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13. SUPPLEMENTARY NOTES

14. ABSTRACT: This proposal addresses the Topic Area of Systemic Lupus Erythematosus (SLE or lupus), specifically lupus nephritis (LN). Lupus nephritis affects between 30-60% of adult SLE patients and is responsible for significant morbidity and mortality. Despite many advances in biologic drug therapy, no effective new therapy for LN has yet emerged and the reason why so many patients fail therapy is not known. Novel molecular datasets are beginning to be generated from single cells isolated from human LN kidney biopsies. In the first aim, we are successfully generating parallel datasets from the mouse models so that as pathways of interest are identified in the human samples they can quickly be modeled and their function clarified in the appropriate lupus prone mouse. Our second aim addresses the role of autophagy and metabolism in renal macrophages. We have found that deficiency of Rubicon protects the lupus mice from LN and death and are in the process of determining which immune cells are responsible for this protection. We have also investigated the role of PGC-1α in metabolic programming of kidney macrophages in LN. In this instance we have not been able to demonstrate a significant role for this transcriptional regulator in macrophages of LN kidneys.

15. SUBJECT TERMS: SLE, macrophages, autophagy, Rubicon, ATG14, PGC1alpha, single cell genomics, lupus nephritis, lysosome associated phagocytosis

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1. INTRODUCTION:

Our goal is to use a systems approach to understand key features that are relevant to the diagnosis and treatment of human lupus nephritis. Renal infiltration with macrophages is one of the few histologic features associated with poor prognosis in humans – therefore our proposal focuses on these cells. We proposed both a discovery and a functional component to our studies. In the discovery component we have been using single cell RNA sequencing to determine the heterogeneity of renal macrophage subsets and the changes that occur when they enter the kidneys. We have successfully generated data from two lupus strains that have both similarities and differences to each other and have pending data from 2 more strains. We have shown overlap with data from human kidneys generated by the Accelerating Medicines Partnership allowing us to start to predict which mouse models correlate best with human disease. In the functional component, we are studying the role of autophagy and mitochondrial dysfunction in these cells with the long term goal of understanding how targeting of dysfunctional macrophages in LN can lead to improved outcomes and a decrease in progression to chronic renal impairment. We found, unexpectedly, that Rubicon deficiency protects mice from disease and further studies are pending. Our studies of PGC1alpha have been more disappointing as we have been unable so far to detect differences in the function of renal macrophages from mice either overexpressing or underexpressing this gene.

2. KEYWORDS

SLE, macrophages, autophagy, Rubicon, ATG14, PGC1alpha, single cell genomics, lupus nephritis, lysosome associated phagocytosis

3. ACCOMPLISHMENTS

What were the major goals of the project?

Aim 1: To use state of the art single cell RNA sequencing technology to understand the heterogeneity of macrophages and DCs in the inflamed lupus nephritis kidney and apply novel systems biology approaches to compare the profiles of single cells from our mouse models with profiles from the analogous cells from human LN kidneys

Aim 2: To examine pathways of interest involved in renal macrophage autophagy and metabolism

a: Characterize the metabolic abnormalities in LN macrophages and explore the role of classical vs. non-classical (LAP) autophagy in renal macrophages by generating bone marrow chimeric mice in which 30% of macrophages in the effector tissue are deficient in either of these pathways.

b. Determine whether overexpressing PGC1 alpha in macrophages will correct the abnormal macrophage phenotype and improve the outcome of LN

Specific Aim 1 is now 60% complete. The 10X experiments are completed but we still need to complete the tracking experiment. Data analysis is in progress and a manuscript needs to be written.

Specific Aim 2 is 50% complete. The required mouse strains have been generated and outcomes evaluated. Functional studies of macrophages need to be completed and manuscripts written.

Major Tasks Specific Aim 1	Single cell RNASeq (Broad) and data processing (NYGC) Two new lupus strains added and data analysis in process including cross comparisons with human data
Molecular characterization of single cells	
Manuscript in preparation	
Changes in macrophage function over time	Methods are established and experiment needs to be completed.
Major Tasks Specific Aim 2A	The mice are generated and analyses of Rubicon mice are being completed. ATG KO mice are being followed – ages 3-8 months currently.
Generate Rubicon and ATG14 deficient mice and follow for nephritis onset. Accelerate disease if necessary	
Manuscript in preparation (unexpected effect of Rubicon deficiency on B cells)	
Major Task 3 Specific Aim 2B	Macrophage function assays need to be done
Subtask 1: Seahorse assays isolated macrophages	
Subtask 2: Sorting of cells from Rubicon chimeras for Seahorse and metabolic assays	
Subtask 3: Arginase and NO assays	
Major Task 4 Specific Aim 2C	Clinical analyses complete Macrophage functional assays are completed Low input mRNASeq and/or single cell RNASeq and data processing remains to be done
Analysis of mice overexpressing PGC1 alpha	
Manuscript planned	

What was accomplished under these goals?

We have made excellent progress on all three aims of this grant in the last year.

Aim 1A: In this first section we used single cell PCR to define the heterogeneity of macrophage subsets in the lupus kidney in several mouse models and compare this with data from human kidneys. We successfully performed two 10X experiments with hashing at NYGC using NZB/W and Sle1.Yaa mice. This allowed us to pool 4 samples to decrease the risk of batch effects. In each experiment we used PBMC from blood, renal myeloid cells from young mice and renal myeloid cells from nephritic mice (2 pools). QI of the cDNA was excellent for both experiments. Analysis of the first two strains has shown remarkable heterogeneity of the myeloid subset in the mice. Since we had >5000 cells from each strain our data is much deeper than reported in humans where we were able to differentiate 4 macrophage subsets and at least one dendritic cell subset (Arazi et al Nat Immunol. 2019 Jul;20(7):902-914). In the NZB/W strain we identified 9 major clusters of myeloid cells that include several subclusters. More complex clusters were found in Sle1.Yaa mice. Important findings are

1. Resident macrophages from young and nephritic mice cluster separately from each other;
2. There are several subpopulations of dendritic cells;
3. Myeloid cells from peripheral blood cluster separately from renal cells;
4. Two small subpopulations of IFN hi and inflammatory macrophages may be pro-inflammatory;
5. Trajectory analysis shows several populations with mixed features of macrophages and dendritic cells;
6. DCs in nephritic kidneys organize lymphoid infiltrates and foster antigen presentation;
7. Macrophages in nephritic kidneys acquire a phagocytic/pro-fibrotic phenotype;
8. We were able to identify the mouse counterparts of the subsets we have described in humans.

