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Influenza A (H1N1) Virus Infection Triggers Severe Pulmonary Inflammation in Lupus-Prone Mice following Viral Clearance

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Abstract

Each year, up to one fifth of the United States population is infected with influenza virus. Although mortality rates are low, hundreds of thousands are hospitalized each year in the United States. Specific high risk groups, such as those with suppressed or dysregulated immune systems, are at greater danger for influenza complications. Respiratory infections are a common cause of hospitalizations and early mortality in patients with systemic lupus erythematosus (SLE); however, whether this increased infection risk is a consequence of the underlying dysregulated immune background and/or immunosuppressing drugs is unknown. To evaluate the influenza immune response in the context of lupus, as well as assess the effect of infection on autoimmune disease in a controlled setting, we infected lupus-prone MRL/MpJ-Fas^{lpr} mice with influenza virus A PR/8/34 H1N1. Interestingly, we found that Fas^{lpr} mice generated more influenza A virus specific T cells with less neutrophil accumulation in the lung during acute infection. Moreover, Fas^{lpr} mice produced fewer flu-specific IgG and IgM antibodies, but effectively cleared the virus. Further, increased extrinsic apoptosis during influenza infection led to a delay in autoimmune disease pathology with decreased severity of splenomegaly and kidney disease. Following primary influenza A infection, Fas^{lpr} mice had severe complications during the contraction and resolution phase with widespread severe pulmonary inflammation. Our findings suggest that influenza infection may not exacerbate autoimmune pathology in mice during acute infection as a direct result of virus induced apoptosis. Additionally, autoimmunity drives an enhanced antigen-specific T cell response to clear the virus, but persisting pulmonary inflammation following viral clearance may cause complications in this lupus animal model.

Keywords

Influenza; SLE; Pulmonary Inflammation; Lupus; MRL-Fas^{lpr}

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1. Introduction

Influenza viruses are a primary cause of respiratory tract infections resulting in the highest number of hospitalizations and mortalities of any viral respiratory pathogen [1]. Three types of influenza virus, A, B, and C, belong to the family *Orthomyxoviridae*. Influenza A virus (IAV) and influenza B virus (IBV) are both pathogenic to humans, but IAV is the most predominant virus type worldwide that contributes to seasonal epidemics [2]. During a primary IAV infection, antigen-specific T cells are recruited to the lung following initial priming in secondary lymphoid organs to control and clear the virus [3, 4]. Although the balance of Th1 CD4+ T cells, B cells, neutrophils and other immune cells all contribute to a successful immune response against influenza, IAV-specific CD8+ effector T cells are essential for effective viral clearance through multiple redundant mechanisms [5]. Indeed, the expression of perforin/granzyme B, Th1 cytokines and proapoptotic TNF family ligands all contribute to the wide range of effector mechanisms by T cells during influenza infection [6-8]. Following primary IAV infection, complete resolution of infection involves contraction of T cell responses and a decline in inflammation in the lung and periphery, which is largely driven by activation induced cell death [9]. Although the proinflammatory effector functions involved with IAV infection may be highly effective in pathogen clearance, these mechanisms may also be dangerous to the host in driving tissue damage and even triggering autoimmunity.

Infection is a leading cause of mortality in individuals with systemic lupus erythematosus (SLE), a complex autoimmune disease with ill-defined etiology [10]. Previous studies indicate infections contribute to 20-55% of SLE patient mortalities [10]. Further, infections contribute to 14-50% of hospitalizations in SLE patients [11]. The high incidence of infection is believed to be due to underlying immune system dysregulation and/or immunosuppressive or immunomodulating agents used for SLE. Although numerous studies have worked to elucidate the mechanism of certain infections as early triggers for autoimmunity [reviewed in [12, 13]], the effect of infections on active SLE are just beginning to be defined. Several studies indicate that treatment of lupus-prone mice with bacterial components, such as bacterial lipopolysaccharide or CpG oligodeoxynucleotide, aggravate glomerulonephritis by increased immune complex deposition that persists long after exposure [14-16]. Further, following chronic parasitic infection with *Schistosoma mansoni*, increased IgG and C3 deposits were observed in the capillaries of the kidney with severe glomerulonephritis reported [17]. In contrast, chronic infections of lupus-prone mice with murine gammaherpesvirus, *Mycobacterium avium*, and *Toxoplasma gondii* were shown to reverse kidney disease symptoms suggesting the effect of infection on autoimmune disease symptoms is variable [18-20].

To understand the immune response to IAV, a transient infection, in SLE, we utilized the Fas^{lpr} model. Interestingly, our results demonstrate that IAV infection does not exacerbate autoimmune disease symptoms during acute infection. Rather, the virus significantly reduced splenomegaly and kidney disease with no effect on autoantibody production. The reduction in splenomegaly can be attributed to increased viral-infected cell killing and activation induced cell death through the caspase-8 pathway. Further, the decrease in autoimmune disease symptoms was proven to be a transient effect, as cells began to

accumulate in the spleen and kidney disease began to worsen following resolution of infection. Lupus-prone mice generate a more directed immune response to IAV with reduced pulmonary inflammation during acute infection from decreased neutrophil accumulation and more TNF α producing IAV-specific CD8 $^{+}$ and CD4 $^{+}$ T cells. However, following IAV clearance, Fas lpr mice develop worsened pulmonary inflammation suggesting lupus patients may develop more severe pathology after, rather than during influenza infection.

