Published in final edited form as:

Clin Immunol. 2008 October ; 129(1): 19–30. doi:10.1016/j.clim.2008.05.010.

A role for Fli-1 in B cell proliferation: implications for SLE pathogenesis

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Abstract

Transgenic overexpression of Fli-1 in normal mice leads to SLE-like disease and increased expression was reported in SLE-affected human and murine lymphocytes. Reducing Fli-1 expression in MRL/*lpr* mice decreased antibody production, proteinuria, renal pathology, and mortality. Compared to those with wild-type expression of Fli-1, we report here that proliferative responses of Fli-1-deficient naïve B cells to several mitogens were reduced in lupus-prone and control mice. Expression of mitogen receptors, including BCR, TLR4, and TLR9, was not significantly impacted in Fli-1-deficient naïve B cells. IL12a transcripts were upregulated and NFAT transcripts were downregulated in Fli-1-deficient MRL/*lpr* B cells. These results demonstrate that Fli-1 deficiency affects B cell proliferative responses to mitogens, independent of BCR and TLR expression. IL12a and NFAT, known to influence proliferation, were identified as potential mediators of this effect. This may be a mechanism by which overexpression of Fli-1 contributes to B cell hyperactivity and subsequent SLE pathogenesis.

Keywords

B cell; BCR; Fli-1; IL12; NFAT; proliferation; SLE; TNF beta; TLR

Introduction

Systemic lupus erythematosus (SLE) is a complex chronic autoimmune disease characterized by numerous immunological abnormalities [1–3]. The multifaceted etiology of this disease is incompletely understood, but is mainly attributed to genetic susceptibility, while epigenetics, stochastic events, infections, exposure to toxins, and medications are thought to also be influential factors [4–16]. The immunological abnormalities observed in SLE can be categorized into three types: production of pathogenic autoantibodies, impaired ability to process and eliminate immune complexes, and lack of regulation regarding T and B lymphocyte function. B cell hyperactivity, both *in vivo* and *in vitro*, is a classic characteristic of SLE, and is likely a consequence of abnormal signaling events [17–19].

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Friend leukemia insertion site 1 (Fli-1) is a DNA-binding protein capable of transcriptional activation or repression [20–23]. Based on the high homology of its DNA binding domain with that of Ets-1, and the central GGAA motif of the sequence to which it binds, Fli-1 is classified as a member of the ETS family of transcription factors [24]. C57BL/6 mice possessing a single targeted disruption of *Fli-1* (*Fli-1*^{+/-}) express 50% less Fli-1, but display a normal phenotype, while *Fli-1*^{-/-} embryos suffer neural tube hemorrhage and die by 12.5 days of gestation [25]. Evidence suggests dysregulation of genes encoding extracellular matrix proteins that contribute to vascular integrity, and those that encode genes related to megakaryocyte development and function is responsible for *in utero* death. Interestingly, humans bearing a chromosomal deletion, which includes *Fli-1*, have thrombocytopenia [26–29]. Mice with a disrupted *Fli-1* functional domain (*Fli-1*^{ΔCTA/ΔCTA}) are viable and produce a truncated protein, lacking the carboxy-terminus transactivation domain, which exhibits 50% less transactivation activity than native Fli-1 [21]. This model is useful because the similarity of many ETS binding sites may allow one ETS factor to substitute for another when it is absent or deficient. For example, Erg-3 has a DNA-binding sequence that is 98% homologous to that of Fli-1. In the case of *IgH* regulation, Erg-3 and Fli-1 bind the enhancer with seemingly equal affinity and activate it with equal efficiency [30]. In the *Fli-1*^{ΔCTA/ΔCTA} model, Fli-1 retains the ability to bind its target genes, minimizing the possibility of other ETS factors influencing expression of Fli-1 target genes.

Transgenic outbred CD1 mice that overexpress Fli-1 developed spontaneous glomerulonephritis, and died prematurely due to renal failure similar to standard models of spontaneous SLE [31]. B cells of mice possessing the Fli-1 transgene exhibited *in vivo* hyperplasia and hyperproliferation in response to *in vitro* stimuli. Baseline expression of Fli-1 was elevated in lymphocytes of murine models of SLE and human patients [32,33]. In patients, Fli-1 expression correlated with disease severity. Autoantibody production, proteinuria, and renal pathology were significantly reduced, and survival was markedly extended in *Fli-1*^{+/-}MRL/*lpr* mice [33]. These results confirm a key role for Fli-1 in murine SLE.

To determine potential mechanisms of the role of Fli-1 in disease, we evaluated the effect of Fli-1 deficiency on B cell proliferation, mitogen receptor expression, and the expression of their related signaling and effector proteins in three models – lupus-prone *Fli-1*^{+/-} MRL/*lpr* mice, and non-diseased *Fli-1*^{+/-} and *Fli-1*^{ΔCTA/ΔCTA} C57BL/6 mice.

