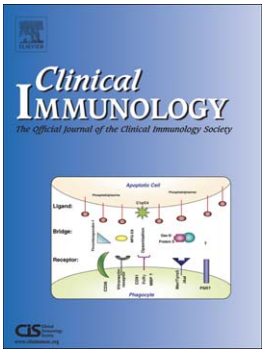


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# Btk Inhibition Treats TLR7/IFN Driven Murine Lupus

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**ABSTRACT**

Bruton's tyrosine kinase (Btk) is expressed in a variety of immune cells and previous work has demonstrated that blocking Btk is a promising strategy for treating autoimmune diseases. Herein, we utilized a tool Btk inhibitor, M7583, to determine the therapeutic efficacy of Btk inhibition in two mouse lupus models driven by TLR7 activation and type I interferon. In BXSB-*Yaa* lupus mice, Btk inhibition reduced autoantibodies, nephritis, and mortality. In the pristane-induced DBA/1 lupus model, Btk inhibition suppressed arthritis, but autoantibodies and the IFN gene signature were not significantly affected; suggesting efficacy was mediated through inhibition of Fc receptors. In vitro studies using primary human macrophages revealed that Btk inhibition can block activation by immune complexes and TLR7 which contributes to tissue damage in SLE. Overall, our results provide translational insight into how Btk inhibition may provide benefit to a variety of SLE patients by affecting both BCR and FcR signaling.

**Keywords**: Lupus, Bruton's Tyrosine Kinase, Interferon, TLR7

**Abbreviations**: Btk, Bruton's Tyrosine Kinase; TLR7, Toll-like receptor 7; BCR, B cell receptor; FcR, Fc receptor; SLE, Systemic lupus erythematosus; IFN, interferon; FACS, fluorescence-activated cell sorting; MMF, mycophenolate mofetil;

## 1. INTRODUCTION

Bruton's tyrosine kinase (Btk) is expressed in a large variety of immune cells including monocytes, macrophages, basophils, mast cells, and B cells. Btk regulates signaling downstream of the B cell receptor (BCR) and Fc receptors (FcR) and may also play a role in toll-like receptor (TLR) signaling [1, 2]. Previous studies using Btk deficient mice and Btk inhibitors have demonstrated that loss of Btk activity can ameliorate disease in animal models of autoimmune disorders including rheumatoid arthritis [3] and systemic lupus erythematosus (SLE) [4-7]. Consequently, Btk is considered a promising drug target for treatment of these diseases.

SLE is an extremely heterogeneous disease that manifests with a wide variety of symptoms including fatigue, rash, arthritis, nephritis, and neurological dysfunction. The pathogenesis and etiology of the disease are also diverse with patients showing different environmental exposures, genetic predispositions, autoantibody profiles, and various cellular dysfunctions. Given the high degree of heterogeneity in SLE, it would be decidedly beneficial to use preclinical animal models that represent different patient populations to learn how inhibition of a certain pathway or target affects disease. One drug target of interest is Btk, as this enzyme regulates signaling in several pathways relevant to autoimmune disease. Btk inhibitors have previously been tested and found to be efficacious in the NZB/W [4, 6], MRL/*lpr* [7], and B6.Sle1 [5] mouse models of SLE. However, these models do not recapitulate all the different subsets of lupus patients, and therefore there is a gap in knowledge as to how Btk inhibition may affect disease driven by TLR activation and type I interferon (IFN). Thus, we wished to test the potential for Btk inhibition to provide therapeutic benefit in SLE mouse models that are more TLR7-driven and have a higher IFN involvement.

Substantial evidence exists implicating TLR7 [8] and IFN [9] in the pathogenesis of human lupus and anti-IFN agents are currently being evaluated in clinical trials [10]. The IFN gene signature, which serves as a marker for high IFN activity, has been shown to be elevated in as much as 50% of all lupus patients. Polymorphisms in these pathways have been found to increase the risk for developing SLE [11] and females have demonstrated higher sensitivity to TLR7 activation [12], consistent with their greater propensity for SLE development. Additionally, there have been correlation studies of high IFN in SLE patients and those with high IFN are likely to develop more severe disease [10]. However, these patients have not been well represented in prior preclinical studies testing Btk inhibition and little is known about how Btk blockade might affect TLR7 and IFN-driven disease processes.

In the studies presented herein the BXSB-*Yaa* and pristane-DBA/1 models of lupus were utilized to test the potential efficacy of Btk inhibition for treating TLR7 and IFN-driven SLE. Previous results in related models have suggested that genetic Btk deficiency may affect disease in the context of *Yaa* duplication [13] or after pristane injection [14] and we wished to determine if a reduction in Btk activity by treatment with a pharmacological inhibitor could have similar efficacy. BXSB-*Yaa* mice have a duplication of the *Yaa* locus, which results in increased expression of TLR7 and spontaneous

development of disease [13, 15]. The lupus-like disease that develops is characterized by production of autoantibodies, lymphoid hyperplasia, and nephritis which leads to early mortality. Proteinuria is an early marker of nephritis in these mice and histological analysis of kidneys reveals evidence of severe glomerulonephritis. In the pristane-DBA/1 model, DBA/1 strain mice are injected in the peritoneum with the hydrocarbon pristane which induces disease development. The disease has mechanistically been characterized as highly TLR7 and IFN dependent [16] and involves generation of autoantibodies and development of arthritis [17]. The arthritis that develops is autoantibody-mediated and has similarities to that of SLE patients as it manifests as a mild erosive disease [18]. The pristane model is one of the very few mouse lupus models that has been demonstrated to display a robust IFN gene signature [17] and it therefore may be particularly relevant as a model for IFN high lupus patients. The autoantibody production in pristane-DBA/1 mice is biased toward reactivities against RNA-binding proteins such as RiboP and SmRNP, more so than against dsDNA or other DNA associated molecules, similar to what has been observed for SLE patients with high IFN signatures as they demonstrate a biased increase in RNA-binding reactivities [19, 20]. Even though these two models both are highly TLR7 dependent, they may represent distinct subsets of lupus as they have different pathogenesis, autoantibody profiles, and end organ disease manifestations. Thus, utilizing these two different models allows for interrogating the effect of Btk inhibition on TLR7-driven disease with a variety of readouts.

In our studies we utilized a small molecule Btk inhibitor, M7583, as a tool to assess the potential for Btk inhibition to treat lupus in the BXSB-*Yaa* and pristane-DBA/1 mouse lupus models. We first present a characterization of the compound to demonstrate its suitability as an effective Btk specific inhibitor suitable for in vivo use, and then demonstrate the efficacy of Btk inhibition for treatment of disease in the two models. We also performed studies using FACS, gene expression analysis, and human immune cells to further characterize the mechanism of action of Btk inhibition efficacy and how this might translate in humans. This work demonstrates the potential for Btk inhibitors to provide benefit to lupus patients of different disease subsets and provides mechanistic insight as to how Btk inhibitors may affect multiple immunological processes mediating disease pathogenesis.

## 2. METHODS

### *2.1 Mouse Lupus Models*

All procedures using animals were performed in accordance with the EMD Serono Institutional Animal Care and Use Committee (IACUC) and all local and national laws and regulations regarding animal care. Female DBA/1 mice used for pristane model studies were purchased from Jackson Labs. To induce disease development DBA/1 mice were injected with 0.5 ml of pristane (Sigma) i.p. at 11-12 weeks of age. Beginning at 2 months after pristane injection mice were fed chow formulated with M7583 at a concentration of 25 mg of compound/kg of chow. The consumption of chow was measured weekly and body weights were recorded and these two values were used to calculate the dose of compound received per mouse which averaged 3.35 mg/kg over the course of the study. Mice were also fed chow formulated with mycophenolate mofetil (MMF) (Selleck Chemicals) at a concentration of 500 mg/kg of chow which resulted in a dosage of 63.6 mg/kg. Vehicle group mice were fed chow of the same diet (Harlan Teklad 2018) without any added compounds. Chow formulations were made by Research Diets, Inc.. At 6 months after pristane injection mice were euthanized via $CO_2$ asphyxiation and blood was collected via the vena cava. Spleens were collected and split in half for FACS analysis or gene expression analysis, and paws were preserved in formalin for histology analysis.

Male BXSB-*Yaa* mice were purchased from Jackson Labs and at 9 weeks of age treatment was initiated using the same formulated chows described above fed to the DBA/1 mice. Based on chow consumption and body weights, BXSB-*Yaa* mice received on average 3.7 mg/kg of M7583 and 74.4 mg/kg of MMF. At 20 weeks of age mice were euthanized via $CO_2$ asphyxiation and blood was collected via the vena cava. Spleens were collected and split in half for FACS analysis or gene expression analysis, and kidneys were preserved for histology analysis.

### *2.2 Arthritis and Nephritis Assessments*

Arthritis development in pristane-injected mice was monitored over time and clinical scores were assigned based on inflammation and swelling similar to a scoring system previously detailed [21]. Mice were scored on a scale of 0-4 per paw with 4 being the most severe arthritis, and scores from all 4 paws were summed for a total score. At the conclusion of the study paws were removed, fixed in formalin and shipped to HistoTox Labs where they were processed for toluidine blue staining and scored for histological evidence of damage by a trained pathologist. For monitoring of proteinuria in BXSB-*Yaa* mice, urine was collected by bladder massage in the morning on 2 consecutive days and the samples were pooled. The levels of albumin and creatinine in the urine were determined using the Advia 1800 clinical chemistry analyzer (Siemens). The urinary albumin-to-creatinine ratio (UACR) was calculated as the ratio of milligrams of albumin per gram of creatinine per deciliter of urine. At the conclusion of BXSB-*Yaa* studies kidneys were collected, fixed in formalin and shipped to HistoTox Labs where they

were processed for hematoxylin and eosin staining and scored for histological evidence of damage by a trained pathologist. The scoring system used was modified from a previously published system [22] and evaluates kidney sections based on glomerular crescents, protein casts, interstitial inflammation, and vasculitis and a total histology score is obtained based on a composite score of these parameters.

