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Tofacitinib ameliorates murine lupus and its associated vascular dysfunction

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Abstract

Objectives—Dysregulation of innate and adaptive immune responses contributes to the pathogenesis of systemic lupus erythematosus (SLE) and its associated premature vascular damage. To date, no drug targets both systemic inflammatory disease and the cardiovascular complications of SLE. Tofacitinib is a Janus kinase (JAK) inhibitor that blocks signaling downstream of multiple cytokines implicated in lupus pathogenesis. While clinical trials have shown that tofacitinib exhibits significant clinical efficacy in various autoimmune diseases, its role in SLE and on its associated vascular pathology remains to be characterized.

Methods—MRL/*lpr* lupus-prone mice received tofacitinib or vehicle by gavage for 6 weeks (therapeutic arm) or 8 weeks (preventive arm). Nephritis, skin inflammation, serum autoantibody levels and cytokines, mononuclear cell phenotype and gene expression, neutrophil extracellular trap (NET) release, endothelium-dependent vasorelaxation and endothelial differentiation were compared in treated and untreated mice.

Results—Treatment with tofacitinib led to significant improvement in measures of disease activity including nephritis, skin inflammation, and autoantibody production. In addition, tofacitinib treatment reduced serum levels of pro-inflammatory cytokines and interferon responses in splenocytes and kidney tissue. Tofacitinib also modulated NET formation and significantly

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Contributors YF designed and performed experiments and wrote the manuscript. LB, WZ, CKS, WLT, AMT, LM, SGT, AZ, and GS performed experiments and contributed to statistical analysis. VH, LN and SB analyzed data. ATR, JJO MJK and MG conceived and designed study and wrote the manuscript.

Competing interests YF, MG, MJK, and JJO have received research support based on a Cooperative Research and Development Agreement (CRADA) from Pfizer Inc. WZ, CKS, SB, SGT, WLT, AMT, LM, VH, and ATR have nothing to disclose.

increased endothelium-dependent vasorelaxation and endothelial differentiation. The drug was effective as both preventive and therapeutic strategies.

Conclusions—Tofacitinib modulates the innate and adaptive immune responses, ameliorates murine lupus and improves vascular function. These results indicate that JAK inhibitors have the potential to be beneficial in SLE and its associated vascular damage.

Keywords

Tofacitinib; SLE; vascular function; inflammatory cytokines; NET release

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic, heterogeneous autoimmune syndrome that primarily afflicts young women, targeting many organs and tissues but especially the kidneys and skin. A key feature of SLE is dysregulation of innate and adaptive immune responses associated with loss of tolerance and the production of autoantibodies (1) (2). T cells are considered to play major roles in SLE by amplifying autoimmune responses once tolerance is compromised. Indeed, lupus T cells display aberrant cytokines secretion and cell signaling properties and promote inappropriate recruitment and activation of B cells and dendritic cells (DCs) at sites of inflammation (3). A consistent finding in SLE is exaggerated interferon (IFN) production, thought to play key roles in disease pathogenesis (3) (4) (5) (6). Overproduction of type I IFNs may also play pivotal roles in the premature development of atherosclerosis observed in SLE (7) (8, 9) (10). In addition, the interplay between type I IFNs and aberrant neutrophil function with enhanced capacity to form neutrophil extracellular traps (NETs), has recently been proposed as a key proinflammatory loop that can further propagate immune dysregulation and tissue damage (2). Because of its complexity and clinical heterogeneity, treatment of SLE is challenging and current therapies, while effective, are associated with significant complications. Importantly, no drug to this date has demonstrated efficacy in reducing cardiovascular (CV) risk in SLE.

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway is the major signaling cascade downstream of type I and type II cytokine receptors. JAKs have been shown to be an effective therapeutic target for various autoimmune and inflammatory diseases driven by cytokines (11) (12). Tofacitinib is a first-generation JAK inhibitor (Jakinib) that inhibits JAK1, JAK3, and, to a lesser extent, JAK2 (13) (14). Since common γ chain-using cytokines use JAK1 and JAK3, tofacitinib efficiently blocks their signaling cascades. Tofacitinib also blocks Gp130-using cytokines as well as signaling downstream of type I/II IFNs. Tofacitinib may therefore have diverse effects on cytokines and cells that contribute to SLE pathogenesis, including subsets of $CD4^+$ T cells (helper T cells including Thelper1 (Th1), pathogenic Th17 cells), $CD8^+$ T cells, B cells and innate immune cells (15) (16) (17). Although tofacitinib has been approved by regulatory agencies for the treatment of rheumatoid arthritis (RA) and is in clinical trials exploring its use in psoriasis, psoriatic arthritis, ulcerative colitis, Crohn's disease, and ankylosing spondylitis, its effects in SLE have not been investigated (11). Moreover, while some preliminary studies indicate that the JAK-STAT pathway can modulate vascular repair, the role of JAK inhibitors in SLE-associated vasculopathy is unknown (18) (19).

