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Reduction of CD19 autoimmunity marker on B cells of paediatric SLE patients through repressing PU.1/TNF-α/BAFF axis pathway by miR-155


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ABSTRACT
microRNA-155 (miR-155) is implicated in regulating B-cell activation and survival that is important in systemic lupus erythematosus (SLE) pathogenesis. PU.1, a target for miR-155, is a crucial regulator of B-cell development and enhances Tumour-Necrosis-factor-alpha (TNF-α) expression. TNF-α induces the expression of B-cell-activating-factor (BAFF). BAFF is reported to increase the expression of the autoimmunity marker; CD19. This study aimed to investigate the regulation of expression of PU.1 in pediatric-systemic-lupus-erythematosus (pSLE) patients by miR-155, and hence evaluate its impact on TNF-α/BAFF/CD19 signalling pathway. Screening revealed that PU.1 is upregulated in PBMCs and B-cells of pSLE patients. PU.1 expression directly correlated with systemic-lupus-erythematosus disease-activity-index-2 K SLEDAI-2K. Ectopic expression of miR-155 and knockdown of PU.1 suppressed PU.1, TNF-α and BAFF. Finally, miR-155 decreased the proportion of BAFF-expressing-B-cells and CD19 protein expression. These findings suggest that miR-155 suppresses autoimmunity through transcriptional repression of PU.1 and TNF-α, which in turn suppresses BAFF and CD19 protein expression.

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microRNA 155; BAFF; PU.1; TNF-α; B cells; lupus

Introduction
Ongoing research is now focusing on the role of microRNAs (miRNAs, miRs) in the regulation of autoimmunity (Simpson & Ansel, 2015; Garo & Murugaiyan, 2016). miR-155 is among the prominent miRNAs studied in B cell regulation (Costinean et al., 2006; Calame, 2007). The role of miR-155 in SLE remains controversial. In murine lupus models (MRL-lpr and B6-lpr mice), miR-155 was significantly increased in whole splenocytes, as well as in splenic B and T cells (Dai et al., 2010). Deletion of miR-155 reduced autoantigen-induced GC reactions in spleens of lupus mice (Thai et al., 2013). In SLE patients, miR-155 urinary levels were reported to be significantly increased compared to healthy controls and significantly correlated with proteinuria and systemic lupus erythematosus disease activity index (SLEDAI) (Wang et al., 2012). Paradoxically, that same research group had earlier reports indicating that the serum level of miR-155 was lower in SLE patients and correlated with glomerular filtration rate estimates, red blood cell count, platelet count, lymphocytic count and the C-reactive protein (Wang et al., 2010). miR-155 expression levels also showed significant reduction in the plasma of SLE patients (Wang et al., 2012). On the other hand a recent study revealed that miR-155 expression was reduced in CD4+T cells from SLE patients versus healthy controls, and upon forcing its expression there was an increase in STAT3 phosphorylation and accordingly an increase in IL-21 production, therefore they hypothesized that the decreased levels of miR-155 serves in regulating the pathologically increased IL-21 in SLE (Rasmussen et al., 2015). Similarly, our research group has demonstrated that miR-155 expression is significantly down-regulated in PBMCs from juvenile SLE patients versus age-matched healthy controls, and there was a negative correlation between miR-155 expression and SLEDAI. Moreover, we have shown that forcing the expression of miR-155 reduced PP2Ac (Protein phosphatase 2A homologues, catalytic domain) expression and accordingly increased IL-2 levels in PBMCs of juvenile SLE patients (Lashine et al., 2014).