*2.3 In Vitro Assays*

The potency for M7583 against Btk was determined using purified rBtk (Carna Biosciences). The Btk protein was diluted in buffer to a final of 0.05 ng/μl with 75 μM ATP and 1 μM of the KinKDR peptide FITC-AHA-EEPLYWSFPAKKK-NH2 (Tufts Core Facility, Boston MA). Various concentrations of M7583 were also included. Reactions were performed at 25 C for 90 minutes and halted by addition of stop solution containing 0.5 M EDTA. Plates were then read on the Caliper LabChip 3000 (Caliper Life Sciences) and the data was loaded into GeneData Screener for generation of $IC_{50}$ curves.

Kinase selectivity for M7583 was determined in the Kinase Profiler™ screening panel (EMD Millipore) that tested the inhibitory activity of the compound at 1 μM against 270 kinases. The biological selectivity of M7583 was assessed in vitro using primary human cells with BioMap® profiling by BioSeek Inc.. The activity of the compound was assessed using a concentration range of 1 nM to 1 μM in 12 different primary cell co-culture assay systems according to previously published methods [23].

The ability of M7583 to block FcR signaling was determined using basophils in whole blood. Human blood was collected with citrate as an anticoagulant and transferred to 96 well plates. Blood was pre-treated for 30 min at 37 C with dilutions of M7583 before activation with anti-IgE (Beckman Coulter) added to a final of 2 μg/ml and incubated at 37 C for 5 minutes. After activation, cells were stained for 15 min with anti-CD63-FITC (BD Biosciences) and then PBS-EDTA (20 mM) was added followed by fixative/lysing buffer and cells were fixed in formaldehyde prior to FACS analysis. The MFI for CD63 expression was determined after first gating for $CD123^{+}HLA\text{-}DR^{-}$ cells using the Canto II instrument (BD Biosciences). The ability of M7583 to block BCR signaling was determined using B cells in whole blood. Human blood was collected with citrate as an anticoagulant and transferred to 96 well plates. Blood was pre-treated for 60 min at 37 C with dilutions of M7583 before activation with goat anti-human IgM $(Fab')_2$ (Dianova) added to a final of 20 μg/ml and incubated at 37 C overnight. After activation, cells were stained for 45 min with anti-CD69-APC (BD Biosciences) and anti-CD19-PerCP-Cy5.5 and then lysed using FACS lysis solution (BD Biosciences) and resuspeneded in PBS prior to FACS analysis. FACS analysis was performed on the Canto II instrument and cells were first gated on CD19 and the percent of $CD19^{+}$ cells that were also positive for CD69 was determined.

Human PBMCs were isolated from buffy coat preparations of healthy donors (New York Blood Center) using Ficoll Paque Plus (GE Health Sciences) according to the manufacturer's instructions. Monocytes were purified by adherence to plastic for 90 minutes and subsequently differentiated to macrophages

by culture with 100 ng/ml GM-CSF (Sargramostim, Sanofi) in RPMI 1640 (Gibco) containing Pen/Strep and 10% heat inactivated fetal bovine serum (Corning). Complexes of human IgG-biotin (Jackson ImmunoResearch) and streptavidin-coated beads (0.3 μm in size from Thermo Scientific) were formed by mixing the two and gently rotating for 1 hr at 4 C. Beads were also mixed with the same IgG, but non-biotin tagged as a control. After rotation, the beads were washed once to remove unbound IgG and the complexes were then added to cells in RPMI 1640 containing 10% ultra-low IgG FBS and incubated at 37 C. After 24 hr the media was removed for measurement of TNF-α production by ELISA (R&D Systems).

PBMCs were stimulated to produce IFN by treatment with a purified human anti-RNP IgG preparation. Anti-RNP (Immunovision Inc.) was combined with necrotic lysate from HEK cells subjected to 4 cycles of free thawing to -80 C. The anti-RNP was combined with the lysate at a final concentration of 5% in media, and as a control lysate was also pre-treated with 1 μg/ml RNAse (Fisher) for 30 min at 37 C to demonstrate the RNA dependency of the stimulation. PBMCs were treated with the anti-RNP complexes in media (RPMI 1640 + 10% FBS + pen/strep + L-glut) at a concentration of 2.5 x $10^5$ cells/ml in a total volume of 200 μl in 96 well plates for 16 hr at 37 C. After incubation, cell supernatant was collected and 150 μl was applied to HEK293 ISRE-*luc* reporter cells (generated in house) and incubated for 6 hr. Afterwards, media was removed and 50 μl of Steady-Glo® luciferase substrate was added and the cells were incubated for 10 min and the luciferase signal was quantitated on the Infinite M1000 Pro plate reader (Tecan).

*2.4 Pharmacokinetics and Btk occupancy*

C57BL/6 mice (Charles River Labs) were used to determine the pharmacokinetics and pharmacodynamic activity of M7583. M7583 was formulated in 20% Kleptose HPB (Roquette) in 50 mM Na-Citrate buffer pH 3.0. Compound was administered by oral gavage, and at timepoints after dosing blood was collected via the vena cava into tubes treated with heparin as an anti-coagulant. A portion of the blood was used for determination of Btk occupancy and the remainder was centrifuged for isolation of plasma which was used for measurement of compound concentrations by LC-MS.

Btk occupancy was determined using a biotinylated-probe (MSC2527393) which is a Btk inhibitor that competes with M7583 for binding to the active site on Btk. This methodology is similar to a previously reported assay for measuring occupancy [24]. Briefly, 80 μl of blood collected from mice was aliquoted into tubes and the red blood cells were lysed by addition of 800 μl of RBC lysis buffer (Roche Diagnostics). Cells were then washed once with 400 μl of RBC lysis buffer and the remaining white cells were incubated in RPMI 1640 with 1 μM of the biotinylated probe for 1 hr at 37 C. The cells were then lysed by the addition of 120 μl of MPER lysis buffer (Pierce) and used in a streptavidin-capture ELISA to assess probe binding to Btk.

To perform the ELISA Btk occupancy assay streptavidin-coated 96 well plates (R&D Systems) were blocked with blocking buffer (PBS + 2% BSA) 200 μl/well at room temperature for 1 hr. After blocking, plates were washed once 200 μl/well with washing buffer (PBS/0.05% tween 20) and then 100 μl/well of standards or probe-treated cell lysate were added per well of the plate diluted in PBS + 1 mg/ml BSA and incubated for 2 hr at room temperature with gentle shaking. Recombinant human Btk (Carna Biosciences) previously treated with the probe was used to construct a standard curve. Plates were then washed 3 x 200 μl/well and a rabbit anti-Btk antibody (Pierce) was added 100 μl/well at 1 μg/ml in PBS and plates were incubated for 1.5 hr with gentle shaking. Plates were then washed 3 x 200 μl/well and a donkey anti-rabbit HRP-tagged antibody (Jackson ImmunoResearch) was added 100 μl/well at 0.16 μg/ml in PBS and plates were incubated for 1.5 hr. Plates were next washed 3 x 200 μl/well and TMB substrate (BD Biosciences) was added 100 μl/well. After sufficient color development the reaction was halted by the addition of 1 N $H_2SO_4$ (Sigma). Plates were immediately read at 450 nm using a SpectraMax® M5 microplate reader (Molecular Dynamics).

*2.5 Autoantibody assays*

Autoantibodies were measured in mouse plasma samples using a custom manufactured 5-plex Meso Scale Discovery (MSD) plate for assessment of five different autoantibodies: anti-dsDNA, anti-histone, anti-Ro/SSA, anti-Sm/RNP, and anti-RiboP. Standard binding plates were coated by MSD with activated calf thymus DNA, histones purified from chicken RBCs, Ro/SSA from bovine spleen, Sm/RNP from calf spleen or thymus, and RiboP from bovine thymus. DNA was coated at 50 μg/ml and was from Sigma while all other antigens were from Immunovision and coated at 50 U/ml except for histones which was at 50 μg/ml. To perform the assay, plates were blocked overnight with PBS + 5% BSA (Sigma) and then washed once with washing buffer (PBS + 0.05% tween 20). Plasma samples were diluted 1:100 with PBS + 0.5% BSA and loaded 100 μl/well. Plasma samples from MRL/*lpr* mice with high autoantibody titers were pooled to create a standard which was used for all assays. Serial dilutions of the standard were prepared to create a standard curve to allow for quantitative comparison of the lupus study plasmas and values were expressed as autoantibody units per ml of plasma (U/ml). The most concentrated standard was arbitrarily assigned a value of 100 U/ml. Plates were incubated with samples and standards for 2 hr with gentle shaking and were then washed 3 x 200 μl/well. An anti-mouse-SULFO-TAG antibody (MSD) was then added at a 1:500 dilution and incubated for 2 hr with gentle shaking. Plates were then washed 3 x 200 μl/well and 2X Read Buffer (MSD) was added and the plates were immediately read on the MSD Sector Imager 600 and data was processed using the MSD Discovery Workbench 2.0 software.

*2.6 FACS analysis*

At the conclusion of studies mice were euthanized and spleens were collected as described above. One half of each spleen was added to 5 ml of ice cold PBS with 2% heat inactivated fetal bovine serum (HIFCS) (Corning). Homogenization was performed using the gentleMACS Octo Dissociator (Miltenyi Biotech). Red blood cells were lysed using ACK lysis buffer (Lonza) and the remaining spleenocytes were

washed and resuspended in PBS containing 2% HIFCS. Spleenocytes (1 x $10^6$ per well in 96 well plates) were incubated with anti-CD16/CD32 antibody (BD Biosciences) to block IgG-Fc receptors, followed by 20 min incubation with antibodies against cell surface markers or control IgG. Antibodies used for staining were all from BD Biosciences and were: anti-CD16/CD32 (2.4G2), PerCP-Cy™5.5-conjugated anti-B220 (RA3-6B2), BV421-conjugated anti-CD138 (281-2), PE-conjugated anti-CD69 (H1.2F3), APC-conjugated anti-CD27 (LG.3A10), FITC-conjugated anti-CD38 (90/CD38), PE-CF594 conjugated-anti-CD11c(HL3), APC-H7- conjugated-anti-CD8a (53-6.7), BV510-conjugated anti-CD11b (M1/70), BV650 conjugated anti-CD4(GK1.5), AlexaFlor700-conjugated anti-Ly6C(AL-21),ratIgG2a (R35-95), rat IgG2b (R35-38), rat IgM (R4-22), hamster IgG (A19-3). After staining, cells were washed twice in PBS containing 2% HIFCS and were evaluated on a LSR Fortessa X-20 (BD) and the percentage of cells positive for different markers was analyzed using FlowJo software (Tree Star, Inc.).