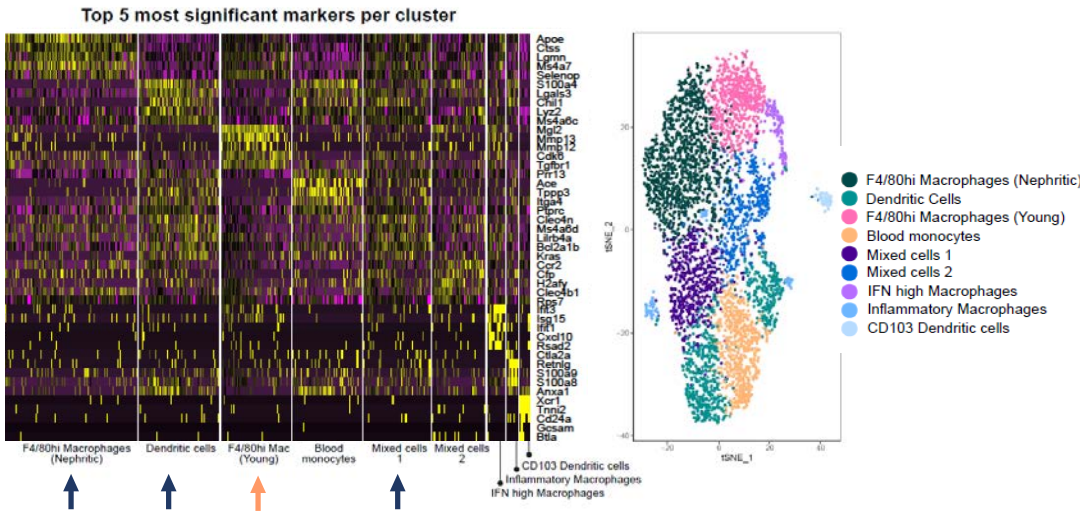

Figure 1: Single cell sequencing shows nine clusters of myeloid cells in NZB/W kidneys. Blue arrows show clusters found predominantly in nephritic mice. Orange arrow shows a cluster found only in young mice.

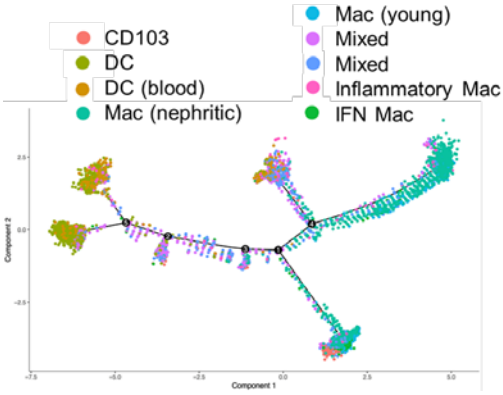

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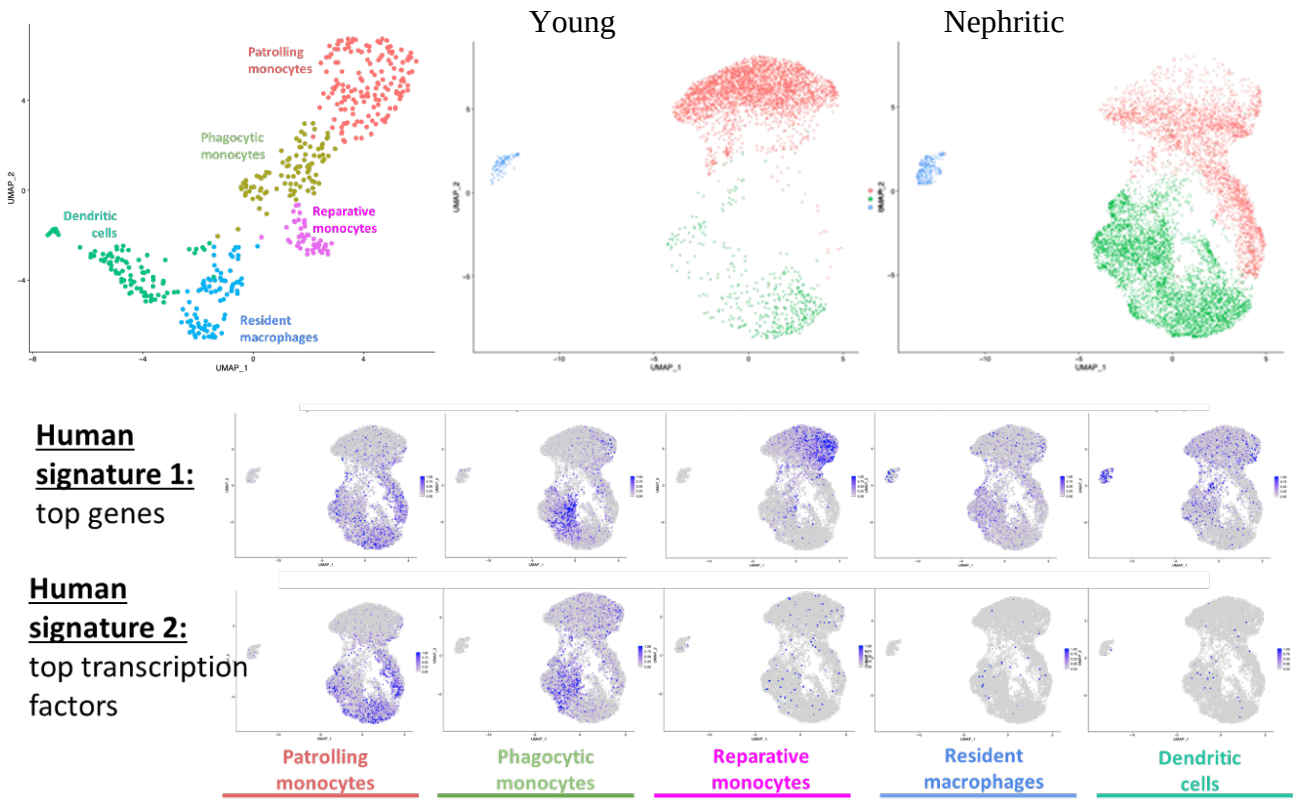
Figure 2: Trajectory analysis of the 9 clusters shows separation of macrophages (blue) from DCs (green) and several populations of cells that display a mixed profile.

This work was reported at the American College of Rheumatology Annual meeting in November 2019 as an oral presentation as well as an invited presentation. Another invited presentation at the 2020 AAI meeting is pending and a manuscript is in preparation. Because our collaborator Dr Hacohen is funded by the Lupus Research Alliance for a complementary project, we have been able to expand our studies in collaboration with his group to include NZW/BXSB and MRL/lpr mice as well; his grant is covering the cost of the added 10X experiments and sequencing and his group is contributing to data analysis

Aim 1B: We had also proposed in this Aim to use intra-bone marrow transplant to trace myeloid cells newly arriving in the kidney so that these could be analyzed for their gene expression profile. The first approach that we proposed, to use GFP donors did not work because the GFP is a cytoplasmic antigen and was taken up by many macrophages. The second approach was to use beads for labeling phagocytic macrophages. Although initially we thought this was working, after careful gating of the data only very few cells in the kidney are labeled and only few of these are macrophages. The last technique is to use transfer of CD45 allotype mismatched bone marrow cells. This has been working in Sle1.Yaa mice but since the numbers of cells are small and the number of genes we retrieve with low input RNASeq is still limited, we are changing to a technique in which we irradiate only the mouse tail and legs and then transfer the bone marrow to give us larger numbers of transferred cells.

