loss of balance between inhibitory B-10 cell and activated B-2 cells, enhancing the autoimmunity in B/W mice. IFN λ although initially caused B-10 expansion pre-disease, the effect was not sustained with disease progression.

3.4. MicroRNA-15a was expressed differentially in splenic B cell subsets

Decreased miR-15a has been linked to preferential B-1 clonal expansion [49] and distinct microRNA signatures have been identified in different human lymphocyte subsets [50]. Due to differences in B cell subpopulations following *in vivo* treatment with IFNs, the expression of miR-15a in these B cell subsets was determined. FACS sorting of splenocytes from B/W into B-2, B-10 and B-1 (B-1 without B-10 cells) (Fig. 5A) was performed on untreated B/W mice that were proteinuria-free or proteinuria-3+, followed by realtime PCR measuring miR-15a (Fig. 5B). B-10 cells, regardless of disease status, showed the highest expression of miR-15a among the subsets. Interestingly, B-2 miR-15a level was lowest among the 3 subsets before proteinuria occurring, yet it reversed to be higher than B-1 when proteinuria occurred. Thus the increased miR-15a levels observed in total spleen following the development of proteinuria may be, in part, due to preferential expression of miR-15a in B-10 and B-2 subpopulations. This indicates the importance of miR-15a in directing the balance of splenic B cell subsets and finally the outcome of autoimmunity in B/W mice.

4. Discussion

The present study identified the interplay between splenic B cell subsets in the level of autoantibody production in interferon treated B/W mice. In this study, we compared the effects of IFN λ in this lupus model to IFN α effects, since the latter interferon is an

established key mediator of lupus nephritis [12]. Much of the current knowledge about IFN λ is focused on its anti-viral and anti-tumor activities [51–54]. Despite the many similarities shared by IFN α and IFN λ , the latter differs from IFN α both in the cellular receptors, the target cells and kinetics of bioactivities [17, 18]. As a result of treatment, IFN α high dose caused significant acceleration of disease in B/W mice. In contrast, the dose of IFN λ employed in this study had no significant enhancement of autoimmunity. Similarly IFN α low dose did not augment the development of autoimmunity. However, with the combination of the above two IFNs at those doses, significant effects were achieved. Different effects of IFN λ as a single treatment or in combination with IFN α , suggested that IFN λ could augment the effects of IFN α in accelerating autoimmunity in B/W mice.

Type I IFNs (α/β) regulation of the expression of genes for anti-viral activities has been intensely studied [55, 56]. Because microRNAs (miRs) are important in the regulation of mRNAs, dysregulation of mRNA may be due to microRNA effects. As expected, abnormalities in microRNAs have been identified in human SLE patients and some of the targets of these microRNAs are involved in IFN signaling networks [32, 57]. Moreover, direct effects of IFNs on cellular microRNAs have been reported. IFNs induction leading to anti-viral state is accompanied by alterations in several microRNAs [58, 59]. In addition, resistance to IFN chemotherapy has been associated with microRNA abnormalities [60]. In this present study the effects of IFNs, both IFN α and IFN λ , on the microRNA profile in the B/W lupus model was investigated.

The hypothesis was that in diseased B/W mice an IFN-induced microRNA signature would be found. This was not the case, the PCR array of 88 well characterized microRNAs showed no IFN signature on miR levels, rather similar microRNA changes were observed in all B/W mice upon the development of autoimmunity. On the other hand, there was a temporal signature of microRNA alterations induced by IFNs in that both IFN α and IFN λ , depending on the dose, triggered similar changes of microRNAs at timepoints earlier than those observed in control PBS. Other studies of microRNA arrays have found that the majority of microRNAs which were changed in activated T lymphocytes were upregulated [61]. Consistent with this finding, among all the microRNAs distinguishing pre-disease mice from B/W with disease, more microRNAs were upregulated with disease progression and only a few downregulated. The consistent upregulation of a subset of microRNAs in disease development supports that lupus nephritis involves immune activation.