*2.7 Gene Expression Analysis*

Gene expression analysis was performed using the NanoString platform. At the conclusion of studies mice were euthanized and spleens and kidneys were flash frozen in liquid nitrogen. Blood was collected and 100 μl was preserved in RNAlater (Qiagen) at room temperature and then frozen to -80. Kidneys and spleens were later thawed and added to RLT buffer (Qiagen) and then homogenized in M tubes (Miltenyi) using the gentleMACS Octo Dissociator and program RNA_01. After homogenization, RNA was purified using the Qiagen RNeasy Mini Kit. The RNeasy Protect Animal Blood System was used for blood RNA isolation after samples were preserved in RNAlater. A custom 50 gene panel was designed for NanoString analysis and included 45 disease relevant genes for interrogation and 5 housekeeping genes for normalization (see Supplemental Table 2 for gene list). Purified RNA samples were hybridized overnight to gene specific reporter probes at 65 C and analyzed using the NanoString Prep Station and nCounter instruments according to the manufacturer instructions with 100 ng of RNA as input.

## 3. RESULTS

### *3.1 M7583 is a potent and selective Btk inhibitor*

M7583 is an irreversible Btk inhibitor [25, 26] and in these studies we have utilized it as a tool to advance the understanding of the potential for Btk inhibition to treat a subset of lupus patients not modeled in previous preclinical studies. Before usage in disease model studies, a characterization of the compound was performed to qualify its selectivity, potency, and suitability as a specific Btk inhibitor and in vivo tool.

M7583 covalently reacts in the active site of Btk and thus permanently prevents catalysis. Using purified recombinant protein it was demonstrated that the compound is a potent inhibitor of Btk and has an $IC_{50}$ of 1.48 nM (Supplemental Fig 1A) for the purified enzyme. M7583 was next tested for potency in cellular assays of BCR and FcR signaling. The compound was found to completely and potently block BCR activation (Supplemental Fig 1B) with an $IC_{50}$ of 45.75 nM and also inhibited FcR activation in basophils (Supplemental Fig 1C) with an $IC_{50}$ of 1.01 μM. The potency was less in basophils than for inhibition of the BCR, but it has been reported with other Btk inhibitors as well that there are some differences in potency for different cell types and readouts [27].

As M7583 is an irreversible Btk inhibitor that permanently occupies the active site, it has the potential to have prolonged inhibitory activity given the long half-life of the Btk protein [24]. The duration of Btk occupancy and timecourse for clearance of M7583 in mice was determined after administration of a single oral dose. Btk occupancy was measured in an assay using a biotin-tagged probe and streptavidin-capture ELISA similar to an assay previously described [24]. It was found that dosing mice with M7583 led to a high degree of Btk occupancy and the inhibitory activity persisted for several days, long after the compound had been cleared from the circulation (Supplemental Fig 2). More detailed characterization of the pharmacokinetics of the compound revealed that it has sufficient bioavailability (16%) with a relatively short half-life (45 minutes), and moderate volume of distribution (0.87 L/kg) in mice. These results indicate that M7583 treatment of mice will result in a robust sustained inhibition of Btk.

The compound was tested for selectivity against 270 kinases using the Kinase Profiler Panel (EMD Millipore). At a compound concentration of 1 μM the compound showed an excellent selectivity profile with only 6 other kinases inhibited greater than 50% (ZAP-70, Blk, Bmx, Txk, Itk, SGK) (Fig 2A and Supplemental Table 1). Although there are numerous other kinases the compound was not tested against, the kinase profiling results reveal the compound targets a limited number of kinases other than Btk and should have a limited number of off-target effects. The specificity for the compound against different disease relevant pathways and processes was determined using a BioSeek BioMAP® profiling panel which tested the compound in 12 different co-culture systems using 7 different primary human cell types (Fig 2B). In the 12 different assay systems the compound only showed activity in the B cell/T cell interaction system, which utilizes anti-IgM B cell activation and mild T cell stimulation to mimic T cell dependent B cell activation and class switching. The specificity shown in the BioMap panel is another

indication of the selectivity of the compound for Btk, as aside from the B cell/T cell system none of the other assay systems are likely Btk-regulated processes. Overall, these in vitro and cellular results demonstrate that M7583 is a highly potent and selective Btk inhibitor that can block both BCR and FcR signaling and is qualified as a pharmacological tool for in vivo studies.

*3.2 Efficacy of Btk inhibition in mouse lupus models*

To assess the potential of Btk inhibition as an effective SLE treatment strategy, M7583 was tested in the BXSB-*Yaa* and pristane-DBA/1 mouse lupus models for efficacy. The BXSB-*Yaa* and pristane-DBA/1 models are both driven by TLR7 activation, but are different in that they have dissimilar disease manifestations. Testing candidate SLE therapeutic strategies in multiple models is informative as the disease is heterogeneous and different models may represent disparate patient populations.

BXSB-*Yaa* mice develop severe nephritis and the resulting kidney disease can lead to early mortality, similar to the disease suffered by lupus nephritis patients. When BXSB-*Yaa* mice were treated with M7583 or the standard of care reference drug mycophenolate mofetil (MMF), it was found that both compounds led to a significant reduction in proteinuria (Fig 4A, 4B). This reduction in nephritis resulted in much improved survival for mice treated with either compound (Fig 4C). To confirm that proteinuria and early mortality were a result of inflammation and glomerulonephritis, and compound treatment was affecting this pathology, kidneys were analyzed by histology and this analysis showed that treatment did in fact reduce kidney damage (Fig 4D). The histology results support the conclusion that the improvement in survival with either Btk inhibition or MMF treatment was likely due to a decrease in kidney damage.

DBA/1 mice injected with pristane develop arthritis that is clinically visible and also detectable upon histological evaluation of toluidine blue stained paw sections. This pathology is analogous to the rheumatic symptoms experienced by many SLE patients. Inhibition of Btk with M7583 treatment in this model led to a significant reduction in clinical signs of arthritis and showed greater efficacy than MMF (Fig 5A, 5B). Dosing of mice with M7583 led to a significant reduction in arthritis scores and there was a mild therapeutic effect with MMF as well. Histological analysis of the paws showed that the arthritis was mainly inflammatory in nature and minimal bone destruction was noted. The histological analysis of the paws of these mice confirmed the anti-arthritic activity of Btk inhibition as pathology in the joints was significantly reduced with M7583 treatment (Fig 5C). Thus, in both models there was a reduction in clinical disease (nephritis and arthritis) with M7583 treatment and inhibition of Btk.

*3.3 Effects on autoantibody levels in lupus mice*

After observing a reduction in end organ damage with Btk inhibition, we explored the mechanism by which M7583 reduced disease. Autoantibodies are used both clinically and preclinically as a disease marker and readout for B cell activation. They may also be useful for patient stratification and subsetting. At the conclusion of BXSB-*Yaa* and pristane-DBA/1 lupus studies, autoantibodies were

measured in plasma samples using a custom multiplex assay on the MSD platform that tests for 5 different auto-reactivities (anti-dsDNA, anti-histones, anti-Ro/SSA, anti-Sm/RNP, and anti-RiboP). In BXSB-*Yaa* studies (Fig 6A) mice were sampled just before the start of treatment at 9 weeks of age (Pre-treatment) via a submandibular bleed and also at the end of the study at 20 weeks of age (Post-treatment). It was found that mice of this strain display elevated titers of anti-dsDNA, anti-histone, and anti-Ro/SSA which increase with time. Anti-Sm/RNP and anti-RiboP autoantibodies were not detectable in these mice. The increase in titers of autoantibodies was significantly blocked by inhibition of Btk with M7583. There was a trend toward reduction in autoantibody titers with MMF treatment as well, but the effect did not reach statistical significance for any of the auto-reactivities.

In the Pristane DBA/1 model (Fig 6B) there was an increase in anti-dsDNA, anti-histones, and anti-Ro/SSA with disease (Veh vs PBS), but the increase in titers was modest in comparison to the elevation seen in the BXSB-*Yaa* mice. In contrast, pristane-DBA/1 mice had a large induction of anti-Sm/RNP and anti-RiboP (data not shown) autoantibodies. Treatment of pristane-injected DBA/1 mice with M7583 reduced levels of anti-dsDNA, anti-histones, and anti-Ro/SSA, but not anti-Sm/RNP. MMF treatment did not significantly impact titers of any of the autoantibodies. There have previously been reports indicating that the cell populations responsible for production of anti-RNA binding reactivities may be different from those producing anti-dsDNA and anti-histones. It has been suggested that long-lived plasma cells produce anti-RNA reactivities while plasmablasts are the source of anti-dsDNA and other auto-reactivities [28]. Furthermore, the elimination of cells producing anti-RNA binding reactivities is more challenging [28] and may explain our finding that MMF and Btk inhibitor treatment did not reduce anti-RNA binding reactivities. However, it is unclear which subset of autoantibodies is responsible for disease pathology as levels of plasmablasts and anti-dsDNA titers do tend to track with flare in SLE patients whereas the levels of RNA-binding reactivities remain more constant [29]. Also, in pristane treated mice there has only been reported to be a small increase in autoantibodies against connective tissue antigens [30] which typically mediate arthritis development in RA, and we did not detect a major increase in anti-collagen II titers in our pristane-DBA/1 mice (data not shown). Overall, the autoantibody results demonstrate an ability for Btk inhibition to affect B cells and ultimately antibody-secreting cells to reduce autoantibody production. However, the effect seems to be more significant in the BXSB-*Yaa* model and greater against anti-dsDNA and anti-histone autoantibodies compared to RNA-binding reactivities such as anti-Sm/RNP.

