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TITLE: The Function of Renal Macrophages in Lupus Nephritis

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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, effective new therapies for LN have been slow to emerge 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 successfully generated 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. There is striking overlap between the mouse and human datasets with heterogeneity in the humans that we can model in the mice. Our second aim addressed the role of autophagy and metabolism in renal macrophages. We have found that deficiency of Rubicon (LAP pathway) protects the lupus mice from LN and death due to altered B cell selection whereas deficiency of ATG14 (classical pathway) specifically in macrophages has no effect. ATG14 deficiency in B cells also protects from disease indicating a role for autophagy in the generation of autoreactivity. We also investigated the role of PGC-1 α in metabolic programming of kidney macrophages in LN but were not 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:** This is the final report on this project. We thank the DOD for supporting our work.

Lupus nephritis is a devastating complication of Systemic Lupus Erythematosus for which there is currently no satisfactory treatment. The last few years have seen advancements in therapeutics for lupus but there are still only two new drugs approved for lupus nephritis and neither achieves complete remission in more than 60% of patients. Renal macrophages are associated with poorer outcomes in lupus nephritis. Renal macrophages comprise both resident cells and bone marrow derived infiltrating cells. Macrophages are highly heterogeneous and plastic cells and can be involved in both tissue injury and repair. Therefore, a better understanding of the heterogeneity of these cells in the kidneys of patients with lupus nephritis and a determination of their function, whether pathogenic or protective, is needed in order to target them therapeutically. We proposed both a discovery and a functional component to our studies. The goals of this project were:

a. To characterize renal macrophages in several mouse models of SLE and to compare these with human macrophages so as to identify those mouse models and cells that should be further studied. Our approach was to use a systems approach to understand key features that are relevant to the diagnosis and treatment of human lupus nephritis. In collaboration with Paul Hoover and Nir Hacohen at the Broad Institute we used 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 four lupus strains that have both similarities and differences to each other. 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. We have also been able to map the origins of the various macrophage subsets using trajectory analyses and their location in the kidney. Finally, have identified shared transcription factors in renal macrophages and have generated mouse strains bearing Cas and retroviral vectors with guide RNAs to study their role in vivo. The most important discovery from these experiments has been that we have identified a new subset of macrophages that is associated with disease activity in both mice and humans. Further characterization of this subset is ongoing.

b. In the mechanistic component of this proposal, we proposed to study the role of autophagy and mitochondrial dysfunction in macrophages 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 formed a collaboration with Dr Mark Shlomchik at University of Pittsburgh to analyze the mechanism. Our studies have shown that Rubicon deficiency alters B cell selection in the germinal center. We then generated mice with B cell deficiency of ATG14 to determine the role of classical autophagy in B cells and found that these mice are also protected from autoantibody formation which is surprising since Rubicon regulates ATG14 function. We also generated uMT Sle1 mice so that we can study the role of Rubicon deficiency specifically in B cells and are approaching this using bone marrow chimeras.

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

This goal is 100% completed and follow-up studies are in progress. The first manuscript describing these studies is almost complete and will be submitted shortly. Follow up studies have been funded by a DOD grant and a RO1 proposal received a fundable score and will be funded next year.

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.

We are following up with studies to determine how these pathways influence autoantibody production. A manuscript describing the effect of Rubicon deficiency was published this year.

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

This goal has been completed. There was no effect of PGC1 alpha deletion or overexpression on disease outcome in our model indicating that it likely has redundant functions.

