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B cell- and BAFF-dependence of IFN α -exaggerated disease in SLE-prone NZM 2328 mice¹

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Abstract

IFN α is a potent activator of innate and adaptive immunity, and its administration to pre-autoimmune (NZBxNZW)F1 mice promotes virulent systemic lupus erythematosus (SLE) disease. Given the known contributions of B cells and BAFF to SLE, we evaluated the ability of IFN α administration to induce disease in wild-type (WT), B cell-deficient, and BAFF-deficient NZM 2328 mice. Whereas WT mice rapidly developed proliferative glomerulonephritis (GN), marked proteinuria, and increased mortality in response to IFN α administration, B cell-deficient mice developed neither renal pathology nor clinical disease. Moreover, BAFF-deficient mice, despite developing limited glomerular IgG and C3 deposition, also remained free of histological GN and clinical disease. Strikingly, similar T cell expansion and serum IgG responses were observed in Adv-IFN-treated WT and BAFF-deficient mice despite their disparate pathological and clinical responses, whereas numbers of activated B cells increased in WT mice but not in BAFF-deficient mice. Nonetheless, B cell, plasma cell, and T cell infiltration of the kidneys in Adv-IFN-treated WT mice was similar to that in WT mice treated with Adv-control. Its ability to promote SLE disease in WT mice notwithstanding, IFN α administration failed to drive the preferential expansion of CD4⁺ memory T cells that occurs during the natural course of disease, and glomerular infiltration of macrophages failed to associate with development of disease. These results collectively suggest that therapeutic targeting in SLE of BAFF and/or B cells in SLE could be successful even in states of IFN α overexpression. Moreover, our results document important biological differences between IFN α -driven and spontaneous “natural” SLE disease.

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Keywords

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Introduction

The clinical diagnosis of systemic lupus erythematosus (SLE³) is based on a constellation of signs, symptoms, and clinical laboratory-based abnormalities. Perturbation of distinct single genes can lead to SLE-like manifestations in mice (1-6), and human linkage and association studies have identified multiple SLE susceptibility genes (7-14). Accordingly, SLE represents a collection of disorders which likely stem from a plethora of discrete etiologies and pathogenetic pathways.

This inherent heterogeneity notwithstanding, certain common threads tend to run across all “varieties” of SLE. One of these is the IFN α signature, defined as the preferential increase in expression of those genes up-regulated by IFN α (15-17).

Several observations support a contributory role for IFN α in SLE. Systemic administration of IFN α has promoted *de novo* SLE-like disease in several patients (18). Moreover, circulating IFN α levels in SLE are frequently elevated (17), corticosteroid-induced remissions are paralleled by the extinguishing of the IFN α signature (16), and the risk allele of the *STAT4* gene, which codes for a transcription factor activated by IFN α signaling, is associated with increased sensitivity to IFN α signaling (19). In several murine SLE models, blockade of IFN α signaling by genetic deletion of the type I IFN receptor (IFNR) attenuates disease (20-22). Most strikingly, administration of IFN α promotes the rapid onset of virulent clinical disease in young (pre-autoimmune) (NZBxNZW)F1 (BWF1) female mice but has no pathogenic effects on age- and sex-matched non-SLE-prone BALB/c mice (23).

B cells indispensably contribute to spontaneous SLE not just by autoantibody-dependent means but by autoantibody-independent means as well, as highlighted by the complete absence of SLE features in B cell-deficient MRL-*lpr* mice (24) and the partial restoration of

³Abbreviations

Adv	adenovirus
BAFF	B cell activating factor belonging to the TNF family
BWF1 mice	(NZBxNZW)F1 mice
DC	dendritic cells
GN	glomerulonephritis
IFNR	IFN receptor
M/N ratio	memory cell to naive cell ratio
mDC	myeloid DC
SLE	systemic lupus erythematosus
Tg	transgenic
TNFR	TNF receptor
vp	viral particles
WT	wild-type

disease following genetic reconstitution of these mice with B cells incapable of secreting Ig (25). Indeed, B cell-depletion therapy in human SLE leads to clinical improvement in many patients despite limited effects on circulating autoantibodies (26-31).

B cell activating factor belonging to the TNF family (BAFF) also vitally contributes to SLE. BAFF-transgenic (Tg) mice that are otherwise not autoimmune-prone develop SLE-like features, including elevated circulating titers of multiple autoantibodies and immune-complex glomerulonephritis (GN) (32-34). Furthermore, constitutive BAFF overexpression leads to accelerated glomerular pathology in mice with an incomplete diathesis to SLE (35), and SLE-like features develop in otherwise non-autoimmune-prone BAFF-Tg mice even in the complete absence of T cells (as long as MyD88-mediated signaling is intact) (36). Conversely, treatment of BWF1 or MRL-*lpr* mice with a BAFF antagonist retards disease progression and improves survival (34,37-39), and disruption of the *Baff* gene in SLE-prone NZM 2328 (NZM) mice (an inbred recombinant strain derived from BWF1 mice which closely mirrors the parental SLE phenotype and shares many features with human SLE [40]) markedly attenuates development of clinical disease (41). In humans, treatment with an anti-BAFF mAb ameliorates disease activity in “seropositive” SLE patients (42-46).

Importantly, BAFF expression is affected by IFN α . In human dendritic cell (DC) cultures, IFN α up-regulates BAFF expression (47), whereas treatment of SLE patients with an anti-IFN α mAb down-regulates BAFF expression (48). In both SLE-prone BWF1 mice and non-autoimmune-prone BALB/c mice, circulating BAFF levels increase following IFN α administration (23).

Given that clinical trials in human SLE have been, and are continuing to be, conducted with agents that deplete B cells or neutralize BAFF, the dependence of IFN α -driven pathogenicity on B cells or BAFF in SLE becomes a clinically important topic. The ability of IFN α to drive exaggerated disease in a SLE-prone host has previously not been tested in the context of B cell or BAFF deficiency. Moreover, the faithfulness of IFN α -driven disease to the spontaneous disease has previously not been sufficiently assessed. To address these issues, we induced IFN α overexpression through an adenovirus (Adv) construct containing murine IFN α (Adv-IFN) and studied SLE-prone NZM mice as well as NZM mice deficient in B cells (NZM.JHD), in BAFF (NZM.*baff*^{-/-}), in the type I IFN α chain (NZM.*ifnar*^{-/-}), or in both TNF receptors (TNFR) 1 and 2 (NZM.*tnfr1*^{-/-}.*tnfr2*^{-/-}).

Materials and methods

General

All reported studies were approved by the University of Southern California IACUC.