*3.4 Cell populations affected by Btk inhibition in lupus mice*

To try to gain greater insight into which cell types Btk inhibition may be affecting, FACS analysis was performed on spleenocytes from lupus model mice. Spleens from BXSB-*Yaa* mice (Fig 7A) were analyzed for percentages of B cells ($B220^+$) and T cells ($CD4^+$ and $CD8^+$) expressing CD69. With both M7583 and MMF treatment there was a reduction in B and T cells expressing CD69, indicating the compounds had reduced activation of these cell populations. Furthermore, plasma cells ($B220^+CD38^{INT}CD138^{HI}$) were significantly reduced with M7583 treatment. This reduction in plasma cells could explain the decreased levels of autoantibodies seen with compound treatment. There was no change in total percentages of

$B220^+$ and $CD4^+$ cells with compound treatment, but there was a slight elevation of $CD8^+$ cells with both M7583 and MMF treatment (Supplemental Fig 1). The same FACS analysis was performed on spleens at the conclusion of pristane-DBA/1 studies (Fig 7B). M7583 increased the percentage of B cells expressing CD69, but did not have an impact on T cell CD69 expression. There was also a dramatic reduction in $B220^+CD38^{INT}CD138^{HI}$ cells with M7583 treatment in the pristane-DBA/1 model. MMF had a negligible effect on these cell populations. The interpretation of the spleen FACS analysis results in the pristane-DBA/1 model is confounded by the fact that disease may be more driven by cell activation in the peritoneum, where pristane generates lipogranulomas that function as ectopic lymphoid tissue and are a major site of IFN and autoantibody production [16]. However, there seems to be a reduction in B cell activation and numbers of plasma cells with Btk inhibition in both models as is to be expected given the major dependence of BCR signaling on Btk.

*3.5 Gene expression analysis of lupus mice*

To learn more about which pathways and processes are contributing to disease in the two models and how they are affected by Btk inhibition, gene expression in the blood and different organs of lupus mice was measured by NanoString analysis. At the conclusion of BXSB-*Yaa* and pristane-DBA/1 studies, blood, spleens, and kidneys were collected and RNA was isolated and subsequently analyzed on the NanoString platform using a 50 gene panel comprised of markers for various cell types, inflammation, and tissue damage (see gene list in Supplemental Table 2).

Gene expression analysis was performed on BXSB-*Yaa* mice (Fig 6) from the vehicle group, M7583 treated group, and young healthy mice which served as a control group (Ctl). Fold changes in expression from the control group were calculated to determine which genes were increased with disease (Veh vs Ctl) and which genes were subsequently affected by Btk inhibitor treatment (M7583 vs Veh). There were a large number of genes found to be statistically changed with disease in the kidney. The majority of these genes were markers of infiltrating immune cells (i.e. *CD4, ITGAM, CD68, PSMB9*) or inflammatory cytokine genes (i.e. *CCL2, TNF, CXCL1*). The induction of these genes was greatly reduced by Btk inhibitor treatment. In the spleen, there were changes in many B cell and plasma cell markers. Interestingly, in diseased mice, increases in the plasma cell markers *SDC1* (CD138) and *XBP1* were noted while decreases in general B cell markers such as *EBF1*, *BANK1*, and *MS4A1* (CD20), were found. Similarly, changes in B cell numbers and plasma cell proportions have also been found in human SLE [31-33]. Btk inhibitor treatment reversed these changes and actually decreased *SDC1* and *XBP1* expression to below the level of the Ctl group. These effects on plasma cell gene expression are consistent with the FACS results (Fig 5A) demonstrating an increase in $CD38^{INT}CD138^{HI}$ cells with disease and reduction in their numbers with M7583 treatment. Only 4 genes were found changed with disease in the blood of BXSB-*Yaa* mice. Of note, *BCL2A1A* was increased with disease and significantly decreased with Btk inhibition. Previously, *BCL2A1* has been found to be Btk inhibitor responsive in humans after dosing [34] and after Btk siRNA treatment of human cells in vitro [35]; suggesting *BCL2A1A* may be a useful biomarker for Btk inhibitor activity.

Blood and spleens from mice in pristane-DBA/1 model studies were also analyzed for gene expression (Fig 6). In both the blood and spleen there was a massive induction in expression of interferon-regulated genes (*USP18*, *IRF7*, *IFIT1*, *OAS3*) and neutrophil marker genes (*MPO*, *NGP*, *C3AR1*) with disease. The increase in these genes was not blocked by compound treatment. Similar to the BXSB-*Yaa* model, in pristane-DBA/1 mice there was also a decrease in expression of general B cell marker genes in the spleen (*EBF1*, *MS4A1* (CD20), *BANK1*, *BTK*). In the blood there was not a change in B cell markers, but markers for other immune cells were altered (i.e. *FCGR3*, *ITGAM*, *CCR5*, *PSMB9*). *BCL2A1A* was again found to be increased with disease and significantly affected by Btk inhibitor treatment. The effect of Btk inhibition on gene expression in the pristane-DBA/1 model was very minimal and *EBF1* in the blood was the only disease gene that was statistically impacted by treatment.

To make a quantitative comparison of the levels of IFN in mice from the two different models, an "IFN gene signature score" was calculated based on the expression of 5 genes known to be interferon regulated (*USP18*, *IRF7*, *IFIT1*, *OAS3*, *BST2*). A significant increase in scores was found with disease for pristane-DBA/1 mice in both the spleen and blood (Supplemental Fig 4). In contrast, there was not an increase in scores for BXSB-*Yaa* mice in the blood or spleen, although there was a modest increase in scores in the kidney. The differences in the systemic expression and magnitude of increase of IFN gene signature scores between the two models indicates the existence of much higher levels of circulating interferon in pristane-DBA/1 mice compared to BXSB-*Yaa* mice. Clinical studies have found the IFN gene signature in humans to be relatively stable over time and resistant to change with most treatments, and this seems to be the case in our pristane model studies.

*3.6 The effect of Btk inhibition on activation of human immune cells with SLE relevant stimuli*

To better understand the mechanism of action of Btk inhibition in the two models, and to determine the ability of Btk inhibition to block FcR activation of myeloid cells, M7583 was tested in vitro using human immune cells activated with representative immune complexes. First, the ability of Btk inhibition to block activation of IFN production by anti-RNA binding autoantibodies was tested in PBMCs. When SLE patient-derived purified anti-RNP IgG was combined with necrotic cell lysate to form immune complexes, it stimulated IFN production (Fig 7A), presumably through RNA-mediated activation of TLR7 in pDCs which are recognized as high IFN producing cells in SLE [36]. The stimulatory effect of the immune complexes was abolished by treatment of the lysate with RNAse, further suggesting that the activation was mediated through RNA activation of TLR7 (data not shown). The TLR7 agonist R848 was used as a positive control IFN-inducing stimulus. When PBMCs were pre-treated with M7583 it was found that Btk inhibition did not block IFN production (Fig 7B), except at the 10 μM concentration, which showed evidence of cell toxicity (data not shown). This seems to be consistent with the results of the pristane model studies where the IFN gene signature was not affected by M7583 treatment (Fig 6 and Supplemental Fig 4). To test if Btk inhibition could be reducing end organ disease in lupus mice through a localized effect, human monocytes differentiated to macrophages with GM-CSF were used as a representative myeloid cell type that is activated by pathogenic immune complexes. These cells could be activated to produce TNF-α by treatment with complexes of IgG-biotin bound to streptavidin beads (Fig 7C), which is designed to mimic an immune complex. Btk inhibition with M7583 could block

activation of monocyte-derived macrophages with these complexes and also activation of the cells with the TLR7 agonist R848. However, the potency for the compound for blocking these pathways was higher than what was observed with B cell activation. Inhibition of these signaling pathways in macrophages could explain the efficacy of Btk inhibition in the pristane model in the absence of an effect on autoantibodies or the IFN gene signature. Overall, these results with human immune cells indicate that Btk inhibition can block FcR activation in myeloid cells. Thus, there are multiple SLE-relevant disease pathways that may be affected by Btk inhibition and augur beneficial effects for lupus patients of various subsets.

## 4. DISCUSSION

Btk inhibitors are being developed for treatment of autoimmune diseases as well as hematological cancers dependent on BCR signaling. Ibrutinib was the first FDA approved Btk inhibitor and is currently clinically used for treating mantle cell lymphoma and chronic lymphocytic leukemia. Other Btk inhibitors are currently in development for oncology as well as autoimmune indications [3, 37]. In our studies we demonstrated that MSC248820 is a highly potent and selective Btk inhibitor with a long duration of action in vivo. Because of these properties, we used the compound as a tool to determine how inhibition of Btk activity may impact disease in mouse models of lupus and how the preclinical activity may translate to SLE treatment.

The vast majority of preclinical drug candidate evaluation for lupus has been performed in the MRL/*lpr* and NZB/W mouse lupus models [38, 39]. However, with the growing appreciation for the heterogeneity in lupus has come the recognition that testing candidate therapeutics in a wider variety of models that mimic different patient populations may help better predict clinical utility for blocking a pathway or specific target. Despite the fact that TLR7 and IFN likely play a role in the disease process for a number of SLE patients, there is a dearth of studies using mouse models that mimic these patients. Thus, we chose to test the effect of Btk inhibition in the BXSB-*Yaa* and pristane-DBA/1 models. Prior studies using genetic knockouts have demonstrated the dependence of the BXSB-*Yaa* model on TLR7 [40, 41] and the pristane-DBA/1 model on both IFN [42, 43] and TLR7 [44, 45] signaling. These models have not been used extensively for drug evaluation, although in the pristane model an anti-IFN antibody [46], an anti-CD80 antibody [47] and several standard anti-rheumatic agents [48] have demonstrated varying degrees of efficacy. The assessment of Btk inhibition in our studies provides new information on the mechanisms of disease pathogenesis in these models and further characterizes how they may be used to evaluate candidate drugs. By providing this characterization we hope that these models will become more useful for others aiming to develop lupus treatments for this patient population.

