

AWARD NUMBER:

**W81XWH-16-1-0609**

TITLE:

**Novel Small-Molecule Inhibitor of Tyk2: Lucrative Therapeutic Target in Lupus**

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REPORT DATE:

**June 2019**

TYPE OF REPORT:

**Final Report**

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

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1. REPORT DATE <b>June 2019</b>			2. REPORT TYPE <b>Final</b>		3. DATES COVERED <b>1 Sep 2016 - 28 Feb 2019</b>	
4. TITLE AND SUBTITLE <b>Novel Small-Molecule Inhibitor of Tyk2: Lucrative Therapeutic Target in Lupus</b>					5a. CONTRACT NUMBER	
					5b. GRANT NUMBER <b>W81XWH-16-1-0609</b>	
					5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) <b>Abhishek Trigunaite</b> E-Mail: <b>abhishek.trigunaite@sri.com</b>					5d. PROJECT NUMBER	
					5e. TASK NUMBER	
					5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  <b>SRI International</b> <b>333 Ravenswood Avenue</b> <b>Menlo Park</b> <b>CA. 94025</b>					8. PERFORMING ORGANIZATION REPORT	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					10. SPONSOR/MONITOR'S ACRONYM(S)	
					11. SPONSOR/MONITOR'S NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT  Approved for Public Release; Distribution Unlimited						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT This research project explored the use of small molecule inhibitors of Tyk2 and Jak1 for treating lupus like disease pathology in mouse models of lupus. <b>In our findings we report that 6-week treatment of lupus-prone (NZBxNZW)F1 mice with small molecule inhibitor of Tyk2/Jak1 significantly lowers the pathological auto-antibody production.</b> Similar treatment in MRL/lpr model of lupus did not have any effect. The treatment of (NZBxNZW)F1 mice also leads to a drop in the number of germinal centers in the spleen as well as a decrease in spleen weights. Since lupus is a chronic disease, we think that a prolonged treatment (>6 weeks) will show more profound effects on other lupus related disease pathologies like kidney damage. Over all, the treatment has shown potential for significantly improving the disease pathology in (NZBxNZW)F1 mouse model of lupus.						
15. SUBJECT TERMS <b>Lupus, Tyk2, Jak1, Inhibitor, cytokine signaling, Auto-antibodies</b>						
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON	
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC	
U	U	U	UU	10	19b. TELEPHONE NUMBER (include area code)	

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## 1. INTRODUCTION:

The topic of this research proposal is systemic lupus erythematosus (SLE), commonly known as lupus, in the Peer Reviewed Medical Research Program. Lupus is a disease of the immune system characterized by autoantibody production, often against nuclear constituents. Patients suffer from damage to different organs like- kidney, heart and nervous system due to immune complex deposition and subsequent activation of immune responses. The chronic inflammation in lupus is mediated by key cytokines such as - interferon-alpha (IFN-alpha), interleukin-6 (IL-6) and interleukin-23(IL-23). These key cytokines promote auto-antibody production that drives tissue damage. Signaling through IFN-alpha, IL-6 and IL-23 receptors is heavily dependent on Janus kinase family members Tyk2 and Jak1 which makes them a potential therapeutic target in lupus. The current research project focuses on the use of small molecule inhibitor of Tyk2/Jak1 as a therapeutic for lupus. The experiments will test the hypothesis that Tyk2/Jak1 inhibition will reduce the levels of pathogenic antibodies (anti-dsDNA) in two mouse model for lupus, which results in less kidney damage and increased lifespan. The proposed work will evaluate the therapeutic efficacy of Tyk2/Jak1 inhibitors in a spontaneous mouse model (NZBxNZW) F1 as well as the MRL/lpr mouse model.