2. Material and Methods

2.1 H1N1 Infection in Mice

MRL/MpJ-Fas lpr , MRL/MpJ, and C57BL6 mice were purchased from the Jackson Laboratory and maintained at the Oklahoma Medical Research Foundation. All mice utilized for experiments were female and were age matched for each experiment between 10-12 weeks of age. All experiments were performed in accordance with the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation. Influenza A PR/8/34 H1N1 (IAV) was propagated in chicken eggs as previously described [21]. Mice were infected with 200 EID50 (50% egg infective dose) of influenza A PR/8/34 H1N1 (in 40 μ l of sterile PBS) to produce a non-lethal infection or given control PBS by intranasal instillation. Mice were weighed at the same time daily following IAV infection and percent starting weight was calculated by dividing weight at day 0 post infection. At given time points, mice were euthanized by intraperitoneal injection with a combination of ketamine and xylazine. Terminal blood collection was procured by cardiac puncture.

2.2 Viral Real time PCR Detection

RNA was extracted from lung tissue [22], reverse transcribed, and amplified for viral matrix protein as described previously [23]. Briefly, RNA was collected from the right, middle lobe of the lung (Qiagen), treated with DNase and reverse transcribed (Applied Biosystems). cDNA was then amplified with a FAM-labeled probe 5'-CTCAGTTATTCTGCTGGTGCACCTGCCA-3', PCR forward primer 5'-GGACTGCAGCGTAGACGCTT-3' and reverse primer 5'-CATCCTGTTGTATATGAGGCCCAT-3' targeting the influenza M protein on the ABI PRISM 7500 detection system. The specific gene expression was calculated relative to GAPDH expression. The primer and probe sequences targeting influenza M1 protein have been previously published [24].

2.3 Histologic Analysis

Mice were sacrificed at the indicated time points, and lungs were inflated and fixed with 10% neutral buffered formalin (Sigma-Aldrich) and embedded in paraffin. Kidneys were cut and collected in formalin for paraffin embedding. Lung sections (5 μ m thickness) were stained with hematoxylin and eosin and kidney sections (3 μ m thickness) were stained with Periodic acid-Schiff stain for morphometric analysis. Terminal dextrynucleotidyltransferase dUTP nick end labeling (TUNEL) staining was performed with the Dead End Fluorometric TUNEL system from Promega. Images were acquired using a Zeiss Axioplan Microscope and TUNEL staining was quantitated based on cells per 20X field of view.

2.4 Lung Injury and Kidney Scoring

To score pulmonary inflammation and kidney disease, each slide was independently evaluated by two blinded investigators. For each lung section, the entire lobe was analyzed for the percentage of alveolar tissue with inflammation, based on the following scale: 0: no inflammation, 1: 1% to <25%, 2: 25% to <50%, 3: 50% to <75%, 4: 75% to 100% inflammation as previously described [25]. Kidney sections were scored based on both acute and chronic glomerulonephritis on a scale of 0-10. Acute glomerulonephritis was scored based on mesangial matrix expansion, focal necrosis and glomerular hypercellularity. Chronic glomerulonephritis score was calculated based on interstitial fibrosis, medullary inflammation, glomerulosclerosis, and tubular atrophy as previously described [26]. Scores were combined for each kidney section to give a cumulative acute and chronic kidney disease score.

2.5 Lung and Spleen Single Cell Preparation

Lung and spleen tissue were prepared as previously described [22]. In short, spleens were weighed and single cell suspensions were generated by digesting lung tissues or spleen and diffusing cells through a 70- μ M tissue strainer (BD Falcon). Red blood cells in the cell suspension were lysed using Gey's solution (ammonium chloride), washed and centrifuged twice in PBS. The collected leukocytes were used in the downstream assays.

2.6 Flow Cytometry

Single cell suspensions were stained in 96-well plates using fluoro-chrome labeled antibodies specific for CD3 (500A2), CD4 (RM4-5), CD8 (53-6.7), CD44 (IM7), B220 (RA3-6B2), CD19 (1D3), CD11c (HL3), CD11b (M1/70), GR1 (RB6-8C5), F4/80 (BM8), IFN γ (XMG1.2), TNF α (MP6-XT22) and granzyme B (NGZB) or relevant isotype control antibodies. For the intracellular analysis of cells, cells were stimulated for either 5 hours with PMA (50 ng/ml) and ionomycin (750 ng/ml) cocktail with GolgiStop (BD Biosciences-Pharmingen) or stimulated overnight with formalin-inactivated Influenza A PR/8/34 H1N1 [27]. For IAV-virus stimulated cells, GolgiStop was added after overnight stimulation and cells were incubated for an additional 6 hours. Cells were surface stained and permeabilized with Cytofix-Cytoperm solution (BD Biosciences-Pharmingen) and stained with intracellular markers. Data was collected using a BD LSR II flow cytometer using FACS Diva software. All cells were gated on forward-by-side scatter characteristics and analyzed using FlowJo (TreeStar, Inc).