We found that blockade of Btk activity with the Btk inhibitor M7583 very effectively treated end-organ disease in both BXSB-*Yaa* and pristane-DBA/1 mice as it reduced kidney disease and arthritis respectively in the two models. This is notable as both of these pathologies are manifested in SLE patients. Although Btk inhibition was therapeutic in both models, the mechanism of action by which Btk inhibitor treatment provided benefit may have differed. In the BXSB-*Yaa* model Btk inhibition resulted in a reduction in nearly all aspects of the disease as autoantibody titers were decreased, gene expression changes were reversed, and the activation of immune cell populations was prevented. This may indicate an effect on a very proximal aspect of disease pathogenesis, such as inhibition of BCR activity and autoantibody production. In contrast, although there was a complete suppression of arthritis in the pristane-DBA/1 model, there was only a reduction in select autoantibody reactivities and there was no effect on gene expression changes, particularly for IFN-regulated genes. The disconnect between end organ efficacy and systemic disease may be explained by an effect of the compound via a different mechanism of action from that in the BXSB-*Yaa* model and likely a more localized effect in the diseased paws. However, we cannot exclude the possibility that the differences are a result of the

treatment in the two models beginning at different phases in the timecourse of disease as disease may already be more established in the pristane model and B cell activation at a more difficult to treat time.

The pathogenesis of SLE in humans and mice is thought to involve generation of autoantibodies which form immune complexes that become deposited in tissues and cause inflammation and organ damage [49]. As Btk regulates multiple signaling pathways involved in this process, Btk inhibition may provide therapeutic benefit by more than one mechanism. Btk inhibition could provide benefit by inhibiting BCR activation and reducing the production of autoantibodies and other B cell inflammatory activities such as cytokine production or presentation of antigen. This may be the mechanism of action of M7583 treatment in the BXSB-*Yaa* model. Additionally, Btk inhibition may reduce activation of myeloid cells by immune complexes which bind to Fc receptors. This inhibition may decrease inflammation in tissues where the immune complexes are deposited and therefore reduce organ damage. This could explain how M7583 reduces joint arthritis without reducing several other upstream disease processes in the pristane-DBA/1 mice. Btk inhibition may effectively suppress inflammation triggered by immune complexes activating Fc receptors and this hypothesis is supported by our in vitro studies with macrophages demonstrating the ability of Btk inhibition to block TNF-$\alpha$ production after treatment with IgG-bead complexes. However, there may be additional mechanisms by which Btk inhibition provided benefit, such as through inhibition of TLR signaling. Reports have suggested that Btk regulates toll-like receptor (TLR) signaling, although some conflicting studies exist. Btk has been implicated to play a role in TLR3 [50], TLR4 [51, 52], TLR7 [53, 54], TLR8 [55] and TLR9 [53, 55] signaling. Additionally, a prior study demonstrated that Btk is expressed in synovial tissue from RA patients and the Btk inhibitor RN486 could block production of some cytokines by RA patient synovial explants or human macrophages stimulated with IgG-bead complexes, CD40L, or LPS [56]. Curiously, we found that anti-RNP IgG (complexed with necrotic cell lysate) stimulated IFN production from PBMCs was not prevented by Btk inhibition. Although both FcR and TLR7 activation by R848 in macrophages were inhibited by M7583, there may be differences in regulation of these pathways in pDCs that leads to IFN production compared to TNF-$\alpha$ production from macrophages and thus a differential effect of Btk inhibition between the two cell types. In support of this hypothesis, Wang et. al, have reported that BTK regulates TLR9 but not TLR7 signaling in human pDCs [57]. Nonetheless, when taken together, the data indicate that by acting through multiple mechanisms Btk inhibitors may provide benefit to a wide range of lupus patients.

Given the wide heterogeneity in lupus symptoms and pathogenesis, a treatment that blocks multiple disease mediating mechanisms would offer the best chance for providing therapeutic benefit. Btk inhibition may be a therapeutic approach that can benefit a large proportion of lupus patients because of its ability to act through several different mechanisms. The ability of Btk inhibitors to target B cells responsible for autoantibody production as well as myeloid cells mediating end organ inflammation is a major advantage for this therapeutic approach. Effectively blocking more than one disease process may provide a high degree of efficacy, and even if not all disease processes are blocked, therapeutic benefit may still be accomplished if success in only one area is achieved. This point may be underscored by the efficacy seen with Btk inhibition in the pristane model and lack of effect of MMF treatment. MMF

(CellCept®), an inhibitor of inosine-5'-monophosphate dehydrogenase, is a currently used standard of care drug for lupus. MMF mechanistically acts as an anti-proliferative against immune cells and has shown benefit for treatment of lupus patients. In our studies MMF was nearly as effective in the BXSB-*Yaa* model as Btk inhibition. However, in the pristane-DBA/1 model MMF failed to demonstrate the same anti-arthritic efficacy as Btk inhibition. As Btk inhibition can affect a larger number of pathogenic processes, it may have a clinical advantage over other drugs such as MMF in addition to its greater selectivity.

In summary, the results presented here demonstrate that Btk inhibition may be an effective therapy for lupus patients with TLR7 and IFN-driven disease in addition to other lupus subtypes. Furthermore the efficacy may be achieved through more than one mechanism of action, making Btk inhibitors a promising therapeutic approach for lupus. Additionally, these results provide an increased characterization of two mouse lupus models that may facilitate their use in future candidate therapeutic studies.

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**FIGURE LEGENDS**

Fig. 1 – **M7583 kinase and cellular selectivity**. Selectivity of M7583 was determined in the Millipore Kinase Profiler screen and shown are the results in a kinase tree format with the activity of the molecule at 1 μM against individual kinases displayed by the size of the red circle (***A***). The specificity of M7583 was tested by BioSeek in a BioMAP® panel of 12 assays that represent different disease relevant systems/pathways using 7 different human cell types (***B***). The compound was tested at concentrations ranging from 1 nM to 1 μM.

Fig. 2 – **Efficacy of Btk inhibition in the BXSB-*Yaa* mouse lupus model**. Beginning at 9 weeks of age BXSB-*Yaa* mice were dosed with M7583 (**M7583**) or mycophenolate mofetil (**MMF**) via formulated chow and vehicle group mice were fed normal chow (**Veh**). Urine samples were collected over time and proteinuria was determined by measuring the urinary albumin to creatinine ratio (UACR) (***A*** and ***B***) and the values for mice at the 20 week timepoint are shown individually (***B***). Treatment with either M7583 or MMF increased survival (***C***). Histological analysis of kidneys collected at 20 weeks of age was performed to evaluate nephritis and results are presented as a Histology Score which is a composite assessment of several parameters (***D***). Young 9 week of age pre-diseased (**Pre**) mice kidneys were collected and served as a healthy control group. Representative pictures of the histological results are shown (***E***). Results presented are a compilation of 2 separate studies. For survival, **significantly different from Veh $p<0.01$ or *significantly different from Veh $p<0.05$ (Mantel-Cox test). For UACR, **significantly different from Veh $p<0.01$ or *significantly different from Veh $p<0.05$ (Kruskal-Willis, Dunn's). For Histology score, **significantly different from Veh $p<0.01$ (ANOVA, Dunnett's).

Fig. 3 – **Efficacy of Btk inhibition in the Pristane DBA/1 mouse lupus model**. Mice were dosed starting at 8 weeks after pristane injection with M7583 (**M7583**) or mycophenolate mofetil (**MMF**) via formulated chow and vehicle group mice were fed normal chow (**Veh**) while mice not injected with pristane served as non-diseased controls (**Ctl**). Mice were scored for observable signs of arthritis over time (***A***) and the maximum arthritis score achieved for each mouse is shown (***B***). At the end of the study, 24 weeks after pristane injection, histological damage in the T-blue stained paws was scored (***C***). Representative pictures of the histological results are shown (***D***). Results presented for the timecourse are representative of one study and the other results are a compilation of 2 separate studies and are means $\pm$ SEM. **Significantly different from Veh $p<0.01$ or *$p<0.05$(ANOVA, Dunnett's).

Fig. 4 – **Autoantibody production in lupus mice is reduced by Btk inhibition**. Lupus mice were treated with M7583 (**M7583**) or mycophenolate mofetil (**MMF**) via formulated chow and vehicle group mice

were fed normal chow (**Veh**). Blood samples were collected from BXSB-*Yaa* mice at 9 weeks of age just before the start of treatment (**Pre-treatment**) and at 20 weeks of age at the conclusion of the study (**Post-treatment**) and autoantibodies in the plasma were measured using a custom multiplex autoantibody MSD assay (***A***). The same multiplex assay was used to measure autoantibodies in plasmas of Pristane DBA/1 mice at 6 months after pristane injection at the conclusion of the study (***B***). For the pristane-DBA/1 model, mice injected with PBS instead of pristane (**Ctl**) serve as non-diseased controls. Results presented are a compilation of 2 separate studies and medians are shown. **Significantly different from Veh $p<0.01$ or *significantly different from Veh $p<0.05$ (Kruskal-Willis, Dunn's).