Major Tasks Specific Aim 1	Single cell RNASeq (Broad) and data processing (NYGC)
Molecular characterization of single cells	
<i>Data analysis is finished and manuscript almost complete. We performed multiple new analyses over the last year and set up a flow cytometry protocol to isolate each of the major cell subsets. A new DOD grant was awarded for continuation of these studies and a new RO1 received a fundable score (priority score 1)</i>	
Changes in macrophage function over time	We decided to use a strain with a more predictable disease onset for these studies. The mice for this experiment have now been generated and the single cell experiment needs to be completed.
Major Tasks Specific Aim 2A	The mice are generated and analyses of Rubicon mice are completed and manuscript published.
Generate Rubicon and ATG14 deficient mice and follow for nephritis onset. Accelerate disease if necessary	ATG14 macrophage specific KO mice have no change in renal outcome. B cell deficient ATG14 KO mice have been generated and are also protected. New grant planned to study B cell autophagy in lupus
<i>Manuscript published</i>	
Major Task 3 Specific Aim 2B	uMT knockouts generated and chimeras have been made and are being followed These experiments are still in progress
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	No changes in function found in either KO or overexpressing mice
Analysis of mice overexpressing PGC1 alpha	
<i>Completed</i>	

What was accomplished under these goals?

The bioinformatics analyses have been time consuming but we are now certain of the myeloid subsets in each strain. We identified 13 subsets of myeloid cells in the 4 strains. These are shown in Figures 1 and 2.

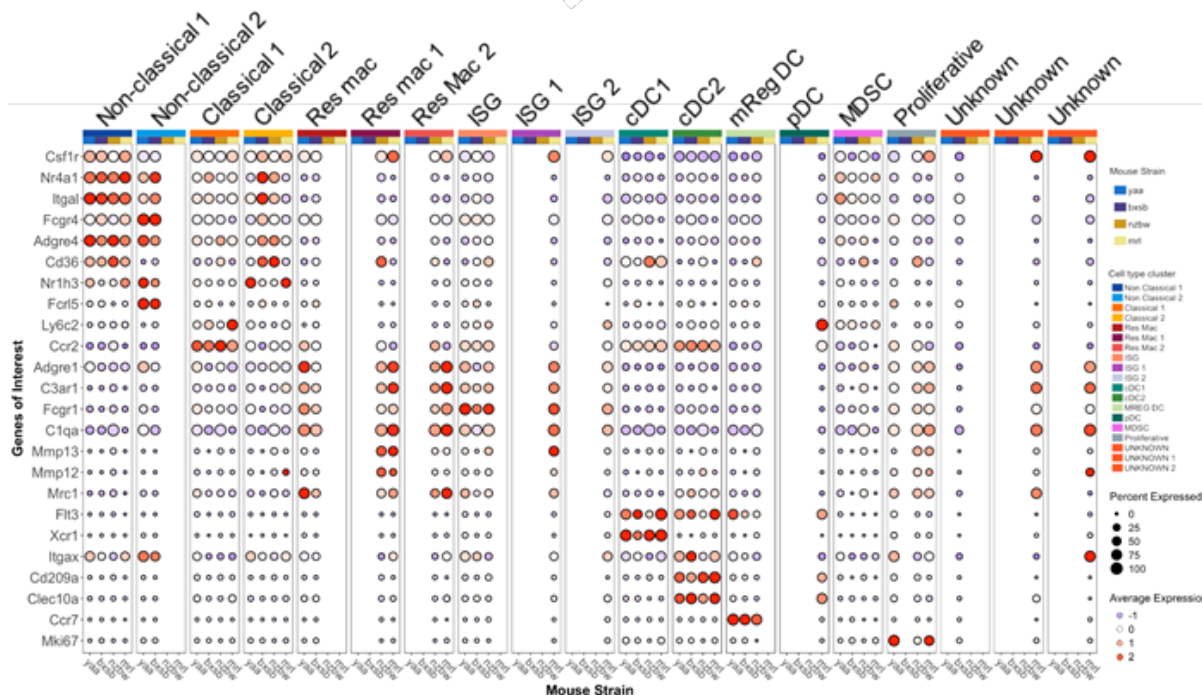


Figure 2:
(left)
Informative
genes that
distinguish the
myeloid cell
subsets and
their
expression in
each strain

1. The two TR7 over expressing strains have a novel monocyte subset that we have termed non-classical type 2 to distinguish it from the non-classical type 1 subset found in all 4 strains (light blue in Figure 1). This subset

is localized in glomeruli and has a unique expression pattern with overrepresentation of genes involved in lipid synthesis and efflux. These cells derive from circulating Ly6Clo patrolling monocytes by trajectory analysis. Our hypothesis, based on prior work by the Geissmann laboratory, is that overexpression of TLR7 in endothelial cells attracts these cells and that they then adhere to the endothelium respond to ingestion of nucleic acid material from circulating immune cells and local apoptotic material by expressing a program induced by phagocytosis.