2.7 Directed Protein Evaluation by ELISA

Mouse ELISA antibody pairs and standards from eBioscience were used to detect IFN γ , TNF α and IL-10 protein levels in lung supernatants according to the manufacturer's protocol.

2.8 Influenza specific IgG and IgM ELISAs

High binding, 96-well plates were coated with whole PR8 influenza virus at 500,000 EID₅₀/well, and stored overnight at 4°C. Sera samples were serially diluted 1:100 to 1:102,400. Sera from an MRL infected mouse known to be positive for PR8 antibodies was used as a

positive control (1:400). Alkaline phosphatase conjugated anti-mouse IgG (Sigma), anti-mouse IgM (Sigma) and 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma) were used to detect the flu-specific antibodies. The optical densities of the plates were read at 410 nm when the positive control had an average OD of 1.0. Endpoint titers were calculated using a negative cutoff values determined by separately screening and averaging the OD values of PBS treated B6, MRL and MRL-Fas^{lpr} mice. The endpoint titer was calculated by taking the reciprocal of the highest dilution that had a reading 2 standard deviations above the cutoff.

2.9 Hemagglutination Inhibition (HAI) Titer Assay

Mouse serum samples were treated with receptor destroying enzyme (RDE, Denka Seiken Co.) at 1:3 dilution for 18 hours at 37°C. Following incubation, samples were heated to 56°C for 60 minutes to inactivate complement. Starting with a 1:10 dilution of RDE-treated serum in PBS, samples were serially diluted in a 96 well plate. PR8 virus at 8 hemagglutinating units were added to each well and incubated for 15 minutes at room temperature. Turkey red blood cells diluted to 0.5% in PBS were added to each well and incubated for ~60 minutes until PBS-only wells had settled to form a button. HAI antibody titer was calculated as the reciprocal of the highest well that did not exhibit hemagglutination of the red blood cells.

2.10 Total IgG and Anti-Nuclear Antibody ELISAs

Blood was incubated on ice for approximately 4 hours and centrifuged for 10 minutes at 1200 rpm, followed by collection and storage at -80°C of serum. Mouse serum for all antibody assays were diluted to 1:1,000,000 for total IgG and 1:100 for anti-nuclear antibodies (ANA) and anti-dsDNA antibodies in manufacturer's dilution buffer. Total IgG was analyzed using plates pre-coated with mouse IgG specific antibody and the manufacturers protocol was followed (Abcam, Cambridge, MA). After incubation of standards and samples, plates were incubated with HRP-conjugated anti-mouse IgG and developed with TMB substrate solution. Quantitative ELISA tests for ANA and dsDNA antibodies were performed on mouse serum using commercial kits containing a 96-well plate pre-coated with either mouse dsDNA or extracted nuclear proteins, and detected with anti-mouse IgG-HRP conjugate and chromogenic substrate based on manufacturers guidelines (Alpha Diagnostic, San Antonio, Texas).

2.11 Creatinine Assay

For measurement of serum creatinine, a colorimetric assay was used following manufacturer's instructions (Abcam, Cambridge, MA). Briefly, 25 ul of undiluted serum was incubated for 1 hour at 37 degrees Celsius with creatininase to create creatine. Plates were then read on a plate reader at 570 nm.

2.12 Statistical Analysis

Statistical analysis was performed using a two-tailed student T test for unpaired samples to determine differences in the means of experimental groups. Differences were considered significant when P values were less than or equal to 0.05.

3. Results

3.1 Lupus-prone MRL/MpJ-Fas^{lpr} mice develop enhanced influenza-specific cellular immunity and clear influenza infection

To examine the effects of influenza infection in SLE, MRL/MpJ (MRL) and MRL/MpJ-Fas^{lpr} (MRL-Fas^{lpr}) mice were challenged with 200 EID₅₀ influenza A PR8/34 H1N1 virus (IAV) at age 10-12 weeks, an age where autoimmune MRL-Fas^{lpr} mice have developed autoantibodies and glomerulonephritis [28]. Further, C57BL/6 (B6) mice were also included for comparison as a non-autoimmune model as MRL control mice do develop SLE, although much later than MRL-Fas^{lpr} mice. Following viral challenge, we weighed mice as a measurement of morbidity and found the lupus-prone MRL-Fas^{lpr} mice had decreased weight loss and accelerated recovery throughout the course of infection (Figure 1a). Infection of B6 mice led to a peak in weight loss at day 9, consistent with previous reports [29], whereas MRL-Fas^{lpr} and MRL mice had delayed weight loss with peaks at day 10 and 11, respectively. We suspected differences in lupus-prone MRL-Fas^{lpr} mouse morbidity might be due to changes in viral burden. To determine variability in viral load in the lung, we measured the influenza M1 gene by RT-PCR from lung tissue on days 7, 10, and 20 post infection. Interestingly, despite the decrease in morbidity, lupus-prone mice had a similar viral load to control MRL mice and effectively cleared the virus (Figure 1b). Taken together, these data suggest lupus-prone mice are able to tolerate influenza infection as they effectively clear the influenza virus and recover from morbidity.