## 2. KEYWORDS:

Lupus, Tyk2, Jak1, Small-molecule Inhibitor, cytokine signaling, Auto-antibodies

## 3. ACCOMPLISHMENTS:

### Major project goals and accomplishments:

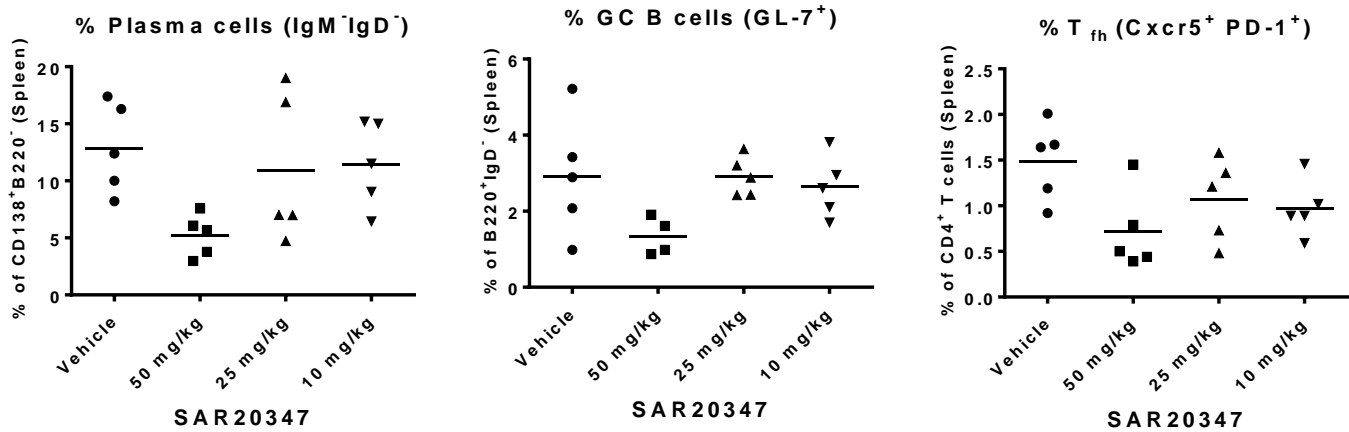
**Aim 1. To determine the potential of Tyk2/Jak1 small molecule inhibitor in ameliorating lupus like pathologies in mouse models of SLE.**

It is well known that IFN- $\alpha$ , IL-6 and IL-23 play a central role in disease development, progression and severity in lupus, and all these cytokines depend on Tyk2/Jak1 for their effector function. Our initial animal studies were designed to assess the impact of treating lupus prone mice with different doses of SAR-20347, a Tyk2/Jak1 inhibitor, to identify a physiological relevant dose of the compound. We were guided by previous work demonstrating that oral administration of SAR-20347 successfully ameliorates psoriasis symptoms in a mouse model and suppresses IFN- $\alpha$  signaling in vitro. Based on those studies, mice were dosed with 50 mg/kg, 25mg/kg, 10mg/kg of SAR20347 or vehicle respectively (n=5 for each group). We expected a reduction in lupus-like disease by oral administration of SAR-20347 in mouse model of lupus.

The (NZBxNZW) F1 mouse is a spontaneous model of SLE, and as early as 12 weeks of age the animals start showing signs of lupus like disease - hypergammaglobulinemia and elevated levels of IFN- $\alpha$  in serum. We obtained 16 weeks old female (NZBxNZW) F1 animals then measured total serum IgG levels to ensure that mice were exhibiting hypergammaglobulinemia that is characteristic of the disease. Mice were randomized them into different treatment groups based on total serum IgG levels. The mice were then dosed 2x daily with either drug or vehicle at doses described above. The animals were treated for a total of six weeks and after that the animals were euthanized and spleens, blood, bone marrow, and kidneys isolated for further analysis.