miR-155 has several downstream targets among which is PU.1 that has been previously validated as a
potential target for miR-155, PU.1 is a member of the Ets family of transcription factors, that has been identified as a vital transcriptional factor for lymphoid and myeloid development (McKercher et al., 1996; Carotta et al., 2010). PU.1 overexpression has been correlated with SLE pathogenesis (Hikami et al., 2011), a recent study reported that PU.1 was overrepresented in the promoters of genes linked to SLE susceptibility (Dozmorov et al., 2014). Additionally, PU.1 was demonstrated to directly activate the promoter of tumour necrosis factor alpha (TNF-α) (Niwa et al., 2008; Fukai et al., 2009). The contribution of TNF-α to SLE pathogenesis has been also debatable; although initial findings conveyed TNF-α to have a protective effect against autoimmunity, it was not until later when other studies reported its expression to be directly associated with inflammation, sequential tissue injury, and organ destruction in SLE (Jacob, 1992; Aringer & Smolen, 2003), therefore, TNF-α has been recently considered as a target for molecular therapy in SLE (Xiong & Lahita, 2011). Interestingly, TNF-α was evidenced to increase the mRNA and protein levels of B cell-activating factor (BAFF) (Lee et al., 2013).

BAFF overexpression has been recognized as a crucial factor in systemic lupus erythematosus (SLE) pathogenesis; mice over-expressing BAFF exhibit increased autoantibody production by B cells which leads to a progressive autoimmune disease similar to SLE (Mackay et al., 1999). BAFF was reported to induce the expression of CD19, a major component of the B-cell coreceptor complex (Hase et al., 2004). Overexpression of CD19 has been linked to abnormalities in the immune system; it disrupts peripheral tolerance in B cells and thereby induces autoantibody production (Sato et al., 2000; Inaoki et al., 1997).

Hence, the regulation of the PU.1/TNF-α/BAFF axis seems to hold a potential role in controlling SLE disease activity.

Therefore, this study aimed at further highlighting the role of miR-155 in SLE pathogenesis, through investigating its impact on PU.1 transcriptional factor and consequently on TNF-α/BAFF/CD19 signalling, in B cells of paediatric SLE (pSLE) patients.

### Patients and methods

#### Patients

Thirty-four patients with SLE were recruited from the Department of Rheumatology, Aboelreesh Pediatric Hospital, Cairo University Medical School. All study-recruited patients were under the age of 18 and fulfilled the American College of Rheumatology (ACR) 1997 revised classification criteria for SLE (Hochberg, 1997). Disease activity of each patient was assessed at the time of sample collection using systemic lupus erythematosus disease activity index SLEDAI-2K, and was used to divide patients into active and inactive groups where active SLE disease was defined as SLEDAI-2 K score of ≥6 and inactive SLE disease was defined as SLEDAI-2K score of <6 (Khanna et al., 2004). Absolute lymphocyte counts (ALC) were recorded; lymphopenia was defined as ALC <1000/L. Additional clinical data of the pSLE patients involved in this study is shown in Table 1.

Healthy, age-matched blood donors with no history of autoimmune diseases or treatment with immunosuppressive agents served as controls. Legal guardians of all patients and controls provided a written informed consent before collection of peripheral blood samples. The study was approved by the German University in Cairo and Cairo University’s ethical review committees and experiments were performed in accordance with the Helsinki Declaration of 1975.

#### Collection of samples and separation of PBMCs

Ten millilitres of venous peripheral blood were withdrawn from all SLE patients and healthy controls in the presence of the anticoagulant EDTA. Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood using the Ficoll density gradient centrifugation method within 6 h of blood withdrawal. The isolated PBMCs were cryopreserved and stored at −80 °C.

#### B cell isolation

PBMCs of 6–7 patients were pooled and purified B cells were subsequently isolated by negative selection.