The lupus mouse model MRL/MpJ-Fas$^{lpr/lpr}$/J (MRL/*lpr*) recapitulates several of the clinical manifestations and immune dysregulation observed in human SLE. Indeed, these mice develop immune-complex glomerulonephritis, inflammatory skin disease, aberrant T cell responses and dysregulated inflammatory cytokine synthesis and an enhanced type I IFN signature (20) (21) (22). Furthermore, MRL/*lpr* mice also represent a good model to study vascular dysfunction and neutrophil dysregulation characteristic of lupus (23). Therefore, this animal model was used to explore the effects of tofacitinib in preventing and treating murine lupus. We found that pharmacologic inhibition of the JAK-STAT pathway significantly ameliorated the lupus clinical phenotype and modulated features of dysregulated innate and adaptive immunity characteristic of this disease. Importantly, tofacitinib significantly improved vascular parameters suggesting a potential modulatory role in CV risk in this disease.

METHODS

Mice and treatment

Female MRL/*lpr* (#000485 The Jackson Laboratory, Bar Harbor, ME) and haplotype-, age- and sex-matched control MRL/MpJ (#000486, The Jackson Laboratory) were maintained in specific-pathogen-free conditions and used in accordance with NIH guidelines under the NIAMS-approved animal study protocol #A015-02-01. Tofacitinib (Pfizer Research Laboratories) was suspended in 0.5% methylcellulose/0.025% Tween 20 (Sigma-Aldrich, St. Louis, MO) for *in vivo* studies or in DMSO for *in vitro* use. MRL/*lpr* mice received daily vehicle or tofacitinib (10mg/kg) for 6 weeks (therapeutic treatment trial) or 8 weeks (preventive treatment trial) (Figure 1A) by oral gavage.

Assessment of lupus nephritis and histopathology

Kidneys and dorsal skin samples were harvested after perfusion with PBS and fixed with 10% buffered formalin (Sigma, St. Louis, MO) and sections stained with hematoxylin and eosin (H&E). Clinical scores for nephritis and skin inflammation were obtained in a blinded fashion. Histological analysis for glomerular pathological features included: inflammation, proliferation, crescent formation, and necrosis. A minimum of thirty glomeruli, of at least 10 mice per group, were scored. For each glomerulus, a score from 1 to 5 (1, normal; 2, moderate; 3, severe; 4, severe with crescent formation; 5, necrosis) was used. Scores from each individual mouse were added and averaged to yield the glomerulonephritis score. A total score of glomeruli was measured as sum of the severity of hypercellularity of glomeruli, distribution of hypercellularity of glomeruli, and sclerotic glomeruli. For assessment of skin pathology, a score from 0 to 3 (0, normal; 1, mild: 2, moderate; 3, severe) in epidermis and dermis were given and evaluated separately. Total skin clinical score was indicated as sum of epidermis and dermis inflammation. For both kidney and skin histology samples, scores from each individual mouse were added and averaged to achieve a clinical score. Immune complex deposition in kidney sections was processed and quantified as previously described (23) (24). Urinary albumin and creatinine concentrations were measured using a mouse albumin ELISA (Alpha Diagnostic Intl. San Antonio, TX) and a creatinine assay (R&D systems, Minneapolis, MN), respectively. Urine albumin:creatinine

ratios were then calculated. Blood urea nitrogen (BUN) and blood creatinine were measured by NIH hematology section.

Cell isolation and flow cytometry

Spleens were harvested, weighed and splenocytes isolated as previously described (24). Total splenocyte numbers were counted using a Nexcelom automatic cell counter. Lymphocyte populations were gated, (100,000 cells of the lymphocyte populations) and counted by flow cytometry. CD3+ T cell population and $B220^{+}$ B cell population were counted after exclusion of the NK cell population. Antibodies were all from commercial sources and are listed below. Flow cytometry acquisition was done with a FACSCanto (BD Biosciences, San Jose, CA). Data analysis was performed using the Flowjo software (Tree Star Inc.). TCRβ-FITC, CD3-APC-Cy7, CD4-V500, CD8-AF700, B220-PECy7, CD19-PerCPCy5.5, TCRβ-PerCPCy5.5, CD4-V500 were obtained from BD Bioscience. CD11c-APC eF780 was obtained from eBioscience (eBioscience, San Diego, CA).

Quantification of serum autoantibodies and cytokines

Serum antinuclear antibodies (ANA) and anti-dsDNA antibodies (ds-DNA) were quantified by ELISA following manufacturer's instructions (Alpha Diagnostic International, San Antonio, TX). Serum cytokines were quantified with the BioPlex Pro Mouse Cytokine Assay (BioRad, Hercules, CA).

Assessment of IFN-inducible genes (ISGs) and JAK-STAT related gene expression in total splenocytes and kidney

Following euthanasia, kidney tissues were harvested and immediately frozen in RNAlater Stabilization Solution (Thermo Fisher Scientific, Waltham, MA). Total RNA was isolated from kidney tissues and splenocytes using the *mir*Vana miRNA kit (Life Technologies, Frederick, MD) and quantified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). For qPCR, cDNA was synthesized using a Taqman reverse transcription kit (Life Technologies, Frederick, MD) and analysis performed with a CFX96 Real Time Detection System (BioRad, Hercules, CA) with specific primers and probes (Life Technologies, Frederick, MD). The comparative threshold cycle and an internal control (18S rRNA) were used to normalize the expression of the target genes. RNA from MRL/MpJ mice was used as a control. The fold induction in gene expression was calculated by dividing ΔΔct values from vehicle-treated MRL/lpr vs MRL/MpJ or tofacitinib-treated MRL/lpr vs MRL/MpJ. The following primers and probes were utilized: *Isg15*, Mm01705338_s1; *CXCL10*, Mm00445235_m1; *MX1*, Mm00487796_m1; *STAT1*, Mm00439531_m1; *STAT2*, Mm00490880_m1; *IFIT1*, Mm00515153_m1. Expression levels of 547 immune-related genes were analyzed using the nCounter Mouse Immunology codeset (NanoString Technologies, Seattle, WA) following the manufacturer's protocols. Data were normalized based on internal reference genes provided by the manufacturer.