Mice

Female mice were housed in specific pathogen-free quarters. Five congenic NZM strains were utilized: wild-type (WT); NZM.JHD; NZM.*baff*^{-/-}; NZM.*ifnar*^{-/-}; and NZM.*tnfr1*^{-/-}.*tnfr2*^{-/-}. The generation of NZM.*baff*^{-/-}, NZM.*ifnar*^{-/-}, and NZM.*tnfr1*^{-/-}.*tnfr2*^{-/-} mice have previously been described (22,41,49).

To generate NZM.JHD mice, B cell-deficient JHD mice were backcrossed into NZM mice using a marker-assisted selection protocol (41,50,51). These markers were chosen to include those regions identified as susceptibility loci in NZM mice (52). To detect the disrupted *JH* gene fragment (containing a *neo* insert), genomic DNA extracted from mouse tail clippings was PCR-amplified for 35 cycles at 94°C for 40 sec, 62°C for 40 sec, and 72°C for 1 min. The primer sequences were:

JH sense: 5'-GGACCAGGGGGCTCAGGTAC-3'

JH anti-sense: 5'-GAGGAGACGGTGACCGTGGTCCCT-3'

neo sense: 5'-CCTTGCGCAGCTGTGCTCGAC-3'

neo anti-sense: 5'-GCCGCATTGCATCAGCCATGATGGA-3'.

Band sizes for the intact and disrupted *JH* gene fragments are ~200 bp and ~120 bp, respectively. A *neo* band was detected only in mice with a disrupted gene fragment.

IFN α treatment and clinical assessment

The recombinant Adv vector containing murine IFN α subtype 5 under the control of the CMV promoter/enhancer (Adv-IFN) and the control Adv vector lacking the IFN α insert (Adv-control) have been previously described (23). Mice received single i.v. injections containing $1-1.5 \times 10^9$ viral particles (vp) of either Adv-IFN or Adv-control and were assessed for proteinuria twice weekly on a scale of 0 to 4+ with AlbuStrip assay strips (Bayer, Elkhart, IN). Severe proteinuria was defined as $\geq 3+$ on two consecutive examinations. Blood was collected prior to Adv injection and at days 8 and 28 post-injection.

Serum IFN α determination

Serum IFN α levels were determined by ELISA (PBL Biomedical Laboratories, Piscataway, NJ) according to the manufacturer's instructions. The lower limit of detection is 12.5 pg/ml. Samples with values <12.5 pg/ml were arbitrarily plotted as 12.5 pg/ml.

Cell surface staining

Murine spleen mononuclear cells were stained with fluorochrome-conjugated mAb specific for murine CD3, CD4, CD8, CD44, CD62L, CD45R (B220), CD19, CD21, CD23, CD69, CD80, or CD86 (BD PharMingen, San Diego, CA, or eBioscience, San Diego, CA) and analyzed by flow cytometry (49).

Serum total IgG and IgG autoantibody determinations

Serum levels of total IgG and IgG anti-chromatin and anti-dsDNA antibodies were determined by ELISA as previously described (53,54). Autoantibody OD values were normalized to the mean OD of serum from 5-month-old MRL-*lpr/lpr* mice, the latter being arbitrarily assigned a value of 100 U/ml.

Kidney histology

Individual sections of formalin-fixed kidneys were stained with H&E and examined by light microscopy. Each section was assessed for glomerular hypercellularity, mesangial matrix expansion, interstitial cellular infiltration, and tubular atrophy or dilation.

Kidney immunofluorescence

Five-micron sections of snap-frozen kidneys were stained for total IgG deposition using FITC-conjugated goat anti-mouse IgG antibodies (SouthernBiotech, Birmingham, AL), for individual IgG isotype deposition using FITC-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, or IgG3 antibodies (SouthernBiotech), and for C3 deposition using FITC-conjugated goat anti-mouse C3 antibodies (MP Biomedicals, Solon, OH). To document infiltration of T cells, B cells, or plasma cells into the kidneys, FITC-conjugated rat anti-CD3 (BD Bioscience, San Diego, CA), PE-conjugated anti-CD20 (eBioscience, San Diego, CA), and PE-conjugated anti-CD138 (BD Bioscience) antibodies were used.

Kidney immunohistochemistry

Four-micron sections of de-parafinized formalin-fixed kidneys were subjected to antigen retrieval with an antigen unmasking solution (Vector, Burlingame, CA). The sections were then blocked with hydrogen peroxidase and normal rabbit serum and stained for macrophages using rat anti-mouse Mac-2 mAb (Biolegend, San Diego, CA). Biotin-conjugated rabbit anti-rat IgG (Vector) was used as the secondary antibody, followed by avidin-streptoavidin-peroxidase complex (Vector). Diaminobenzidine (Vector) was used as the substrate for color development.

Statistical analysis

All analyses were performed using SigmaStat software (SPSS, Chicago, IL). Parametric testing between two matched or unmatched groups was performed by the paired or unpaired t test, respectively. Parametric testing among three or more groups was performed by one-way ANOVA. When the data were not normally distributed or the equal variance test was not satisfied, non-parametric testing was performed by the Mann-Whitney rank sum test between two groups and by Kruskal-Wallis one-way ANOVA on ranks among three or more groups. Correlations were determined by Pearson's product-moment correlation for normally distributed interval data.

Results

NZM.JHD mice develop neither clinical nor pathological SLE

B cell-deficient MRL-*lpr* mice are completely protected from development of clinical or pathological SLE (24). Nevertheless, the defect in MRL-*lpr* mice in CD95 (Fas)-mediated signaling and the attendant massive lymphadenopathy and expansion of B220⁺CD3⁺CD4⁻CD8⁻ T cells (features typical of neither other murine SLE models nor human SLE) *a priori* raises the possibility that the consequences of B cell deficiency in other SLE mice may differ from those in MRL-*lpr* mice.

To that end, we turned to NZM mice, which phenotypically resemble human SLE more closely than do MRL-*lpr* mice (40), and generated B cell-deficient NZM.JHD mice. In sharp contrast to B cell-sufficient NZM mice in which severe proteinuria begins to develop by 5-6 months of age and which experience >90% mortality by 11-12 months of age (41,49,55), NZM.JHD mice (n > 40) neither developed severe proteinuria nor died prematurely through 12 months of age. Moreover, no glomerular pathology developed in NZM.JHD mice, in sharp contrast to the diffuse proliferative GN that develops in WT NZM mice (41,49).