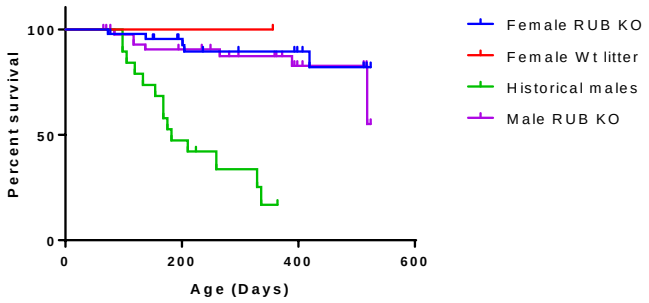
Aim 1C: Initial comparisons with the human data from the Accelerated Medicine Partnerships is being performed using the Phase 1 AMP data. A poster was presented at the American College of Rheumatology Annual meeting in November 2019 and at the Lupus Research Alliance Meeting in October 2019

Figure 3: Upper Left: Myeloid cell populations in human kidneys (Arazi et al Nat Immunol. 2019 20:902-914). **Upper Right:** Myeloid clusters from young and nephritic Sle1.Yaa mice. **Lower panel:** Overlay of mouse and human signatures



Aim 2A and 2B: In this aim we planned to use Rubicon deficient and ATG14 (macrophage) deficient mice to explore the roles of canonical autophagy and LAP in renal macrophages in the Sle1.Yaa model. We have successfully bred the Rubicon deficient mice into Sle1.Yaa and generated 12 male and 16 female bone marrow chimeras. The original reports of the Rubicon deficient mice by Dr Green's group suggested that they develop nephritis and that this is due to the generation of inflammatory rather than anti-inflammatory macrophages. Last year we reported that we did not observe acceleration of the disease course in Rubicon deficient Sle1.Yaa mice: this observation held up and we can now report that not only do the original Rubicon KO mice not develop autoantibodies, but the Sle1 and Sle1.Yaa mice are actually protected.

Figure 4: Survival of Rubicon KO and wild type Sle1 (female and Sle1.Yaa (male) mice. There is no diosease acceleration in females and the males are protected.



When we analyzed the chimeras using flow cytometry we found that the chimeric B cells do not compete well for germinal center entry suggesting that they have a defect in activation. Repertoire analysis is in process to determine whether those cells that do enter are able to undergo mutation and class switching or whether they have an intrinsic defect. A manuscript describing these findings is planned in collaboration with Dr Mark Shlomchik who has

complementary findings in a different lupus model. Further studies of B cells are beyond the scope of this DOD grant and alternate funding will be sought to continue these studies.

Figure 5: ELISA assays of Sle1 Rubicon KO mice (black) shows lower titers of autoantibodies (anti-Sm, anti-cardiolipin, anti-DNA and anti-chromatin) than wild type mice (grey). No increase in autoantibodies is seen in C57BL/6 Rubicon KO mice in contrast to what has been previously reported.

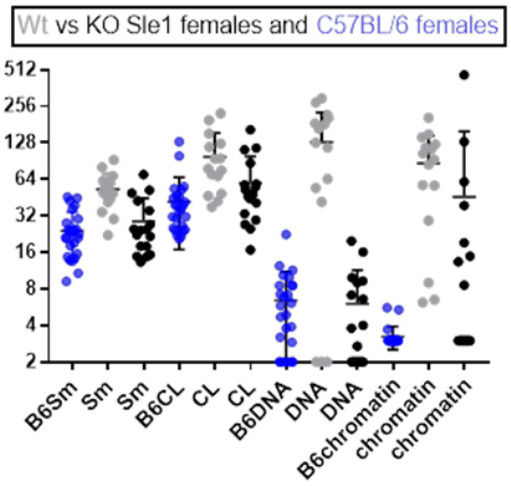
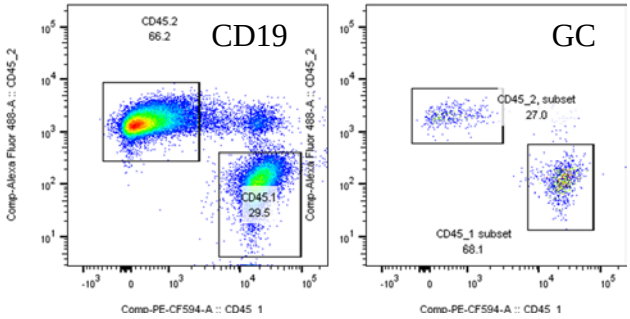


Figure 6: Flow cytometry of chimeric mice shows a ratio of 2:1 KO:Wt B cells but this ratio is reversed in germinal centers. The same data was obtained in all chimeras studied.



Because the rubicon KO mice did not develop nephritis as we had expected, we harvested Rubicon deficient renal macrophages from mixed chimeric mice containing 50% Sle1.Yaa and 50% Sle1.Yaa Rubicon deficient bone marrow. In these chimeras the presence of the Sle1.Yaa bone marrow conferred autoantibodies on the chimeras. These macrophages have been sent for low input RNASeq.

The ATG14 KO mice took longer to breed than the Rubicon deficient mice but are now successfully established and large cohorts are being observed. We will have full results from these mice in this upcoming year. More chimeras will be established for the macrophage functional studies this year but there may be some delay while personnel are being replaced. I have replaced Dr Mishra with Mr Ke Lin who is a senior technician now expert in renal perfusion and macrophage cell preparation and am looking for a replacement for Dr Giannouli.

Aim 2C: In this aim we are studying mice conditionally overexpressing PGC1α in macrophages using LysMCre as the promoter for expression of the PGC1α or Cre transgenes. As described last year we made these mice instead of using the retroviral constructs initially proposed that had low transduction efficiency. We have confirmed the genotype of these mice and showed that macrophages correctly overexpress the mRNA. 3 founders were propagated for our studies. We have now followed a large cohort of PGC1α overexpressing mice for more than a year and they are not protected from disease and have equivalent autoantibody production and mortality to their wild-type counterparts. Analysis of metabolic functions using Seahorse and mitochondrial

stains further did not demonstrate a difference between wild type and overexpressing mice. We have also generated a cohort of PGC1α deficient mice and these do not appear to be more susceptible to disease.

Dr. Giannouli extensively characterized bone marrow macrophages from both these strains and has not been able to detect a difference from wild type in their response to LPS by Seahorse assays or their mitochondrial function by flow cytometry. Our next plan for these mice is to analyze their immune cell phenotypes by flow cytometry at 9 months of age when controls have developed nephritis and to sort renal macrophages for RNASeq to determine whether there are any changes in gene expression. If we fail to find any differences we will report these negative findings as a manuscript. So far, we conclude that PGC1α has no unique role in maintaining renal macrophage function and may be redundant with other related transcriptional regulators.

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

Dr Rakesh Mishra and Dr Christina Giannouli are the post-doctoral fellows who worked on this project. Dr Giannouli was hired specifically for this project. Both learned new techniques in the laboratory including advanced flow cytometry, generation of chimeric mice, intra-bone marrow adoptive transfer and single cell RNASeq. Dr Mishra attended the annual AAI meeting in 2019. He has recently left the laboratory for a job in Pharma. Dr Giannouli attended a meeting on autophagy at NY Academy of Sciences and attended a Keystone myeloid cell meeting. Unfortunately, due to family reasons she needed to return to her native Greece in the Fall of 2019.

How were the results disseminated to communities of interest?

Results were reported at the American College of Rheumatology Meeting in 2019 and the Lupus Research Alliance Meeting in 2019 (see below). Dr Davidson also presented the concepts related to Aim 1 of this proposal to a lay audience of lupus patients and other community members at the Lupus Research Alliance in 2019

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

Aim 1: We plan to continue as per the SOP and complete the analyses of the single cell analyses of the 2 other mouse strains as well as the mouse-human comparisons of the single cell data. Tissue has been stored for analysis of proteins of interest using immunohistochemistry. Once new staff is hired we will complete the timed experiments in Aim 1B.