2. In MRL/lpr and NZB/W mice the dominant myeloid subset is the resident macrophage subset that is localized around the glomerular border and in the interstitium. In these two strains there is a marked shift in gene expression in this subset in the nephritic mice with acquisition of a pro-fibrotic program. This shift occurs much earlier in the two TLR7 expressing strains.

We also identified a novel classical macrophage subset that we have termed Classical 2. Using the markers identified by RNASeq we designed a flow cytometry panel to distinguish the major myeloid subsets in kidneys of nephritic mice. The TSNE plot to the right shows the major CD11b subsets in NZB/W mice and illustrates the infiltration of NC1 and NC2 and classical 2 cells in the kidneys of nephritic mice as well as the shift in phenotype of resident macrophages. ().

Aim 1B: Here we proposed 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. We have optimized our technique in which we shield the kidney during irradiation and then transfer the bone marrow to give us larger numbers of transferred cells. We have also established an exchange transfusion protocol. Using our established flow cytometry protocols to identify renal subsets will allow us to determine the half-life and origins of each subset in the chimeric mice as well as to sort the cells for low input RNA sequencing.

Aim 1C: We now have the AMP Phase 2 data available for comparisons with the mouse data. This is a very rich dataset and it is clear that there are multiple myeloid subsets in human lupus kidneys. We previously reported using data from Phase 1 of the Accelerating Medicine Partnerships program (23 biopsies studied), there are at least 5 major myeloid subsets in lupus kidneys (CM0 – inflammatory CD16+ monocytes; CM1 - phagocytic monocytes; CM2 – resident population; CM3- dendritic cells; CM4 – alternatively activated reparative monocytes). Myeloid cell subsets have now been analyzed using the data from Phase 2 of the Accelerating Medicine Partnerships program. 155 renal biopsies were processed for the single cell analyses and >20,000 myeloid cells were obtained. These now separate into > 20 clusters with the major subsets being CD14+ monocytes, CD16+ monocytes (inflammatory and phagocytic), resident macrophages, and infiltrating DCs (cDC1 and cDC2). There is substantial overlap with the mouse models, such as between non-classical monocytes and human CM0, classical 2 monocytes and CM1 phagocytic cells, resident macrophages and human CM4, classical 1 monocytes and human CD14+ monocytes and correspondence of the dendritic cell subsets. We have further localized CM0 and CM1 cells to glomeruli in both mice and humans and CM4 cells to the interstitium in both species.

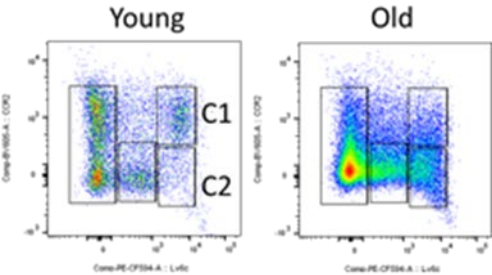


Figure 4: Flow cytometry characteristics of classical 1 and classical 2 macrophages

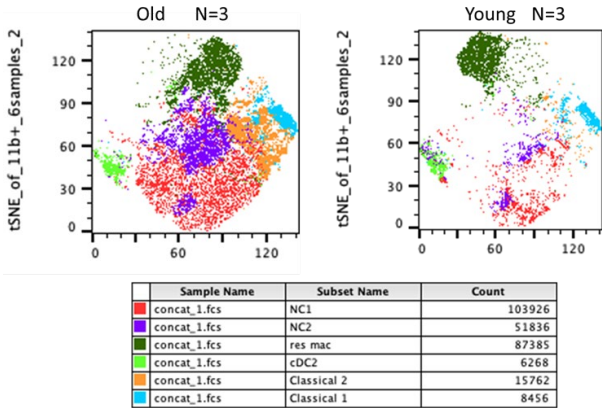


Figure 3: Flow cytometry identification of major monocyte/DC subsets. Neutrophils and cDC1 were gated out prior to analysis.