Further, a reduction in pulmonary inflammation would explain lessened morbidity in MRL-Fas^{lpr} mice. Examination of lung sections by hematoxylin and eosin (H&E) staining for histopathological characteristics confirmed a reduction in interstitial lung inflammation at days 7, 10 and 20 post infection in lupus-prone mice (Figure 1 c-d, Supplemental Figure 1). The areas of inflammation in the MRL-Fas^{lpr} mice were condensed and affected a smaller area of the lung compared to MRL mice, which had increased inflammation by day 7 post infection (Figure 1 c-d) and widespread inflammation by day 10 and 20 post infection (Supplementary Figure 1). To further assess the cellular composition of the lung, flow cytometry was utilized. In general, MRL-Fas^{lpr} mice accumulated more CD8⁺ and CD4⁺ T cells with fewer neutrophils, B cells, and myeloid cells (Figure 1e). Since neutrophil recruitment during influenza infection is implicated in severe disease pathology due to increased reactive oxygen intermediates [24], a decrease would further explain the reduced morbidity seen early in lupus-prone mice.

During influenza virus infection, antigen-specific T cells are critical for containment and elimination of the virus [3, 5]. Lupus-prone MRL-Fas^{lpr} mice already accumulate more CD8⁺ and CD4⁺ T cells in the lung (Figure 1e). To determine whether T cells were influenza-specific, pulmonary cells were stimulated with inactivated IAV and cells were intracellularly stained for TH1 cytokines and granzyme B. MRL-Fas^{lpr} mice accumulated more influenza-specific CD8⁺ and CD4⁺ T cells that produced Tumor Necrosis Factor-alpha (TNFα), at day 7 post infection with similar numbers of granzyme B producing CD8⁺ T cells and Interferon-gamma (IFN-γ) producing CD4⁺ T cells compared to MRL control mice (Figure 2 a-d). Recently, soluble TNFα was found to be required in early influenza

infection for limiting the magnitude of the immune response and mitigating lung injury [30]. In the lung, we found MRL-Fas^{lpr} mice inherently have higher levels of TNF α and IFN γ (Figure 2 e-f). Appropriately, at day 7 post infection the lung homogenates of lupus-prone mice had higher total protein levels of TNF α , but normal IFN γ and IL-10 cytokine responses compared to infected MRL control mice (Figure 2 e-g). These data indicate that MRL-Fas^{lpr} mice generate an enhanced IAV specific cellular immune response with more TNF α producing T cells that direct an efficient anti-viral response.

3.2 Humoral immunity to influenza is impaired in MRL-Fas^{lpr} mice while autoantibody levels remain steady during IAV infection

Cellular immunity was heightened in lupus-prone mice during IAV infection. To examine the other arm of the immune response, humoral immunity, total influenza specific IgG and IgM antibodies were analyzed by ELISA. In contrast to the cellular response, endpoint titers of both IgM (Figure 3a) and IgG (Figure 3b) flu-specific antibodies were significantly lower in MRL-Fas^{lpr} mice than in both B6 and MRL infected mice at both day 10 and day 20 post infection. Further, to assess the efficacy of antibodies in neutralizing the virus, hemagglutinin-inhibiting (HAI) titers were determined in serum samples. Both B6 and MRL influenza-infected mice had a greater neutralization capacity than lupus-prone mice at day 10 and day 20 post-infection with an HAI titer of approximately 128 in B6 and MRL mice compared to approximately 16 in lupus-prone mice. Further, IgG titers remained lower in MRL-Fas^{lpr} mice compared to MRL and B6 mice at day 40 post infection, even though IgM and IgG titers had significantly decreased in all IAV-infected mice compared to day 10 and day 20 post infection (Supplementary Figure 4i, j). Despite deficiencies in the flu specific antibody response in MRL-Fas^{lpr} mice, these mice cleared the virus with reduced pulmonary pathology compared to MRL control mice (Figure 1). These data are consistent with the literature that indicates innate and T cell responses are critical for clearance of primary infection [reviewed in [31]].

Previous reports suggest that infection may contribute to the exacerbation of autoimmune disease activity [reviewed in [32]]. To test whether influenza A infection exacerbates autoimmune disease symptoms, autoantibodies were assessed at day 10 and day 20 post infection in MRL-Fas^{lpr} mice. At 10-12 weeks of age, MRL-Fas^{lpr} mice already produced an increased amount of IgG (Figure 3c) and antibodies directed against nuclear autoantigens (ANA) (Figure 3d) and dsDNA (Figure 3e). However, no significant difference in antibody concentration occurred during influenza infection (Figure 3c-e). Thus, IAV appears to not affect the humoral autoimmune response in lupus-prone MRL-Fas^{lpr} mice during acute infection.

A transient increase in autoantibodies has been observed during specific viral infections and it has long been hypothesized that viral infections may act as an environmental trigger for SLE. Using the B6 mouse model, gammaherpesvirus 68 infection caused a transient increase in autoantibodies during acute infection [18]. Interestingly, during IAV infection an increase in anti-dsDNA antibody is observed in the B6 mice at day 10 post infection during the acute phase of infection (Figure 3e). By day 20 post infection, anti-dsDNA antibodies were decreased and non-significant compared to PBS treated B6 mice suggesting this is only a

transient effect. Further, no differences were observed in the levels of anti-dsDNA antibody at day 10 post infection in control MRL mice suggesting genetic background and/or low autoantibody titers are required to detect an increase in autoantibodies during infection (Figure 3e). Based on these results, we conclude that IAV does not exacerbate autoantibody production in this lupus-prone strain of mice, but may cause transient increases in autoantibodies in select genetic backgrounds.