Aim 2A and 2B. The findings in the rubicon deficient mice were unexpected and have led us to new avenues that will be used to write additional grants related to B cells. The studies of Rubicon and ATG14 deficient macrophages are ongoing. Once staff are replaced, we will complete the macrophage functional studies proposed in chimeras

Aim 2C. These studies will be completed this year and reported.

4. IMPACT:

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

We have completed Year 2 of this project and have the technologies that we need working. The major finding so far is that we can perform single cell analyses of kidney macrophages, the cells that are involved in disposal of dead and damaged tissue but that are abnormally activated in lupus and may contribute to kidney scarring. We have identified complex sub-populations of these cells of which some are similar to the abnormal populations found in human SLE kidneys. Understanding the functions of these cells is important for knowing how they contribute to kidney damage and for designing new approaches to change their function from that of scarring to that of healing. To this end we have been studying the pathways downstream of the important macrophage function of dead cell disposal. Unexpectedly, we found that one of these pathways appears to be required for generating the autoantibodies that cause lupus tissue injury. A specific gene that is an important coactivator of a second pathway that supports energy to the macrophages as they are activated does not seem to play a role by itself in kidney macrophage function.

What was the impact on other disciplines?

The single cell analysis methods can be used by others to study other organs and diseases and is spurring follow up studies by others. The role of autophagy in B cells is a new topic of interest with respect to tolerance in SLE. Pathways downstream of macrophage phagocytosis are of general interest in infection and inflammation.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

We have not made major changes this year. The following minor changes are
a. We have been able to add more mouse strains to the single cell experiments to reflect the heterogeneity of lupus nephritis;
b. We have needed a more extensive characterization of the Rubicon KO mice due to their unexpected phenotype.

Actual or anticipated problems or delays and actions or plans to resolve them

There has been a bit of a slowdown in the second half of the year due to the departure of Dr Mishra (planned) and Dr Giannouli (unplanned). We already have one replacement staff member and hope to get a second soon.

Changes that had a significant impact on expenditures

We have carried over some funds to this year due to the delays conferred by staff leaving and the need to find replacements.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

Publications, conference papers, and presentations

Journal publications.

Davidson A, Aranow C, Mackay M. Lupus nephritis: challenges and progress. Curr Opin Rheumatol. 2019 Nov;31(6):682-688. doi: 10.1097/BOR.0000000000000642 PMID: 31389814. (Federal support acknowledged)

Books or other non-periodical, one-time publications.

Presentations at 2019 ACR Meeting

1. Single Cell Analysis of Renal Myeloid Cells from NZB/WF1 Mice with Lupus Nephritis Reveals Multiple Subsets with Altered Functions (oral presentation). Rakesh Mishra [1], Celine Berthier [2], eather Geiger [3], Weijia Zhang [4] and Anne Davidson [5], [1]Feinstein Institute for Medical Research, Manhasset, NY, [2]University of Michigan, Ann Arbor, MI, [3]New York Genome Center, New York, NY, [4]Mount Sinai School of Medicine, New York, NY, [5]Feinstein Institutes for Medical Research, Manhasset

2. The Single-cell Transcriptomic Landscape of NZB/W Murine Lupus at Early and Late Stages of Disease (poster). Paul Hoover[1], Tom Eisenhaure [2], David Lieb [2], Anne Davidson [3] and Nir Hacohen [2], [1]Brigham and Women's Hospital, Boston, MA, [2]Broad Institute, Cambridge, MA, [3]Feinstein Institutes for Medical Research, Manhasset

3. Using human data sets to inform choice of mouse lupus nephritis models (invited presentation). Anne Davidson, Paul Hoover, Celine Berthier, Heather Geiger, Zeguo Sun, Weijia Zhang, Nir Hacohen

Presentation at Lupus Research Alliance Meeting 2019

1. The Single-cell Transcriptomic Landscape of NZB/W Murine Lupus at Early and Late Stages of Disease (poster). Paul Hoover[1], Tom Eisenhaure [2], David Lieb [2], Anne Davidson [3] and Nir Hacohen [2], [1]Brigham and Women's Hospital, Boston, MA, [2]Broad Institute, Cambridge, MA, [3]Feinstein Institutes for Medical Research, Manhasset

Other

Dr Davidson has been invited to present these findings at a Symposium at the AAI meeting in 2020.

Website(s) or other Internet site(s)

None to report

Technologies or techniques

None to report

Inventions, patent applications, and/or licenses

None to report

Other Products

None to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Christina Giannouli

Project Role: *Post-doc*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *11*

Contribution to Project: *Dr Giannouli worked on Aims 2 and 3.*

Rakesh Mishra

Project Role:	*Post-doc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Dr Mishra worked on Aim 1*
Funding Support:	*Other 6 months of effort funded by the Lupus Research Alliance*

Haiou Tao

Project Role:	*Mouse technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Mouse technician performs all breeding and husbandry and assists with clinical monitoring*
Funding Support:	

Ke Lin

Project Role:	*Senior technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Working on Aim 1*
Funding Support:	*Also partly funded by Feinstein funds to the Davidson laboratory*

Celine Berthier

Project Role:	*Collaborator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Bioinformatics and cross species analyses*
Funding Support:	*Also partly funded by the Kretzler laboratory*

Heather Geiger

Project Role:	*Collaborator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*10X genomics of myeloid cells and bioinformatics*
Funding Support:	*New York Genome Center*

Nir Hacohen

Project Role:	*Collaborator*

Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*

Contribution to Project: *10X genomics of whole kidney cells in different mouse strains and bioinformatics*
Funding Support: *Effort funded by Lupus Research Alliance*

Paul Hoover

Project Role: *Post-doc in the Hacohen laboratory*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *3*

Contribution to Project: *10X genomics of whole kidney cells in different mouse strains and bioinformatics*
Funding Support: *Lupus Research Alliance*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

U19 AI144306-01 (multi-PI: Davidson) 5/01/2019 – 4/30/2024
Heterogeneous pathways to autoantibody production: implications for prognosis and therapeutic targeting.
This proposal examines the Ig repertoire of patients taking TNF inhibitors.

Lupus Research Alliance (PI: Davidson) 12/01/2019 – 11/30/2021
Circadian dysregulation of immune function in SLE
This proposal examines the consequences of circadian dysregulation in macrophages in lupus mice.

What other organizations were involved as partners?