Quantification of NET release, endothelial cell differentiation, and assessment of endothelium-dependent vasorelaxation

Tibias and femurs were collected at euthanasia. Bone marrow neutrophils were obtained as previously described and plated at a density of 0.25 $\times 10^6$ cells/cm^2 in RPMI 1640 without phenol red (23). For the Sytox assay (see below) cells were seeded on poly-lysine coated coverslips for a 15 minutes adherence step. Upon adhesion, neutrophils were left untreated or treated with lipopolysaccharide (LPS, 500 ng/mL, Sigma, St. Louis, MO) for 5 hours before fixation with 4% PFA.

Fluorescence microscopy

Coverslips were stained with anti-myeloperoxidase (1:500, Dako, Carpinteria, CA) and Hoechst 33342 (1:10,000, Technologies, Frederick, MD) before mounting with ProLong Gold Antifade (Life Technologies). Cells were analyzed by fluorescent microscopy using a Zeiss LSM780 confocal microscope (Zeiss). Pictures were taken using the 40X objective (×400 total magnification). Percent of neutrophils forming NETs were counted using Adobe Photoshop CS6 (San Jose, CA). EPCs were cultured and stained as previously described (25).

Sytox green uptake assay

Untreated or LPS-treated neutrophils were incubated with 0.2 μM Sytox Green (Life Technologies, Frederick, MD) for 5 hours, then fixed as stated above. The resulting fluorescence from extracellular DNA (NETs) was quantified (excitation 485 nm, emission 520 nm) using a FLUOstar Omega spectrophotometer (BMG Labtech, Offenburg, Germany).

Assessment of endothelium-dependent vasorelaxation

After euthanasia, thoracic aortas were excised, cleaned, and cut into 2-mm length rings. Endothelium was left intact, and aortic rings were mounted in a myograph system (Danish Myo Technology A/S). Vessels were pre-contracted with potassium sorbate sphere (PSS) containing 100 mM potassium chloride (KPSS) and then returned to PSS. Cumulative concentrations of phenylephrine (PE) (10^{-9} M to 10^{-6} M) were then added to the bath to establish a concentration-response curve. A PE concentration corresponding to 80% maximum was added, and contraction was allowed to reach a stable plateau. To examine endothelium-dependent relaxation, acetylcholine (Ach, 10^{-9} M to 10^{-6} M) was added cumulatively to the bath and a curve was generated. Finally, a normal vascular smooth muscle response was confirmed by removing PE and Ach.

Plasma Lipid and Lipoprotein Analysis

Plasma obtained at euthanasia was separated by centrifugation for 10 min at 3000×g at 4°C, and analyzed for total and free cholesterol levels using Cholesterol-E and Free Cholesterol-E kits (Wako Diagnostic, Mountain View, CA). Cholesteryl ester was calculated for each sample by subtracting free cholesterol from the total cholesterol for each sample. To calculate HDL-C levels, LDL was precipitated using the Cholesterol, HDL Test and the precipitating reagents (Stanbio Laboratory, Boerne, TX). Levels of total and free cholesterol

were determined as for the complete serum samples. LDL-C levels were calculated by subtracting the amount of HDL-C from the total serum cholesterol. Free cholesterol and cholesteryl esters were calculated in a similar manner.

Statistical analysis

To calculate statistical significance, a nonparametric Mann-Whitney test was used, unless otherwise specified. A value of $p < 0.05$ was considered statistically significant. For endothelium-dependent vasorelaxation, curves were first analyzed using an asymmetric (five parameters) logistic equation, and significance of each individual data point was determined by two-way ANOVA. Unless otherwise specified, results are represented as mean ± SEM.

RESULTS

Tofacitinib preventive treatment limits lupus manifestations in MRL/*lpr* mice

MRL/*lpr* mice spontaneously develop a severe SLE-like phenotype characterized by autoantibodies to nuclear antigens, glomerulonephritis, and skin inflammation. To assess if JAK inhibition prevented immunopathology, MRL/*lpr* mice started tofacitinib treatment at 10 weeks of age when they lacked any overt lupus manifestations (Figure 1A). At 18 weeks in the preventive treatment, control, vehicle-treated MRL/*lpr* mice had high levels of anti-ANA and anti-dsDNA antibodies compared to control MRL/MpJ mice, whereas tofacitinib treatment blocked the appearance of autoantibodies (Figure 1B). MRL/*lpr* mice also developed renal disease as manifested by glomerular hypercellularity and immune complex deposition. Tofacitinib prevented renal pathology as judged by changes in histopathologic scores and immune complex deposition (Figure 1C). Renal pathology can also be assessed by measuring proteinuria (urine albumin:creatinine ratio). In tofacitinib treated MRL/lpr mice, the level of proteinuria was significantly reduced compared with the controls (Figure 1D). Additionally, we found a reduction in blood urea nitrogen (BUN) serum levels in tofacitinib treated MRL/*lpr* compared with control mice, although this was not significant (Supplemental Figure 1). MRL/*lpr* mice typically develop an erythematous, scarring rash reminiscent of human lupus (26) (27). Vehicle-treated mice developed facial and dorsal skin hyperplasia, rash, and alopecia more frequently than tofacitinib-treated mice (Supplemental Figure 2). Furthermore, histologic analysis showed significantly reduced hyperplasia and skin inflammatory infiltrates in tofacitinib-treated mice compared to vehicle-treated mice (Figure 1E). Overall, tofacitinib significantly limited renal damage and skin inflammation in the preventive arm treatment.