### Table 1. Clinical data of SLE patients recruited in the current study.

<table>
<thead>
<tr>
<th>Sex (male/female)</th>
<th>10/24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) mean ± SEM</td>
<td>12.15 ± 0.5791</td>
</tr>
<tr>
<td>Disease duration (years) mean ± SEM</td>
<td>3.447 ± 0.5128</td>
</tr>
<tr>
<td>SLEDAI-2K (mean ± SEM)</td>
<td>5.676 ± 0.6482</td>
</tr>
<tr>
<td>Activity (active/inactive)</td>
<td>14/20</td>
</tr>
<tr>
<td>ANA a [+]ve/-ve</td>
<td>20/14</td>
</tr>
<tr>
<td>Lupus nephritis [+]ve/-ve</td>
<td>20/14</td>
</tr>
<tr>
<td>Lymphopenia [+]ve/-ve</td>
<td>14/20</td>
</tr>
<tr>
<td>Neuropsychiatric manifestations [+]ve/-ve</td>
<td>3/31</td>
</tr>
<tr>
<td>Prednisone intake [+]ve/-ve</td>
<td>17/17</td>
</tr>
<tr>
<td>Cyclophosphamide intake [+]ve/-ve</td>
<td>4/30</td>
</tr>
<tr>
<td>Azathioprine intake [+]ve/-ve</td>
<td>19/15</td>
</tr>
<tr>
<td>Hydroquinone intake [+]ve/-ve</td>
<td>31/3</td>
</tr>
</tbody>
</table>

aAnti-dsDNA antibody – anti-double stranded DNA antibody.

bANA – anti-nuclear antibody.

cANC, cANAC anti-nuclear antibody.

dAnti-dsDNA antibody.

### Patients

Thirty-four patients with SLE were recruited from the Department of Rheumatology, Aboelreesh Pediatric Hospital, Cairo University Medical School. All study-recruited patients were under the age of 18 and...
using the human B Cell Isolation Kit II, human (MACS, MiltenyiBiotec, San Diego, CA) according to manufacturer’s recommendations.

**Cell culture and transfection**

PBMCs or B cells were cultured in RPMI 1640 media (Lonza, Visp, Switzerland) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and Mycozap (Lonza, Visp, Switzerland). Cells were transfected with scrambled miRNAs or mimics and inhibitors of miR-155 (Qiagen IDs: MSY0000646 and MIN0000646 respectively) or PU.1 siRNA (Qiagen ID: number SI00047971) using HiPerfect transfection reagent (all supplied by Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Transfected cells were incubated for 48 h or 72 h until analysis.

PBMCs or B cells referred to as mock were those treated with the transfecting agent without the oligonucleotides. After each transfection, the levels of miR-155 were assessed in cells transfected with miR-155 mimics compared to mock cells to confirm transfection efficiency; transfection resulted in an average increase in miR-155 levels of more than 300-fold in PBMCs and more than 500-fold increase in B cells.

**mRNA and miRNA quantification**

Total RNA was extracted using mirVana extraction kit according to the manufacturer’s protocol (Ambion Applied Biosystems, Drive Foster City, CA). The extracted mRNA and miRNA fractions were reverse-transcribed into single stranded complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Archive kit (Applied Biosystems, Life Technologies, Drive Foster City, CA). The relative expression of the genes of interest as well as the miRNA was then quantified using StepOne™ Real-Time PCR instrument (Applied Biosystems). Expression of PU.1, TNF-α and BAFF was normalized to 18S housekeeping gene. miR-155 expression was normalized to RNU6B housekeeping small RNA. All PCR reactions were carried out in triplicates.

**Flow cytometric analysis**

BAFF and CD19 cell-surface expression was quantified using a Coulter Epics XL flow cytometer (Beckman-coulter, Miami, FL). Cell aliquots were incubated for 30 min at 4 °C with FITC conjugated anti-BAFF (monoclonal mouse IgM) and PE-CY5 conjugated anti-CD19 (monoclonal mouse IgM) or matching isotype control (all supplied by Santa Cruz Biotechnology, Dallas, TX), then washed with PBS supplemented with 2% FCS. The mean fluorescence intensities (MFI); which denotes the intensity of expression; as well as the percentages of the cells expressing CD19 or BAFF were analysed using Kaluza software (Beckman-coulter, Miami, FL).