Inability of IFN α to promote pathological or clinical disease in NZM.JHD mice

Administration of a single dose of replication-incompetent Adv-IFN (1×10^{10} vp), but not Adv-control, to pre-autoimmune (9-11 weeks of age) BWF1 mice promoted rapid and virulent GN, manifested as severe proteinuria as early as 3 weeks post-injection and 100% mortality by 2 months post-injection (23). Of note, a lower single dose of the same Adv-IFN ($1-2 \times 10^9$ vp) led to the identical virulent clinical and pathological outcomes in pre-autoimmune NZM mice (22).

Development of disease consequent to overexpression of IFN α in these mice could have been either B cell-dependent or B cell-independent. That is, IFN α might drive a pathogenic pathway that directly or indirectly requires B cells, in which case overexpression of IFN α in a B cell-deficient host would have no pathogenic ramifications. Alternatively, IFN α might up-regulate a pathogenic pathway that does not involve B cells, in which case overexpression of IFN α in B cell-deficient hosts that are otherwise SLE-prone should elicit

clinical disease and end-organ (kidney) pathology. To test these possibilities, we compared the effects of Adv-IFN in B cell-sufficient NZM and B cell-deficient NZM.JHD mice.

In accord with our previous experience (22), pre-autoimmune NZM mice injected with Adv-IFN rapidly developed clinical disease. Severe proteinuria developed in 50% of the mice by 6 weeks post-injection, and all the mice developed severe proteinuria by 10 weeks post-injection (Figure 1A). Mortality ensued shortly after development of severe proteinuria, with 50% of the mice dead by 7 weeks post-injection and 100% of the mice dead by 12 weeks post-injection (~5 months of age; Figure 1B). To put this into perspective, 50% mortality among unmanipulated NZM mice is not reached until ~8 months of age, and 95% mortality is not realized until 11-12 months of age (41). In contrast to the potent pathogenic effects following Adv-IFN administration, Adv-control-treated pre-autoimmune NZM mice failed to develop any clinical disease (Figure 1A-B). Importantly, clinical sequelae also did not develop in Adv-IFN-treated (or Adv-control-treated) NZM.JHD mice.

Kidney histology paralleled the clinical findings. Adv-control-treated NZM mice showed some mesangial hypercellularity and glomerular focal and segmental hyaline thrombi (Figure 2A), consistent with the early pathologic changes in the kidneys of these mice as the onset of clinical disease approaches. In contrast, Adv-IFN-treated mice developed florid diffuse proliferative GN, including crescents, wire loops, and interstitial inflammatory cells (Figure 2B-C). In agreement with the absence of clinical disease, no glomerular or interstitial pathology was detected in NZM.JHD mice treated with Adv-IFN (or Adv-control; Figure 2D-E), and, consistent with the absence of B cells, no renal IgG deposition in these mice was appreciated (data not shown).

Inability of IFN α to promote meaningful immunopathological or clinical disease in NZM.*baff*^{-/-} mice

The most likely explanation for the inability of IFN α to drive disease in NZM.JHD mice is that the absence of B cells extinguished any underlying pathogenic autoimmune process. To address this possibility, we turned to NZM.*baff*^{-/-} mice which harbor B cells that, despite their low absolute numbers, do differentiate into autoantibody-producing cells and lead to serological autoimmunity and renal immunopathology over time (41).

This extant autoimmune process notwithstanding, NZM.*baff*^{-/-} mice failed to develop overt clinical disease even by 16 weeks following injection with Adv-IFN (Figure 1A-B). This does not appear to be due differential *in vivo* production of IFN α by the injected Adv-IFN, since serum IFN α levels in Adv-IFN-treated NZM.*baff*^{-/-} mice were identical to those in Adv-IFN-treated NZM mice (Figure 1C). As expected, serum IFN α levels were below the limit of detection in all tested Adv-control-injected mice.

In agreement with the absence of clinical disease, neither Adv-control-treated nor Adv-IFN-treated NZM.*baff*^{-/-} mice developed renal pathology (Figure 2F-G). Whereas Adv-IFN-treated NZM mice uniformly developed greater glomerular IgG than did control NZM mice, only very limited glomerular IgG deposition was detected in either Adv-control- or Adv-IFN-treated NZM.*baff*^{-/-} mice (Figure 3A-D). This dichotomy between NZM mice and NZM.*baff*^{-/-} mice uniformly held true for deposition of each of the individual IgG subclasses and C3 (Figure 3G-J, M-P, S-V, Y-BB, EE-HH). Of note, infiltration of (CD3⁺) T cells and (CD20⁺) B cells into the renal parenchyma was uniformly substantial in both Adv-control-treated and Adv-IFN-treated NZM mice and was qualitatively similar in each (Figure 3KK-NN) despite the glaring difference in development of GN (Figure 2A-C, F-G). In contrast, infiltration of (CD138⁺) plasma cells in Adv-IFN-treated or Adv-control-treated NZM mice was negligible at most (Figure 3WW-ZZ). In addition, essentially no T cell, B

cell, or plasma cell infiltrates were detected in the kidneys of any of the NZM.*baff*^{-/-} mice (Figure 3MM-NN, SS-TT, YY-ZZ).

It remained possible that resistance of NZM.*baff*^{-/-} mice to Adv-IFN was due to the absence of overt kidney pathology in these young (2-3 months of age) mice at the time of Adv-IFN injection. That is, IFN α may have required not only an underlying autoimmune process to exaggerate disease but some established end-organ (kidney) pathology as well.

We, therefore, turned to 9-month-old NZM.*baff*^{-/-} mice in which both serological autoimmunity and kidney immunopathology had already developed (41). Kidney pathology, highlighted by prominent hyaline thrombi, was appreciated in both Adv-control- and Adv-IFN-treated mice, with glomerular hypercellularity being somewhat more prominent in the latter (Figure 2H-I). Glomerular deposition of total IgG, the individual IgG subclasses, and C3 was very modestly greater in the Adv-IFN-treated mice than in the corresponding Adv-control-treated mice (Figure 3E-F, K-L, Q-R, W-X, CC-DD, II-JJ), but renal infiltration of T cells, B cells, or plasma cells was very limited at most (Figure 3 OO-PP, UU-VV, AAA-BBB). In agreement with the paucity of renal immunopathology, Adv-IFN treatment failed to promote any clinical disease even after 17 weeks in any of the 9 older NZM.*baff*^{-/-} mice tested.