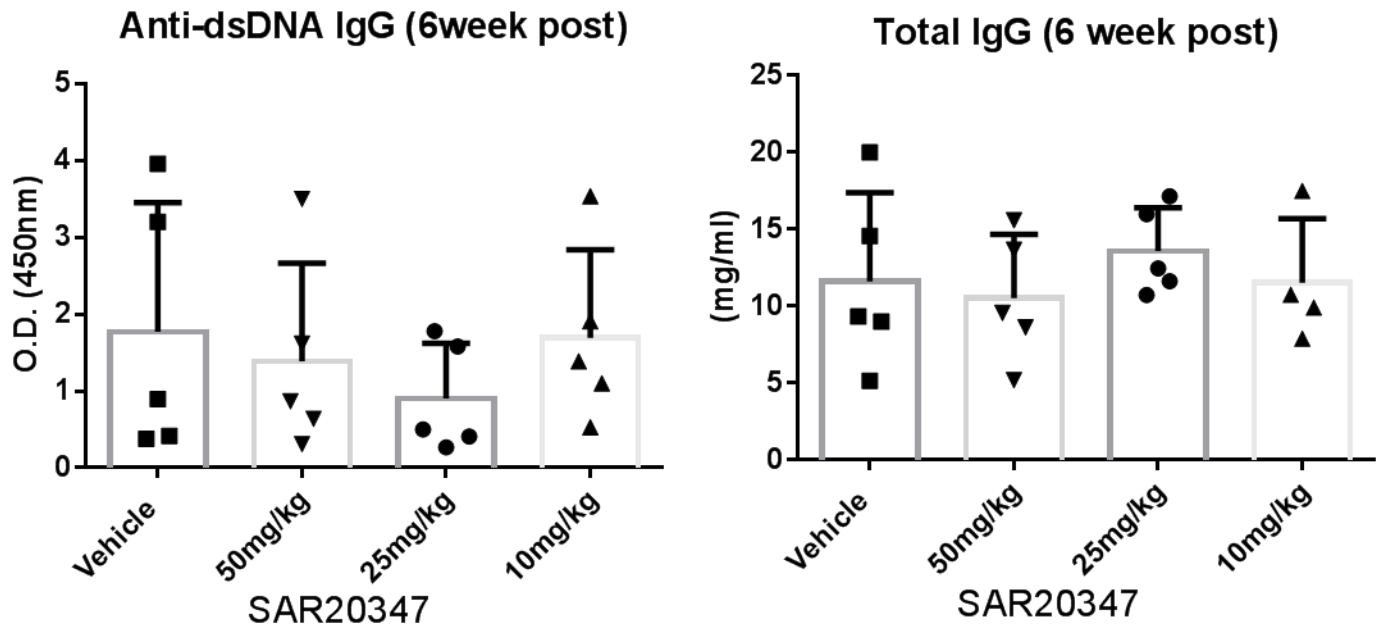
Flow cytometry was performed on spleens and analyzed for presence of plasma cells, Tfh cells and germinal center (GC) B cells. The Tfh cells are a specialized class of T cells that help drive the germinal center formation and promote the differentiation of naïve B cells into antibody secreting plasma cells. In (NZBxNZW) F1 animals plasma cells, Tfh cells and (GC) B cells are elevated as the animals get more severe lupus-like disease. In the **Figure 1** below that plasma cells, (GC) B cells and Tfh cells are decreased in animals that were treated with 50 mg/kg SAR-20347. The effect was not detectable at either 25 mg/kg or 10mg/kg compound. While the results were striking at 50 mg/kg, there is concern that some activity might be due to off target effects. In addition, efficacy was observed at lower doses in an arthritis model, further suggesting that there

may be off target effects at the higher dose. These data are very encouraging as we see a decrease in all three disease driving cell populations with Tyk2/Jak1 inhibition. Additionally, we did not see changes in the overall T and B cell populations in the treated animals which strengthens our hypothesis that we can target specific signaling pathways promoting these pathological cell types. This experiment gave us a clear indication that the effective doses of SAR-20347 optimal for use in long term treatment studies will be 50mg/kg or 25 mg/kg.



**Fig 1. Tyk2/Jak1 inhibition by SAR-20347 decreases plasma cells, Tfh and GC B cells in lupus prone mice.** Mice were treated for 6 weeks and spleens were analyzed by flow cytometry. Results are summarized in the graphs.

We collected serum from the mice and analyzed it for the levels of anti-dsDNA antibodies or total IgG. There was no significant difference found in total anti-dsDNA antibodies or total IgG levels between vehicle and drug treated animals (**Figure 2**). Given the 3-week half-life of antibodies, it may take several additional weeks before a clear decrease in titer is obtained.