**Statistical analysis**

The relative expression of the genes is expressed in relative quantitation (RQ = 2^ΔΔCT). All values are represented as the mean ± standard error of the mean (SEM). Mann–Whitney test was used for statistical analysis. Calculations were performed using GraphPad Prism 5.00 software (GraphPad Software Inc., La Jolla, CA). Each experiment was independently performed at least three times. All statistical tests were two tailed and a p value < .05 was considered statistically significant. For correlation analysis, Pearson’s correlation coefficient (r) was used; where r value shows if there is a positive or negative correlation between the 2 variables and (p) indicates whether this correlation is significant or not.

**Results**

**PU.1 is up-regulated in PBMCs and B cells of pSLE patients**

PBMCs were isolated from individual pSLE patients, while B cells were isolated from pooled PBMCs of pSLE patients and age-matched healthy controls. The relative expression of PU.1 was quantified using real-time PCR and was found to be markedly up-regulated in PBMCs of pSLE patients (n = 15) compared to healthy controls (n = 7) (p = .0289) (Figure 1(A)). Similarly, the expression of PU.1 was significantly higher in pooled B cells of pSLE patients (n = 5) compared to healthy controls (n = 7) (p = .0057) (Figure 1(A)).

To determine whether PU.1 expression was associated with disease activity, a correlation analysis was performed between PU.1 expression in PBMCs of pSLE patients and their SLEDAI-2K, and results revealed a significant positive correlation (r = .5804) (p = .0233) (Figure 1(B)). Furthermore, patients were sub-grouped into active (SLEDAI-2K >6) and inactive (SLEDAI-2 K6) pSLE patients, and their PU.1 relative expression was then compared. Interestingly, active pSLE patients (n = 6)
had significantly higher levels of PU.1 compared to inactive pSLE patients (n = 9) (p = .0256) (Figure 1(C)).

Since PU.1 is an important regulator of lymphopoiesis (Iwasaki et al., 2005), it was interesting to associate its transcript levels with lymphopenia in pSLE patients. Therefore, patients were divided into two groups according to the presence or absence of lymphopenia. The relative expression of PU.1 was markedly elevated in lymphopenic pSLE patients (7) compared to patients without lymphopenia (n = 8) (p = .0304) (Figure 1(D)).

**Figure 1.** Expression of PU.1 mRNA in pSLE patients and its association with disease activity and lymphopenia. (A) PU.1 was significantly up-regulated in PBMCs of pSLE patients (n = 15) compared to healthy controls (n = 7) (p = .0289). Likewise, the expression of PU.1 was significantly higher in pooled B cells of pSLE patients (n = 5) compared to healthy controls (n = 7) (p = .0057). Mann–Whitney was used to assess differences between two samples. ****p < .0001, ***p < .001, **p < .01, *p < .05. All PCR reactions were carried out in triplicate. (B) Spearman’s analysis showed a positive correlation analysis between SLEDAI-2K and PU.1 expression in PBMCs of pSLE patients (r = .5804) (p = .0233). (C) Active pSLE patients (SLEDAI-2k > 6) (n = 6) had significantly increased transcripts of PU.1 compared to inactive pSLE patients (SLEDAI-2k ≤ 6) (n = 9) (p = .0256). Mann–Whitney was used to assess differences between two samples. ****p < .0001, ***p < .001, **p < .01, *p < .05. All PCR reactions were carried out in triplicate. (D) The relative expression of PU.1 showed a significant increase in PBMCs of pSLE patients suffering from lymphopenia (n = 7) compared to those without lymphopenia (n = 8) (p = .0304). Mann–Whitney was used to assess differences between two samples. ****p < .0001, ***p < .001, **p < .01, *p < .05. All PCR reactions were carried out in triplicate.

**TNF-α and BAFF are up-regulated in PBMCs and B cells of pSLE patients**

Since PU.1 is a positive regulator of TNF-α transcription (Niwa et al., 2008) and TNF-α in turn induces BAFF expression (Lee et al., 2013; Ittah et al., 2006) and both TNF-α and BAFF contribute to SLE pathogenesis (Jacob, 1992; Aringer & Smolen, 2003; Zhang et al., 2001; Stohl et al., 2003), hence, it was intriguing to assess TNF-α and BAFF mRNA levels in PBMCs and B cells of pSLE patients. Results showed higher expression of TNF-α and BAFF in PBMCs of pSLE
patients \((n = 15)\) versus age-matched healthy controls \((n = 7)\) \((p = .0145)\). Consistently, pooled B cells of pSLE patients \((n = 5)\) had higher TNF-\(\alpha\) expression compared to pooled B cells of healthy controls \((n = 7)\) \((p = .0025)\). Mann–Whitney was used to assess differences between two samples. 