University of Michigan
Ann Arbor Michigan
Partner's contribution to the project
- *Collaboration with Celine Berthier*

New York Genome Center
New York NY
Partner's contribution to the project
- *Facilities - 10X genomics*
- *Collaboration - Collaboration with bioinformatics team*

Organization Name: Broad Institute
Boston MA
Partner's contribution to the project

- *Facilities - 10X genomics of additional mouse strains*
- *Collaboration - Collaboration with Nir Hacohen*

8. SPECIAL REPORTING REQUIREMENTS

N/A

9. APPENDICES:

ABSTRACT NUMBER: 72

The Single-cell Transcriptomic Landscape of NZB/W Murine Lupus at Early and Late Stages of Disease

Paul Hoover[1], Tom Eisenhaure [2], David Lieb [2], Anne Davidson [3] and Nir Hacohen [2], [1]Brigham and Women's Hospital, Boston, MA, [2]Broad Institute, Cambridge, MA, [3]Feinstein Institutes for Medical Research, Manhasset

Meeting: 2019 ACR/ARP Annual Meeting

Keywords: glomerulonephritis and systemic lupus erythematosus (SLE), Lupus, Lupus nephritis, Mouse model

SESSION INFORMATION

Date: Sunday, November 10, 2019

Session Type: Poster Session (Sunday)

Session Title: SLE – Animal Models Poster

Session Time: 9:00AM-11:00AM

Background/Purpose: Lupus nephritis is a complex and heterogeneous disease characterized by infiltrating immune cells in damaged kidney tissue. While mouse models have enabled mechanistic studies of lupus nephritis, immune and kidney cell types and states are incompletely characterized. Thus, shared cells and pathways between mouse and human lupus nephritis remain unrecognized and could provide the basis for targeted studies as well as engineering existing mouse models to better reflect human disease. To provide a comprehensive view of active leukocytes in a common model of mouse lupus nephritis, NZB/W, we measured the transcriptomes of single cells from murine kidneys and as well as spleens at early and late stages of kidney disease.

Methods: NZB/W female mice were generated and followed clinically. Kidneys and spleens from NZB/W mice were harvested for analysis from two 22-week-old non-proteinuric and two 37-week-old proteinuric mice. Single immune and parenchymal cells from digested kidney, and immune cells from spleen, were collected by flow cytometric cell sorting. We used the 10X Chromium 5' kit for single-cell encapsulation into droplets for library generation and next-generation sequencing for ~50,000 reads per cell. After normalization, single-cell transcriptomes passing quality metrics (>500 genes and < 25% mitochondrial content) were analyzed with unsupervised clustering (Seurat 3.0).

Results: We profiled 32,969 single cells collected from kidneys and spleens from NZB/W lupus mice at early and late stages of kidney disease (pre- and proteinuric, 2 mice per disease stage). Using unsupervised transcriptome analysis we discovered 27 distinct cellular clusters. Of these, 15 were immune cell clusters reflecting distinct subsets of T, B, and myeloid cells in both damaged kidney tissue and the spleen; the remaining 12 cell clusters were non-hematopoietic cells reflecting kidney cell types and states in early and late stages of kidney disease. Comparing cells from the same organ type (kidney or spleen) harvested from different mice at identical disease stages revealed highly similar immune and non-hematopoietic cell clusters, indicating reproducibility across animals and minimal batch effect.

Conclusion: By measuring the transcriptomes from single cells collected from damaged murine kidney tissue and spleens at early and late stages of kidney disease (pre-proteinuric and proteinuric), this study provides a comprehensive view of active leukocytes of lupus nephritis in a common model, NZB/W. We discovered 27 distinct murine cellular clusters reflecting multiple T, B, and myeloid cell subsets, as well as non-hematopoietic kidney cells types. We will compare NZB/W immune and

kidney cellular clusters to those recently discovered in human lupus nephritis patients by single cell transcriptome profiling (Arazi et. al, Nature Immunology, 2019; Der et. al. Nature Immunology, 2019). We expect to identify new and better understand existing cells and pathways shared between human mouse lupus nephritis. We anticipate this approach can incorporate other common mouse models of lupus nephritis to better understand how each is related to human disease.

Disclosure: P. Hoover, None; **T. Eisenhaure**, None; **D. Lieb**, None; **A. Davidson**, None; **N. Hacohen**, None.

ABSTRACT NUMBER: 1784

Single Cell Analysis of Renal Myeloid Cells from NZB/WF1 Mice with Lupus Nephritis Reveals Multiple Subsets with Altered Functions

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Background/Purpose: Renal infiltration with macrophages and dendritic cells is associated with poor prognosis in humans with lupus nephritis (LN). However, the precise pathogenic and/or protective functions of these cells in the LN context are still unknown.

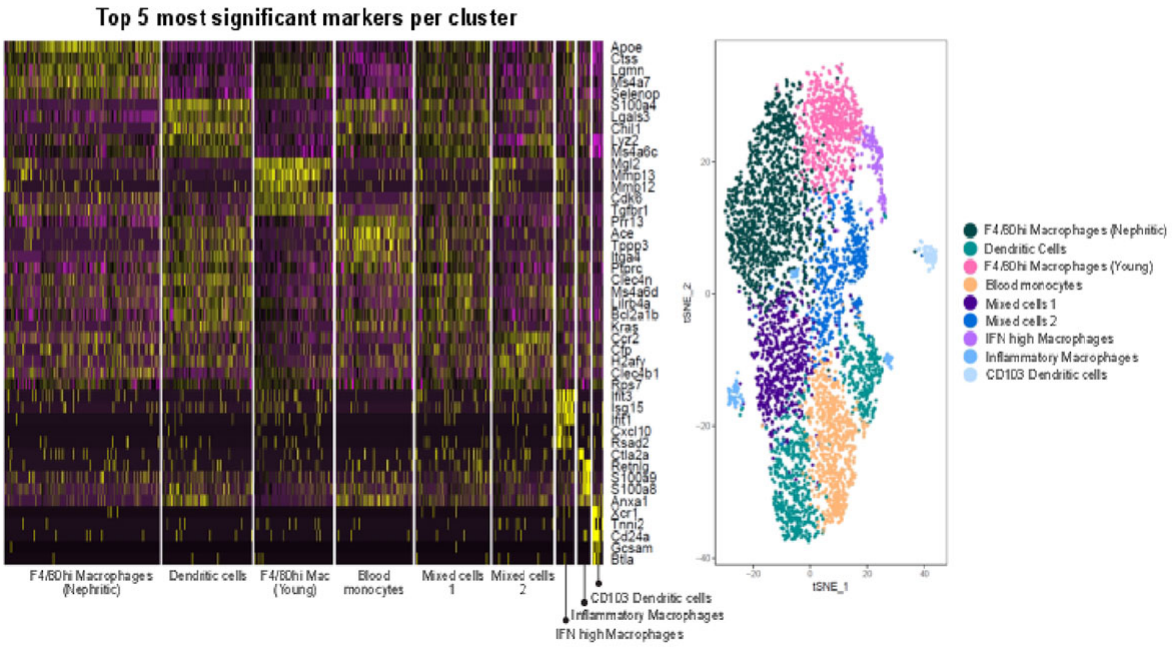
Methods: To characterize the myeloid subsets infiltrating the LN kidney we performed RNASeq of 4 subsets of isolated renal macrophages and dendritic cells from kidneys of nephritic (classical DCs, CD103 DCs, F480hi macrophages, F4/80lo monocytes) and prenephritic (F4/80hi macrophages only) NZB/W mice and Ly6Clo peripheral monocytes of nephritic mice. Single cell RNASeq was performed on renal CD11b+/CD11c+ cells from 2 nephritic and 3 young NZB/W mice and peripheral blood monocytes from nephritic mice using 10X genomics technology and labeling with hashtag olionucleotides to allow subsequent deconvolution of samples. Doublets and cells with >1 hashtag, < 500 expressed genes and/or >15% mitochondrial content were excluded.