3.3 Splenomegaly and kidney disease are temporarily reduced in lupus-prone mice during IAV infection

Splenomegaly in MRL-Fas^{lpr} mice is a result of lymphoproliferation of activated B and T cells due to a Fas deficiency [33]. At 10-12 weeks of age, MRL-Fas^{lpr} mice had a 3-fold increase in spleen size compared to MRL control mice with a substantial increase in all compartments of CD8+ T cells, CD4+ T cells, B cells, and double negative (DN) T cells (Figure 4a, Supplementary Figure 2). Interestingly, following influenza A infection in MRL-Fas^{lpr} mice splenomegaly was significantly reduced compared with MRL-Fas^{lpr} PBS treated mice at day 10 and 20 post infection (Figure 4a). The decrease in spleen size was the result of specific cell population reductions in CD4+ T cells, DN T cells and myeloid cells (Supplementary Figure 2). However, CD8+ T cells and the B cell compartment had only minor and non-significant reductions following IAV infection (Supplementary Figure 2 b,e). The spleen was still smaller by day 20 post infection following complete clearance of the virus suggesting IAV infection may improve some aspects of autoimmune disease symptoms.

A major risk associated with SLE pathogenesis is renal failure due to overproduction of autoantibodies, immune-complex deposition in the kidney and complement activation. In MRL-Fas^{lpr} mice, the mice ultimately succumb to SLE as a result of fatal glomerulonephritis by 28 weeks [28]. To further understand the effect of infection on autoimmune disease, kidney function was examined by assessing glomerulonephritis (GN) and serum creatinine. By day 20 post infection, MRL-Fas^{lpr} influenza infected mice actually had decreased serum creatinine (Figure 4b). Correlating with decreased serum creatinine, Periodic-Acid Schiff (PAS) stain of formalin fixed kidney sections at day 20 post infection revealed infected MRL-Fas^{lpr} mice had reduced glomerulosclerosis and fibrosis compared to PBS treated mice (Figure 4c). However, glomeruli still had substantial disease involvement and did not return to normal as compared to MRL control mice (Figure 4d-e). Acute versus chronic GN revealed the most significant improvement in kidney disease was in chronic GN with major reductions in interstitial fibrosis, medullary inflammation, glomerulosclerosis, and tubular atrophy. Our data suggest that influenza A infection in lupus-prone MRL-Fas^{lpr} mice does not lead to exacerbated autoimmune disease symptoms, but decreases splenomegaly and renal damage during acute infection.

3.4 Increased extrinsic apoptosis during IAV infection decreases lymphoproliferation in lupus-prone mice

In SLE, abnormal lymphocyte activation and cellular death are largely responsible for disease pathology [reviewed in [34, 35]]. Under normal conditions, autoreactive T and B cells are targeted for apoptosis through activation induced cell death (AICD) that utilizes the

extrinsic apoptotic pathway of FasL, TRAIL and TNF α [9]. However, lymphocytes in autoimmune disorders have defects in AICD and an increase in unprogrammed apoptosis, which likely contribute to the persistence of activated cells [35]. Accordingly, polymorphisms in Fas and FasL gene are associated with increased risk of SLE and lower Fas/FasL expression has been noted in SLE patients [36]. Irregularities in TRAIL expression are also associated with SLE disease activity [37].

During influenza infection, FasL, TNF α and TRAIL are significantly increased and used during the primary immune response to trigger extrinsic apoptosis of IAV infected cells [6, 30, 38]. Although MRL-Fas^{lpr} mice are unable to activate Fas dependent apoptosis, we hypothesized the increase in TRAIL and TNF α expression during IAV infection would permit AICD of autoreactive cells in the spleen and decrease autoimmune disease pathology in MRL-Fas^{lpr} mice. To test this hypothesis, formalin fixed spleen sections from day 10 post infected mice were stained for DNA fragments using Terminal Deoxynucleotidyl Transferase dUTP nick end labeling (TUNEL) to observe apoptosis. IAV infected MRL-Fas^{lpr} mice contained significantly more apoptotic cells compared to both PBS treated MRL-Fas^{lpr} mice and IAV infected MRL mice (Figure 5a, b). No significant differences in apoptosis were observed in the lung and kidney of MRL-Fas^{lpr} mice (data not shown). However, lymph nodes were also significantly smaller by weight (Supplementary Figure 3a), similar to the spleens, in MRL-Fas^{lpr} infected mice and also had increased TUNEL staining, indicating increased apoptosis is not restricted to the spleen. (Supplementary Figure 3b,c). Following activation of FAS, TNFR and TRAILR, the receptors cross-link and organize the death-induced signaling complex, which allows for the cleavage of pro-caspase 8 to the activated form, caspase 8. To determine whether apoptosis was truly a result of increased extrinsic apoptosis triggered by the TNF receptor family, lupus-prone mice were treated with a caspase 8 selective inhibitor (Z-IETDFMK) or a negative control (Z-FA-FMK) for a total of 6 days, started at 4 days post infection. Inhibition of caspase-8 significantly impeded contraction of splenic cells during influenza infection (Figure 5c). These results suggest that influenza infection circumvents AICD dysfunction in lupus to improve splenomegaly.