\(* * * p < .0001, ** p < .01, * p < .05.\) All PCR reactions were carried out in triplicate.

\[\text{BAFF mRNA expression in PBMCs of pSLE patients} \quad (n = 15) \quad \text{versus healthy controls} \quad (n = 7) \quad (p = .024)\]. Comparably, pooled B cells of pSLE patients \((n = 5)\) had increased expression of BAFF mRNA compared to pooled B cells of age-matched healthy controls \((n = 7)\) \((p = .0025)\). Mann–Whitney was used to assess differences between two samples. 

\(* * * p < .0001, ** p < .01, * p < .05.\) All PCR reactions were carried out in triplicate.

\[\text{Pearson’s correlation test revealed a direct correlation between mRNA expression of both TNF-\(\alpha\) and BAFF in PBMCs of pSLE patients} \quad (r = .7631) \quad (p = .0006).\]

\[\text{miR-155 suppresses PU.1, TNF-\(\alpha\) and BAFF expression in PBMCs and B cells of pSLE patients}\]

In order to confirm good transfection efficiency, the change in expression of miRNA, relative to mock cells treated with transfection reagent alone, was determined by TaqMan real-time quantitative PCR, normalized to RNU6B endogenous control. PBMCs and B cells transfected with miR-155 mimics showed higher levels of miR-155 expression compared to mock cells \((p < .0001 \quad \text{and} \quad p = .007, \quad \text{respectively})\) \((\text{Figure 3(A))}\).

\[\text{In order to assess the effect of miR-155 on PU.1, TNF-\(\alpha\) and BAFF mRNA, the relative expression of the three genes was analysed after miR-155 mimicking and antagonizing in PBMCs of pSLE patients. Results showed that ectopic expression of miR-155 suppressed the expression of PU.1, TNF-\(\alpha\) and BAFF in PBMCs}\]
of pSLE patients compared to mock cells (p < .0001 and p = .008, respectively) (Figure 3(B–D)). Interestingly knockdown of PU.1 by siRNAs remarkably reduced transcripts of PU.1, TNF-α and BAFF in PBMCs of pSLE patients compared to mock cells (p = .0058, p = .0485 and p = .007, respectively) (Figure 3(B–D)). Similarly, transfection of miR-155 mimics in B cells of pSLE patients significantly down-regulated PU.1, TNF-α and BAFF expression compared to mock B cells (p < .0001, p = .0238 and p = .004, respectively) (Figure 3(B–D)). PU.1 siRNAs resulted in a reduced expression of PU.1, TNF-α and BAFF in B cells of pSLE patients (p = .0007, p = .0357 and p = .0286, respectively) (Figure 3(B–D)). On the other hand, PBMCs and B cells treated with miR-155 inhibitors had mRNA levels of PU.1, TNF-α and