A characteristic immunopathological feature of disease in MRL/*lpr* mice is massive splenomegaly and lymphadenopathy caused by hyperproliferation of T and B cells and expansion of double-negative (DN) $CD3^+$ $CD4^-$ $CD8^-$ T cells (28). Therefore, we next compared splenic cell subsets in the preventive tofacitinib-versus vehicle-treated groups. Spleens from the tofacitinib-treated mice were reduced in size and total cell number when compared to vehicle-treated mice (Figure 2A left panel and Supplemental Figure 3A). Moreover, total numbers of T cells, (Figure 2A right panel) $CD8^+$ T cells and DN T cells were significantly reduced, whereas $CD4^+$ T cells were not (Figure 2B) (29). In contrast, $CD4^+$ T cells, total B cells, $CD138^+$ B cells. IgD^+ B cells or dendritic cells were not

significantly reduced (Figure 2C and Supplemental Figure 3B). Our data suggested that tofacitinib had the greatest effects on T cells homeostasis in the preventive model.

Because tofacitinib affects cytokines that can impact hematopoiesis including red blood cells (11), we also examined this outcome. Significant reduction in red blood cells, hemoglobin concentration and hematocrit were evident after 8 weeks of tofacitinib treatment (Table 1). No changes were seen in neutrophils, monocytes, eosinophils, and basophils numbers. Overall, these results indicate that tofacitinib ameliorates the clinical features of murine lupus with expected effects on erythrocytes and lymphoid cells but not myeloid cells.

Tofacitinib decreases pro-inflammatory cytokines and modulates type I IFN responses in MRL/lpr mice

Overproduction of an array of cytokines is an important contributor to immunopathology in human and murine models of lupus (3). Vehicle-treated MRL/*lpr* mice had elevated levels of proinflammatory cytokines including TNF-α, IFN-γ, IL-17A, IL-2, and MIP1-α (CCL3) and the preventive treatment with tofacitinib normalized cytokine levels (Supplemental Figure 4). Overproduction of IFNs is also a hallmark of lupus and can be measured by expression of IFN-stimulated genes (ISGs) in tissues (7) (30). Expression of multiple ISGs was significantly reduced in the splenocytes of tofacitinib-treated mice compared to the vehicle-treated mice in the preventive trial (Figure 3A and 3B). Furthermore, increased expression of ISGs was observed in the renal tissue of MRL/lpr mice, and tofacitinib administration resulted in significant decrease of ISGs expression (Figures 3C). Overall, these results indicate that preventive administration of tofacitinib was effective at suppressing IFN-dependent and JAK-STAT-related genes and significantly limited synthesis of proinflammatory cytokines all events implicated in lupus pathogenesis.

Tofacitinib significantly decreases NET release

Recent evidence implicates dysregulated NET formation and clearance as a mechanism leading to enhanced exposure of modified nuclear autoantigens, amplification of type I IFN and other proinflammatory responses, and induction of vascular damage in human and murine SLE. Indeed, we previously showed that MRL/*lpr* mice display enhanced spontaneous NETosis that promotes pDC activation leading to increased type I IFN responses (2) (23). As the JAK/STAT pathway is functional in neutrophils and cytokines can prime NETosis, we determined if tofacitinib modulates NET formation in bone marrow-derived neutrophils. While the numbers of total neutrophils in the peripheral blood and in the bone marrow were unchanged with treatment (Table 1 and data now shown), both spontaneous and LPS-induced NETosis were significantly decreased in neutrophils obtained from tofacitinib-treated mice (Figure 4A and 4B).

Tofacitinib administration improves endothelium-dependent vasorelaxation, endothelial cell differentiation and lipoprotein profiles in MRL/lpr mice

We previously demonstrated that MRL/*lpr* mice exhibit impairments in endothelium-dependent vasorelaxation, suggesting endothelial dysfunction (23). Moreover, type I IFNs have been implicated in premature vascular damage in human and murine lupus as well as in the induction of abnormal phenotype of endothelial progenitor cells (EPCs) involved in

vascular repair (31) (32). Given the effect of tofacitinib on several cytokines potentially involved in vascular dysfunction, we assessed if the drug would modulate vascular function and repair (18) (19) (33). Indeed, prophylactic tofacitinib treatment resulted in significant improvements in endothelium-dependent vasorelaxation (Figure 4C). Furthermore, the capacity of EPCs to differentiate into mature endothelial cells *in vitro*, following proangiogenic stimulation, was significantly increased in the tofacitinib group (Figure 4D and 4E).

Recent studies in RA patients indicated that tofacitinib has beneficial effects on the lipoprotein metabolism by modifying cholesterol ester catabolism and high density lipoprotein (HDL) function (34). Similarly to the human RA studies, we observed that total HDL cholesterol was significantly increased in the tofacitinib-treated mice, with a more prominent effect on HDL cholesteryl ester, indicating that tofacitinib treatment restored lipoprotein homeostasis (Supplemental Figure 5). Overall, these results indicate that JAK/STAT inhibition improves lupus vasculopathy and modulates deleterious lipoprotein profiles in murine lupus.