Fig. 5 – **FACS analysis of the effect of Btk inhibition on splenocytes**. Lupus mice were treated with M7583 (**M7583**) or mycophenolate mofetil (**MMF**) via formulated chow and vehicle group mice were fed normal chow (**Veh**). Spleens were collected at 20 weeks of age from BXSB-Yaa mice (***A***) or at 6 months after pristane injection from DBA/1 mice (***B***). For the pristane-DBA/1 model, mice injected with PBS instead of pristane (**Ctl**) serve as non-diseased controls. Spleenocytes were subsequently analyzed by FACS for cell populations and expression of various cell surface markers. Results shown are a compilation of two separate studies and means $\pm$SEM are presented. **Significantly different from Veh $p<0.01$ (ANOVA, Dunnett's)

Fig. 6 – **Gene expression analysis of Btk inhibitor treatment of Pristane-DBA/1 and BXSB-*Yaa* mice**. Blood and spleens were collected from DBA/1 mice 6 months after pristane injection and 4 months of treatment with M7583 formulated in chow (**M7583**) or normal chow (**Veh**). Blood and spleens were also collected from age and sex matched mice injected with PBS instead of pristane to serve as a healthy control group (**Ctl**). Blood, spleens, and kidneys were collected from BXSB-*Yaa* mice at 20 weeks of age after treatment with M7583 formulated in chow (**M7583**) or normal chow (**Veh**). The same three tissues were collected from BXSB-*Yaa* mice at 9 weeks of age to serve as a pre-disease control group (**Ctl**). RNA was isolated and then analyzed by NanoString for expression of 50 genes. Fold changes for the Veh and M7583 groups relative to the Ctl group were calculated. Shown are heat maps of genes that were significantly changed ($p<0.05$ by t-test) with disease (Veh vs Ctl) or with compound treatment (M7583 vs Veh). Results are a compilation of 2 studies and the heat maps show the fold change for the mean of each group relative to the Ctl group.

Fig. 7 – **Inhibition of human immune cell activation by M7583**. Human immune cells were pre-treated with M7583 for 60 min prior to activation with various stimuli designed to mimic FcR activation. PBMCs were stimulated for 16 hr with complexes of purified human SLE patient-derived anti-snRNP and necrotic lysate. Supernatants were harvested and assayed for type I IFN production on a HEK293 interferon stimulated response element (ISRE)-driven luciferase reporter cell line (***A***). Reporter cells were treated with media alone (**No cells**), media from untreated PBMCs (**No Stim**), R848 (1 μM), the

anti-RNP complexes (**Stim**), and complexes after RNAse treatment. PBMCs were treated with M7583 in different concentrations prior to anti-RNP complex treatment and activation of IFN reporter cells by their sups was determined (***B***). Simulation and compound treatment was tested in 4 or more donors in 3 or more independent experiments. Human monocytes differentiated to macrophages with GM-CSF were activated with either complexes of IgG-biotin-SA-beads or SA-beads combined with a non-biotinylated IgG (***C***). Macrophages were also activated with 1 μM R848 (***D***). TNF-α production was measured after 24 hr of macrophage activated by either stimulation. Macrophage stimulation results show are combined data from 3 separate experiments using 3 different donors for ***C*** and 2 experiments with 2 donors for ***D*** and presented as means ± SEM. **Significantly different from Veh $p<0.01$ or *significantly different from Veh $p<0.05$ (ANOVA, Dunnett's).

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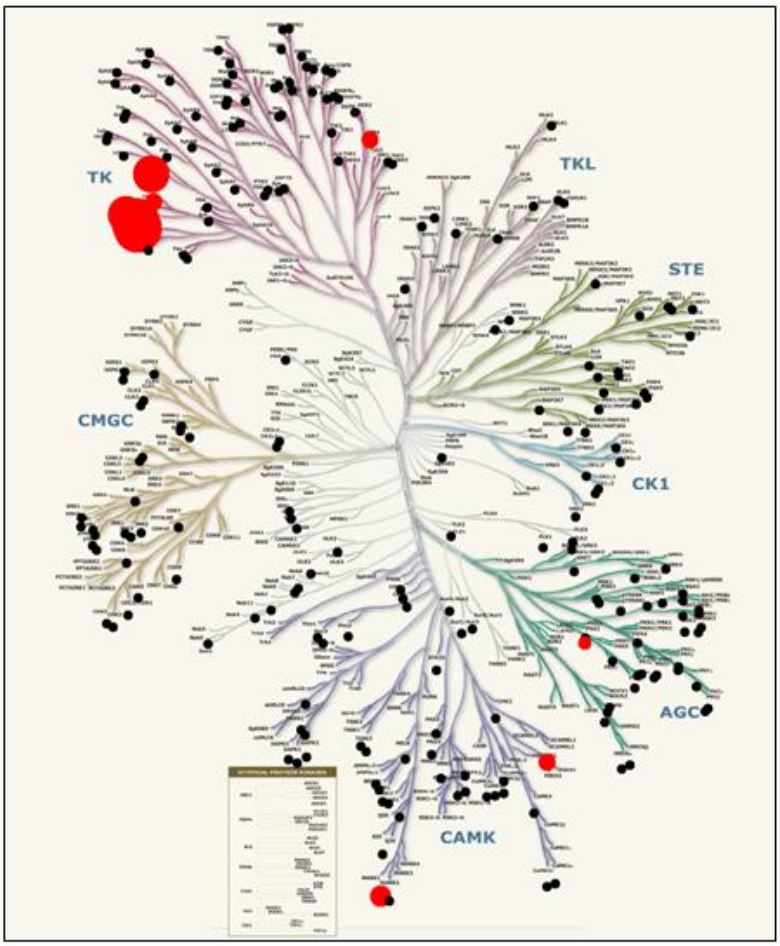
**Fig. 1**