Figure 3. Transfection efficiency of miR-155 oligonucleotides and its impact on PU.1, TNF-α and BAFF mRNA Expression in pSLE patients. (A) Transfection of miR-155 mimics in PBMCs and B cells of pSLE patients increased miR-155 levels significantly compared to Mock cells (p < .0001 and p = .007, respectively). miR-155 expression was determined by TaqMan RTqPCR and normalized to RNU6B endogenous control. All PCR reactions were carried out in triplicate. (B) Ectopic expression of miR-155 reduced PU.1 transcripts in PBMCs and B cells of pSLE patients compared to mock cells (p = .0029 and p < .0001, respectively). Moreover, knocking down PU.1 by siRNAs suppressed PU.1 mRNA in PBMCs and B cells of pSLE patients (p = .0058 and p = .0007, respectively). Mann–Whitney was used to assess differences between two samples. ***p < .0001, **p < .001, *p < .01, *p < .05. All PCR reactions were carried out in triplicate. (C) miR-155 down-regulated TNF-α mRNA expression in PBMCs and B cells of pSLE patients compared to mock cells (p = .0121 and p = .0238, respectively). PU.1 siRNAs reduced transcripts of TNF-α in PBMCs and B cells of pSLE patients (p = .0485 and p = .0357, respectively). Mann–Whitney was used to assess differences between two samples. ***p < .0001, **p < .001, *p < .01, *p < .05. All PCR reactions were carried out in triplicate. (D) miR-155 reduced BAFF mRNA in PBMCs and B cells of pSLE patients compared to mock cells (p = .008 and p = .004, respectively). Intriguingly, siRNAs against PU.1 reduced BAFF mRNA in PBMCs and B cells of pSLE patients (p = .007 and p = .0286, respectively). Mann–Whitney was used to assess differences between two samples. ***p < .0001, **p < .001, *p < .01, *p < .05. All PCR reactions were carried out in triplicate.
BAFF comparable to mock cells. To assess the effect of miR-155 on BAFF protein expression in B cells, PBMCs were stained with FITC-conjugated anti-BAFF and PE-CY5 conjugated anti-CD19 antibodies. The MFIs of CD19 and BAFF were analyzed by flow cytometry. BAFF surface expression was significantly reduced on CD19+ B cells treated with miR-155 mimics or PU.1 siRNA (p = .0095 and p = .0190, respectively) (Figure 4(A,B)).

**CD19 expression is increased in pSLE patients**

CD19 is an important regulator of B cell function and autoimmunity (Hasegawa et al., 2001). Flow cytometric analysis showed that CD19 expression is significantly increased in pSLE patients (n = 10) compared to healthy controls (n = 4) (p = .033) (Figure 4(C,D)).

**miR-155 reduces CD19 expression in pSLE patients**

In order to investigate the impact of miR-155 and PU.1 on CD19 protein expression in pSLE patients, PBMCs were transfected with miR-155 mimics/inhibitors or PU.1 siRNAs. CD19 was markedly suppressed on miR-155 mimicked cells compared to mock cells (p = .0186). Interestingly, cells treated with PU.1 siRNAs also had reduced CD19 protein expression compared to mock cells (p = .0167), while miR-155 antagonized PBMCs showed levels of CD19 similar to mock cells (Figure 4(E)).

**miR-155 reduces BAFF + B cells in pSLE patients**

The proportion of BAFF-positive cells were analyzed in mock, miR-155 mimicked/antagonized and PU.1 siRNA-treated CD19+ B cells. Results revealed that miR-155 as well as PU.1 siRNA dramatically decreased the proportion of BAFF + B cells (p = .0095 and p = .0095, respectively) (Figure 5(A,B)).

**Discussion**

The controversial role of miR-155 in SLE triggered our interest to evaluate the impact of this miRNA on PU.1 transcription factor, one of its novel downstream targets that is highly involved in B cell development and regulation (Vigorito et al., 2007; McKercher et al., 1996). We have recently revealed miR-155 to be underexpressed in pSLE patients (Lashine et al., 2014).

Therefore, the current study initially analysed PU.1 expression profile in pSLE patients. Our results showed that PU.1 expression is remarkably increased in both PBMCs and B cells from pSLE patients versus age-matched healthy controls. This may be supported by previous data reporting PU.1 gene to be hypomethylated in SLE (Javierre et al., 2010), it also stands in line with data stating that PU.1 transcription factor is interlinked with the promoters of genes related to SLE pathogenesis (Dozmorov et al., 2014).