Therapeutic treatment with tofacitinib limited lupus manifestations in MRL/*lpr* mice

Finally, to determine whether tofacitinib could reverse established disease, drug administration was delayed until signs of active lupus (proteinuria) were evident in all the animals (14 weeks of age), and treatment was continued for 6 weeks (therapeutic treatment arm; Figure 1A). Given that, in the preventive treatment, autoantibody production, proteinuria, endothelial cell differentiation and induction of ISGs were the most significantly altered in response to tofacitinib, we focused our analysis on these parameters for the therapeutic treatment arm. Notably, in the tofacitinib-treated group, we observed significant reduction in anti-dsDNA levels whereas, ANAs were also reduced but not significantly (Figure 5A). Proteinuria was significantly reduced (Figure 5B), and serum BUN and creatinine levels were reduced in the tofacitinib-treated group (Supplemental Figure 6).

Tofacitinib-treated mice displayed a significant increase in endothelial differentiation, and a trend of improvement in endothelial dependent vasorelaxation although in these mice, which were only treated for 6 weeks, the improvement in the vasorelaxation did not reach statistical significance (Figure 5C and Supplemental Figure 7). Importantly, as we previously observed in the preventive treatment, gene expression analysis showed a significant reduction in ISGs in the tofacitinib-treated group. In particular, type I IFN-driven ISGs (*Mx1, Stat1, Isg15, and Ifit1*) were the most significantly reduced (Figure 5D) (35) (36). Taken together, these results indicate that tofacitinib administration results in amelioration of disease-associated features when administered to mice with established lupus features.

DISCUSSION

While the treatments for SLE have improved over the last several decades, current therapies are still suboptimal, promote significant side effects and, to this date, have not shown to consistently modify both lupus disease activity, and the enhanced CV risk characteristic of this disease. We now show that the use of oral Jakinibs, and specifically tofacitinib, a drug that preferentially targets JAK1 and JAK3, has pleiotropic beneficial effects in murine lupus

including: a) significant amelioration of clinical phenotype and organ damage; b) modulation of innate and adaptive immune dysregulation; c) improvement in vascular functions and lipoprotein profiles. The observed effects of tofacitinib on innate and adaptive immune dysregulation likely play key roles in modifying clinical responses and vasculopathy. Indeed, pharmacologic JAK inhibition resulted in significant abrogation in IFN responses, other proinflammatory cytokines considered crucial in lupus pathogenesis, NETosis, and T lymphocyte subsets associated with lupus pathogenicity.

The main effects induced by tofacitinib on adaptive immune cells were a reduction of $CD8^+$ and DN T cells. This is consistent with other inflammatory models driven by cytotoxic $CD8^+$ lymphocytes, where JAK inhibition strongly suppressed these responses (16) (37) (17). In SLE, higher numbers of cytotoxic, effector $CD8^+$ T cells are associated with disease activity and may lead to autoantigen generation through perforin/granzyme-related pathways (38). DN T cells are expanded in the peripheral blood and tissues from SLE patients as well as murine models. These cells synthesize pro-inflammatory cytokines and induce antibody production through promotion of B-cell differentiation (39) (40) (41) (42). Given the significant reductions in splenic DN T cells induced by tofacitinib and the decreases in circulating cytokine levels, it is likely that some of the beneficial effects of this drug in the lupus phenotype, including vasculopathy, are due to repression of such responses (43).

Synthesis of type I IFNs driven by endogenous nucleic acids is considered an early event that primes the immune system for immune dysregulation and development of autoimmunity. As such, strategies that target the type I IFN pathway are actively being investigated (44). As type I IFNs signal through the JAK/STAT pathway, tofacitinib blocked the biological responses to these cytokines that was reflected in significant decreases in the type I IFN signature in these mice. It is likely that inhibition of this pathway by tofacitinib led to pleiotropic effects limiting dysregulation of innate and adaptive immune responses including roles in priming neutrophils to undergo NET formation, alterations in B cell ontogeny, as well as improvements in lupus vasculopathy.

NETs have been proposed to represent an important source of immunostimulatory molecules and modified autoantigens that can trigger loss of tolerance in predisposed hosts. Furthermore, NETs are involved in the induction of type I IFN responses through activation of plasmacytoid DCs. Cytokines, autoantibodies, and immune complexes can trigger enhanced NETs formation and may be involved in neutrophil dysregulation in SLE (2). As tofacitinib treatment led to decreases in all of these lupus-associated features, it is possible that this is an important mechanism by which this drug improves the clinical phenotype as well as vascular dysfunction. We previously showed that human and murine lupus are characterized by profound impairments in endothelium-dependent vasorelaxation and inability of the damaged vasculature to undergo proper vascular repair (45). Importantly, tofacitinib administration led to significant improvements in these parameters. While the mechanisms leading to amelioration of the vasculopathy with tofacitinib are as yet unclear, the significant modulation that this drug promoted in both type I IFN responses and NET formation, both considered important factors in premature endothelial damage, make it likely that these are the key downstream pathways by which this drug was effective (18) (46) (47) (48).