Understanding the role of Fli-1 in B cell function may lead to novel methods of treatment, targeting Fli-1 itself or, more likely, its pertinent target genes.

Materials and methods

Manipulation of the murine Fli-1 gene

Derivation of *Fli-1*^{+/-} MRL/*lpr*, *Fli-1*^{+/-} C57BL/6, and *Fli-1*^{ΔCTA/ΔCTA} C57BL/6 mice was previously described [25,33]. Briefly, a floxed *neo* cassette was used to disrupt exon IX of *Fli-1*. Resulting C57BL/6 *Fli-1*^{+/-} mice were backcrossed to the MRL/*lpr* (The Jackson Laboratory, Bar Harbor, Maine) background, using speed congenic techniques to monitor inheritance of disease susceptibility loci for seven generations, to develop MRL/*lpr* *Fli-1*^{+/-} mice. LoxP-/Cre-mediated excision of the disrupted exon was used to generate C57BL/6 *Fli-1*^{ΔCTA/ΔCTA} mice, expressing the truncated protein. Table 1 outlines the strains, genotypes, and disease status of mice used in the studies described here. As no gender-specific effects were noted in any of the three Fli-1-deficient models, both male and female mice were used in all experiments.

DNeasy DNA purification kit (Qiagen) was used to extract DNA from tail snips according to the manufacturer's protocol [34]. Extracted DNA was subject to PCR as previously described [25].

B cell isolation

Under sterile conditions, lymphocyte suspensions were prepared by crushing fresh spleens between frosted glass slides in RPMI 1640 cell culture medium (Mediatech). Following a 5 min centrifugation at 1200 rpm, medium was removed, and red blood cells were lysed by resuspension in a 9:1 mixture of 0.16M ammonium chloride (Sigma) and 0.17M Tris, pH 7.6. Cells were washed twice, resuspended in RPMI 1640, and counted.

For proliferation and FACS assays, naïve B cells were isolated by negative selection with CD43 Microbeads (Miltenyi Biotec) according to the manufacturer's directions [35]. For PCR arrays and real-time RT-PCR assays, splenic B cells were isolated by negative selection using CD90 Microbeads (Miltenyi Biotec) according to manufacturer's directions and cultured with or without 10 g/ml LPS for ~2.5 days [36].

Purity of isolated cells was confirmed by staining with PerCP- or APC-labeled anti-B220 or anti-CD19 (BD Pharmingen) and PE-labeled anti-CD43 (Miltenyi Biotec or BD Pharmingen) and detection by a FACSCalibur™ instrument (BD Biosciences). Each sample was determined to consist of ≥90% naïve B cells.

Proliferation assays

Naïve B cell cultures were prepared, as published elsewhere, in duplicate at 2×10^5 cell/ml with 0.5, 1, 5, or 10 µg/ml LPS (Sigma), 1, 5, or 10 µg/ml anti-IgM (Jackson ImmunoResearch), 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) and 0.25, 0.5, or 1 µg/ml ionomycin (Sigma), or 1, 5, or 10 µg/ml CpG oligonucleotides (Cell Sciences) [37,38]. Cultures were incubated at 37°C in 5% CO₂ for 48 hours prior to addition of 5 µCi/ml tritiated thymidine and incubation at 37°C in 5% CO₂ for 14 hours [39]. Cells were harvested and proliferation determined by a Packard TopCount scintillation counter (PerkinElmer).

FACS analysis of receptor expression

2×10^7 naïve B cells/ml were resuspended in sterile FACS buffer (PBS with 1% sodium azide (Sigma) and 0.1% FBS). 1 µl anti-CD16/CD32 (BD Pharmingen) per 10^6 cells was added and incubated on ice for 20 minutes. 0.5 µg/ml PE-labeled anti-CD43 (Miltenyi Biotec, BD Pharmingen), APC-labeled anti-CD19 (BD Pharmingen), PerCP-labeled anti-B220 (BD Pharmingen), APC-labeled anti-IgM (eBioscience, BD Pharmingen), unlabeled or APC-labeled anti-CD79a (Anaspec, BD Pharmingen), biotin- or FITC-labeled anti-TLR4 (eBioscience, Imgenex), and/or unlabeled or FITC-labeled anti-TLR9 (eBioscience, Imgenex) were added to $0.5-1 \times 10^6$ cells and incubated on ice for 20 minutes, protected from light. Following two washes in FACS buffer, 0.5 µg/ml FITC-labeled anti-rabbit IgG (Abcam), and/or APC-labeled streptavidin (BD Biosciences) were added where appropriate and incubated on ice for 20 minutes, protected from light. Following two washes in FACS buffer, fluorescence was detected by a FACSCalibur™ flow cytometer.