Results: Different cell subpopulations clustered separately by PCA analysis, confirming differences in gene expression between each subset and between young and nephritic mice. Functional analysis of gene expression using GO analysis showed a molecular profile of infiltrating myeloid DCs in nephritic mice that supports lymphoid neogenesis and lymphocyte activation suggesting that these cells help to organize and regulate the inflammatory infiltrates. The profile of F4/80hi macrophages reflects cell metabolism and repair; nevertheless macrophages from nephritic mice had a more inflammatory profile than those from young mice. Single cell analyses were performed on 2096 renal and 1091 blood cells from nephritic and 1449 renal cells from young NZB/W mice. Cluster analysis revealed 9 clusters of myeloid cells of which 7 could be identified as myeloid and CD103 DCs, F4/80hi macrophages (young and nephritic clustered separately), inflammatory macrophages, blood monocytes and a small subset of macrophages with a high IFN signature. The other two subsets comprising < 25% of the cells appeared to contain a mixture of subpopulations including CD209a expressing DCs. Subclustering of the larger clusters revealed that the blood cells and young F4/80hi macrophages were quite homogeneous, whereas F4/80hi macrophages from nephritic mice could be separated into 3 subclusters. Myeloid dendritic cells could be split into 3 subclusters and the two

unknown subsets could also each be split into 3 further subclusters. Importantly a significantly higher percentage of IFN high macrophages expressed inflammatory chemokines than the other subsets.

Conclusion:

We found several subsets of renal macrophages in nephritic mice whereas those in young mice were more homogeneous. An interferon high macrophage subset may contribute disproportionately to renal inflammation. Dendritic cells appear to play an important role in lymphoid neogenesis but also displayed considerable heterogeneity. An understanding of the heterogeneity of infiltrating myeloid cell types in LN may allow more precise targeting of those cells that are most likely to be causing renal damage while preserving the protective function of resident cells.



For Anne_ACR abstract_06032019

Cluster analysis of all myeloid cells from NZB/W F1 kidneys

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REVIEW

Lupus nephritis: challenges and progress

Anne Davidson, Cynthia Aranow, and Meggan Mackay

Purpose of review
The management of lupus nephritis remains unsatisfactory due to insufficiently effective treatment regimens and the dearth of reliable predictors of disease onset or progression to guide individualized therapeutic decisions. This review summarizes new findings related to lupus nephritis over the last 18 months and discusses clinical needs that should be considered to advance trials of mechanism-based therapeutic strategies.

Recent findings
Collaborative teams are addressing how to improve disease definitions and are developing predictive models for disease onset, disease response and risk of flare in individual patients. More attention is being paid to clinical trial design. Advanced technologic approaches are allowing the analysis of small amounts of human tissue and urine in unprecedented detail so as to discover new pathogenic mechanisms and identify disease biomarkers. Novel therapies continue to be tested in disease models and include new strategies to protect renal tissue from cell damage and fibrosis.

Summary
The collaborative efforts of patients, clinical and translational researchers, the pharmaceutical industry and funding sources are needed to advance therapies for lupus nephritis. Specialized clinical centers can then deliver optimal and more personalized patient care that will improve patient outcomes.

Keywords
lupus nephritis, pathogenesis, systemic lupus erythematosus, treatment

INTRODUCTION

Lupus nephritis is a severe complication of Systemic Lupus Erythematosus that progresses to ESRD in ≈10% of patients within 5 years of onset. Current standards for diagnosis and treatment of lupus nephritis are unsatisfactory and it is not possible to accurately predict responsiveness to therapy or the long-term outcome of individual patients. Although there has been a recent decrease in the severity of lupus nephritis in European patients [1], perhaps reflecting a more comprehensive approach to lupus management, the risk of lupus nephritis-related ESRD has remained unchanged in the US population since the 1990s [2]. Immune-mediated inflammation is a major initiator of lupus nephritis, but pathogenic mechanisms leading to ESRD are poorly understood and cannot be therapeutically addressed in a patient-specific manner. There is as yet no successful biologic therapy for lupus nephritis and many unsolved problems in clinical trial design impact the interpretation of trial outcomes. In this article, we will review recent advances in clinical and mechanistic approaches to lupus nephritis and consider what is needed for translation of new information into successful clinical trials.

DIAGNOSIS AND OUTCOME MEASURES

Renal biopsy is the gold standard for diagnosis of lupus nephritis. Treatment decisions are based on the International Society of Nephrology/Renal Pathology Society classification of glomerular involvement [3] and indices for active inflammation and chronicity. Importantly, although long-term renal outcomes are worse for proliferative disease, better predictive models for risk and outcomes are needed to direct therapeutic decisions. It is still unknown whether preemptive treatment of serologic flare will prevent subsequent lupus nephritis onset [4▪▪] – this needs larger controlled studies and the development of models that predict the risk of renal flare [5,6]. Predictive models are also needed to

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KEY POINTS

- Lupus nephritis remains a significant unmet need that causes morbidity and mortality in patients with systemic lupus erythematosus.
- There is a pressing need for the development of noninvasive predictors of lupus nephritis risk and prognosis that can only be addressed using clinical registries with optimal collection of patient data and biospecimens.
- Using advanced technologies, human tissue can now be examined in unprecedented detail, yielding new insights into disease mechanisms.
- The successful development of new therapies will need more attention to clinical trial design and the consideration of individual patient features including genetic polymorphisms and tissue characteristics.
- Improvement of disease outcomes will need universal patient access to specialized clinics that monitor disease progression and medication adherence and deliver optimal access to both standard care and new therapies.

define a uniform composite short-term treatment response that can predict long-term outcomes and be used either as a surrogate endpoint in clinical trials or as a guide for decreasing maintenance immunosuppression. As a start in this direction, a proteinuria cutoff of less than 0.7–0.8 g/dl at 12 months after lupus nephritis onset has been confirmed as a biomarker of good long-term outcome in several studies [7▪▪,8,9] and a set of hazard index tools incorporating clinical data from the first 12 months of treatment, have been shown to predict long-term outcomes [7▪▪]. A longitudinal study comparing the accuracy of spot urines to 24-h collections strongly advocates use of 24-h collections for accurate results [10]. Because large patient numbers are needed to test and develop predictive models, the establishment of lupus nephritis registries with prospectively collected data and biospecimens will be essential to refine current models (Table 1). An international lupus nephritis registry would also address the problem of multiple small studies in distinct ethnic and racial groups that lack generalizability.