Lipoprotein dysregulation is associated with the development of atherosclerosis and CVD. This is particularly relevant in patients with chronic inflammatory diseases that have enhanced risk for vascular complications (49). Previous evidences have been conflicting with regards to tofacitinib promoting beneficial versus detrimental lipoprotein modulation. Tofacitinib has been reported to increase HDL (34) and recent studies indicate that this drug can restore lipoprotein homeostasis and improve cholesterol efflux capacity in RA patients. (50) (34). The effects of tofacitinib on cholesterol transport was proposed to be resulting from an increase in cholesterol ester production rate through augmented activity of the lecithin-cholesterol acyltransferase (LCAT) (34). Given the changes observed in lipoprotein profiles of tofacitinib-treated mice and in particular the reduction in the fraction of free cholesterol, which has recently been shown to be proatherogenic, the lipoprotein changes induced by tofacitinib could be an additional mechanism by which JAK inhibition could be vasculoprotective in systemic autoimmunity (51). Nevertheless, the molecular events leading to change of lipid balance by JAK inhibition need to be further investigated. Future studies should address the role of tofacitinib in modulation of vascular health and their effects on the endothelium in human SLE.

Various JAK isoform-selective inhibitors are currently being developed and tested in preclinical models and early-stage clinical trials (14). The availability of such reagents will allow us to better define the molecular events leading to the efficacy of tofacitinib and new Jakinibs in SLE and SLE-associated CVD. While the mice in this study tolerated the drug well despite a drop in RBCs, presumably due to JAK2-mediated erythropoietin inhibition (52), in future studies, it will be important to assess the effect of Jakinibs on predisposition to infections and overall immune surveillance.

In summary, tofacitinib administration to lupus-prone mice resulted in significant improvements in clinical phenotype, immune dysregulation and vasculopathy characteristic of this disease. These results suggest that modulation of the JAK/STAT pathway has the potential to be beneficial in the treatment of SLE and its associated vascular damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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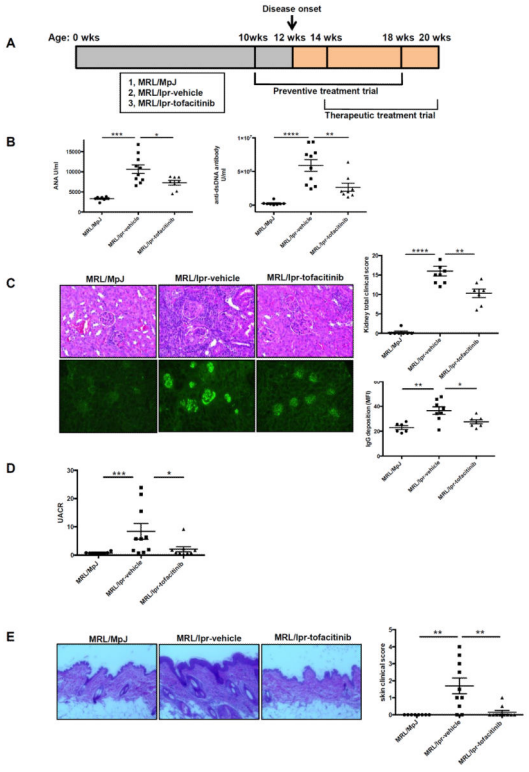

Figure 1. Tofacitinib improves clinical parameters of murine lupus

(A) Schematic representation of tofacitinib treatment regimens. MRL/*lpr* mice were treated with either vehicle or tofacitinib by gavage once daily for 8 weeks between 10 weeks and 18 weeks of age for the preventive treatment, and for 6 weeks between 14 weeks and 20 weeks of age for therapeutic treatment. (B) ANA and anti-dsDNA serum levels in the preventive treatment group. (C) Representative H&E microphotographs of MRL/*lpr* kidneys (preventive treatment) (top panel) and immunoglobulin G (IgG) deposition staining (bottom panel) are shown (MRL/MpJ: left panel, vehicle-treated: center panel, and tofacitinib-treated: right panel). Images were visualized using a 20x (0.75NA) magnification. Glomerulonephritis clinical score (top right, detail described in materials and methods) and mean fluorescence intensity (MFI) level of the IgG deposition (bottom right). MFI was evaluated using the ImageJ software. (D) Urine albumin:creatinine ratio of the preventive treatment group. (E) Representative H&E microphotographs of dorsal skin biopsies. Images were visualized using a x20 (0.45NA) magnification. Skin clinical score (right, detail described in materials and methods). For all experiments, n=7 to 13 mice per group. Results represent mean ± SEM. Statistical significance was calculated using the Mann-Whitney test: *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