We further aimed at examining the association between PU.1 expression and clinical features in SLE patients. Interestingly, PU.1 expression demonstrated a positive correlation with SLEDAI-2K. Similarly, active SLE patients and those suffering from lymphopenia showed notably higher expression of PU.1 than their inactive or non-lymphopenic counterparts. These findings provide further support for a previous study that suggested PU.1 up-regulation to play a role in the pathogenesis of SLE (Hikami et al., 2011).

This study investigated the effect of manipulating miR-155 on PU.1 expression in PBMCs and B cells of pSLE patients and its consequent impact on B cell pathology. Our results revealed that forcing the expression of miR-155 has markedly reduced PU.1 mRNA expression in PBMCs and B cells of pSLE patients compared to mock cells. Indeed, the suppressive effect of miR-155 on PU.1 was comparable to the effect of knocking down PU.1 by siRNAs.

PU.1 overexpression has been suggested to enhance TNF-α transcription (Niwa et al., 2008). This notion was further validated by a study demonstrating that knocking down PU.1 noticeably reduced TNF-α protein in dendritic cells, while increasing PU.1 led to an increased production of TNF-α. Therefore, it was proposed that TNF-α promoter is directly activated by PU.1(Fukai et al., 2009). For these reasons, it was of our interest to analyze the expression profile of TNF-α in pSLE patients and to explore the effect of manipulating PU.1 expression by miR-155 on the expression of TNF-α. Our Results showed considerable augmentation of TNF-α baseline expression in PBMCs and B cells of pSLE patients. This finding is consistent with studies reporting that TNF-α is markedly increased in SLE (Gabay et al., 1997; Sabry et al., 2006; Studnicka-Benke et al., 1996) and that anti-TNF therapy achieved a good outcome in arthritis and nephritis SLE patients (Aringer & Smolen, 2008). Nevertheless, it may contradict previous data that have reported TNF-α levels to be higher in SLE patients with inactive disease compared to patients with active disease (Gomez et al., 2004).

Interestingly, our study demonstrated a notable inhibitory effect of miR-155 on TNF-α expression in PBMCs and B cells of pSLE patients suggesting that
Figure 4. Flow cytometric analysis of BAFF and CD19 cell surface expression in pSLE patients. (A) Flow cytometric histogram showing BAFF and CD19 expression (MFI; x-axis) on PBMCs of pSLE patients after different manipulations. PBMCs were stained with anti-CD19 antibody conjugated to PE-CY5 and anti-BAFF antibody conjugated to FITC. CD19 and BAFF levels were decreased on cells treated with miR-155 mimics or those treated with PU.1 siRNA compared to Mock cells. While miR-155 antagonized cells had similar expression of CD19 as Mock cells. (B) Percentages of MFI, as measured by flow cytometry, of BAFF protein expression assessed on CD19 + B cells of pSLE patients. miR-155 and PU.1 siRNA notably reduced the expression of BAFF on CD19 + B cells of pSLE patients (p = .0095 and p = .0190, respectively). Mann–Whitney was used to assess differences between two samples. **p < .0001, ***p < .001, **p < .01, *p < .05. The experiment was done in triplicate. (C) Flow cytometric histogram showing CD19 expression on PBMCs of pSLE patients and healthy controls. Background fluorescence was determined with an irrelevant isotype-matched antibody. SLE patients had higher CD19 protein expression compared to healthy controls. (D) Percentages of MFI of CD19 expression were increased on PBMCs of pSLE patients (n = 10) compared to healthy controls (n = 4) (p = .033). Mann–Whitney was used to assess differences between two samples. **p < .0001, ***p < .001, **p < .01, *p < .05. The experiment was done in triplicate. (E) miR-155 mimicked and PU.1 knocked-down PBMCs had decreased CD19 expression (p = .0166 and p = .0167, respectively) compared to Mock cells. miR-155 inhibitors did not affect CD19 expression. Mann–Whitney was used to assess differences between two samples. **p < .0001, ***p < .001, **p < .01, *p < .05. The experiment was done in triplicate.
this is the impact of repressing PU.1 which was confirmed by bioinformatics analysis that did not predict miR-155 to have any binding sites on the 3'UTR of TNF-α. Therefore, for further confirmation that TNF-α reduction is due to PU.1 repression, PU.1 was knocked down using siRNAs and a suppressive effect on TNF-α expression similar to that obtained by miR-155 mimics was detected in both PBMCs and B cells of pSLE patients. Hence, miR-155 may be an indirect regulator of TNF-α mediating its action through PU.1.