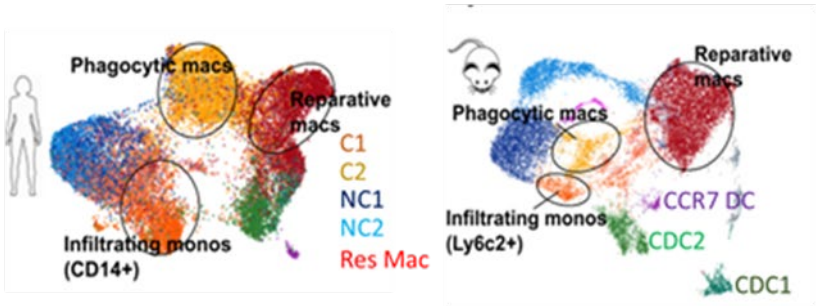


Figure 5: Comparison of scRNASeq data of human and mouse LN kidneys. Similar subsets are given the same color in each species.

Furthermore, there are several subsets that correlate with activity and chronicity scores. In particular, classical 2 macrophages, but not the other subsets, are associated with disease activity in both mice and humans, whereas resident macrophages and cDC2 are associated with chronicity. We are now able to isolate these cells using flow cytometry and further studies of their function are ongoing.

We have additionally identified transcription factors that are shared between the mouse models, that overlap with the human subsets and that distinguish the subsets from each other. In particular, there are clear differences in the transcription factors that drive the CM0 vs the CM4 program in both species. This will allow us to proceed with functional experiments to screen for the function of these transcription factors in vivo or in vitro. We have generated lentiviral vectors expressing guide RNAs for 15 of these transcription factors and are in the process of optimizing lentiviral transduction into LSK cells.

Our data has led us to several different hypotheses that we will be testing in new studies. First, we believe that monocytes enter the kidneys either via the glomeruli or the interstitium. Those entering the glomeruli differentiate to CM1 cells whereas those entering the interstitium differentiate to CM4. This is regulated by transcription factors induced in the different microenvironments. The most pressing task our end is to determine the function of each of the myeloid subsets, to determine which subsets are pathogenic and which are protective. These experiments are the functional experiments that will form the basis for our new RO1 grant.

Aim 2A and 2B: In this aim we proposed 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. Both these strains were successfully bred. As we reported previously, we found that the Rubicon deficient mice Sle1.Yaa did not develop nephritis contrary to what was expected based on the literature. We worked with the Shlomchik laboratory on these mice and the manuscript has now been published in JCI insight. Of note, the original publication by the Green lab that reported induction of lupus in Rubicon deficient mice was retracted this year.

We have also generated uMT Sle1.Yaa mice and reconstituted them with Rubicon deficient B cells to study whether this effect is B cell intrinsic. These mice are being followed. Finally, we generated Rubicon deficient uMT mice. We will reconstitute them with Rubicon sufficient B cells. This will allow us to study the effect of Rubicon deficiency in renal macrophages once the mice develop nephritis.

We completed the analysis of ATG14 macrophage specific KO mice. These have a modestly decreased survival compared with littermates suggesting that there may be a protective effect of canonical autophagy. We completed the autoantibody profiles in these mice and there are no differences between KO mice and wild type mice. There was also no difference in renal macrophages. We next generated ATG KO mice using a B cell Cre. These mice are protected from lupus nephritis and autoantibody formation but they still develop spontaneous germinal centers. They mount a normal response to immunization with exogenous antigen but have smaller germinal centers and fewer TFH than their wild type counterparts. Rubicon and ATG14 are thought to have counterregulatory functions but our data show that both are needed for autoantibody production. If the effect of Rubicon deficiency is B cell intrinsic, we will further analyze their effects on B cell autophagy.