3.5 IAV infected lupus-prone mice have exacerbated pulmonary inflammation following extended recovery

AICD is a critical step, not just for balance of autoreactive cells, but in immune cell contraction following infection of a pathogen [9]. Further, the measure of a successful immune response also includes the contraction of effector T cell populations in the lung. To examine the long-term recovery of lupus prone mice, MRL-Fas^{lpr} mice were IAV infected and pulmonary pathology was inspected at day 40 post infection. Surprisingly, H&E stains revealed that lupus-prone mice have significantly more pulmonary pathology with accumulation of more effector cells capable of producing Th1 cytokines (Figure 6a,b, Supplementary Figure 4). In contrast to primary infection, the lungs of lupus-prone mice contained more CD19+B220+ B cells and myeloid cells in addition to CD8+ and CD4+ effector T cell populations (Supplementary Figure 4b) that were antigen-specific and capable of producing TNF α and IFN γ (Supplementary Figure 4 c-f). Interestingly, IAV infected MRL-Fas^{lpr} mice produced similar amounts of TNF α and IFN γ to PBS treated

MRL-Fas^{lpr} mice suggesting these antigen-specific cells are not being exposed to the viral antigen to stimulate the production of Th1 cytokines (Supplementary Figure 4g,h). Further, by day 40, IgM and IgG flu titers were significantly reduced compared to day 10 and day 20 titers (Supplementary Figure 4i,j). MRL-Fas^{lpr} mice still had reduced IgG flu titers by day 40 post infection further supporting the decreased ability of MRL-Fas^{lpr} mice to generate IAV specific antibodies. These data support the role of Fas for pulmonary *in vivo* T cell contraction and suggest the possibility of complications arising following influenza infection in lupus from exacerbated and unresolved pulmonary inflammation after viral clearance.

Next we asked whether lupus disease symptoms increased following long-term clearance of the infection. Indeed, following infection, the spleen accumulated leukocytes (Figure 6c) and chronic glomerulosclerosis increased in severity to that of PBS MRL-Fas^{lpr} mice (Figure 6d-e). These data further support our hypothesis that a temporary improvement in disease symptoms is a result of the transient increase in influenza-induced AICD.

4. Discussion

In this study, we asked whether IAV infection exacerbated autoimmunity in lupus-prone mice and if lupus-prone mice were capable of generating a protective immune response to IAV. Surprisingly, we found that influenza did not worsen acute autoimmune disease symptoms, but caused severe pulmonary inflammation following viral clearance due to persistence of inflammatory cells. Influenza infection transiently improved both splenomegaly and kidney disease. A significant decrease in both spleen size and cell numbers in lupus-prone mice followed IAV infection. Further, chronic GN was reduced by day 20 post infection. A decrease in spleen size was correlated to increased extrinsic apoptosis through the caspase-8 pathway. IAV infected MRL-Fas^{lpr} mice had more apoptotic cells in the spleen and anti-caspase 8 treatment reversed the beneficial effects of IAV infection in the spleen. However, blockade of the caspase-8 pathway had no effect on glomerulonephritis suggesting multiple pathways contribute to autoimmune disease improvement. It is important to note that autoimmune disease symptoms in IAV infected MRL-Fas^{lpr} mice may be delayed rather than improved depending on how quickly the disease progressed during the course of infection. However, autoimmune disease symptoms were not exacerbated in MRL-Fas^{lpr} mice and autoimmunity did not affect the ability of mice to develop an antigen-specific T cell response and clear the virus, but did significantly affect the ability of mice to develop antigen-specific IgM and IgG neutralizing antibodies.

TNF α is a pleiotropic cytokine produced during influenza infection that acts as both an inflammatory mediator and an inducer of extrinsic apoptosis. Although controversial, TNF α plays a role in the pathogenesis of SLE with increased levels of the cytokine circulating in the serum of patients with elevated disease activity [39]. Further, high TNF α levels in SLE patients may be related to heritable factors as unaffected first degree relatives also had significantly higher TNF α levels compared to healthy unrelated control subjects [40]. Interestingly, rheumatoid arthritis patients treated with TNF blockers can develop drug-induced lupus with ANA and anti-dsDNA antibodies, and lupus-prone mice that are TNF deficient develop accelerated autoimmunity [41, 42]. In MRL-Fas^{lpr} mice treated with proteins that block the TNF receptor signaling pathway, autoimmune disease symptoms did

not improve and actually worsened kidney damage with increased cellular infiltrates and glomerulonephritis [43]. During IAV infection, MRL-Fas^{lpr} mice have increased TNF α levels and decreased kidney damage. Spleen size may be reduced by increased TNF α driven extrinsic apoptosis and kidney disease is limited by higher TNF α that decreases medullary inflammation and glomerulosclerosis. Further, these data suggest that systemic TNF α , due to pleiotropic effects of the cytokine, does not contribute to worsening autoimmune disease pathology in the MRL-Fas^{lpr} mouse model and it may be TNF α associated pathogenesis in SLE could be better targeted by directing therapy to specific dysfunctional cells.