T cell, B cell, and IgG responses to Adv-IFN in NZM and NZM.*baff*^{-/-} mice

Importantly, the absence of clinical findings in Adv-IFN-treated NZM.*baff*^{-/-} mice cannot be attributed to an inability to respond to IFN α . Young NZM mice or NZM.*baff*^{-/-} mice were injected with Adv-IFN, and their spleens were harvested 3-4 weeks later (when some Adv-IFN-injected NZM mice started becoming clinically sick). Control mice were either injected with Adv-control or not injected with any Adv. Since no differences were appreciated between Adv-control-treated mice and untreated mice in each mouse cohort, the results were pooled. Marked splenomegaly was visually obvious in both NZM and NZM.*baff*^{-/-} mice that had been injected with Adv-IFN in comparison to corresponding control mice. In fact, expansions of spleen total mononuclear cells, CD3⁺ cells, total CD4⁺ cells, CD4⁺ naive (CD44^{lo}CD62L^{hi}) cells, CD4⁺ memory (CD44^{hi}CD62L^{lo}) cells, and CD8⁺ cells in Adv-IFN-injected NZM.*baff*^{-/-} mice were no less than the corresponding expansions in Adv-IFN-injected NZM mice (Figure 4A-F), with the expansion of CD8⁺ cells in Adv-IFN-injected NZM.*baff*^{-/-} mice being greater than that in the corresponding NZM mice ($p = 0.008$). Expansion of spleen total mononuclear cells and individual T cell subsets was not as robust in Adv-IFN-injected old NZM.*baff*^{-/-} mice as in young NZM.*baff*^{-/-} mice ($p \leq 0.004$ for each comparison), although expansion of CD4⁺ memory cells was substantially greater in the former than in the latter ($p = 0.011$).

To assess whether the variability in expansion of these T cell subsets was related to differential IFN α expression, we identified 4 Adv-IFN-treated NZM mice and 4 Adv-IFN-treated NZM.*baff*^{-/-} mice that had been bled on day 8 post-injection and subsequently analyzed for both lymphocyte phenotype and serum IFN α levels. Among the former mice, numbers of CD3⁺, CD4⁺, CD8⁺, and CD4⁺ naive cells (but not CD4⁺ memory cells) correlated with serum IFN α levels ($p \leq 0.028$). In contrast, no such correlations were observed among the latter mice.

In contrast to the considerable IFN α -driven T cell expansion, serum levels of total IgG and IgG autoantibodies even at day 28 post-injection were similar in Adv-IFN-treated 3-month-old NZM mice or 3-month-old NZM.*baff*^{-/-} mice as in the corresponding Adv-control-treated mice. Only in 9-month-old NZM.*baff*^{-/-} mice was there a trend for greater serum levels of IgG anti-dsDNA antibodies (Figure 5A-C), and this trend involved each of the measured IgG subclasses (IgG1, IgG2a, IgG2b; Figure 5D-F).

Although the total IgG and IgG autoantibody responses in NZM mice were not affected by treatment with Adv-IFN, the number of spleen B cells did modestly increase ($p = 0.021$, Figure 5G). In contrast, no such increase was appreciated in either young or old NZM.*baff*^{-/-} mice. Moreover, the numbers of activated (CD69⁺) B cells significantly expanded in Adv-IFN-treated NZM mice ($p = 0.005$) but not in Adv-IFN-treated NZM.*baff*^{-/-} mice (Figure 5H). This expansion in CD69⁺ B cells was accompanied by a similar expansion in CD86⁺ B cells ($p = 0.031$) but not in CD80⁺B cells. In addition, the dramatic reductions in follicular and marginal zone B cells that are characteristic of NZM.*baff*^{-/-} mice (41) were not affected by Adv-IFN treatment (data not shown). No correlations between serum IFN α levels and any B cell populations were observed among either Adv-IFN-treated NZM or Adv-IFN-treated NZM.*baff*^{-/-} mice.

Failure of IFN α to preferentially expand the activated memory CD4⁺ cell population

BWF1 mice undergo an ordered progression from serological autoimmunity to renal immunopathology to clinical disease as they age, with the entire process taking as long as 11 months. This ordered progression is condensed into a much shorter time frame in young pre-autoimmune BWF1 mice injected with Adv-IFN (23), raising the hope that the exaggerated disease in Adv-IFN-treated mice could serve as a rapid, yet faithful, model of the “natural” disease.

This does not appear to be the case. NZM mice undergo a virtually identical ordered progression from serological autoimmunity to renal immunopathology to clinical disease as they age. A biomarker that parallels this progression is the ratio of spleen CD4⁺ memory cells to spleen CD4⁺ naive cells (M/N ratio). This ratio is low in clinically healthy mice but markedly rises with development of clinical disease (proteinuria). For example, in 5-month-old healthy WT NZM mice ($n = 4$), the mean M/N ratio was 0.22, whereas in 9-month-old proteinuric WT mice ($n = 6$), the mean M/N ratio was 1.99 (Figure 4G, $p = 0.003$). In NZM.*ifnar*^{-/-} mice, in which disease onset is delayed and severity is relatively mild (22), the change in M/N ratio over time was less striking, albeit still significant ($p = 0.020$), with the mean M/N ratio rising from 0.39 in 6-month-old NZM.*ifnar*^{-/-} mice ($n = 3$) to 0.95 in 12-month-old mice ($n = 3$). In NZM.*tnfr1*^{-/-}.*tnfr2*^{-/-} mice, in which disease is accelerated (49), the mean M/N in proteinuric 5-month-old mice ($n = 4$) was already very high (2.81).

In contrast to the association between M/N ratios and clinical disease in NZM, NZM.*ifnar*^{-/-}, and NZM.*tnfr1*^{-/-}.*tnfr2*^{-/-} mice, no such associations were noted in Adv-IFN-treated mice. Even in Adv-IFN-treated NZM mice which either had or were soon to develop lethal GN, M/N ratios remained very low and were no different than those in control WT mice.

Lack of correlation between glomerular macrophages and clinical disease among Adv-IFN-treated mice

As NZM mice age and develop their “natural” spontaneous disease, increasing numbers of macrophages progressively infiltrate the glomeruli. Few, if any, such cells are detectable “pre-autoimmune” mice (3 months of age); some cells are present in “autoimmune but clinically healthy” mice (6 months of age); and many cells are present in “clinically sick” mice (9 months of age) (Guo S et al, manuscript in preparation). Our observations in NZM are consistent with those in BWF1 mice, in which an association between activated renal macrophages and clinical disease has been reported (56).