Aim 2C: In this aim we studied mice conditionally overexpressing PGC1 α in macrophages using LysMCre as the promoter for expression of the PGC1 α or Cre transgenes. We found that neither PGC1 overexpressing or underexpressing mice differed from controls with respect to any of the assays that we tried or in the lupus outcome, leading us to conclude that it plays either no role or a redundant role in macrophages. As discussed above, the CRISPR library approach is a much wider approach that will allow us to identify those transcription factors that are important for renal macrophage function and are relevant to human disease. This is much preferable to the targeted approach that can fail as we showed here.

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

Dr. Paul Hoover is our junior collaborator at the Broad Institute, working with Dr Hacohen. This project is his main project and he submitted a KO8 application that was scored and is being revised this year. He obtained Bridge funding from the Rheumatology Research Foundation this year. He presented this work at the ACR

meeting in 2020 and at an NIH meeting on mouse models of lupus in 2021. He has been invited to speak at a symposium at the ACR meeting this year

My student Mr. Chirag Raparia was admitted to the Graduate School at Feinstein and is working on the renal myeloid cell function. He also worked on the Rubicon and ATG deficient mice. He attended the AAI meeting in 2021 and 2022, a Keystone macrophage meeting this year and will attend the ACR meeting this Year. He has also been appointed to the T32 Training Grant at our Institution.

How were the results disseminated to communities of interest?

Abstracts were presented at the ACR meeting in 2020, 2021 and 2022 (pending). Both Dr Davidson and Dr Hoover have given plenary talks at national lupus specialty meetings and the ACR Meeting.

One manuscript is published and the other is almost complete. I have also been asked to write several reviews on renal macrophages and the chapter on lupus nephritis in the upcoming new edition of the Dubois Lupus textbook.

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

N/A

4. IMPACT: What was the impact on the development of the principal discipline(s) of the project?

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

We have shown both complexity and heterogeneity of the myeloid cell response in the kidneys and are starting functional studies to determine the role of each myeloid cell subset. With respect to autophagy, we have novel findings that correct a current misconception in the field and will be able to go on to determine the role of autophagy in B cell tolerance in general. Importantly, previous papers by others describing exacerbation of lupus by rubicon deficiency were retracted this year and our work corrects the record.

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. Once our data is published it will provide a resource for others wishing to do different analyses. The role of autophagy in B cells is a new topic of interest with respect to tolerance in SLE and this should be of interest to the wider immunology community.

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:

Nothing to report

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

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report.

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:

Journal publications.

Maria NI and **A Davidson**. Emerging areas for therapeutic discovery in SLE. *Curr Op Immunology*. *Curr Opin Immunol*. 2018 Sep 20;55:1-8.

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.

Maria NI, **Davidson A**. Protecting the kidney in systemic lupus erythematosus: from diagnosis to therapy. *Nat Rev Rheumatol*. 2020 May;16(5):255-267. doi: 10.1038/s41584-020-0401-9. Epub 2020 Mar 19.

Davidson A. Renal Mononuclear Phagocytes in Lupus Nephritis. *CR Open Rheumatol*. 2021 Jul;3(7):442-450. doi: 10.1002/acr2.11269. Epub 2021 Jun 1. PMID: 34060247

Moore E, Reynolds JA, **Davidson A**, Gallucci S, Morel L, Rao DA, Young HA, Putterman C. Promise and complexity of lupus mouse models. *Nat Immunol*. 2021 Jun;22(6):683-686. doi: 10.1038/s41590-021-00914-4

Rubicon promotes rather than restricts murine lupus and is not required for LC3-associated phagocytosis. Rachael A. Gordon, Christina Giannouli, Chirag Raparia, Sheldon I. Bastacky, Anthony Marinov, William Hawse, Richard Cattley, Jeremy Tilstra, Allison M. Campbell, Kevin M. Nickerson, **Anne Davidson** and Mark J. Shlomchik.