Apoptosis is closely related to pathologic effects in influenza infection, being important not only for viral clearance and T cell contraction, but also responsible for tissue damage in the lung [44, 45]. Likewise, in SLE, increased apoptosis and defects in apoptotic clearance have been implicated in disease pathogenesis [46, 47]. However, failure to remove autoreactive cells in SLE by activation induced apoptosis can also contribute to autoimmune disease symptoms [35]. Increased apoptosis in the spleen and lymph nodes of MRL-Fas^{lpr} without worsening of disease symptoms in the kidney suggests aberrant mechanisms of apoptosis in SLE may be circumvented during IAV infection to improve splenomegaly and lymphadenopathy. As autoimmune patients and numerous lupus-prone mouse models have interference in the expression of apoptotic factors, such as Fas, FasL, TRAIL, TNF α , Bcl2/Bim, and programmed cell death 1, the correction of aberrant apoptotic effector mechanisms in SLE by viral infection may provide insights to the cause and potential therapies in SLE [40, 48-51].

During IAV infection in MRL-Fas^{lpr} mice, we further found that lupus-prone mice had decreased pulmonary inflammation during acute infection. Concurrently, these mice also had more IAV specific TNF α producing T cells, more total TNF α in the lung, and fewer neutrophils. During influenza infection, a deficiency in TNF α , specifically soluble TNF α , is known to increase morbidity and immunopathology in the lung suggesting TNF α acts as a strong immunoregulator during infection [30, 52]. Further, it was found TNF α was important during IAV infection for directing a more proficient CD8⁺ T cell response although TNF α was dispensable for viral clearance [30, 52]. Similarly, in IAV infected MRL-Fas^{lpr} mice, decreased morbidity and increased antigen-specific TNF α producing CD8⁺ and CD4⁺ T cells were not accompanied by a decrease in viral burden. Our data suggests that lupus-prone mice develop an enhanced and more direct immune response against influenza that limits pulmonary pathology due to an increased ability of lupus T cells to produce TNF α .

Strikingly, virus-specific antibody titers of IgG and IgM in the serum were markedly lower in lupus-prone mice compared to MRL and B6 control mice. Although influenza specific antibodies have multiple mechanisms in controlling influenza, virus neutralization is the most efficient [53]. Accordingly, we show that decreased influenza specific antibodies in MRL-Fas^{lpr} mice translated into decreased virus neutralization as determined by HAI titer. The role of influenza specific antibodies in the prevention of reinfection is known to be critical and well documented; however, during primary infection, the cellular response is essential [31]. Thus, it appears the innate and adaptive cellular response of MRL-Fas^{lpr} mice are sufficient for controlling initial, acute IAV infection.

Our findings indicate that lupus-prone mice develop a strong antigen-specific T cell response to IAV infection, not only clearing infection, but also not acutely worsening lupus pathogenesis. However, lupus-prone mice do have worsening pulmonary inflammation and associated morbidity over a month after the acute infection is cleared. Further, the understanding of IAV infection in lupus-prone mice may provide novel mechanisms to test for relevance in explaining some increased infectious complications in SLE patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- MRL-Fas^{lpr} lupus-prone mice developed enhanced cellular immunity, but reduced humoral immunity to influenza A virus.
- Influenza A virus infection did not exacerbate autoimmune disease symptoms in lupus-prone mice.
- Splenomegaly and kidney disease severity were transiently reduced during influenza infection.
- After influenza A viral infection, lupus-prone mice developed severe pulmonary inflammation.
- Our findings offer new insights into the mechanisms underlying autoimmune pathogenesis and the protective immune response in SLE.

Figure 1. MRL-Fas^{lpr} mice clear IAV infection with reduced morbidity and less pulmonary pathology

Ten to 12 week old B6, MRL, and MRL-Fas^{lpr} mice (females only) were infected i.n. with 200 EID₅₀ of PR8 IAV or left uninfected. All PBS mice in Figure 1 are MRL-Fas^{lpr} mice. Weights were measured daily and the percent starting weight was calculated and plotted over time for 20 days (a). In separate experiments, on days 0, 7, 10, or 20 post infection, the middle right lobe was collected to access viral burden by expression of the influenza M1 gene by RTPCR (b). Formalin-fixed paraffin embedded (FFPE) serial sections were stained with hematoxylin and eosin (H&E) at day 7 post infection to examine pulmonary pathology. Representative images were captured with .5X and 10X objective with original magnification at x5 and x100, respectively. Black boxes in .5X image represent the area of 10X image (c). The severity of pulmonary inflammation was blindly scored on a scale of 0-4 on day 7 post infection, with a score of 4 being the most severe (d). The number of specific cell populations in the lung was determined at day 10 post infection by flow cytometry (e). CD4+ and CD8+ T cells were gated using forward by side scatter, then by gating on CD3+ cells following by gating on the respective CD4 or CD8 marker. B cells were gated on forward by side scatter and CD19+ and B220+ cells. Total myeloid cells were gated by size using forward by side scatter and CD11c+ and/or CD11b+ expression and neutrophils were further calculated based on CD11b+ and GR1+ expression. Total frequencies were used to back calculate total numbers per lung. The data points represent the mean (±SD) of values from 4-6 mice (a-e). ns=not significant, *p 0.05, **p 0.005, ***p 0.0005.