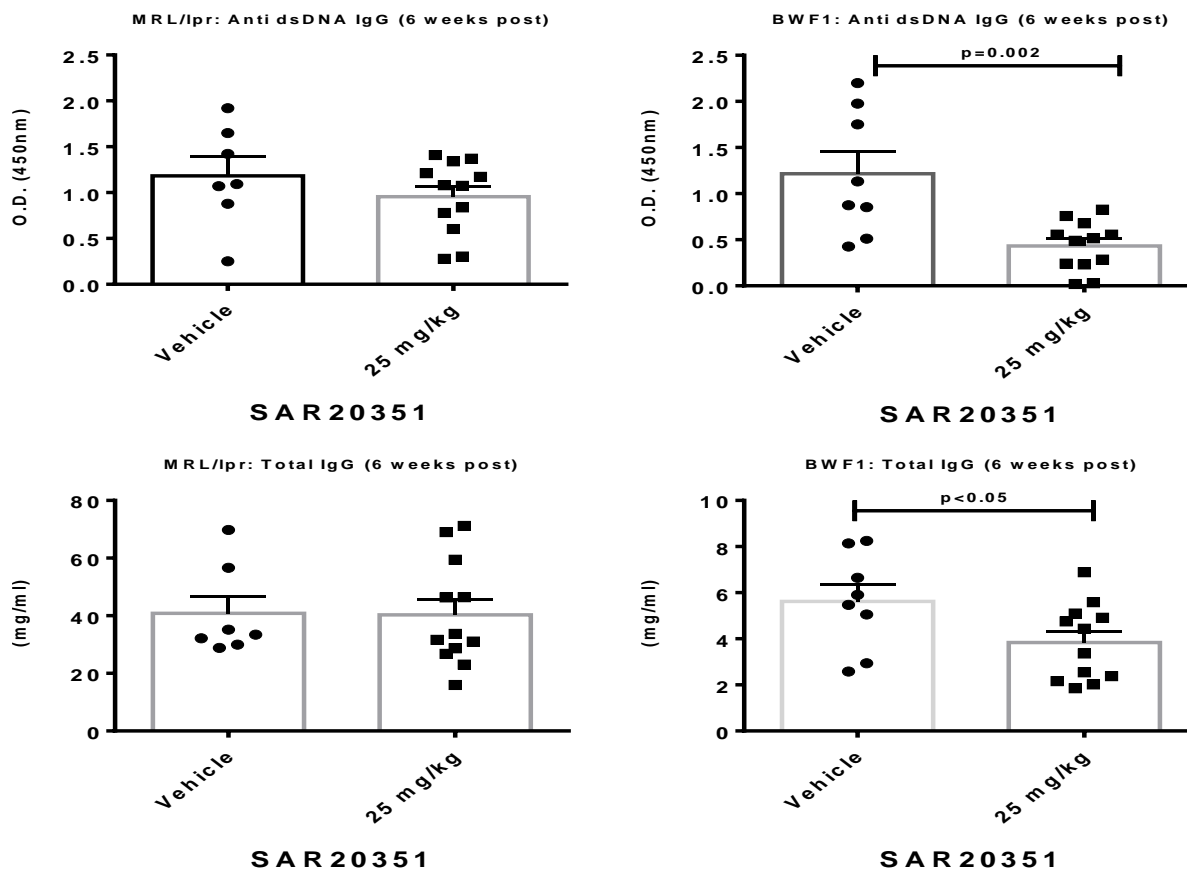
**Fig 2. SAR20347 treatment does not decrease auto-antibody production in (NZBxNZW) F1 mice.** Mice were randomized at the beginning of treatment based on total IgG and anti-dsDNA antibodies. After 6 weeks of treatments with SAR20347 serum was collected and analyzed by ELISA.

## Developing next generation Tyk2/Jak1 inhibitor:

During the treatment period of the first cohort of animals we tested several new Tyk2/Jak1 small molecules in biochemical as well as cell-based assays. We found that the new compound SAR20351 has similar inhibitory profile against Tyk2/Jak1 as SAR20347, but SAR20351 showed a marked improvement in terms of bio-availability and had a superior pharmacokinetic profile compared to SAR20347. Moreover, SAR20351 was tested in CIA (collagen induced arthritis) mouse model and showed very good in vivo efficacy even at doses as low as 10mg/kg and 4mg/kg. Based on this evidence we decided to use SAR20351 as our inhibitor/drug of choice for the remainder of this study. Since SAR20351 shows efficacy at lower doses in the CIA model, we decided to dose the animals with 25mg/kg of SAR20351 for further animal studies.

## Comparative study of two mouse models of lupus:

During the scientific review of this proposal it was suggested to use more than one mouse model of lupus. We performed a comparative study using MRL/lpr mouse and (NZBxNZW) F1 mouse models of lupus. In contrast to (NZBxNZW) F1, the disease progression is faster and more severe in MRL/lpr mouse model. The MRL/lpr mouse model lacks the Fas receptor which results in the accumulation of autoreactive immune cells in lymphoid tissues (spleen and lymph nodes) leading to auto-antibody production and severe tissue damage. MRL/lpr mice develop high titers of auto-antibodies as early as 12 weeks of age and show signs of lupus.