One of the most upregulated microRNAs associated with autoimmunity was miR-15a, which targets important genes involving cell cycle (e.g cyclin D1) [62] or cell apoptosis (e.g. bcl-2) [63]. Decreased expression of mature miR-15a has been linked to malignant B-1 cell expansion [49]. One of the mechanisms for this uncontrolled B-1 proliferation could be the partial loss of regulation of cyclin D1 by miR-15a/16 in B-1 cells as exogenous replacement of miR-15a/16 in malignant B-1 cells leads to cell cycle arrest, cyclin D1 decrease and apoptosis [62]. Overexpression of miR-15a/16 via lentiviral vectors *in vivo* in NZB mice lead to a significant decrease in malignant B-1 cells [64] and more interestingly, the anti-dsDNA autoantibodies were also increased (data not shown, manuscript in preparation). The findings that systemic *in vivo* elevation of miR-15a/16 in NZB lead to increased autoantibody levels is consistent with the findings in this present study in B/W. PAX5 may play a role in the upregulation of miR-15a in B/W mice with full-blown autoimmunity, considering that PAX5 is downregulated in plasma cells [46] and PAX5 has been shown to suppress miR-15a expression [47], the downregulation of PAX5 in antibody producing plasma cells would relieve the suppression of miR-15a expression, resulting in an increase in miR-15a in B-2 cells. Other studies have found elevated miR-16 levels in a subpopulation of rheumatoid arthritis (RA) patients which further supports that miR-15a/16 levels maybe involved with autoimmunity [65]. We found a highly correlated upregulation of plasma

autoantibodies with increased splenic and plasma miR-15a levels in the B/W lupus mouse model treated with IFNs. The plasma elevation of miR-15a may indicate preferentially packaging of cellular microRNAs for trafficking to extracellular compartments including the plasma.

The increase in miR-15a may lead to apoptosis since one of the known targets of miR-15a is bcl-2, an anti-apoptotic protein [63]. In a previous study, miR-15a was exogenously increased in situ by injection directly into arthritic mouse joints (arthritis induced by autoantibodies) resulting in an increase in apoptosis [66]. However, in intact B/W, the miR-15a-induced apoptosis may occur in the B-10 subset of cells controlling autoantibody production, since these cells have the highest levels of miR-15a following disease onset. The miR-15a-induced loss of this regulatory B cell subset may lead to a more prominent autoantibody production.

Analysis of the splenic B cell subsets in the IFNs treated B/W mice indicated an effect on the ratios of these subsets. IL-10 is a well-known inhibitory cytokine in the immune system [67] and in the B cell compartment, B-10 cells are the major producers of IL-10. The expansion of splenic B-10 cells in the pre-disease B/W mice compared with C57BL/6 mice may be due to a regulatory feedback in the ongoing autoimmune response in the B/W mice [21, 68]. During the disease course, the CD5⁺CD1d^{hi} B cell subset, which includes B-10 cells, was also found to be expanded in B/W mice by comparing 28wk old versus 12wk old mice [23]. However, the expanded B-10 subpopulation failed to prevent B/W from developing autoimmune disease indicating that either the relative B-10 numbers were still not enough to combat the overwhelming immune activation in B/W mice, or an underlying intrinsic B-10 cell defect which compromised the expansion or function of the B-10 cells. Previous studies from mouse models have shown that these B cells can suppress T cell help [69], and these regulatory B cells which produce IL-10 are B-10 cells [22]. However, the B cell production of pathogenic autoantibodies in B/W mice is mainly T-cell dependent [70, 71], and these B cells residing in the B-2 subpopulation which are less capable of producing IL-10 (Fig. 4A). Flow analysis of splenic B cell showed that B-10 cells were drastically inhibited by high dose IFN α treatment and lesser by low dose IFN α in combination with IFN λ . IL-10 seemed to be a target of IFNs effects since IL-10 expression in B-10 cells was also inhibited by high dose IFN α treatment. Such an effect took place very early (IFN α 10ug pro-free) after treatment and was sustained throughout disease progression indicating that, due to the early loss of B-10 in the peripheral, the acceleration of autoimmunity occurred. In contrast, IFN λ single treatment caused an early B-10 expansion, yet this effect was lost with autoimmunity progression, and the mechanisms remain unknown. However, it is possible that exogenous IFN λ also can promote IFN α signaling, which eventually results in a similar pathogenic signaling pathways. In addition, the B-10 versus B-2 ratio decreased with IFNs-induced disease, and the loss of balance between regulatory cells and effector cells could be the key to the development of autoimmunity.

Studies on depleting B cells using anti-CD20 mAb between 12 wk and 28 wk of age in B/W mice showed delayed onset of disease symptoms and prolonged survival [21]. Since anti-CD20 targeted vast majority of peripheral B cells including splenic B cells [72], and depletion of these B cells resulted in reversing the disease outcome, it is reasonable to suggest that most splenic B cells in this window of age were pathogenic. However, in the pre-disease stage, B-10 cells producing IL-10 are present. Thus, the relative ratio of immunoregulatory B-10 cells to the pathogenic B-2 cells could be critical during this period such that even small decrease in B-10 could cause great loss in inhibition of the pathogenic B cell activities. Disease outcome and splenic B cell subpopulation ratios changed in B/W mice (age between 17 wk and 29 wk) following IFN-induced disease acceleration. This indicates that disease induction is very sensitive to a relative loss of B-10 cells.