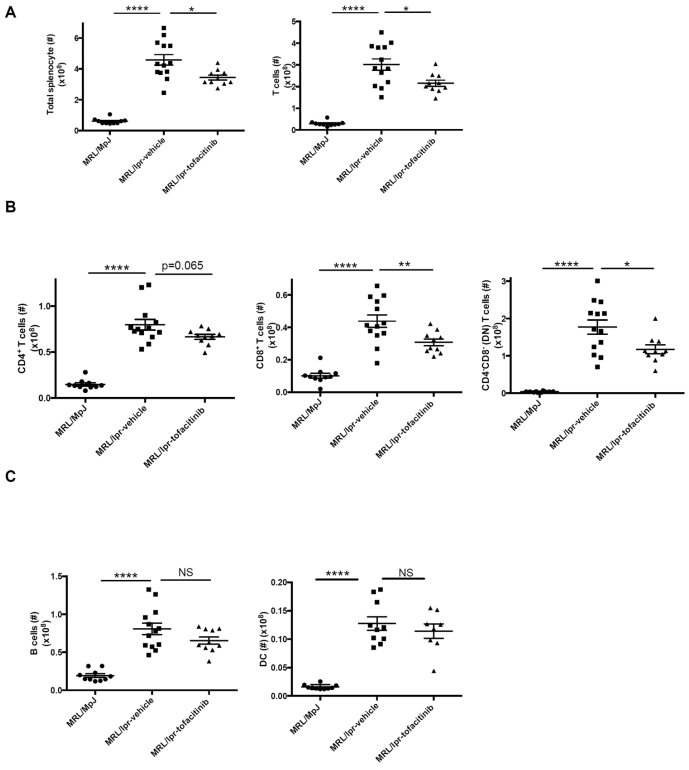

Figure 2. Tofacitinib modulates the number of lymphocytes and dendritic cells in the spleen

Cells were counted at the end of 8 weeks of treatment at 18 weeks of age of the preventive treatment. (A)Total numbers of splenocytes (upper left), T cells (upper right panel). (B) Total numbers of CD4+ T cells (left panel), CD8+ T cells (center panel), and $CD4^{-}CD8^{-}$ (right panel) T cells. (C) B cells (left panel), and DCs (right panel). For all experiments, n=7 to 13 mice per group. Results represent mean ± SEM. Statistical significance was calculated using the Mann-Whitney test : $*p < 0.05$, $**p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

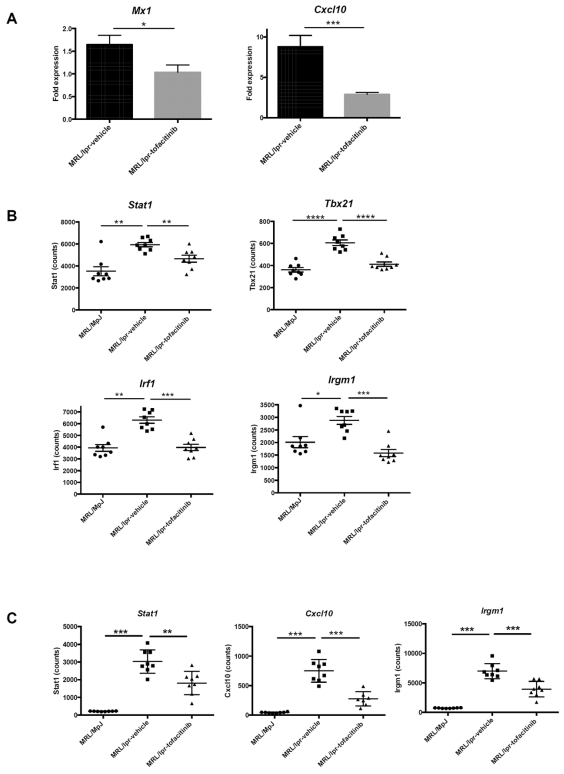

Figure 3. Tofacitinib modulates gene expression in murine lupus

(A) ISGs (*Mx1, and Cxcl10*) were quantified by RT-qPCR. (B) and (C) Expression of JAK-STAT-regulated genes and additional ISGs in splenocytes (B) and kidney (C), measured by NanoString Technology. For all experiments, n=8 mice per group. Results represent mean ± SEM. Statistical significance was calculated using the Mann-Whitney test: *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

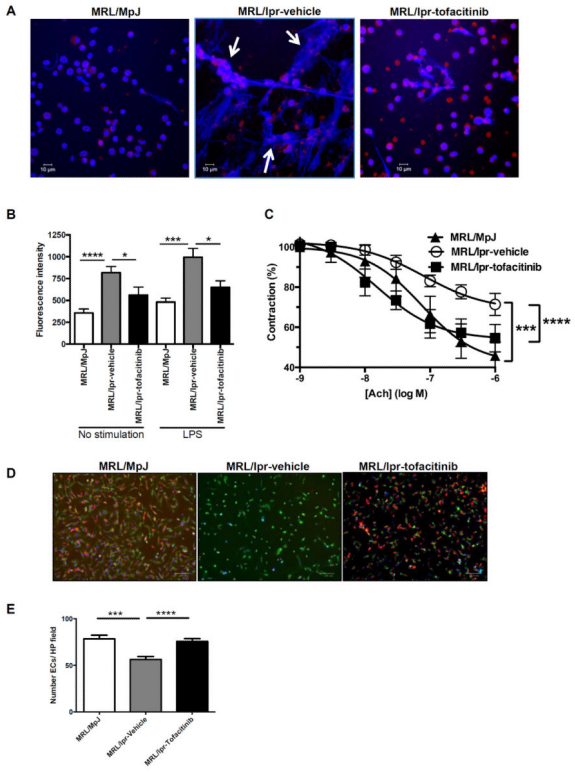

Figure 4. Tofacitinib reduces NET formation and ameliorates endothelium-dependent vasorelaxation and endothelial cell (EC) differentiation in murine lupus