**A.**




**B.**

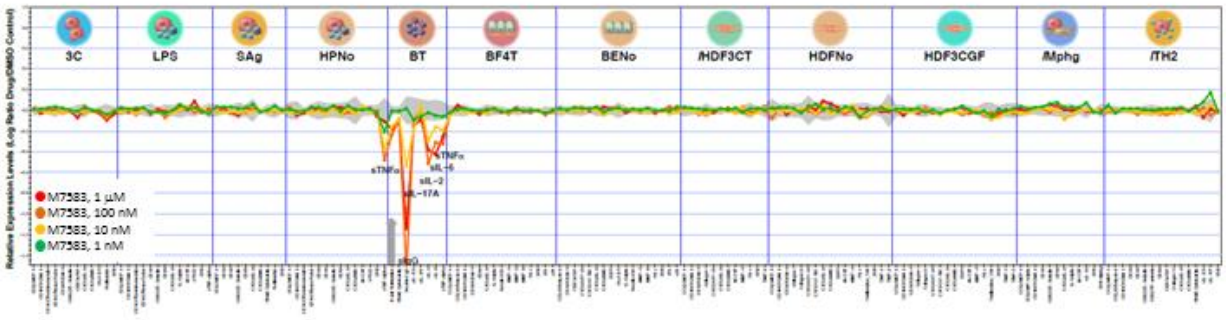


Fig. 2

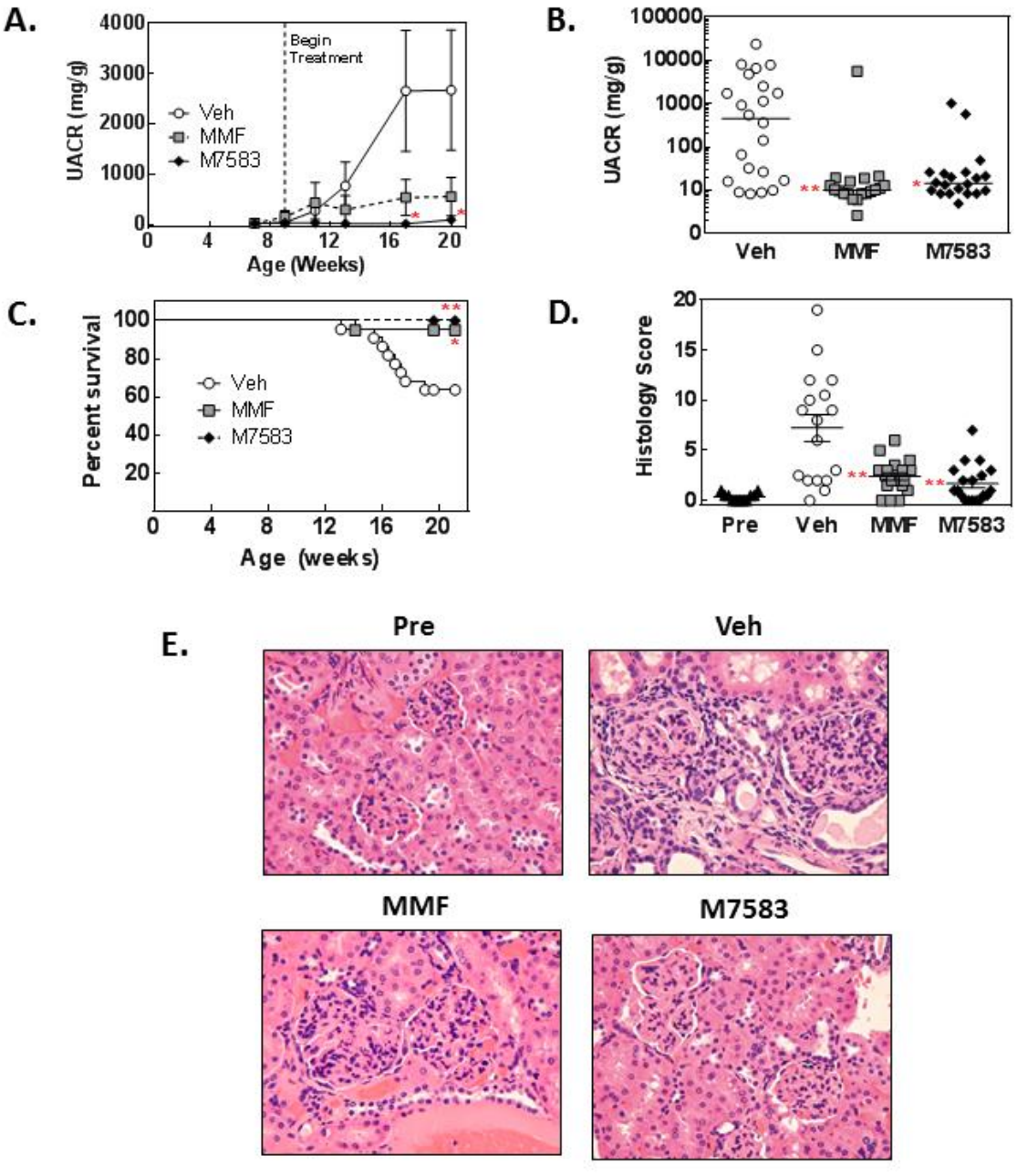


Fig. 3

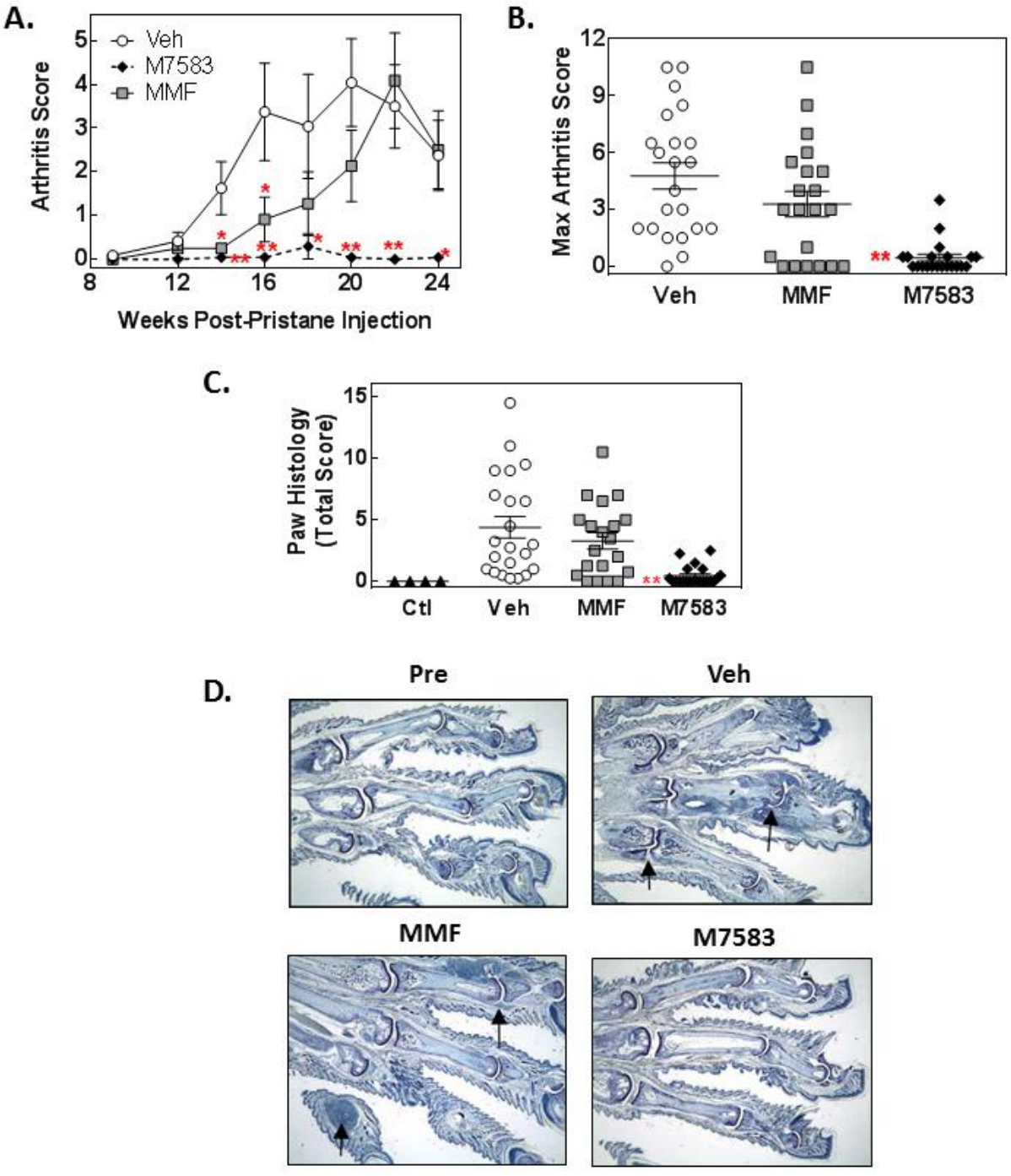


Fig. 4

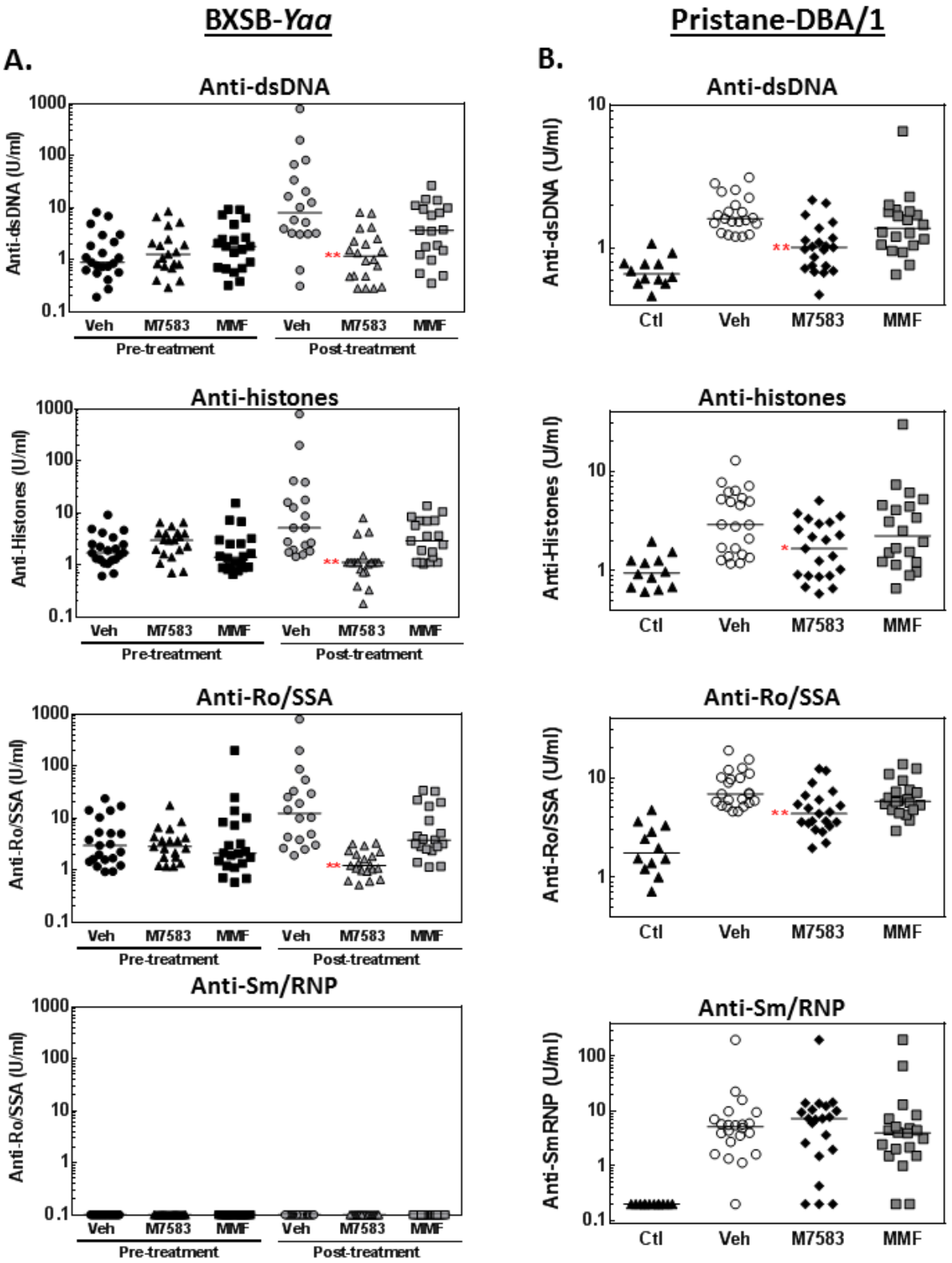
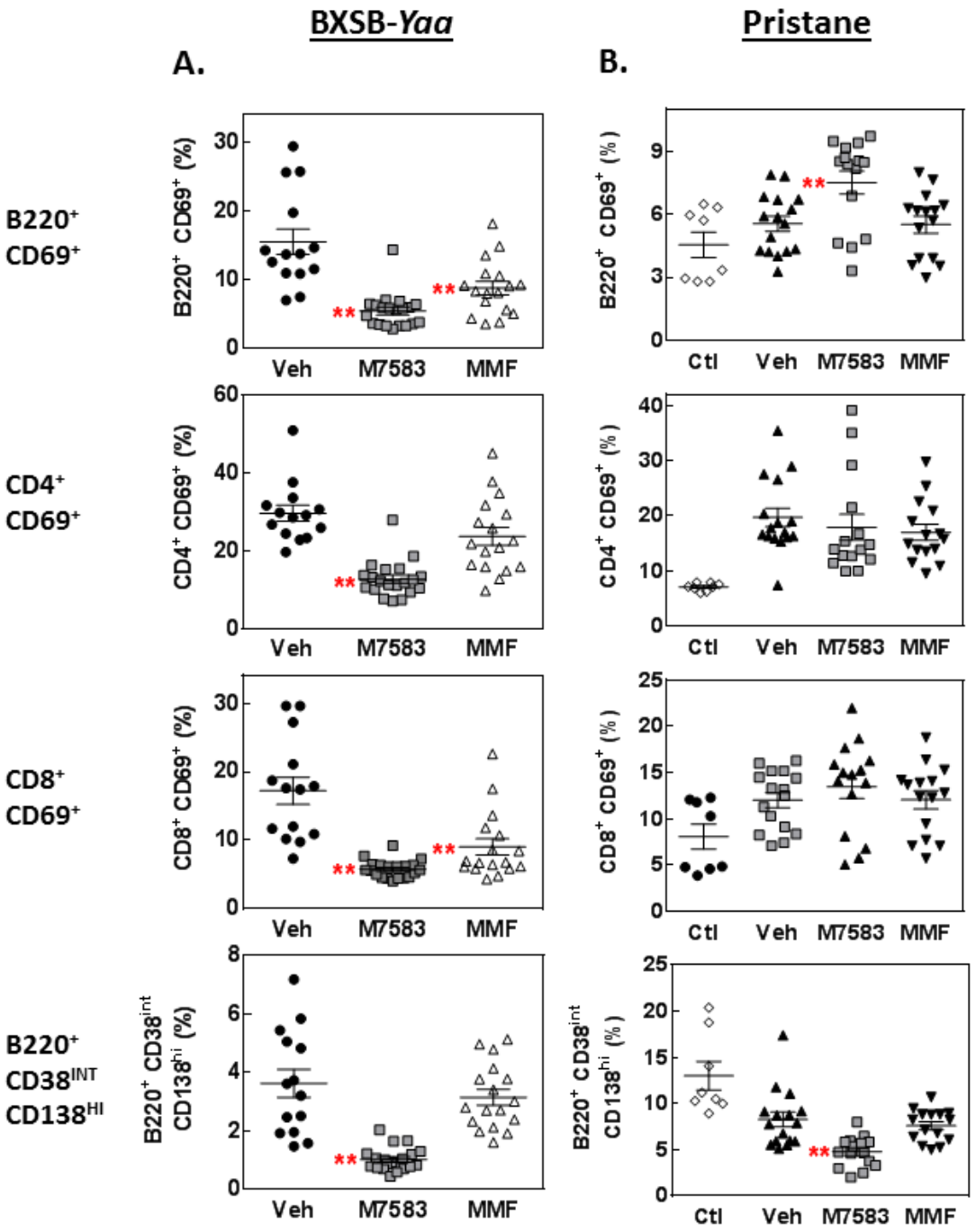