For intracellular staining, cells were resuspended and blocked as described above, then stained with 0.5 µg/ml PE-labeled anti-CD43 and APC-labeled anti-CD19 or PerCP-labeled anti-B220 (BD Pharmingen). Following two washes in FACS buffer, cells were fixed, permeabilized, and stained with unlabeled or FITC-labeled anti-TLR9 according to manufacturer's protocol [40,41]. Staining with FITC-labeled anti-rabbit IgG followed, when appropriate.

Real-time RT-PCR

RNA was extracted from B cells with Trizol[®] reagent (Invitrogen) according to the manufacturer's protocol [42]. RNA was diluted in TE:8 buffer (10mM Tris-hydrochloride, 1mM EDTA, pH 8.0) and scanned by a SmartSpec[™] Plus (Bio-Rad) spectrophotometer to determine RNA concentration and purity according to the manufacturer's instructions [43].

mRNA was amplified according to Invitrogen's SuperScript Indirect RNA Amplification kit protocol, doubling length of incubations throughout [44]. Resulting aRNA yield was determined as described above. RNA or aRNA was converted to cDNA according to manufacturer's instructions for the RT² PCR Array First Strand Kit (SuperArray) [45]. cDNA was subjected to a Mouse RT² RNA QC PCR Array (SuperArray) on a MyiQ real-time PCR detection system (Bio-Rad) according to manufacturer's protocol, to determine reverse transcription efficiency, amplification efficiency, genomic DNA content, and DNA contamination [46]. cDNA was then applied to a Mouse Toll-Like Receptor Signaling Pathway RT² Profiler[™] PCR Array (SuperArray) according to manufacturer's protocol [45]. cDNA was also subject to single RT² qPCR primer assays in triplicate, using primers for murine *csf3*, *elf1*, *il12a*, *lta*, *nfat1c*, *nfkb1a*, *nfkb1*, or *tlr9* (SuperArray) according to manufacturer's protocol [47].

Identification of overrepresented transcription factor binding motifs

MDFrame software was used to identify overrepresented transcription factor binding motifs located in conserved promoter regions of differentially expressed genes [48,49].

Statistical analysis

Analysis of proliferation data by Mann-Whitney test was performed using Prism software (GraphPad). A *p* value of ≤ 0.05 was considered significant. PCR array data was scaled and quantile normalized prior to analysis by T test. A *p* value of 0.05 and a fold-change greater than 2 was considered significant.

Results

Fli-1 deficiency restricts B cell proliferation

To investigate the effects of reduced Fli-1 expression on B cell proliferation in the context of murine SLE, naïve B cells from *Fli-1*^{+/-} and *Fli-1*^{+/+} MRL/*lpr* mice were stimulated with LPS, anti-IgM, CpG DNA, or PMA and ionomycin (PMA/ion), and proliferation was assessed. Presumably, more activated B cells are present in wild-type mice, as disease is more severe and/or progresses faster [33]. Use of naïve B cells for these studies ensures that proliferative activity observed is a result of controlled *in vitro* stimulation, as opposed to *in vivo* activation by unknown stimuli, allowing fair comparisons. Mice were sacrificed at three ages (6, 12, and 20 weeks) representing different stages of disease (pre-, mild, and severe disease, respectively) [50,51]. At 6 weeks of age, naïve B cells from *Fli-1*^{+/-} MRL/*lpr* mice proliferated significantly less in response to LPS, and PMA/ion than those from *Fli-1*^{+/+} MRL/*lpr* mice (Figure 1A). Significantly reduced proliferation of *Fli-1*^{+/-} MRL/*lpr* B cells in response to anti-IgM and LPS at 12 weeks of age, and anti-IgM at 20 weeks of age was also observed (Figure 1B and 1C). Proliferation of naïve *Fli-1*^{+/-} MRL/*lpr* B cells in response to anti-IgM and CpG DNA at 6 weeks, CpG DNA at 12 weeks, and CpG DNA and LPS at 20 weeks of age was also reduced, though these differences did not reach statistical significance (Figure 1).

C57BL/6 mice were sacrificed at ages corresponding to those at which MRL/*lpr* mice were sacrificed, though age was not expected to affect the activity of C57BL/6 B cells. At 6 weeks of age, naïve B cells from *Fli-1*^{+/-} C57BL/6 mice proliferated significantly less in response to LPS and anti-IgM than naïve B cells from *Fli-1*^{+/+} C57BL/6 mice (Figure 2A). Heterozygous

and wild-type responses to CpG DNA, however, were similar. Compared to wild-type, heterozygous cells also proliferated significantly less in response to LPS, CpG DNA, and anti-IgM at 12 weeks of age (Figure 2B). Twenty-week-old *Fli-1* heterozygous B cells proliferated significantly less than wild-type cells in response to PMA/ion and anti-IgM, but not in response to LPS and CpG DNA (Figure 2C).