Bone marrow-derived neutrophils from MRL/MpJ mice, MRL/*lpr* mice treated with vehicle, or MRL/*lpr* mice treated with tofacitinib of the preventive treatment at 18 weeks of age. (A) LPS-induced NETs (white arrows) were detected by extracellular colocalization of DNA (blue) and myeloperoxidase (red) staining (x63 magnification). Representative fluorescent images are shown. (B) Extracellular DNA release by neutrophils was detected using SYTOX green dye. For all experiments, n=10 mice per group. (C) Isolated aortas were exposed to phenylephrine (PE)-induced maximal contraction, followed by increasing concentrations of acetylcholine (Ach), to measure endothelium-dependent vasorelaxation. Statistical significance was analyzed by 2 way ANOVA. $*p < 0.05$, $**p < 0.01$. (D–E) Mature endothelial cells (ECs) were quantified as those displaying dual uptake of Hoechst (blue) acetylated-LDL (red) and BS-lectin (green). Representative fluorescent images are shown. Experiments were performed in triplicates with 3 photomicrographs taken per well (MRL/*MpJ*, vehicle-treated MRL/*lpr*, tofacitinib-treated MRL/*lpr*, scale bar = 100 μm, HP = high-power). For all experiments, n=8 to 10 mice per group. Results represent mean ± SEM. Statistical significance was calculated using the Mann-Whitney test : $*p < 0.05$, $**p < 0.01$, and *** $p < 0.001$.

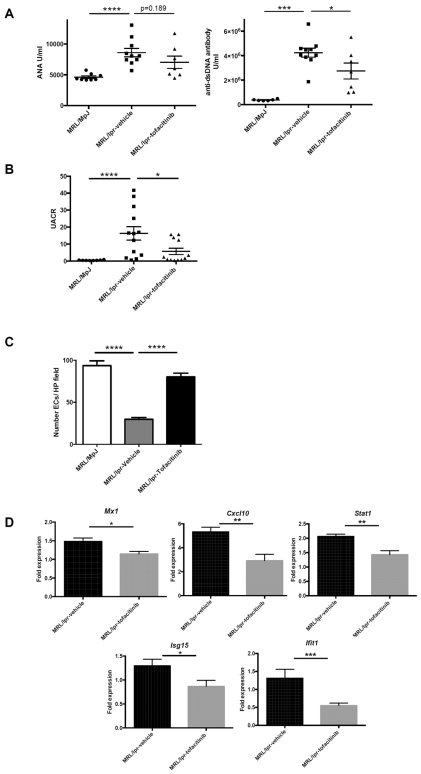

Figure 5. Tofacitinib improves established SLE-like phenotype

(A) and (B) ANA and anti-dsDNA serum levels and albumin:creatinine ratio at 20 weeks of age in the therapeutic treatment group. (C) Mature endothelial cells (ECs) were quantified as those coexpressing Hoechst (blue) acetylated-LDL (red) and BS-lectin (green). Experiments were performed in triplicates with 3 photomicrographs taken per well (MRL/*MpJ*, vehicle-treated MRL/*lpr*, tofacitinib-treated MRL/*lpr*, scale bar = 100 μm, HP = high-power). (D) ISGs were quantified by RT-qPCR. For all experiments, n=7 to 13 mice per group. Statistical significance was calculated using the Mann-Whitney test; *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

Table 1

Complete blood count (CBC) in the preventive treatment arm

	MRL/MpJ	MRL/lpr-vehicle	MRL/lpr-tofacitinib	*p* value vehicle vs tofacitinib
WBC (K/ul)	3.00 ± 1.55	12.77 ± 3.64	10.35 ± 4.31	*p*=0.1909
RBC (M/ul)	8.56 ± 0.55	7.92 ± 0.52	7.32 ± 0.72	*, *p*=0.0431
Hemoglobin (g/dL)	13.76 ± 0.84	11.85 ± 0.7	10.78 ± 1.17	*, *p*=0.0209
Hematocrit (%)	40.33 ± 2.60	34.7 ± 2.16	31.86 ± 2.63	*, *p*=0.016
MCV (fL)	47.11 ± 0.74	43.85 ± 1.26	43.61 ± 0.98	*p*=0.6414
Platelets (K/ul)	779.88 ± 159.74	647.73 ± 168.18	614.78 ± 255.07	*p*=0.7326
Polys (%)	25.73 ± 4.84	12.22 ± 4.23	12.74 ± 8.58	*p*=0.8604
Lympho (%)	47.64 ± 10.84	66.85 ± 10.06	64.20 ± 11.57	*p*=0.5896
Mono (%)	14.36 ± 6.65	12.01 ± 7.07	17.11 ± 8.45	*p*=0.1583
Eosino (%)	2.75 ± 1.52	2.78 ± 6.26	1.67 ± 1.41	*p*=0.6084
Baso (%)	9.55 ± 3.10	6.15 ± 3.31	4.28 ± 4.57	*p*=0.3031
Poly (K/ul)	0.75 ± 0.37	1.57 ± 0.77	1.29 ± 0.79	*p*=0.4275
Lympho (K/ul)	0.75 ± 1.03	1.57 ± 3.22	1.29 ± 3.55	*p*=0.2368
Mono (K/ul)	0.39 ± 0.21	1.40 ± 0.66	1.62 ± 0.74	*p*=0.478
Eosino (K/ul)	0.08 ± 0.06	0.12 ± 0.12	0.16 ± 0.16	*p*=0.4406
Baso (K/ul)	0.26 ± 0.11	0.71 ± 0.34	0.46 ± 0.48	*p*=0.181

CBC count has been done by NIH Hematology Section