**Fig 3. SAR20351 treatment significantly decreased auto-antibody production in (NZBxNZW) F1 mice but not in MRL/lpr mice.** Mice were randomized at the beginning of treatment based on total IgG and anti-dsDNA antibodies. After 6 weeks of treatments with SAR20351 serum was collected and analyzed by ELISA.

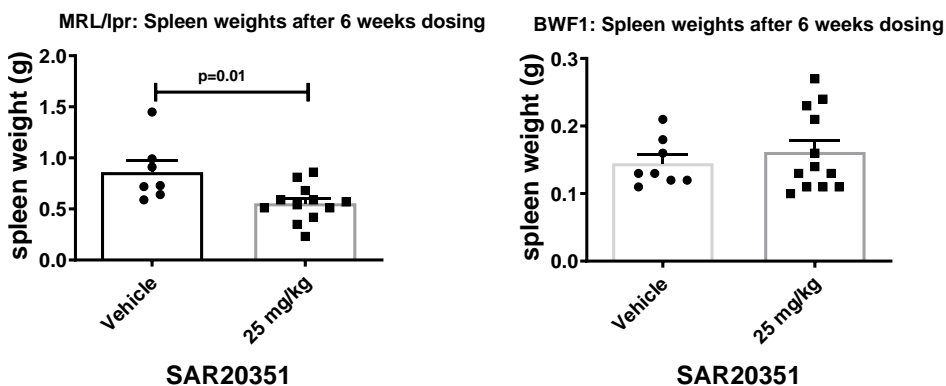
For the second animal study we obtained 12 weeks old female MRL/lpr mice and 20 weeks old female (NZBxNZW) F1. These ages were selected to ensure that mice were exhibiting lupus symptoms. All the animals were randomized based on serum IgG measurements and the mice were then dosed 2x daily with 25 mg/kg of SAR20351 (n=12) or vehicle respectively (n=8) for each group. The animals were treated for a total of six weeks, then the animals were euthanized and spleens, blood, bone marrow, and kidneys isolated for further analysis. Flow cytometry was performed on spleens and analyzed for presence of plasma cells, Tfh cells and germinal center (GC) B cells. We did not observe any significant changes in the cell populations in either of the mouse strains. We also performed ELISA on serum collected from these animals at the end of the 6-week study and found that SAR20351 treatment significantly decreased anti-dsDNA antibodies as well as total IgG (**Figure 3**) in (NZBxNZW) F1 mice (right panels) but had no effect in MRL/lpr mice (left panels).

It is of particular interest that we observed significant differences between SAR20347 (the original compound tested) and the newer compound SAR20351. At 25 mg/kg, neither compound affected the cell types associated with lupus, yet SAR20351, unlike SAR20347, reduced the amounts of pathogenic antibodies. This is instructive on two levels, first it indicates that development or maintenance of the pathogenic cell populations probably are not reliant on Tyk2/Jak1 dependent cytokines, and two, other aspects of the disease, such as Type I IFN signaling which requires Tyk2/Jak1 may help drive auto-antibody production.

These results also demonstrated that there is a difference in the efficacy of Tyk2/Jak1 inhibition in ameliorating lupus like disease in different mouse strains. These data have significant scientific relevance, showing that the same treatment may have different outcomes depending on the animal disease model of lupus. While (NZBxNZW) F1 is a spontaneous mouse model of lupus with multigenic traits giving rise to disease, the MRL/lpr mice is genetically deficient in Fas receptor where immune cells fail to undergo apoptosis and results in autoimmunity. The lupus-like pathology in the (NZBxNZW) F1 mice has a major contribution from key cytokines - IFN-alpha, IL-6 and IL-23, and since our treatment using a Tyk2/Jak1 inhibitor targets the signaling pathways of the above-mentioned cytokines, it is likely that there would be a better response in this mouse model. In contrast, the sheer abundance and accumulation of immune cells in MRL/lpr mice, caused by defective apoptosis, is the major disease driving force in this model. Additionally, lupus development in MRL/lpr mice is very rapid and severe while (NZBxNZW) F1 present a more chronic and slower progression of the disease as seen in humans.