Table 1. Outcome prediction

Predictors of poor outcome at disease onset
Clinical [1,7▪▪]
Male sex
Younger age
Arterial hypertension
Increased serum creatinine
African-American race
Histologic [11,12]
Proliferative disease
High activity and chronicity index
Glomerulosclerosis and crescents
Interstitial inflammation
Tubular injury
Interstitial fibrosis
Biomarkers that need to be further evaluated [13▪,14]
Markers for tubular injury
Nonalbumin proteinuria
Predictors of poor outcome after treatment
Lack of access to a specialized center [2]
Absence of maintenance immunosuppressive therapy [1]
Failure to reach proteinuria threshold of <0.7–0.8 g/dl at 12 months [7▪▪,8,9]
High activity index on second biopsy at 12 months [15▪▪]

CONTROVERSIES IN LUPUS NEPHRITIS MANAGEMENT AND CLINICAL TRIAL DESIGN

Mycophenolate (MMF) or cyclophosphamide combined with high doses of prednisone are standard of care treatment for lupus nephritis [16,17]. Clinical questions remain about the optimal management of lupus podocytopathy, renal microangiopathy and membranous nephritis [18–20]. Because remission rates of lupus nephritis are low even with optimal management, studies using combinations of standard immunosuppressives [21], or the introduction of a calcineurin inhibitor are being considered. Controlled trials in Asian populations suggest that the combination of low-dose MMF with tacrolimus is more effective than MMF alone, but safety and long-term efficacy remain to be established in other populations [16,22]. The new calcineurin inhibitor voclosporin, modified to confer enhanced potency and decreased toxicity, has shown efficacy in combination with low-dose MMF and a rapid steroid taper in two phase 2 trials [23,24▪] – phase 3 studies are in process. Combinations of immunosuppressive agents with biologic drugs have not yet been successful in randomized clinical trials in lupus nephritis patients. The addition of belimumab to cyclophosphamide or rituximab or both is a rational approach to prevent the expansion of autoreactive B cells by high levels of BAFF resulting from B-cell depletion. However, data from the CALIBRATE trial showed no improvement in outcome at 24 or 48 weeks of a regimen of cyclophosphamide, rituximab, prednisone and belimumab compared with the same regimen without belimumab [25]. A study

of belimumab and either MMF or cyclophosphamide is ongoing. Given the increasing reports of decreased general systemic lupus erythematosus (SLE) flare rates over the long term in patients treated with belimumab [26], longer term follow-up will be needed to determine its benefit in lupus nephritis.

MMF is the preferred treatment for remission maintenance [27,28] but there are no data regarding the optimal duration of treatment and no definition of a low disease activity state predicting safe treatment withdrawal [29,30]. Two recent studies have addressed this question by performing repeat biopsies at 6–12 months after induction and have shown an alarming discrepancy between clinical and histologic response. A small prospective study showed that activity on a second biopsy performed 12 months after induction predicted subsequent renal flare following maintenance withdrawal, regardless of clinical parameters [15▪▪]. A second study showed that chronicity at 6 months after induction predicted long-term renal outcome regardless of clinical or histologic remission status [31▪]; larger studies are needed to define the utility of second biopsies for guiding personalized treatment based on pathologic or molecular findings. ~~On the contrary~~, adherence to maintenance therapy among lupus nephritis patients is unacceptably low and new approaches are needed to address the complex contributors to this behavior [32▪▪]. In those patients who develop ESRD, timely referral for transplant is associated with a survival benefit by reducing deaths from comorbidities such as infections and cardiovascular disease [33].

Unfortunately,

Because of the continuing failure of clinical trials of rational therapies for lupus nephritis [16], much thought is being given to clinical trial design [34▪▪]. Limited duration phase 3 trials allow evaluation of remission induction but do not address subsequent flare prevention or long-term renal outcomes. Defining response for clinical care and endpoints in clinical trials remains problematic as there is no consensus on definitions of complete and partial renal response or remission and the utility of second renal biopsies is still unknown. Doubts about the inclusion of high doses of prednisone in lupus nephritis clinical trials and about the robustness of outcome measures have led to difficulty in the interpretation of lupus nephritis trial outcomes. Most biologic agents are tested with MMF, without considering whether each drug targets the same or different immune pathways. Design features that consider the mechanism of action of each drug in the context of genetic polymorphisms or biomarkers have not yet been incorporated into lupus nephritis clinical trials (Table 2).

Table 2. Considerations in trial design

Problems
Confounders
High placebo response to standard of care therapy leads to small effect sizes, necessitating large patient cohorts
Use of high-dose background steroids may mask the effects of new immune therapies
Enrolment criteria do not always reflect patients seen in clinical practice
Optimal outcome measures are still not defined and are not uniform across trials
Long-term outcomes are currently not measured
In the absence of informative biomarkers, diagnosis of residual activity may require a second biopsy
Solutions
When to treat
Current approach – based on clinical evidence of nephritis e.g. proteinuria onset
Future approaches
Prevention based on evaluation of risk
Preclinical initiation of treatment triggered by biomarker change
Medication choice based on risk stratification (genetic, biomarker or OMICs driven)
Drug mechanism should drive trial design
Treatment of active disease vs. flare prevention
Rational choice of standard of care therapy
Stratification for individual patient differences should replace post-hoc analyses e.g. ethnicity, genetic polymorphisms, sex
Uniform trial design may help to compare the effects of agents with similar mechanisms of action
Measurements of compliance need to be incorporated, especially when multiple drugs are being used
Improved understanding of disease pathogenesis should result in development of better diagnostic and therapeutic tools

OMICS: genomics/proteomics/metabolomics

AQ16

MECHANISMS FOR RENAL DAMAGE IN LUPUS NEPHRITIS

An increased understanding of disease pathogenesis may expand treatment strategies beyond global immunosuppression.

Genetics of lupus nephritis

Genetic risk factors for lupus nephritis are only beginning to be described. In SLE patients of European descent, polymorphisms of PDGFRA, sodium glucose cotransporter Slc5a11, hyaluronan synthase 2, TNIP1 and MHC Class I and II alleles are associated with lupus nephritis [35]. Identification of lupus nephritis-associated variants of ITGAM that increase its proinflammatory properties and genetic polymorphisms that decrease renal kallikrein expression have led to the development of therapies

platelet derived growth factor
TNFAIP3 interating protein 1
major histocompatibility complex
intergrin subunit alpha M

that specifically target these pathways [36,37]. A polymorphism that increases the expression of the adapter Dab2 that mediates TGFβ signaling in epithelial cells is associated with CKD in humans [38▪▪]. Epigenetic studies have identified differential methylation of genes regulating the response to tissue hypoxia and interferon-mediated signaling in women with lupus nephritis [39]. European ancestry protects against lupus nephritis [40] and it is therefore important to study genetic risk factors in patients of other ethnicities. APOL1 risk genotypes are associated with poor outcome of most forms of CKD in individuals with African ancestry, with the risk being intrinsic to the kidney. Several pathogenic mechanisms have been suggested, but the relative role of each mechanism is still not known, making the APOL1 risk alleles difficult to target therapeutically. Consideration of APOL1 status in the kidney transplant setting is now being prospectively studied by the APOLLO Consortium [41▪,42].

transforming growth factor
chronic kidney disease
apolipoprotein L1

Cellular composition and gene expression in lupus nephritis kidneys

The kidney harbors multiple cell types and infiltrating immune cells add to the complexity of the microenvironment in the lupus nephritis kidney. Two new technologies are being used to understand the heterogeneity of the renal microenvironment in lupus nephritis patients so as to develop better diagnostic tools and individualized therapy. The first is two-photon microscopy together with cell distance mapping (CDM) to determine relationships between infiltrating renal cells [43▪]. The combination of CDM with staining of more than 20 different antigens using only small amounts of frozen tissue will yield insights into inflammatory responses by revealing how various cell types interact in the kidneys.