Naïve B cells from 6-week-old *Fli-1*^{ΔCTA/ΔCTA} C57BL/6 mice proliferated significantly less, compared to *Fli-1*-wild-type C57BL/6 B cells, in response to anti-IgM and CpG DNA, but not LPS (Figure 3A). At 12 weeks of age, naïve B cells from *Fli-1*^{ΔCTA/ΔCTA} C57BL/6 mice proliferated less than those from *Fli-1*^{+/+} C57BL/6 mice in response to LPS, while differences in response to PMA/ion and CpG DNA did not reach statistical significance (Figure 3B). Proliferation of 20-week-old *Fli-1*^{ΔCTA/ΔCTA} C57BL/6 B cells was significantly reduced in response to anti-IgM, CpG DNA, and LPS (Figure 3C).

BCR expression is independent of Fli-1 expression or activity

Given the decreased B cell proliferative response in *Fli-1*-deficient mice, we next examined whether *Fli-1* deficiency affected mitogen receptor expression. To examine the effect of *Fli-1* deficiency on BCR expression, IgM and Igα expression on naïve B cells from 6-, 12-, and 20-week-old *Fli-1*^{+/+} versus *Fli-1*^{+/−} MRL/*lpr* mice was examined. IgM expression was slightly reduced, and Igα was unchanged, on *Fli-1*^{+/−} cells from 20-week-old mice, while Igα was slightly reduced, and IgM was unchanged, compared to wild-type, on *Fli-1*^{+/−} B cells from 6-week-old mice (Figure 4 and data not shown). Neither IgM nor Igα expression appeared to be affected by *Fli-1* deficiency at 12 weeks of age (data not shown). IgM and Igα are expressed together as the BCR, but Igα is also known to associate with IgD on naïve B cells, and dissimilar IgD expression may account for the observed differences in Igα expression while IgM expression remains unaffected [52]. IgD expression was not altered on *Fli-1* deficient cells from MRL/*lpr* mice of any age (Figure 4 and data not shown).

At 20 weeks of age, IgM expression was slightly reduced on C57BL/6 *Fli-1*^{+/−} B cells compared to wild-type, but appeared to be similar at 6 and 12 weeks of age (Figure 4 and data not shown). IgD expression was also slightly reduced on B cells from 12-week-old C57BL/6 *Fli-1*^{+/−} mice compared to those from wild-type C57BL/6 mice, but was similar at 6 and 20 weeks of age (Figure 4 and data not shown). Igα expression was slightly reduced on *Fli-1*^{+/−} B cells from 6- and 20-week-old C57BL/6 mice, compared to wild-type, but was similar on B cells from 12-week-old mice (Figure 4 and data not shown).

Upon comparison of B cells from C57BL/6 *Fli-1*^{+/+} and *Fli-1*^{ΔCTA/ΔCTA} mice, minimal differential expression of IgM, Igα, and IgD was observed at 6 weeks of age (Figure 4). IgM, Igα, and IgD expression on *Fli-1*^{+/+} and *Fli-1*^{ΔCTA/ΔCTA} cells was similar at 12 weeks of age (data not shown). At 20 weeks, Igα and IgD expression was similar, while IgM expression was slightly increased on *Fli-1*^{ΔCTA/ΔCTA} cells compared to wild-type (data not shown). Similar to BCR expression, Ca²⁺ influx in response to anti-IgM in cells from 12-week-old *Fli-1*^{ΔCTA/ΔCTA} mice was not affected (data not shown).

TLR4 and TLR9 expression is independent of Fli-1 expression or activity

To examine the effect of *Fli-1* deficiency on expression of TLR4 and TLR9, expression of these receptors on naïve B cells from *Fli-1*^{+/+} versus *Fli-1*^{+/−} MRL/*lpr* mice at 6, 12, and 20 weeks of age was determined. Slightly reduced expression of TLR4 was noted on *Fli-1*^{+/−} B cells, compared to wild-type B cells, at the ages of 6 and 20 weeks, while it appeared to be slightly increased at 12 weeks (Figure 5 and data not shown). Much of the current literature asserts that TLR9 is expressed primarily intracellularly, and CpG DNA must be taken into the endosome to initiate signaling [53–55]. While experimental evidence of this has been

established in macrophages, dendritic cells, and B cell lines, TLR9 has also been detected on the surface of primary B cells [56,57]. Therefore, both surface and intracellular expression of TLR9 were assessed. Compared to *Fli-1*-wild-type B cells, surface expression of TLR9 on B cells from *Fli-1*^{+/-} MRL/*lpr* mice was slightly reduced at all three ages and intracellular expression of TLR9 was reduced in *Fli-1*-deficient cells from mice 12 and 20 weeks of age, and unchanged in *Fli-1* deficient cells from mice 6 weeks of age (Figure 5 and data not shown).