Remarkably, no association between glomerular infiltration of macrophages and clinical disease was appreciated in Adv-IFN-treated mice. NZM mice, treated with Adv-IFN at 2.5 months of age and sacrificed 2 months later (when becoming clinically sick), harbored only

few macrophages in their glomeruli which were no greater in number than those harbored by clinically healthy Adv-control-treated NZM mice (Figure 6A-B). Although no glomerular infiltration of macrophages was appreciated in either Adv-control- or Adv-IFN-treated young (age-matched with the WT mice) NZM.*baff*^{-/-} mice (Figure 6C-D), occasional macrophages were detected in the glomeruli of old NZM.*baff*^{-/-} mice treated with Adv-control (Figure 6E). Surprisingly, numbers of such macrophages increased considerably in Adv-IFN-treated old NZM.*baff*^{-/-} mice despite the absence of clinical disease.

Discussion

IFN α has, to date, garnered considerable interest as a contributing factor to SLE pathogenesis. Genetic depletion of the type I IFNR (which blocks IFN α signaling) attenuates disease in several murine SLE models (20-22), and administration of IFN α to pre-autoimmune BWF1 mice promotes the rapid onset of a highly aggressive clinical disease (23). In humans, an IFN α signature is commonly expressed by SLE patients (15-17), and systemic administration of IFN α has induced SLE-like disease (18). These results have prompted many investigators to consider IFN α a viable candidate therapeutic target, and a phase-I clinical trial in SLE with one anti-IFN α mAb has already been completed (48).

These observations notwithstanding, IFN α does not immutably contribute to SLE pathogenesis. In MRL-*lpr* mice, genetic depletion of the type I IFNR not only does not ameliorate disease but actually aggravates it (57). By implication, IFN α may not necessarily be vital to or even contribute to development of SLE in other hosts. Thus, a more complete understanding of the permissive factors that work in concert with IFN α to promote disease is needed.

In this light, our results unequivocally demonstrate that although administration of Adv-IFN can rapidly promote florid clinical and pathological disease in pre-autoimmune NZM mice, identical treatment fails to drive SLE disease in congenic B cell-deficient NZM.JHD mice. Of note, IFN α -sufficient, B cell-deficient NZM and MRL mice are each completely resistant to the spontaneous SLE disease that their respective IFN α -sufficient, B cell-sufficient WT counterparts develop (24), indicating that life-long production of endogenous IFN α even in a SLE-prone environment is incapable of overcoming resistance to disease imposed through the absence of B cells. It should be stressed that these observations do not prove that B cells are the actual pathogenic effectors. B cells may simply be part of a cascade of events that culminate with the generation and/or activation of some pathogenic non-B cell population.

Not only does Adv-IFN treatment not promote disease in NZM.JHD mice, but Adv-IFN treatment also fails to drive SLE disease in either young NZM.*baff*^{-/-} mice (in which overt renal immunopathology has not yet developed) or in old NZM.*baff*^{-/-} mice (in which overt renal immunopathology has already developed). This does not represent a failure of IFN α to effect its biological activity in BAFF-deficient hosts, inasmuch as expansion of all tested T cell subsets in Adv-IFN-treated young NZM.*baff*^{-/-} mice is identical to that in age-matched Adv-IFN-treated NZM mice.

Although T cell expansion was noted in all Adv-IFN-treated mice (both WT and BAFF-deficient), the degree of expansion was variable. One possible contributing factor could have been variability in IFN α expression following Adv-IFN injection. We measured serum IFN α levels to document successful administration of Adv-IFN, and levels in NZM.*baff*^{-/-} mice were identical to those in WT NZM mice. Of note, among the small number of Adv-IFN-treated NZM WT mice in whom both day-8 serum IFN α levels and lymphocyte profiles were determined, serum IFN α levels correlated significantly with CD3⁺, CD4⁺, CD8⁺, and

CD4⁺ naive cells, whereas no correlations were detected in Adv-IFN-treated NZM.*baff*^{-/-} mice.

With regard to these results, there are several important caveats to consider. First, we did not perform comprehensive pharmacokinetic studies. Since the total integrated exposure to IFN α in an individual host is biologically more meaningful than is the serum level at a single point in time, T cell expansion may have better correlated with (unmeasured) total integrated exposure to IFN α over time. Second, the number of mice studied was small ($n = 4$ in each group), so the presence or absence of significant correlations may have reflected “chance” occurrences. Third, since our results are the composite of several individual experiments performed over many months, variability in T cell expansion may have reflected subtle (unmeasured) differences in the local environment, such as changes in the microbiologic profiles over time within the animal room. Fourth, differences in clinical status may have affected T cell expansion among the WT mice. We have long appreciated that spleen cell numbers in NZM mice decline as the course of their spontaneous “natural” SLE reaches the point of overt clinical disease (development of ascites, unkempt fur, lethargy). Indeed, T cell numbers in Adv-IFN-treated NZM mice that were clinically very sick at the time of analysis tended to be lower than in those Adv-IFN-treated NZM mice that had not yet become as sick.

The paucity of IgG (including IgG anti-chromatin and anti-dsDNA) responses by day 28 post-injection in Adv-IFN-treated NZM mice was unexpected, in light of the greater glomerular deposition of IgG and C3 in Adv-IFN-treated NZM mice than in control NZM mice and the increases in serum total IgG levels in BWF1 mice treated with the same Adv-IFN by day 10 post-injection and the increases in serum IgG anti-dsDNA levels by day 21 post-injection (23). Importantly, the dose of Adv-IFN given to these BWF1 mice (1×10^{10} vp) was 7- to 10-fold greater than the dose given to our NZM mice ($1-1.5 \times 10^9$ vp), so the limited IgG responses in young NZM mice may have reflected a dose of Adv-IFN insufficiently high to promote robust IgG responses. Indeed, Adv-IFN at a dose of 3×10^9 vp (a dose closer to the one we used) did not promote either anti-nuclear or anti-dsDNA antibodies in B6.Sle1.Sle2.Sle3 SLE mice despite promoting immune-complex GN (58). These findings do not exclude a pathogenic contribution from autoantibodies at later time points, but they do collectively point to an important autoantibody-independent component for IFN α -exaggerated disease, although it remains possible that nephritogenic autoantibodies with specificities other than chromatin or dsDNA were promoted by IFN α overexpression within the first 28 days post-injection and contributed to development of disease. This apparent uncoupling of circulating autoantibody levels from clinical disease is reminiscent of NZM.*stat4*^{-/-} and NZM.*stat6*^{-/-} mice in which the effects on circulating autoantibody levels were anti-parallel to those on clinical disease (55).