There was a significant correlation between miR-15a level and autoantibodies production in lupus-prone B/W mice. In addition, a decrease in the presence of the immunoregulatory B cell subpopulations, B-10 was observed in the spleens of B/W mice with IFN-induced disease acceleration. To determine the miR-15a level in distinct B cell subpopulations, sorting strategies were employed to obtain microRNAs from B-10, B-2 and B-1 subpopulations. In pre-diseased animals, the miR-15a levels were lowest in B-2 cells. It was consistently found that the B-10 subpopulation was the highest in miR-15a expression and this increased with disease progression. This biased expression of miR-15a in different B cell subsets could be the explanation why B-10 cells were preferentially destined to decrease in IFNs augmented autoimmunity, since one of the targets for miR-15a is the antiapoptotic gene, *bcl-2*. Consistent with this, annexin V was higher in B/W splenic B-10 cells than other B cells (data not shown), indicating B-10 cells were more prone to apoptosis due to the elevated expression of miR-15a in this subpopulation. In addition, evidence for preferential effects of miR-15a in B cell subsets comes from CLL patients and the mouse model of CLL, NZB mice, in which the germline deletion/mutation in *mir-15a/16* loci caused underexpression of miR-15a/16 accompanied with only a B-1 malignant expansion [49, 73]. Moreover, the delivery of lentiviral miR-15a/16 *in vivo* in NZB mice only resulted in a malignant B-1 loss [64]. Thus, the intrinsic higher expression of miR-15a in splenic B-10 cells indicates that B-10 cell fate could be more dependent upon miR-15a effects than other B cell subsets.

5. Conclusions

IFN α *in vivo* treatment of B/W mice resulted in early elevation of miR-15a levels (spleen and plasma), increase in syndecan-1 expression (plasma cell development), decreased PAX5 expression (negative regulator of miR-15a/16), decreased B-10 levels when compared to disease free B/W mice. In contrast, IFN λ at the same dose did not have the same acceleration of disease but could accelerate the effect of low dose IFN α alone. Splenic B-10 cells seemed to be the target of IFNs and these cells were critical in disease progression. Results indicated that miR-15a levels, induced by IFNs, were correlated to autoantibody levels, and also potentially regulated the balance of splenic B cell subsets due to its differential expression in B cell subpopulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We investigated in vivo IFN λ effects in B/W murine model of SLE
- miR-15a increased with disease and correlated with autoantibody levels
- IFN α accelerated disease and an early decrease in B10 levels while IFN λ alone did not
- IFN α increased plasma cells and decreased PAX5 (negative regulator of miR-15a)

Figure 1. IFNs accelerated both autoimmunity and induced microRNA changes in B/W mice
(A) Proteinuria, monitored weekly using urine stripes, was used as the marker for the development of autoimmunity in B/W mice. The graph plots average proteinuria-free survival time up to 29wk of age, when the experiment end-point was reached. The survival time of B/W mice receiving different treatment was compared between groups independently: IFN α 10ug and PBS ($p < 0.0001$), IFN α 1ug + IFN λ 10ug and PBS ($p < 0.0001$), IFN λ 10ug and IFN α 1ug (ns), IFN λ 10ug and PBS (ns). Statistical analysis was performed using unpaired, two-tailed t test. **(B)** Pooled splenic RNA for each treatment group ($n = 3$ for each pool of group) was employed for microRNA arrays. The heat map represents the expression magnitude of selected microRNAs that changed the most across different treatment groups.

Figure 2. Upregulation of splenic miR-15a and autoantibodies following IFNs treatment
(A). Mice were treated with IFN α high dose (10ug/pump) and divided into pre-disease group (5 wk post treatment, proteinuria-free) and diseased group (proteinuria-3+), and age-matched (5 mo) PBS-treated pro-free mice served as control. miR-15a expression was measured by TaqMan realtime PCR assay and reported as relative values to miR-30c. Anti-dsDNA IgG by ELISA was performed on plasma from the same individual sample and reported as OD values. (All results represent mean $\pm$ SEM, $n>3$ in each group, treated groups were compared to PBS control *** $p<0.001$, * $p<0.05$, student's t test). **(B).** Ratio between mRNA levels of Syndecan-1 (plasma cell marker) and PAX5, measured by realtime PCR. Relative expression of genes were normalized to GAPDH. Mice were age-matched between comparison groups. **(C).** Correlation analysis was performed between splenic miR-15a levels and plasma anti-dsDNA IgG levels. Individual samples from each group: IFN α 10ug proteinuria-3+ (pro-3+), IFN α 1+IFN λ 10ug pro-3+, PBS pro-3+ and PBS pro-free, are shown (left panel), and linear regression analysis was performed without regard to treatment group to show the correlation between miR-15a expression and anti-dsDNA IgG production (right panel). **(D)** Plasma levels of miR-15a and anti-dsDNA IgG in PBS treated diseased mice (pro-3+) and UT predisease mice (pro-free). ($n=3$, * $p<0.05$, student's t test).