Comparison of TLR4 expression on *Fli-1*^{+/+} versus *Fli-1*^{+/-} C57BL/6 naïve B cells demonstrated a slight reduction on *Fli-1*-deficient B cells at 6 and 20 weeks of age, comparable to the observations made evaluating TLR4 expression on *Fli-1*^{+/+} versus *Fli-1*^{+/-} MRL/*lpr* B cells (Figure 5 and data not shown). TLR4 expression appeared unaffected on *Fli-1*-deficient cells at 12 weeks of age (data not shown). Surface expression of TLR9 was also slightly reduced on *Fli-1*-deficient B cells at the age of 6 weeks, but similar to wild-type at 12 and 20 weeks, while intracellular expression of TLR9 was slightly reduced at all ages (Figure 5 and data not shown).

Only TLR9 surface expression was slightly reduced on *Fli-1*^{ΔCTA/ΔCTA} C57BL/6, compared to wild-type B cells, at 6 and 20 weeks of age (Figure 5 and data not shown). Unlike *Fli-1*^{+/-} MRL/*lpr* and C57BL/6 B cells, *Fli-1*^{ΔCTA/ΔCTA} cells demonstrated no discernible reduction in TLR4 or intracellular TLR9 expression at any age (Figure 5 and data not shown).

***Il12a*, *Ita*, *nfatc1*, and *tlr9* transcript expression is affected by *Fli-1* deficiency**

Because *Fli-1* genotype had little effect on BCR or TLR4/9 expression, we assessed downstream signaling pathways from BCR and TLR for differences that may account for the observed proliferation differences in B cells with variant *Fli-1* expression. The expression of 84 genes related to TLR signaling was determined in LPS-stimulated or unstimulated splenic B cells isolated from 6-week-old *Fli-1*^{+/+} and *Fli-1*^{+/-} MRL/*lpr* mice. Several of these genes are also known to participate in BCR signaling. Genes identified as more than 2-fold differentially expressed and statistically significant by these arrays, and genes coding for transcription factors which bind the promoters of these differentially expressed genes (*csf3*, *il12a*, *Ita*, *tlr9*, *nfkb1*, *nfkb1a*, *elf1*, and *nfatc1*) were subject to individual RT PCR analysis, using total RNA from stimulated or unstimulated B cells of 6-week-old *Fli-1*^{+/+} versus *Fli-1*^{+/-} MRL/*lpr* mice. Fold change differences in expression from confirmation assays are summarized in Table 2. Expression of *il12a* and *Ita* in unstimulated cells and *tlr9* in stimulated cells demonstrated greater than twofold increases in confirmation assays. Furthermore, expression of *nfat1c* demonstrated a greater than two-fold decrease in stimulated B cells.

Discussion

Fli-1 is overexpressed in lymphocytes of SLE patients and murine models of SLE. Transgenic overexpression in normal mice leads to SLE-like disease [31,32]. Reduction of *Fli-1* expression in the MRL/*lpr* mouse model of SLE results in decreased immunoglobulin production, proteinuria, and renal pathology; and markedly increased survival [33]. These findings suggest that *Fli-1* activity may be a central contributor to SLE pathogenesis. Investigation of this role and discovery of *Fli-1* target genes may provide novel therapeutic targets.

Transgenic mice that overexpress *Fli-1* exhibit innate B cell hyperplasia and exaggerated *in vitro* proliferation of B cells in response to LPS and anti-IgM [31]. Also, spleens from MRL/*lpr* *Fli-1*^{+/-} mice demonstrated lower percentages of B cells and serum exhibited reduced immunoglobulin concentration [33]. It was therefore hypothesized that *Fli-1* deficiency may impair B cell proliferation. Additionally, it was predicted that *Fli-1* activity is integral to the process in normal immunity, outside the context of SLE. Because overexpression of *Fli-1*

preceded the manifestation of disease in Fli-1 transgenic mice, significant effects of Fli-1 deficiency early in the progression of MRL/*lpr* disease was anticipated [31].

Effects of Fli-1 deficiency on B cell proliferation were evaluated in three models – *Fli-1*^{+/-}MRL/*lpr*, *Fli-1*^{+/-} C57BL/6, and *Fli-1*^{ΔCTA/ΔCTA} C57BL/6 mice. Proliferative responses of naïve B cells from Fli-1-deficient mice to several mitogens were significantly reduced compared to those of *Fli-1*-wild-type littermates (Figures 1–3). Proliferation initiated by all these mitogens was affected, suggesting that Fli-1 regulates B cell genes which directly or indirectly influence proliferation.