Fig 5



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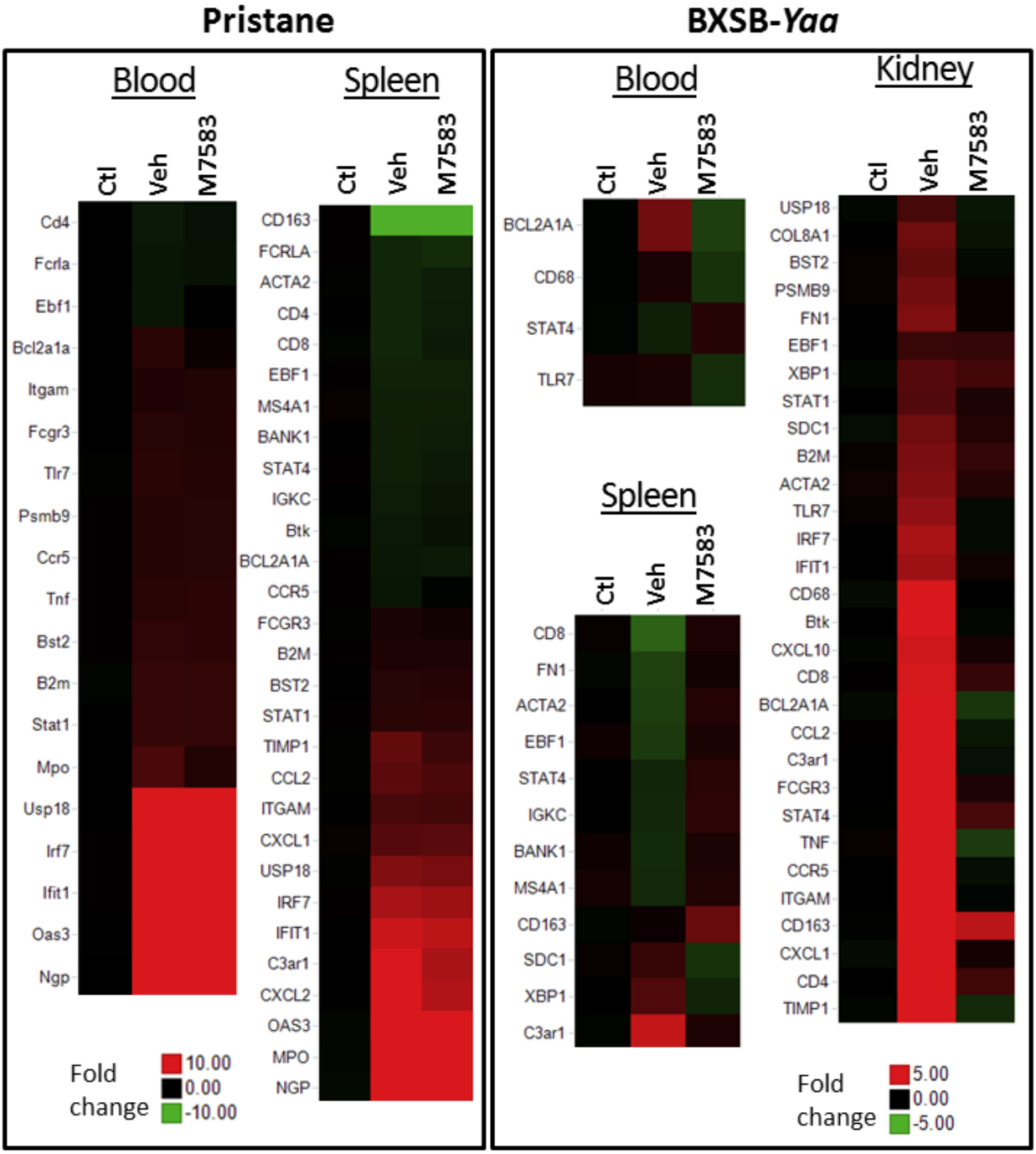
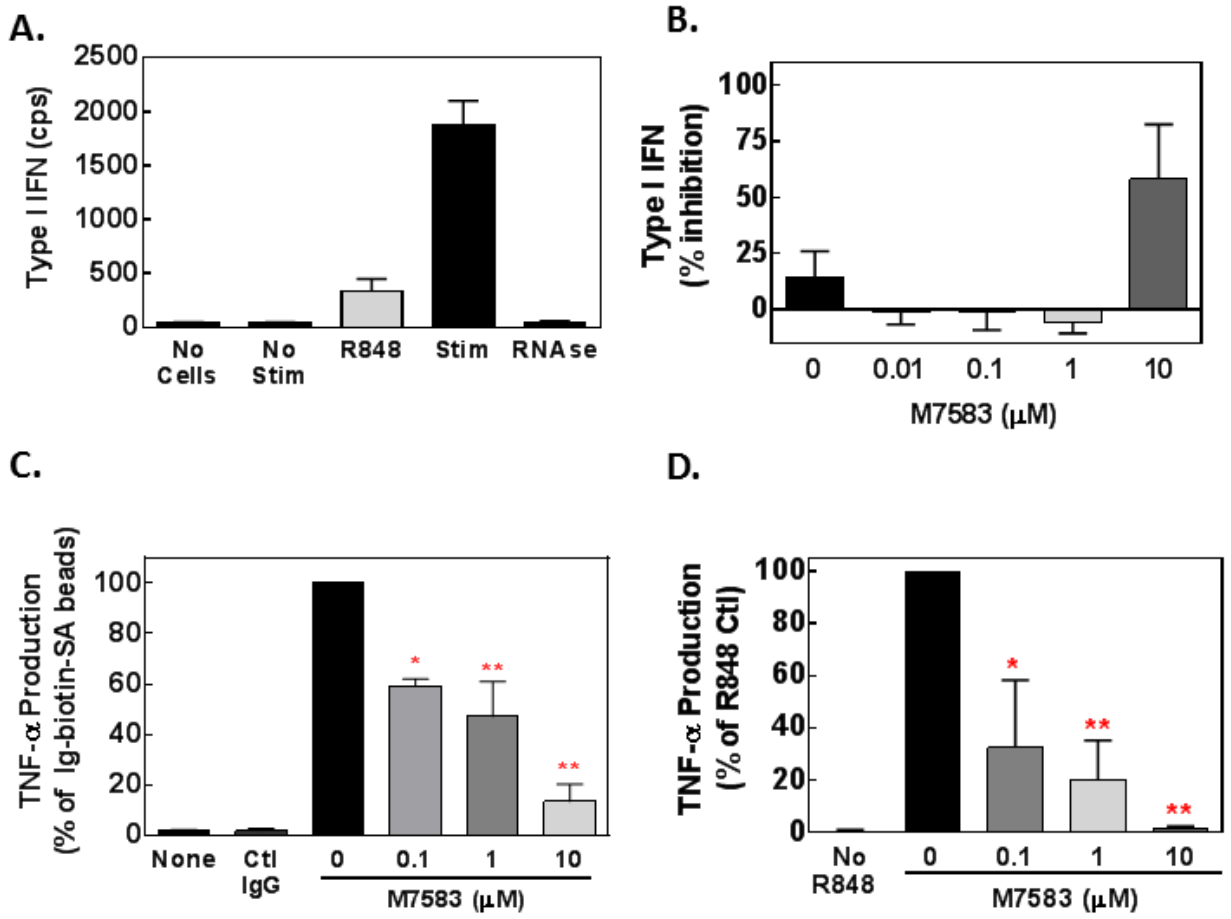


Fig. 7



**Highlights**

- Btk inhibition reduces autoantibodies, nephritis, and mortality in BXSB-*Yaa* lupus mice
- Btk inhibition suppresses arthritis development in pristane-induced mouse lupus
- In lupus mice, arthritis can be suppressed without a significant reduction in autoantibodies or IFN
- Btk inhibition can block activation of human macrophages by immune complexes and TLR7
- Btk inhibition can provide therapeutic benefit to SLE patients by affecting both BCR and FcR signaling