The second technology is single-cell transcriptome analysis of renal biopsies [4[illegible]▪▪]. Although the sequencing depth using this approach is relatively shallow, it allows a full description of the cell types present in individual kidneys and some understanding of cell functions that can be correlated with histologic and clinical variables and outcomes. Phase 1 studies of infiltrating renal cells in 24 lupus nephritis patents and 10 controls revealed multiple subtypes of B, T and myeloid cells in the lupus nephritis kidneys. NK cells and CD8 T cells with cytotoxic activity are the major proliferating immune populations; despite the identification of exhausted CD8 T cells in kidneys from MRL/lpr mice [46], no CD8 T-cell exhaustion phenotype was identified in lupus nephritis biopsies although it was readily identified in PBMCs. T follicular helper cells and activated B cells were present, with the accumulation of plasma cells and B cells with an age-associated B-cell phenotype. These studies failed to show a predominance of IL-17 or IFNγ-producing CD4 T cells with the caveat that most patients had already been treated at the time of biopsy. There is also evidence for activation of the resident macrophage population and for renal infiltration with $CD16^{+}$ inflammatory macrophages that then appear to transition to a M2-like phenotype that may orchestrate migration of other inflammatory cell subsets [45▪▪]. Dysregulated repair function of these cells may contribute to their pathogenic potential [47].

A similar single cell transcriptomic analysis of renal stromal cells from 21 lupus nephritis patients revealed both an interferon signature and a fibrotic signature in the tubular cells, both of which may be associated with poorer response to therapy [44]. Analyses of other tissues such as urine and unaffected skin that are more amenable to repeat sampling may yield information that reflects changes in the kidneys [44,[illegible]▪▪].

natural killer
MRL/MpJ-Faslpr/J
peripheral blood mononuclear cells

Gut microbial diversity and lupus nephritis

An alteration in the composition of the gut microbiota has been associated with the production of antibodies to a particular species, *Ruminococcus gnavus* only in patients with active lupus nephritis [48▪] but not in inactive lupus patients. T and B-cell tolerance to the gut microbiota may be lost if disturbance of the gut epithelial barrier allows bacterial translocation to sites where they may elicit an inflammatory response [49,50]. Alternatively, a change in the composition of the microbiota may induce pathogenic cross-reactive antiself/anticommensal immune responses. It remains to be determined whether the dysbiosis of lupus nephritis is causative or reflects homeostatic disturbances associated with inflammation and immunosuppressive medications. Longitudinal studies are now needed to examine the course of gut dysbiosis in lupus nephritis patients and to test the therapeutic applicability of approaches that restore commensal homeostasis and gut barrier integrity.

LUPUS NEPHRITIS BIOMARKERS

Biomarker discovery in lupus nephritis has progressed from analysis of individual candidate markers to unbiased high-throughput methods such as mass spectrometry [51▪], multiplexed immunoassays, renal imaging [52] and modular transcriptome analyses. Proteomic studies indicate that small panels of biomarkers can distinguish lupus nephritis from healthy control urine and that of active from inactive disease [13▪,53–56]. A set of six biomarkers,

Renal Activity Index for Lupus (RAIL) is associated with nephritis in children and to a lesser extent in adults in cross-sectional studies [57,58]. However, a recent longitudinal study using RAIL and additional biomarkers failed to identify a panel that outperformed GFR or predicted renal functional decline in individual patients [59▪]. Nevertheless it may be possible to develop a home-based assay to be performed between visits to improve early detection of nephritis [60]. Transcriptomic analyses have identified a peripheral blood neutrophil signature as a risk factor for lupus nephritis, although it may not be a robust biomarker of disease response [61,62]. Finally there is still debate about the significance of renal deposition of the terminal Mac complex as a biomarker for complement activation that could be targeted by anti-C5 drugs [63,64].

glomerular filtration rate

NEW APPROACHES TO THERAPEUTIC TARGETING

Mining of existing databases has revealed pathways that could be targeted by available drugs - repurposing of IL12/23 inhibitors, proteasome inhibitors and Jak inhibitors are examples of this approach. Although the concordance of mouse and human interventions for lupus nephritis has historically been poor, a number of new therapeutic targets have been recently tested in murine models. Allogeneic mesenchymal stem cells prevent lupus nephritis in mouse lupus models and multiple mechanisms for their efficacy have been reported (reviewed [65]). Results in humans are conflicting and await randomized trials. Defective production of IL-2 by conventional T cells in patients with lupus and lupus nephritis favors the generation of inflammatory T cells. Correction of this defect by low-dose IL-2 improves survival in lupus mouse models and approaches to enhance IL-2 are now being developed for human use [66]. Immune activation is associated with metabolic changes that favor cell proliferation and differentiation. Inhibition of both glycolysis and oxidative phosphorylation can effectively treat established nephritis in mouse models [67]. Other approaches include targeting of the cholinergic inflammatory reflex with galantamine [68], targeting of renal macrophages by introducing deficiency of IL-34 [69], targeting of inflammatory cytokines by introducing deficiency of inactive rhomboid 2 [70], targeting of Th17 cells and podocyte injury by inhibition of CAMK4 [71▪▪] and targeting of inflammasome mediated renal injury by inhibition of NLRP3 [72,73]. Because some of these therapies may have unacceptable systemic consequences, new approaches are needed to direct such therapies only to the inflamed site. Nanogels are specially formulated drug-containing liposomes that can be targeted to specific cells using antibodies. In a recent proof-of-principle study a nanogel containing a CamK4 inhibitor was directed to podocytes using antinephrin or antipodocin antibodies and prevented podocyte injury and proteinuria in a mouse lupus model [71▪▪]. Development of such site-directed therapeutic agents may enhance our current ability to prevent flares while decreasing the need for chronic global immune suppression.

It is increasingly evident that nonimmune pathways contribute to renal injury in lupus nephritis. One recent question is whether the kidneys can be protected from inflammatory damage by altering the resilience of resident renal cells to oxidative stress [74]. Tubular cell injury is a common feature of all forms of CKD. Tubular epithelial cells rely on mitochondrial fatty acid oxidation for their energy supply and can be protected in mice by strategies that conserve this pathway [75▪]. Tubule-specific deletion of the TGFβ signaling effector Dab2 in mice also protects from renal injury and fibrosis, suggesting another therapeutic approach especially in genetically susceptible individuals [38▪▪]. Since multiple cell types are affected during renal injury, it is expected that multi-OMICs discovery experiments will uncover new pathways for targeting.

OMICs: genomics, proteomics, metabolomics

CONCLUSION

Coordinated approaches that involve all stake holders are needed to prevent and treat lupus nephritis, especially in those ethnic groups whose outcome is historically poor. Collaborative interactions can help to identify knowledge deficits and clinical needs so as to design appropriate multicenter studies. Registries and uniform biospecimen collection will allow testing of hypotheses that can only be addressed with longitudinal data. Mechanistic studies involving individual renal cell types from human samples will allow us to further unravel precise mechanisms in appropriate mouse models and to predict new and/or synergistic therapeutic approaches. Because it is easier to prevent than to treat established lupus nephritis, identification of patients at risk and at home monitoring for early renal changes may improve outcomes. Providing access to care in specialized lupus centers can facilitate early detection, encourage and monitor patient compliance, improve management of ancillary morbidities and enable access to renal transplant so as to improve the long-term outcome of all patients with lupus nephritis.

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Conflicts of interest

There are no conflicts of interest.

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- ■■ of outstanding interest

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kidney injury molecule 1

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