Another point to consider is the striking degree of T cell and B cell infiltration into the kidneys of Adv-control-treated (clinically healthy) NZM mice. In mice treated in parallel with Adv-IFN, renal infiltration of T cells and B cells was qualitatively similar, but the latter mice developed a virulent clinical disease whereas the former mice remained healthy. It may be that IFN α somehow affects the nature or function of these infiltrating cells and, thereby, promotes “conversion” from a clinically quiescent state to a very active one. The absence of such renal T cell and/or B cell infiltrates may help explain the protection of NZM.*baff*^{-/-} mice from the pathogenic effects of Adv-IFN treatment.

Since IFN α is a potent inducer of BAFF (47) and treatment of SLE patients with an anti-IFN α mAb reduces their endogenous expression of BAFF (48), our findings support a pathogenic pathway from IFN α to clinical disease with the vital involvement of BAFF along the way. Given that Adv-IFN administration drives similar robust T cell expansion in WT

and BAFF-deficient NZM mice, the dichotomous clinical and pathological responses in these mice suggest that the role for IFN α in IFN α -exaggerated disease might hinge more on effects on B cells and/or the innate immune system than on T cells.

Regarding B cells, both IFN α and BAFF up-regulate the expression and sensitivity of TLR7 and TLR9 (59-63). Such signaling can promote B cell activation, proliferation, and differentiation (64), and signaling through the downstream MyD88 pathway may be especially relevant to SLE (36,65). Moreover, TLR-mediated signaling promotes production of IL-6 (60,63,66), the administration of which to BWF1 mice being capable of accelerating disease and the neutralization of which being capable of ameliorating disease (67-69). Indeed, Adv-IFN treatment, promoted a modest increase in total B cells and a substantial increase in activated (CD69⁺ or CD86⁺) B cells in NZM, but not NZM.*baff*^{-/-}, mice. One possible explanation is that IFN α -driven activation of B cells in a BAFF-sufficient environment differs from that in a BAFF-deficient environment. That is, engagement of both IFN α and BAFF on the target B cell is required for “complete” activation, and the failure of activated B cells to increase in number in Adv-IFN-treated NZM.*baff*^{-/-} mice truly reflects an “incomplete” activation of B cells. An alternative explanation is that the marked reduction in B cells in a BAFF-deficient environment precludes detection of IFN α -driven activation. That is, B cell activation in Adv-IFN-treated NZM.*baff*^{-/-} mice is “complete”, but we cannot detect it due to issues external to the activation process, such as homing to extra-splenic sites. To aid in distinguishing between these two possibilities, we are currently generating BAFF-deficient NZM mice that selectively overexpress Bcl-2 in their B cells and, therefore, harbor (near)-normal numbers of B cells.

Regarding cells of the innate immune system, IFN α promotes monocyte and immature DC activation and proliferation (70,71). Of note, IFN α promotes increased expression of BAFF in myeloid DCs (mDCs), which also express BAFF receptors (47,72). When stimulated with BAFF, mDCs up-regulate costimulatory molecules and produce chemokines and pro-inflammatory cytokines (73). Conversely, systemic BAFF antagonism reduces DC numbers (39), and when BAFF is silenced in mDCs following treatment with specific siRNA, the mDCs remain immature and fail to produce IL-6 (74). NZM.*ifnar*^{-/-} mice, which cannot generate any IFN α -mediated signaling, harbor reduced numbers of DC subsets that exhibit only limited responses to TLR9 stimulation (22).

It is of interest that both NZM.*baff*^{-/-} mice and NZM.*ifnar*^{-/-} mice are largely (albeit incompletely) protected to similar degrees from spontaneous SLE clinical disease (22,41). It remains to be established whether BAFF-triggered signaling is a vital downstream component of the IFN α -driven pathogenic pathway (that is, reduction in BAFF-triggered signaling plays an important direct role in mediating resistance to disease in NZM.*ifnar*^{-/-} mice) or whether BAFF and IFN α trigger vital pathogenic pathways that work in parallel but are largely independent of each other (Figure 7). Development of SLE disease in NZM.*ifnar*^{-/-} mice following enforced overexpression of BAFF (e.g., by introduction of a BAFF Tg) would point to the first alternative, whereas continued protection from disease would point to the second. Additional investigation into the cellular and molecular underpinnings of the intertwined roles played by BAFF and IFN α is needed.

If IFN α and BAFF each trigger a pathogenic pathway that is largely independent of the other, then therapeutic targeting of IFN α in human SLE is highly rational. However, if BAFF is an indispensable downstream component of the pathogenic pathway triggered by IFN α , then one may question the choice of IFN α as an optimal therapeutic target rather than directly targeting BAFF. Post-hoc analysis of a phase-II trial with belimumab, an anti-BAFF mAb, in SLE demonstrated clinical benefit in the subset of patients who were “seropositive” (serum ANA titer \geq 1:80 or elevated serum anti-dsDNA level) at the time of enrollment into

the study (42,43). Two recent phase-III trials have substantiated such efficacy (44-46). From a clinical standpoint, additional investigation is sorely needed to identify those SLE patients who could benefit more from antagonism of BAFF (or B cells) versus those who could benefit more from antagonism of IFN α .

In addition to the therapeutic implications our results may have for human SLE, our findings have important implications for experimental murine SLE models. The differences in M/N ratios between NZM mice which had developed their natural disease and those mice which had developed IFN α -exaggerated disease were striking. Moreover, the association between glomerular infiltration of macrophages and clinical disease in the natural NZM disease is in stark contrast to the uncoupling of the two in Adv-IFN-treated NZM and NZM.*baff*^{-/-} mice. These disparities clearly demonstrate that SLE disease driven by artificial overexpression of IFN α in a given host may not faithfully reflect all the characteristics of the SLE disease that would otherwise spontaneously develop in that same host. That is, the disease that is promoted by IFN α overexpression in a given host may be subtly, but fundamentally, different from the natural spontaneous disease in that host. In the case of NZM mice, it appears that spontaneous disease is much more T cell-driven than is IFN α -exaggerated disease.

Differences between the natural disease and IFN α -exaggerated disease are not limited to NZM mice but have also been documented in the anti-phospholipid syndrome-related (NZWxBXSB)F1 mouse model (75). Importantly, differences between the IFN α -exaggerated diseases and the natural spontaneous diseases in these hosts in no way negate the likely value of the IFN α -driven models and the insights that can be gleaned from them. Nevertheless, investigators should exercise considerable circumspection prior to extrapolating findings from IFN α -exaggerated models to infer mechanistic pathways in the unmanipulated native hosts.