In SLE patients, serum levels of BAFF were found to be aberrantly high and correlated with disease activity (Zhang et al., 2001; Stohl et al., 2003).
Moreover, Belimumab, a monoclonal antibody against BAFF, has been recently approved for SLE therapy (Navarra et al., 2011).

Our study further quantified BAFF gene expression in PBMCs and B cells of pSLE patients and revealed it to be evidently enhanced in pSLE patients. We have also shown that BAFF mRNA expression is positively correlated with TNF-α mRNA expression in PBMCs of pSLE patients and since TNF-α shown in several studies to enhance BAFF expression (Lee et al., 2013; Ittah et al., 2006; Assi et al., 2007), it was intriguing to detect the impact of miR-155 and PU.1 on BAFF expression. Interestingly, both miR-155 mimics and PU.1 siRNAs considerably suppressed BAFF mRNA and protein levels in B cells and PBMCs of pSLE patients, and also markedly decreased the proportion of BAFF-positive B cells as quantified by flowcytometer. These findings appear to partially oppose previous literature which reported miR-155 inhibitors to significantly reduce BAFF-R expression in B cells; however the latter study was performed on B cells from myasthenia gravis patients (Wang et al., 2014), an explanation for this might be that miRNAs act in a cell-specific or disease-specific manner where Lin et al. found that miR-206 can act in an opposite manner on the expression of the same target (KLF4) in normal versus cancer cells (Lin et al., 2011). This shows that miRNAs action might depend on the pathological status of the cell. In similarity with the TNF-α bioinformatics results, we found no predicted binding regions for miR-155 on the BAFF 3’UTR. Hence, it is plausible that the effect of miR-155 on BAFF is through transcriptional repression of PU.1 as well as TNF-α.

Dysregulated CD19 expression has been associated with abnormalities of the immune system, where upregulation of CD19 was linked to the development of autoantibodies (Sato et al., 2000). In SLE, CD19 was somehow controversial; while a study reported CD19 levels to be highly expressed on B cells from SLE patients versus healthy controls (Mei et al., 2012). Other studies reported about 20% reduction in CD19 expression levels by naive and memory B cells from SLE patients (Sato et al., 2000; Sato et al., 2004). Accordingly, and in a trial to identify the status of CD19 in SLE patients, CD19 expression was assessed using FACS analysis; our results showed that CD19 protein expression is inherently upregulated in pSLE patients compared to healthy controls. This goes in line with the fact that the expression of CD19 on all B cell subsets; memory cells, plasmablasts and naive B cells correlates with each other in SLE patients. However, CD19 expressed by plasmablasts of SLE patients was found to be less than that expressed by memory cells and naive B cells (Mei et al., 2012). Therefore, further investigation is needed to assess CD19 expression in different B cell subsets of SLE patients. CD19 has been reported to be indirectly enhanced by BAFF-R activation. Such activation modulates the transcription factor Pax5 and consequently augments CD19 expression, resulting in an enhanced B cell Receptor signalling (Hase et al., 2004). Therefore, the impact of miR-155 on CD19 expression was analysed. Results showed that forcing the expression of miR-155 as well as knocking down PU.1 by siRNAs significantly reduced CD19 protein expression.

In conclusion, we attempted to further evaluate miR-155 in SLE, through investigating its net impact on autoimmune stimulatory proteins BAFF and CD19. Our results revealed that miR-155 acts as a suppressor of autoimmunity through transcriptional repression of PU.1 and TNF-α, which in turn suppresses BAFF and CD19 protein expression.

Disclosure statement

The authors declare no commercial or financial conflict of interest.

References