It was noted that statistical significance of differences in proliferation were observed more frequently in younger MRL/*lpr* groups than the 20-week-old group (Figure 1). Differences in proliferation of MRL/*lpr* B cells of varying *Fli-1* genotype may be less prominent at 20 weeks because disease in the heterozygotes has progressed to a stage more equivalent to that of wild-type mice. Alternatively, and more likely, significant differences may not be observed because, as wild-type MRL/*lpr* mice age, the ability of their B cells to respond to proliferative stimuli decreases, potentially equalizing the proliferative responses of *Fli-1*^{+/-} and wild-type cells [58–60].

Proliferation of B cells from *Fli-1*^{+/-} and *Fli-1*^{ΔCTA/ΔCTA} C57BL/6 mice demonstrated impairment similar to that of B cells from *Fli-1*^{+/-} MRL/*lpr* mice, indicating Fli-1 participates in B cell proliferation under non-disease conditions (Figures 1 and 2). In the *Fli-1*-heterozygous model, limited availability of Fli-1 protein may allow binding sites to be occupied and activated by other ETS factors, such as Erg-3. As the mutant Fli-1 is assumed to bind the majority of available Fli-1 binding sites, compensation for Fli-1 deficiency may explain why fewer statistically significant differences in B cell proliferation were observed in *Fli-1*^{+/-} C57BL/6 mice than in *Fli-1*^{ΔCTA/ΔCTA} C57BL/6 mice compared to *Fli-1*^{+/+} C57BL/6 mice (Figures 2 and 3). The effect of Fli-1 deficiency on B cell proliferation appeared to be accentuated in MRL/*lpr* mice, perhaps suggesting that aspects of SLE contribute to this effect. Differing genetic background or the lack of functional Fas receptor on MRL/*lpr* B cells may also influence the extent of the effects of Fli-1 deficiency.

B cell proliferation was evaluated in each model of Fli-1-deficiency when they were 6, 12, and 20 weeks old – ages that correspond to progressive stages of MRL/*lpr* disease [50,51]. Proliferative responses of Fli-1-deficient MRL/*lpr* B cells were significantly reduced compared to wild-type B cell responses beginning at the pre-disease stage at 6 weeks of age (Figure 1). Given the development of SLE-like disease in Fli-1-overexpressing transgenic mice, the observed impact of Fli-1 deficiency prior to disease onset suggests that abnormal expression of Fli-1 is likely a contributing factor to causal events, rather than a consequence of disease.

As a transcription factor, Fli-1 is able to regulate expression of multiple genes. Altered expression of mitogen receptors corresponding to the mitogens used to evaluate proliferation was also investigated as a mechanism behind the decreased B cell proliferation associated with Fli-1 deficiency.

A higher proportion of B cells expressing the CpG DNA-binding TLR9 is found in SLE patients with active, versus remitted, disease [61]. Expression of TLR9 also correlates with levels of anti-dsDNA. It was therefore hypothesized that Fli-1 deficiency results in decreased or incomplete expression of these receptors. Surface expression of BCR components IgM and Igα, TLR4, and TLR9, and intracellular expression of TLR9 was only slightly and inconsistently reduced in naïve B cells of Fli-1-deficient mice compared to *Fli-1*-wild-type mice of similar strain (Figure 5 and data not shown). Slight reduction of Igα, without concomitant reduction of IgM or IgD, or vice versa, was also occasionally observed (Figure 4 and data not shown). Were the reduced expression of Igα due to faulty association with IgD

or IgM exclusively, normal expression of either IgM or IgD and reduced expression of the other would be expected. As this was not observed, it is unlikely that Fli-1 deficiency affects Ig α expression in this manner.

Because Ig α is known to be regulated by Fli-1, a more impressive reduction of Ig α expression was expected [62]. As BCR expression is essential to B cell development and survival, expression of Ig α may not be sensitive to the level to which Fli-1 activity is decreased in our models. This may be true not only for Ig α , but also for the other receptors, either because there is enough residual Fli-1 activity for near normal expression, or because Fli-1 plays a minor or nonessential role in regulating expression. Alternatively, our method of analysis may be insufficient to detect subtle, but significant, differences in expression, though we believe this is unlikely.

Additionally, differential expression of a particular receptor did not always coincide with differential B cell proliferation response to the corresponding mitogen. It is thus unlikely that reduced expression of mitogen receptors is the mechanism driving the large differences in B cell proliferation observed. If these results accurately reflect *in vivo* BCR expression, the idea that this minimally reduced expression may contribute to the observed proliferation assay results cannot be completely discounted, though to what extent can not be speculated.