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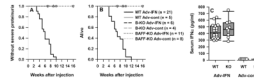


Figure 1. Resistance of NZM.JHD and NZM.*baff*^{-/-} mice to the disease-accelerating effects of Adv-IFN

Panels A-B: Pre-autoimmune (9-11 weeks of age) WT and age-matched B cell-deficient (B-KO) or BAFF-deficient (BAFF-KO) NZM mice were injected with Adv-IFN (black lines and symbols) or Adv-control (Adv-cont; gray lines and symbols). Data are plotted as the fraction of mice over time that did not develop severe proteinuria (panel A) and remained alive (panel B). Panel C: Sera collected on day 8 post-injection with either Adv-IFN or Adv-control were available from 18 Adv-IFN-injected WT mice, 6 Adv-control-injected WT mice, 10 Adv-IFN-injected NZM.*baff*^{-/-} mice, and 3 Adv-control-injected NZM.*baff*^{-/-} mice were assayed for IFN α levels by ELISA. Samples with values <12.5 pg/ml (lower limit of detection) were arbitrarily plotted as 12.5 pg/ml.

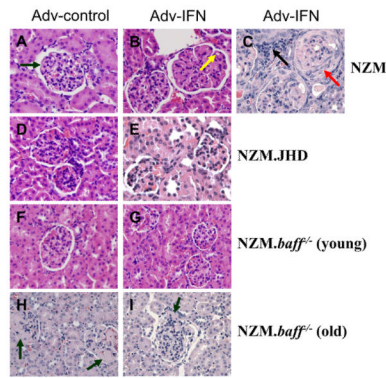


Figure 2. Renal histology in Adv-control-, or Adv-IFN-treated NZM, NZM.JHD, or NZM.baff^{-/-} mice

Representative H&E-stained sections of formalin-fixed kidneys from NZM (panels A-C), NZM.JHD (panels D-E), and NZM.baff^{-/-} mice (panels F-I) that had been injected with Adv-control (panels A, D, F, H) or Adv-IFN (panels B, C, E, G, I). All mice were 2-3 months of age at the time of Adv injection, with the exception of “old” NZM.baff^{-/-} mice, which were 9 months of age at the time of Adv injection. The yellow arrow in panel B points to a glomerular “wire loop”. The black arrow in panel C points to an interstitial infiltrate. The red arrow in panel C points to a glomerular “crescent”. The green arrows in panels H and I point to areas of increased mesangial deposition. Original magnification is 400X.

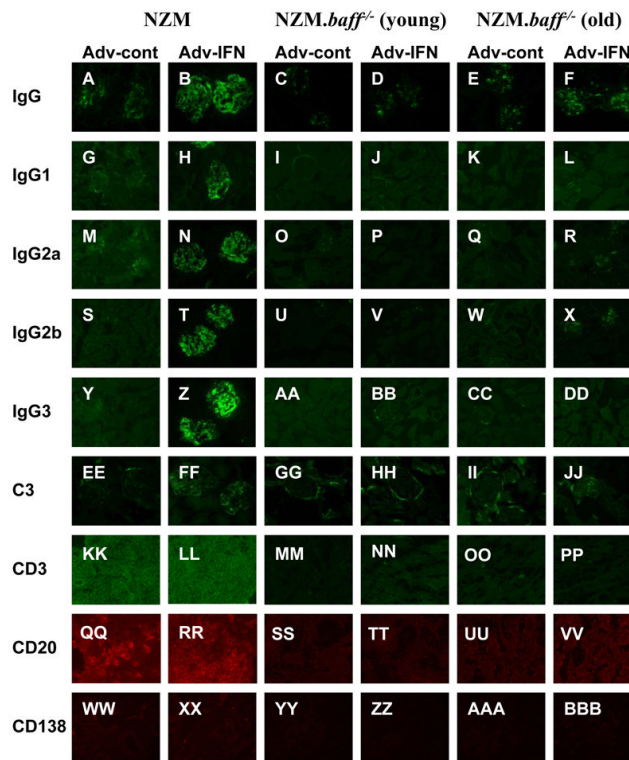


Figure 3. Glomerular IgG and C3 deposition and T cell and B cell infiltration in Adv-control-, or Adv-IFN-treated NZM or NZM.*baff*^{-/-} mice

Representative kidney sections from NZM (panels A, B, G, H, M, N, S, T, Y, Z, EE, FF, KK, LL, QQ, RR, WW, XX) and NZM.*baff*^{-/-} (panels C-F, I-L, O-R, U-X, AA-DD, GG-JJ, MM-PP, SS-VV, YY-BBB) mice that had been injected with Adv-control (panels A, C, E, G, I, K, M, O, Q, S, U, W, Y, AA, CC, EE, GG, II, KK, MM, OO, QQ, SS, UU, WW, YY, AAA) or Adv-IFN (panels B, D, F, H, J, L, N, P, R, T, V, X, Z, BB, DD, FF, HH, JJ, LL, NN, PP, RR, TT, VV, XX, ZZ, BBB). were stained for IgG (panels A-F), IgG1 (panels G-L), IgG2a (panels M-R), IgG2b (panels S-X), IgG3 (panels Y-DD), C3 (panels EE-JJ), CD3 (panels KK-PP), CD20 (panels QQ-VV), or CD138 (panels WW-BBB). All mice were 2-3 months of age at the time of Adv injection, with the exception of “old” NZM.*baff*^{-/-} mice, which were 9 months of age at the time of Adv injection. Original magnification is 400X for panels A-JJ and 200X for panels KK-BBB.

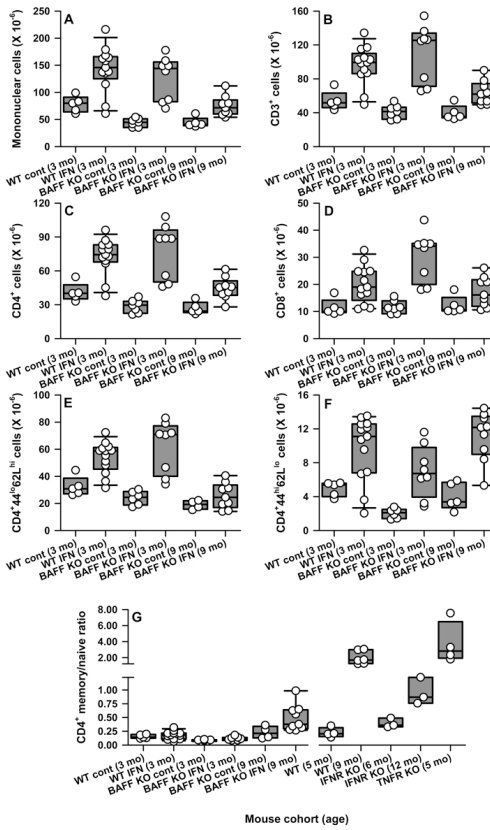


Figure 4. Effect of Adv-IFN administration on spleen T cell populations in NZM and NZM.baff^{-/-} mice