Figure 2. Increased IAV-specific T cells and higher TNFα levels are found in the IAV infected lung of MRL-Fas^{lpr} mice

Mice were infected as in Figure 1. All PBS mice in Figure 2 a-d are MRL-Fas^{lpr} mice. The number of antigen (Ag)-specific cells were determined by flow cytometry on day 7 post infection. The total number CD8+ T cells producing TNFα (a) and granzyme B (c), and CD4+ T cells producing TNFα (b) and IFNγ (d) following stimulation with inactivated IAV are shown. Total cytokine levels of TNFα (e), IFNγ (f) and IL-10 (g) in the lung were determined from lung homogenates by ELISA. The data points represent the mean (±SD) of values from 4-6 mice (a-g). ns= not significant, *p 0.05, **p 0.005, ***p 0.0005.

Figure 3. Virus-specific antibodies are severely reduced while autoantibody production remains unaltered in IAV-infected MRL-Fas^{lpr} mice

All mice were infected as in Figure 1. At days 10 (black) and 20 (white) post infection blood was collected and processed for serum. Serum was either treated with RDE for influenza antibody analysis or used untreated for autoantibody analysis. Total IgM (a) and IgG (b) IAV-specific antibodies were determined by ELISA. Neutralization capacity was determined by the hemagglutination inhibition assay and HAI titer was calculated (c). Dilutions were made and total IgG (d) and autoantibody concentrations for total anti-nuclear antibodies (e) and anti-dsDNA antibodies (f) were analyzed in uninfected and infected mouse sera by ELISA. The data points represent the mean ($\pm$ SD) of values from 4-6 mice (a-f). ns= not significant, *p 0.05.

Figure 4. IAV-infected MRL-Fas^{lpr} mice have temporary reduction in autoimmune organ damage

Splenomegaly and kidney function were assessed at day 10 and day 20 post infection in IAV-infected mice as described in Figure 1. Spleens were taken and weighed before further analysis (a). Serum creatinine was measured in IAV infected and PBS treated mice at day 20 post infection (b). Representative kidney sections stained with periodic acid-Schiff (PAS) from B6, MRL, and MRL-Fas^{lpr} mice that were infected or not infected with IAV at 10-12 weeks of age (c). Representative glomeruli are shown in each section. Kidney sections were scored in a blinded analysis in PAS stained kidney sections based on both acute (mesangial matrix expansion, focal necrosis and glomerular hypercellularity) and chronic (interstitial fibrosis, medullary inflammation, glomerulosclerosis, and tubular atrophy) glomerulonephritis parameters on a scale of 0-10. Scores were combined for each kidney section to give a cumulative acute (d) and chronic (e) kidney disease score. The data points represent the mean ($\pm$ SD) of values from 4-6 mice (a-e). ns= not significant, *p 0.05, **p 0.005, ***p 0.0005.

Figure 5. Extrinsic influenza-induced apoptosis alleviates splenomegaly in MRL-Fas^{Δpr} mice
Mice were infected as previously described (Figure 1). Spleens from infected and uninfected mice at day 10 post infection were formalin-fixed paraffin embedded and terminal deoxynucleotidyltransferase-mediated dUDP nick-end labeling (TUNEL, *green*) stained to determine cells undergoing apoptosis (red arrows point to TUNEL expressing cells). Representative images are shown (20x objective) (a). Original magnification x200. Apoptotic cells were quantitated by counting the number of TUNEL+ cells per 20x field (10 images taken of individual mouse lobes) (b). IAV infected MRL-Fas^{Δpr} mice were treated with caspase-8 inhibitor (Z-IETD-FMK, 0.1 μM) or a negative control (Z-FA-FMK) daily for 6 days starting at day 4 post infection. Mice were sacrificed at day 10 post infection and spleens were weighed (c). PBS treated mice are MRL-Fas^{Δpr} mice (c). The data points represent the mean (±SD) of values from 4-6 mice (a-c). ns= not significant, *p 0.05, **p 0.005.

Figure 6. Lupus prone MRL-FAS^{lpr} mice are unable to resolve pulmonary inflammation following IAV infection

MRL and MRL-Fas^{lpr} mice were infected with IAV PR8 with 200 EID⁵⁰. Mice were sacrificed at day 40 post infection and lung tissue was formalin fixed and paraffin embedded for pathology analysis. Slides were H&E stained (a) and scored for disease severity on a scale of 0-4 (b). Representative pictures were taken using a Zeiss Axioplan Microscope at 0.5x and 10x objective, original magnification of x50 and x100, respectively. Black boxes in .5X image represent the area of 10X image. Spleen weight was measured in grams for each individual mouse spleen (c). Kidneys were formalin fixed and paraffin embedded before staining with PAS. Both acute and chronic disease parameters (as mentioned in Figure 5) were blindly scored for each mouse kidney and scores were combined for a cumulative score of acute (d) and chronic (e) glomerulonephritis. The data points represent the mean ($\pm$ SD) of values from 4-6 mice (a-e). ns= not significant, *p 0.05, **p 0.005, ***p 0.0005.