Significantly reduced proliferative responses to all mitogens tested, including PMA/ion, which bypass interaction with receptors, suggests that multiple pathways are affected by Fli-1 deficiency. Therefore the mechanism is likely multifactorial and may or may not be similar for all affected pathways. Minimal decreases in mitogen receptor expression on Fli-1-deficient cells suggest that more profound effects of Fli-1 deficiency may be downstream of receptor expression. Transcript expression of several TLR and BCR signaling-related genes was evaluated in MRL/*lpr* *Fli-1*^{+/+} versus *Fli-1*^{+/-} B cells. IL12 α and TNF β transcripts were upregulated in unstimulated Fli-1-deficient cells (Table 3). TLR9 transcripts were upregulated and NFAT transcripts were downregulated in stimulated Fli-1-deficient B cells (Table 3).

As Fli-1 is generally recognized as a transcription activator, it was unexpected to find upregulation of genes in Fli-1 deficient cells. There are, however, a number of published examples of Fli-1 acting as a repressor of genes including those that code for collagen type 1 and the retinoic acid receptor [63,64]. Fli-1 itself may not interact with upregulated genes identified here, but may activate expression of a repressor that inhibits their transcription.

IL12 α upregulation may have a protective effect as transcript levels are below normal in SLE PMBC [65]. Also, treatment of SLE PMBC and MRL/*lpr* mice with IL12 reduced anti-dsDNA production [66,67]. In treated mice, lymphadenopathy, splenomegaly, proteinuria, and glomerulonephritis were also alleviated [68]. These effects of IL12 on SLE parameters may contribute to the overall phenotype of *Fli-1*^{+/-} MRL/*lpr* mice. Upregulation of IL12 may also contribute to the effect of Fli-1 deficiency on B cell proliferation, as IL12 treatment reduces proliferation and tumorigenicity of malignant B cells in which IL12 receptor expression is induced [69]. We were unable to detect serum IL12 in MRL/*lpr* mice of either *Fli-1* genotype.

TNF β upregulation may offset the overproduction of immunoglobulin in MRL/*lpr* disease, as reduction of TNF β results in increased immunoglobulin production [70]. Decreased immunoglobulin levels were present in *Fli-1*^{+/-} MRL/*lpr* sera, but TNF β levels remain unknown. Low production of TNF β was associated with SLE and lupus nephritis in a Korean population [71]. The relationship between elevated TNF β transcripts and reduced B cell proliferation in Fli-1-deficient B cells requires further investigation, as literature regarding this cytokine and its effect on proliferation are conflicting.

Though the mechanism is unknown, absence of TLR9 in lupus-prone mice exacerbates disease, suggesting that upregulation of TLR9 is protective [72]. While transcripts were upregulated in Fli-1-deficient MRL/*lpr* B cells, assessment of receptor expression did not reflect this upregulation. Perhaps an alternative method of measuring TLR9 protein expression is needed to accurately address the question of the relationship of Fli-1 with TLR9, and whether this relationship influences B cell proliferation.

As NFAT expression is known to be induced in stimulated B cells and has an established role in lymphocyte proliferation, the finding of decreased NFAT transcripts in Fli-1-deficient B cells is of interest (Table 3) [73–75]. As a known regulator of c-myc and BAFF, decreased NFAT expression could directly result in decreased proliferation [76,77]. In addition, NFAT is known to activate expression of CD23, the IgE Fc receptor [78]. The soluble form of this receptor is elevated in SLE and B-chronic lymphocytic leukemia (B-CLL) patient serum and enhances immunoglobulin production [79–81]. Interaction of soluble CD23 with its receptors stimulates a growth response [82]. Selective increase of surface CD23 prompts resting B-CLL cells to proceed through G1 and S phase of the cell cycle [83]. Blockade of CD23 ligation by anti-CD23 monoclonal antibodies inhibits proliferation of Epstein-Barr virus (EBV)-transformed B cells [84]. Interestingly, B220+ cells from *Fli-1*^{ΔCTA/ΔCTA} C57BL/6 mice demonstrated reduced surface CD23 expression (unpublished data). Confirmation that NFAT expression is reduced in these mice may clarify the mechanism by which Fli-1 deficiency diminishes B cell proliferation.

The results of the studies presented here clearly demonstrate that Fli-1 deficiency affects B cell proliferative responses to mitogens, independent of BCR and TLR expression. It should be considered that the genes evaluated here are not an exhaustive representation of proteins involved in these pathways. However, the upregulation of IL12a and downregulation of NFAT transcripts are prospective points from which to continue exploration of the relationship between Fli-1, B cell proliferation, and SLE.