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

Davidson A. Lupus Nephritis. Dubois Lupus textbook (in press).

Other publications, conference papers, and presentations.

Invited talks (Davidson)

Mouse models of SLE (NIH conference) December 2020.

National SLE meeting Lupus 2021 Sept 2021

CORA meeting Oct 2021

Mayo Clinic Immunology Seminar series Dec 2021

Scleroderma National Meeting Boston July 2022

Lupus 21st Century National Meeting Tucson, AZ September 2022

Invited talks (Hoover)

ACR Meeting Philadelphia 2022

Abstracts accepted

R Mishra, CC Berthier, H Geiger, W Zhang and **A Davidson**. Single Cell Analysis of Renal Myeloid Cells from NZB/WF1 Mice with Lupus Nephritis Reveals Multiple Subsets with Altered Functions. ACR/ARP Annual Meeting Atlanta GA. 2019

P Hoover, T Eisenhaure, D Lieb, **A Davidson** and N Hacohen. The Single-cell Transcriptomic Landscape of NZB/W Murine Lupus at Early and Late Stages of Disease. ACR/ARP Annual Meeting Atlanta GA. 2019

P.J. Hoover, M. Peters, D. Lieb, R. Mishra, N. Hacohen, **A. Davidson**. Single-Cell Transcriptomics of Mouse and Human Lupus Nephritis Identifies Conserved Myeloid Populations Across Species. ACR/ARP Annual Meeting (virtual) 2020.

P.J. Hoover, M. Peters, D. Lieb, R. Mishra, H. Geiger, N. Hacohen, **A. Davidson**. The Identification of Shared and Unique Myeloid Cell States in Pre- and Post-nephritic Lupus Mouse Models, Sle.Yaa1 and NZBW. ACR/ARP Annual Meeting (virtual) 2020.

Hoover P, Peters M, Lieb D, Wang R, Dunlap G, Rao D, Hacohen N, **Davidson A**. An Atlas of Human and Mouse Intrarenal Immune Cells in Lupus Nephritis Reveals Homologous Immune Populations Across Common Mouse Strains and Species [abstract]. Arthritis Rheumatol. 2021; 73 (suppl 10).

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

All techniques will be reported in our manuscripts

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

All molecular data will be deposited in a public database

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Rakesh Mishra

Project Role: Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked:* 3
Contribution to Project: Was involved in the single cell data generation for the first 2 years of the project
Funding Support: Also partly funded by other grants to the Davidson laboratory

Haiou Tao

Project Role: Mouse technician
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 3
Contribution to Project: Breeding, clinical evaluation, bleeding, ELISAs
Funding Support: Also partly funded by other grants to the Davidson laboratory

Ke Lin

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

Chirag Raparia

Project Role: Student
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 12
Contribution to Project: Aim 2 Bioinformatics and cross species analyses
Analysis of Rubicon deficient mice
Flow cytometry
Funding Support: T32 grant and Hofstra Northwell School of Medicine

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: 9

Contribution to Project: 10X genomics of whole kidney cells in different mouse strains and bioinformatics

Funding Support: Lupus Research Alliance

* per year

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

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9/30/21 – 9/29/2024

Modeling the Heterogeneity of Human LN in Mouse Models

What other organizations were involved as partners?

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:

Manuscripts and abstracts

Title: The Identification of Shared and Unique Myeloid Cell States in Pre- and Post-nephritic Lupus Mouse Models, Sle1.Yaa and NZBW

Authors: Paul J. Hoover, Michael Peters, David Lieb, Rakesh Mishra, Heather Geiger, Nir Hacohen, Anne Davidson

Background/Purpose: Poor renal prognosis in lupus nephritis (LN) is associated with an abundance of renal macrophages and dendritic cells (DCs) but the role of these cells is not well understood. Because mouse models provide a system for studying the immune response in LN, we characterized the transcriptomes of myeloid cells during pre- and post-nephritic disease in Sle1.Yaa mice that have an extra copy of TLR7 and NZB/W mice in which an activated adaptive immune system drives disease.