Figure 3. Individual analysis of autoimmunity and splenic miR-15a upregulation for each IFN treatment group

Different IFN-treated samples with proteinuria-3+ (pro-3+) were compared with the age-matched PBS-treated proteinuria-free (pro-free) samples: IFN α 10ug, IFN α 1+IFN λ 10ug and age-matched PBS groups, aged between 5 to 6 months. IFN α 10ug, IFN λ 10ug and age-matched PBS groups, aged between 6.5 to 7 months. Anti-dsDNA IgG was reported as OD values and relative miR-15a expression was normalized to miR-30c (All results represented mean $\pm$ SEM, n>3 in each group, **p<0.01, *p<0.05, student's t test)

Figure 4. Splenic B cell subsets following chronic IFNs treatment *in vivo*

(A) Representative histograms of unstimulated B/W spleen cells stained for CD5 and IgM, which identify total B-1 cells (CD5+, B220+) and B-2 cells (CD5-, B220+) (left panel). The B-1 population was further subdivided into B-10 cells (CD5+, B220+, CD1d+, IL-10+) (middle panel). IL-10 expression in the indicated subpopulations is shown in the histogram overlays (B-2 black, B-10 dashed and B-1 without B-10 filled histogram, right panel). (B) Spleen cells stimulated for 5h with LPIB and analyzed as above. (C) Splenic cells were cultured for 5h and identification of B-10 and B-2 cells determined. The mean B-10/B-2 ratio (region indicated by dashed boxes in (B)) for each B/W treatment group is shown (upper black bars, pro-free and lower open bars, pro-3+). (Bars are mean $\pm$ SEM, $n > 3$ in each group, ** $p < 0.01$, * $p < 0.05$, student's t test.) (D) IL10 expression in B-10 cells after 5hr stimulation (unfilled histogram) and IL10 expression in unstimulated total splenocytes (filled histogram). Delta mean fluorescence intensity (Δ MFI) between samples is indicated. Spleens from indicated treatment groups were proteinuria-free (upper groups) and proteinuria-3+ (lower groups).

Figure 5. Differential miR-15a expression in B cell subsets

(A). Flow Cytometric Sorting Strategy and Representative Histograms: Splenic B cell subsets from B/W mice were sorted from the live lymphoid gate into the following populations: B-2 (IgM+CD5⁻), B-10 (IgM+CD5+CD1d^{hi}) and B-1 without B10 (IgM+CD5+CD1d⁻), as shown in dashed boxes. (B). The sorted B cell subpopulations from untreated mice proteinuria-free (5mo, n=3) and untreated mice with proteinuria-3+ (>7.5mo, n=3) were obtained and RNA isolated. miR- 15a levels are reported as relative expression normalized to miR-30c (mean $\pm$ SEM, **p<0.01, *p<0.05, student's t test).

Splenic B-10 absolute numbers¹

Table 1

Absolute B-10 number in spleen (×10 ⁶)			
	pro-free mean±SEM	pro-3+ mean±SEM	
PBS	1.13±0.10	4.92±0.28	
α10	0.33±0.03 **	0.58±0.19	
λ10	3.30±0.13 ***	2.15±0.21	***
α1λg	1.52±0.51	1.57±0.19	
α1+λ10		1.44±0.25	

¹ Splenocytes were obtained from individual B/W mice receiving interferons or PBS as control. Mice were grouped according to treatment and disease status (pro-free) or after proteinuria (pro-3+). B-10 cells were identified by flow cytometry as B220+CD5+CD1d^{hi}10+, after stimulation with LPS/PMA/Ionomycin/brefeldin (LPiB) for 5h. Absolute numbers were calculated from total spleen weight (estimating 1mg = 10⁶ cells) multiplied by percentage of B-10 cell in the total live spleen gate. (n = 3 in each group). Statistical comparisons between IFN treatment and PBS control within the same disease status was performed using Student's t test (**p<0.01, ***p<0.001).