Acknowledgements

We would like to acknowledge individuals without whom this work would not be possible. Dr. Dennis Watson and members of his laboratory developed the *Fli-1*^{+/-} and *Fli-1*^{ΔCTA/ΔCTA} C57BL/6 mice. We are grateful to Jackie EuDaly, Michelle Lee, Meagan Mollenhauer, and Dr. Omar Moussa for technical assistance. We also thank Dr. Margie Peden-Adams, Dr. Makio Ogawa, and Dr. Haiqun Zeng for use of instruments in their laboratories. We also acknowledge personnel of the Ralph H. Johnson Veterans Affairs Medical Center animal facility for care of study mice.

Abbreviations

CPM	counts per minute
Fli-1	Friend leukemia insertion site 1
NFAT	nuclear factor of activated T cells
PMA	phorbol 12-myristate 13-acetate
SEM	standard error of the mean
SLE	

systemic lupus erythematosus

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Figure 1.

Proliferative response of MRL/lpr *Fli-I*^{+/+} (n = 2 or 3) versus *Fli-I*^{+/-} (n = 2 or 3) B cells to mitogens at (A) 6, (B) 12, or (C) 20 weeks of age. Data from 2 or 3 independent experiments is shown as mean CPM (counts per minute) ± SEM (standard error of the mean). * = $p \leq 0.05$.

Figure 2. Proliferative response of C57BL/6 *Fli-1*^{+/+} (n = 2 or 3) versus *Fli-1*^{+/-} (n = 2 or 3) B cells to mitogens at (A) 6, (B) 12, or (C) 20 weeks of age. Data from 2 or 3 independent experiments is shown as mean CPM ± SEM. * = $p \leq 0.05$.

Figure 3. Proliferative response of C57BL/6 *Fli-I*^{+/+} (n = 2 or 3) versus *Fli-I*^{ΔCTA/ΔCTA} (n = 3) B cells to mitogens at (A) 6, (B) 12, or (C) 20 weeks of age. Data from 2 or 3 independent experiments is shown as mean CPM $\pm$ SEM. * = $p \leq 0.05$.

Figure 4.

Expression of IgM, Igα, and IgD on naïve B cells from 6-week-old MRL/*lpr* *Fli-1*^{+/+} (area filled, n = 12, 7, and 8 respectively) versus *Fli-1*^{+/-} (area open, n = 11, 6, and 7 respectively), C57BL/6 *Fli-1*^{+/+} (area filled, n = 15, 12, and 13 respectively) versus *Fli-1*^{+/-} (area open, n = 14, 11, and 14 respectively), and C57BL/6 *Fli-1*^{+/+} (area filled, 11, 11, and 3 respectively) versus *Fli-1*^{ΔCTA/ΔCTA} (area open, n = 11, 11, and 3 respectively) mice. Data is representative of 2-5 independent experiments. Isotype controls are represented by dotted lines.

Figure 5.

Expression of TLR4 and TLR9 (s - surface, ic - intracellular) on naïve B cells from 6-week-old MRL/lpr *Fli-1*^{+/+} (area filled, n = 7, 4, and 1 respectively) versus *Fli-1*^{+/-} (area open, n = 6, 4, and 1 respectively), C57BL/6 *Fli-1*^{+/+} (area filled, n = 12, 8, and 5 respectively) versus *Fli-1*^{+/-} (area open, n = 11, 9, and 6 respectively), C57BL/6 *Fli-1*^{+/+} (area filled, n = 11, 10, and 10 respectively) versus *Fli-1*^{ΔCTA/ΔCTA} (area open, n = 11, 10, and 10 respectively) mice. Data is representative of 2-5 independent experiments. Isotype controls are represented by dotted lines.

Table 1

Mouse strains, genotypes, and disease status.

strain	MRL/lpr	C57BL/6	
<i>Fli-I</i> genotype	+/+	+/+	ΔCTA/ΔCTA
SLE	YES		NO

Table 2

Expression of selected genes in *Fli-I*^{+/+} (n = 5) versus *Fli-I*^{+/-} (n = 5) MRL/*lpr* B cells. One triplicate experiment per gene.

gene	Fold change in MRL/ <i>lpr</i> <i>Fli-I</i> ^{+/-} B cells	
	Unstimulated	LPS stimulated
<i>csf3</i>	1.5	-0.97
<i>elf1</i>	1.96	1.13
<i>il12a</i>	2.2	1.2
<i>lta</i>	2.58	1.56
<i>mphosph1</i>	2.6	2.1
<i>nfatc1</i>	-1.01	-2.03
<i>nfkb1</i>	1.78	-0.9
<i>nfkbia</i>	-0.5	1.0
<i>tlr9</i>	1.58	2.62