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

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This research project is directed at exploring the use of small molecule inhibitors of Tyk2 and Jak1 aimed at ameliorating the chronic inflammatory milieu and high titers of auto-antibodies in lupus, decrease disease symptoms and prolong survival in the (NZBxNZW)F1 mouse model of lupus. In our initial findings we report that treatment of lupus-prone (NZBxNZW)F1 mice with small molecule inhibitor of Tyk2/Jak1 significantly lowers the pathological auto-antibody production. The treatment also lead to a drop in the number of germinal centers in the spleen (NZBxNZW)F1 mice as well as a decrease in spleen weights. Over all, the treatment has shown potential for significantly improving the disease pathology in (NZBxNZW)F1 mouse model of lupus. We are in the process of performing experiments for the next phase of the proposal.
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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 a mouse model for lupus, which results in less kidney damage and increased lifespan.

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 (NZBxNZW) F1 spontaneous mouse model of SLE.

It is well known that IFN-α, 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, in order 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-α 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-α 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 survival 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.

To confirm these results, we have sectioned frozen spleen samples from vehicle and inhibitor treated animals and are in the process of performing immunofluorescence to determine the frequency and the size of germinal centers. The germinal centers are identified by PNA staining (green) and are located inside the B cell follicles marked by IgD (purple). **Figure 2** shows the preliminary representative images of germinal centers from vehicle and SAR-20347 treated animal. Although this data is preliminary, we observe a marked decreased in germinal center (indicated by arrows) area in the spleen of drug treated animal. We are in the process of performing immunofluorescence on the whole cohort of animals from this study. In addition, we have kidneys frozen for cryosectioning to determine if pathological immune complex deposits are reduced after drug treatment.

**Fig 2. Germinal Center (GC) area is reduced after treatment with SAR-20347.** Spleens were sectioned and stained with indicated markers. The reduction in GC area is consistent with reduction in pathogenic cell populations which promote GC formation and maturation. PNA: marks germinal centers; IgD: marks B cell follicles; CD4: marks CD4 T cells and possibly Tfh cells.
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 3). Given the 3-week half-life of antibodies, it may take several additional weeks before a clear decrease in titer is obtained. We will perform long term survival studies using animals that are further progressed in disease than the animals used for the current study.

![Graph of Anti-dsDNA IgG and Total IgG levels](image)

**Fig 3. 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 bioavailability 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.

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 SR20351 treatment significantly decreased anti-dsDNA antibodies as well as total IgG (Fig 4) 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. Based on these results we have decided to use (NZBxNZW) F1 mice for the remainder of this project as our lupus mouse model.

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

While performing the animal experiments we did some preliminary 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 5**, 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.

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**Fig 5.** 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.

We are in the process of performing other in vitro experiments mentioned in Aim2 and Aim3 of this proposal. Briefly, **Aim2)** Differentiation assays for Tfh cells and antibody secreting B cells in the presence or absence of Tyk2/Jak1 inhibitor and **Aim3)** Effect of Tyk2/Jak1 inhibition on IFN-induced gene scripts in human PBMCs in
the presence of lupus patient serum. Together these experiments will give detailed insights into the mechanism of action of Tyk2/JAk1 inhibitors as a therapeutic in lupus.

Key conclusions (all data are preliminary at this stage):

- 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.
- 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 Trigunaute (PI for this project) various opportunities for thinking as an independent scientist and refining the technical skill set while performing the experiments. Dr. Paul Stein (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

What do you plan to do during the next reporting period to accomplish the goals?

- Perform detailed immuno-histochemistry on the tissues collected from the animal studies. Because of the reduction in antibodies by SAR20351, we are especially interested in determining if there is a decrease in lupus nephritis, as exemplified by less immune complex deposition in the kidney.
- Perform a long term animal study determine the effect of Tyk2/Jak1 inhibition on lupus progression and life span in (NZBxNZW) F1 mice.
- IFN-induced genes are highly up-regulated in lupus patients and are considered a hallmark of the disease. We will perform RT PCR on PBMCs collected from long term treated animals to determine the level of IFN-induced gene scripts.
- Perform ELISA for key cytokines like –IL-17, IL-6, IL-23 and IFN-alpha on the serum collected at different time points from Tyk2/Jak1 inhibitor treated animals.
- Perform in vitro differentiation assays for Tfh cells and antibody secreting B cells in the presence or absence of Tyk2/Jak1 inhibitor.
- Determine the effect of Tyk2/Jak1 inhibition on IFN-induced gene scripts in human PBMCs in the presence of lupus patient serum.

4. IMPACT:

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

We have preliminary 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 current approach may benefit numerous lupus patients and improve their quality of life and productive contribution to society.

5. CHANGES/PROBLEMS:

Nothing to report

6. PRODUCTS:

Nothing to report

7. Participants and Other Collaboration Organizations:

No Change

8. SPECIAL REPORTING REQUIREMENTS:

Not Applicable

9. APPENDICES:

Nothing to report