Panels A-F: NZM (WT) or NZM.baff^{-/-} (BAFF KO) mice were injected with Adv-IFN (IFN) (n = 13 for WT; n = 8 for 3-month-old BAFF KO; n = 9 for 9-month-old BAFF KO) and were assessed for total spleen mononuclear cells (panel A), CD3⁺ cells (panel B), CD4⁺ cells (panel C), CD8⁺ cells (panel D), CD4⁺ naive cells (panel E), or CD4⁺ memory cells (panel F). Control (cont) WT mice were either injected with Adv-control (n = 3) or not injected with any Adv (n = 2); control BAFF KO (3 mo) mice were either injected with Adv-control (n = 4) or not injected with any Adv (n = 2-3); and control BAFF KO (9 mo) mice were injected with Adv-control (n = 5). The composite results are plotted as box plots. The lines inside the boxes indicate the medians; the outer borders of the boxes indicate the 25th and 75th percentiles; and the bars extending from the boxes indicate the 10th and 90th percentiles. Panel G: The ratios of CD4⁺ memory cells to CD4⁺ naive cells (M/N ratios) from the Adv-IFN-treated or control mice in panels A-F as well as the M/N ratios from unmanipulated 5-month-old WT (n = 4), 9-month-old WT (n = 6), 6-month-old NZM.ifnar^{-/-} (IFNR KO; n = 3), 12-month-old NZM.ifnar^{-/-} (n = 3), and 5-month-old NZM.tnfr1^{-/-}.tnfr2^{-/-} (TNFR KO; n = 4) mice are plotted.

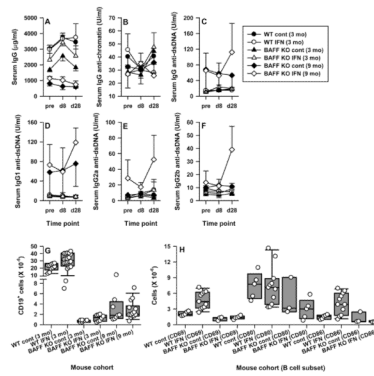


Figure 5. Effect of Adv-IFN administration on serum total IgG and IgG autoantibody levels and on spleen B cells in NZM and NZM.baff^{-/-} mice

Panels A-F: Adv-IFN-injected or control NZM (WT) or NZM.baff^{-/-} (BAFF KO) mice of the indicated ages were assessed for serum levels of total IgG (panel A), IgG anti-chromatin (panel B), IgG anti-dsDNA (panel C), IgG1 anti-dsDNA (panel D), IgG2a anti-dsDNA (panel E), and IgG2b anti-dsDNA (panel F) immediately prior to injection (pre) or at days 8 and 28 (d8, d28). Open symbols indicate Adv-IFN-injected mice, and closed symbols indicate control mice. WT 3-month-old NZM mice are depicted by circles; young (3-month-old) NZM.baff^{-/-} mice are depicted by triangles; and old (9-month-old) NZM.baff^{-/-} mice are depicted by diamonds. Each cohort contained 4-8 mice. Panel G: Adv-IFN-injected or control NZM (WT) or NZM.baff^{-/-} (BAFF KO) mice of the indicated ages were assessed for spleen total B (CD19⁺) cells. Each cohort contained 7-20 mice. Panel H: Splens from Adv-IFN-injected or control NZM (WT) or NZM.baff^{-/-} (BAFF KO) mice were assessed for the indicated subset. Each cohort contained 3-11 mice.

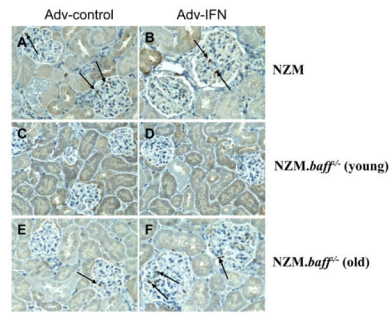


Figure 6. Glomerular infiltration of macrophages in Adv-control- or Adv-IFN-treated NZM and *NZM.baff^{-/-}* mice

Representative sections of formalin-fixed kidneys stained for Mac-2⁺ macrophages from NZM (panels A-B) young *NZM.baff^{-/-}* mice (panels C-D), and old *NZM.baff^{-/-}* mice (panels E-F) that had been injected with Adv-control (panels A, C, E) or Adv-IFN (panels B, D, F). All mice were 2.5 months of age at the time of Adv injection, with the exception of “old” *NZM.baff^{-/-}* mice, which were 9 months of age at the time of Adv injection. The black arrows point to individual Mac-2⁺ macrophages. Original magnification is 200X.

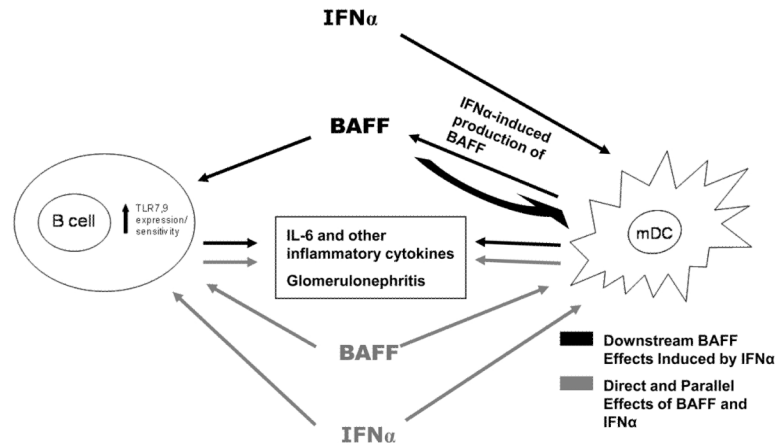


Figure 7. Role for BAFF in IFN α -driven exaggerated disease in NZM mice

Upper half: Overexpression of IFN α activates myeloid DC (mDC) to increase BAFF production. This increased BAFF production further activates mDC and promotes increased B cell survival and activation, the latter leading to increased B cell responses to endogenous and/or exogenous TLR ligands. Collectively, this results in production of IL-6 and other proinflammatory cytokines and culminate in exaggerated disease. Lower half: Overexpression of IFN α directly activates both mDC and B cells. Such activation does not require BAFF as an intermediary, but a distinct BAFF-driven pathway in parallel must be present for exaggerated disease to develop.