#### Splenic changes in (NZB/NZW) F1 and MRL/lpr mice treated with Tyk2/Jak1 inhibitor:

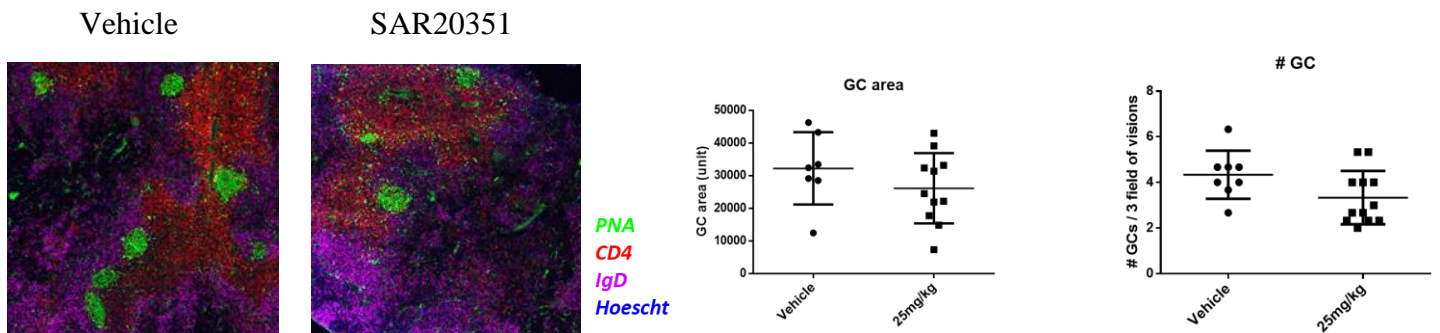
Both the (NZB/NZW)F1 as well as MRL/lpr lupus prone mice develop splenomegaly as disease progresses. This is accompanied by an increase in germinal centers, which are sites where B cells differentiate into antibody secreting cells. Over time, the size of germinal centers also increases in the lupus prone mice. The spleen weights of the experimental animals were examined after six weeks of treatment with SAR2051 or vehicle. There was a significant decrease in the spleen weight of MRL/lpr mice after six weeks of treatment while the spleen weights for (NZB/NZW)F1 did not show any significant change post treatment (**Figure 4**).



**Fig 4. SAR20351 treatment significantly decreased splenomegaly in MRL/lpr mice but not in (NZBxNZW) F1 mice.**

The difference seen in the splenomegaly between the two lupus prone mice strains may be attributed to the differential disease progression between the (NZB/NZW)F1 and MRL/lpr animals. By the age of 25 weeks the MRL/lpr mice are highly progressed in lupus like disease pathology and almost terminally sick whereas the (NZB/NZW)F1 mice are still progressing in disease pathology. Since, we did not see any decrease in auto-antibody production in MRL/lpr mice on treatment with SAR20351, we speculate that the decrease in splenomegaly in MRL/lpr mice may be due to the general diseased state of the animals and not an affect of the treatment.

We also sectioned frozen spleen samples from (NZB/NZW)F1 vehicle and inhibitor treated animals and performed immunofluorescence to determine the frequency and the size of germinal centers. The MRL/lpr animals were not responsive to treatment for reducing pathogenic auto-antibodies, hence we did not perform immunohistochemistry of tissues obtained from the MRL/lpr mice. The germinal centers are identified by PNA staining (green) and are located inside the B cell follicles marked by IgD (purple). **Figure 5** shows the representative images of GCs from vehicle and SAR-20351 treated animal as well as the quantification of GC area and numbers.



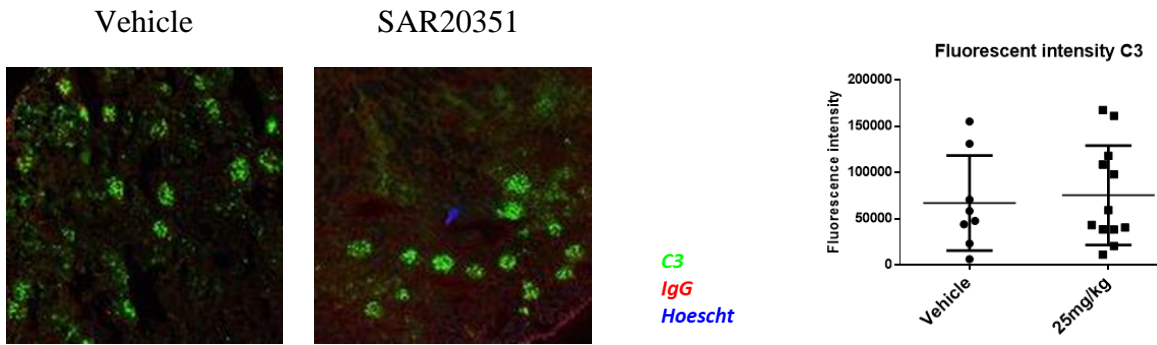
**Fig 5. Quantification of germinal center area and numbers in spleens of (NZBxNZW) F1 mice.** Spleens were sectioned and stained with markers indicated on panel. PNA: marks germinal centers; IgD: marks B cell follicles; CD4: marks CD4 T cells, possibly Tfh cells. The number of PNA+ germinal centers (sites of antibody producing B cells) were counted in 3 fields from vehicle or SAR20351 treated animals and the average was plotted. The area of germinal centers was determined using ImageJ and the total average area was plotted.

Although we noticed a slight decrease in the number as well as the area of GCs in the SAR20351 treated animals but there is no statistical significance.

### Immune complex deposition and fixation in kidney of (NZBxNZW) F1 mice:

Lupus nephritis is one of the major complications of lupus in patients with lupus and is one of the major causes of mortality. Lupus nephritis is caused by immune complex (IC) deposition and fixation in the kidney glomeruli which results in damage to the glomeruli. In (NZBxNZW) F1 mice IC deposition and fixation is a major lupus like pathology. Complement C3 (C3) plays an important role in kidney damage in (NZBxNZW) F1 mice. Under normal condition, C3 is distributed in the interstitial space between glomeruli but as the animals progress in disease and develop nephritis, the C3 binds immune complexes and localizes inside the glomeruli. The resultant complement fixation leads to glomerular damage. We analyzed the distribution of IgG and C3 in kidneys of SAR20351 treated and vehicle treated animals. As C3 fixation in the glomeruli of the kidney is the main cause of renal disease, we stained frozen-sectioned kidney samples with IgG (red) and C3 (green) and further calculated the fluorescence intensity of C3 (indicative of lupus nephritis). **Figure 6** shows the representative images of kidney sections from vehicle and SAR-20351 treated animal as well as the quantification of fluorescence intensity of C3.



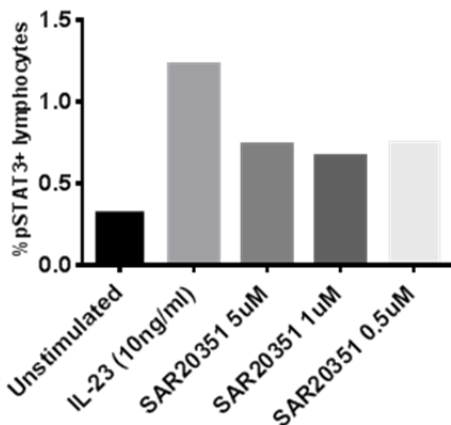


**Fig 6. Quantification IC deposition in kidneys of (NZBxNZW) F1 mice.** Kidneys were sectioned and stained with markers indicated on panel: anti-IgG (red) and complement C3 (green). Fluorescent intensity of C3 present in the glomeruli was calculated and plotted.

There is no significant difference in the IC deposition and fixation in the glomeruli of (NZBxNZW) F1 treated animals versus vehicle treated animals. It is possible that these differences will be more prominent if the animals could reach a later and more severe state of the disease which is around 8-9 months of age. All the animals in this experimental group were 6-7 months of age at the end of the treatment. Unfortunately, due to lack of funds we were not able to perform a long term survival study of (NZBxNZW) F1 mice.

**Aim 2: To determine the mechanism of Tyk2/Jak1 small molecule inhibitor in inhibiting antibody production in vitro.**

We performed in vitro cell based studies directed towards the mechanism of action of Tyk2/Jak1 inhibition in ameliorating lupus. As mentioned in the earlier section that IFN-alpha, IL-6 and IL-23 play critical role in lupus, we have already shown (unrelated studies) that SAR20351 has inhibitory activity against IFN-alpha and IL-6 signaling in vitro. In the current study we tested if SAR20351 can inhibit IL-23 signaling in vitro. Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood and were pre-treated with SAR20351 or DMSO control for 15 min and subjected to a 20-minute stimulation with IL-23. IL-23 signaling activity was measured by performing flow cytometry for pSTAT3. We observed that pre-treatment with SAR20351 reduced the amount of pSTAT3 after IL-23 stimulation by at least 50%. As seen in **Figure 7**, IL-23 signaling could be inhibited even at low doses of 1uM and 0.5uM SAR20351. These data provide us with clear evidence that our Tyk2/Jak1 inhibitor (SAR20351) can successfully inhibit- IFN-alpha, IL-6 and IL-23 signaling in vitro. IL-23 signaling drives TH17 cells which are pathogenic in lupus. Th17 cells are potent IL-6 producers which drives the germinal center reaction and auto-antibody production. Hence, the inhibition of IL-23 signaling by SAR20351 might play a significant role in the decrease of auto-antibodies seen in (NZBxNZW) F1 with SAR20351 treatment.



**Fig 7. SAR20351 inhibits IL-23 induced pSTAT3 in human PBMCs.** Isolated human PBMCs were pre-treated with different concentrations of SAR20351 or DMSO and treated with 10ng/ml of IL-23 for 20 min. to initiate STAT3 activation. pSTAT3 positive lymphocytes were detected by flow cytometry.

More detailed experimentation with human PBMCs can give better insights in to the mechanism of action SAR20351 downstream of other inflammatory mediators like IL-6 and IL-17.

## **Key conclusions:**

- Treatment with Tyk2/Jak1 inhibitor (SAR20351) significantly reduces auto-antibody levels in 20 weeks old female (NZBxNZW) F1 mice.
- There is no significant effect of Tyk2/Jak1 inhibition on disease parameters in MRL/lpr mice.
- SAR20351 treatment tends to decrease GC activity in (NZBxNZW) F1 mice but it does not reach statistical significance.
- The IC deposition in glomeruli of (NZBxNZW) F1 mice is not affected by SAR20351.
- Our Tyk2/Jak1 inhibitor blocks IL-23 signaling in human PBMCs.

## **What opportunities for training and professional development has the project provided?**

Performing and designing the experiments for this project provided Dr. Abhishek Trigunaite (PI for this project) various opportunities for thinking as an independent scientist and refining the technical skill set while performing the experiments. Dr. Toufan Parman (Mentor for Dr. Trigunaite) has provided excellent guidance to Dr. Trigunaite in multiple aspects of the project like – data analysis, data interpretation and to perform experiments in an efficient manner boosting his professional development as an independent investigator.

## **How were the results disseminated to communities of interest?**

Nothing to report

## **4. IMPACT:**

### **What was the impact on the development of the principal discipline(s) of the project?**

We have evidence of reducing pathogenic auto-antibodies in mouse model of lupus without effecting the normal balance of the immune cell subsets. This approach is novel and may lead to development of a therapy for lupus which doesn't involve systemic immuno-suppression with harmful side effects.

### **What was the impact on other disciplines?**

Since the compounds can block Th17 associated cytokines (e.g. IL-6 and IL-23), these results could influence treatments aimed at other Th17 mediated diseases, such as arthritis and psoriasis.

### **What was the impact on technology transfer?**

Nothing to report

### **What was the impact on society beyond science and technology?**

This approach may open up additional treatment approaches for lupus. The standard of care is little changed in 50 years, so there is a need for new treatment options. Lupus particularly targets women in their most productive years (child bearing age) of life. Our results, though not very conclusive and only based of short-term treatment, have significant indications that our approach can be used as a therapeutic target in a chronic disease like lupus and may have positive outcomes. Our current approach may benefit numerous lupus patients and improve their quality of life and productive contribution to society.

## **5. CHANGES/PROBLEMS:**

We received a no cost extension for this project in April 2018. The PI on this project, Dr. Abhishek Trigunaite, had to travel to his home country (India) for immigration related issues (VISA stamping). Because of the unanticipated length of the processing time, he was required to stay in India for nearly

3 months and only able to return close to the termination date of the project. This loss of effort meant that we were unable to perform the long-term dosing studies prior to the termination date of the grant. Additionally, at the beginning of this award we had only proposed to look at only one strain of lupus prone mice ((NZBxNZW) F1 mice) but later during the scientific review of the proposal, it was decided to include a second mouse model (MRL/lpr) in the study. This increase in the animal number for the short term (4-6 week) treatment did not leave us with enough funds to perform a long-term survival study.

**6. PRODUCTS:**

Nothing to report

**7. Participants and Other Collaboration Organizations:**

No Change

**8. SPECIAL REPORTING REQUIREMENTS:**

Not Applicable

**9. APPENDICES:**

No Appendices