Methods: We sorted PBMCs and dissociated renal cells from pre- and post-nephritic Sle1.Yaa and NZB/W mice (3-4 per group) using (i) CD45 beads/antibodies to isolate all immune cells or (ii) CD11b/CD11c to enrich for myeloid cells. We performed single cell transcriptomic profiling using 10x genomics and analyzed droplets that contained >500 genes and UMIs after doublet removal. We performed coarse and fine clustering and differential expression (DE) (Seurat v3.0); gene set enrichment; trajectory (analysis Monocle v2.0 and Velcyto); and integration (Harmony). We identified residential vs. infiltrating cells by co-clustering intrarenal with blood data and by using published residential and infiltration gene signatures. We further identified macrophage and DCs subsets by scoring clusters using gene signatures generated from bulk RNASeq of intrarenal myeloid cells sorted by CD11b/CD11c and F4/80 expression.

Results: Several myeloid clusters were identified using data from >5000 cells from each mouse strain. Several novel subclusters of macrophages and DCs were identified in nephritic mice. Macrophages from pre- and post-nephritic mice clustered separately. Trajectory analysis of these cells indicated the acquisition of activation and inflammatory genes in nephritic mice including IL1b, AP1 (Fos and Jun), chemokines, cytoskeleton remodeling genes and polarization towards an alternative phenotype. Loss of MMP13 and increased Mmp12 expression further suggests acquisition of a profibrotic phenotype. Fcrl5, Fcrg4 patrolling monocytes were found only in nephritic Sle1.Yaa mice, confirming their expansion in TLR7 overexpressing models. Putative cluster functions were based on the top DE genes and gene set enrichment: macrophages had characteristics of resident cells and were enriched for phagocytosis, lysosomal function and antigen processing/presentation whereas DCs were enriched for transendothelial migration, chemokine signaling and cell adhesion genes, all characteristics of infiltrating cells. pDCs were enriched in genes in the glycolysis pathway whereas CD103 DCs were highly enriched for genes involved in oxidative phosphorylation.

Conclusions: Resident macrophages are the major myeloid subpopulation in pre-nephritic kidneys in both models and acquire an activated and profibrotic phenotype during nephritis. Post-nephritic kidneys from both models contained novel subclusters of expanded residential and infiltrating macrophages and DCs. Only Sle.Yaa1 kidneys contained patrolling monocytes. These analyses generate new hypotheses about the development of tissue damage in lupus kidney disease and can be used to identify molecular features shared with myeloid cells from human lupus kidneys for targeted follow-up studies.

Title: Single-Cell Transcriptomics of Mouse and Human Lupus Nephritis Identifies Conserved Myeloid Populations Across Species

Authors: Paul J. Hoover, Michael Peters, David Lieb, Rakesh Mishra, Nir Hacohen, Anne Davidson

Background/Purpose: We recently identified novel immune cell states in the kidneys of lupus nephritis patients (Arazi et al, Nature Immunology 2019). To determine the similarities with lupus mouse models, we compared human immune cell transcriptomes to those from Sle1.Yaa and NZBW mouse kidneys. We focused on macrophages and DCs since intrarenal myeloid abundance may drive tissue damage in lupus patients.

Methods: We collected myeloid cells from dissociated kidneys from pre- and post-nephritic Sle1.Yaa and NZBW mice (3-4 mice per group). Using 10x Genomics we performed single cell transcriptomic profiling and analyzed droplets that contained >500 genes and UMIs after doublet removal. To identify intrarenal mouse immune cells equivalent to those in humans, we leveraged immune cell transcriptomes collected from the kidneys of lupus patients in AMP Phase 1 to generate state-specific gene signatures from each human myeloid cell cluster composed of: (i) the top 10 to 40 most highly and ubiquitously differentially expressed (DE) genes, (ii) the top 4-10 DE transcription factors, or (iii) modules of co-varying genes from non-negative matrix factorization. After clustering mouse myeloid cells (Seurat v3.0), we calculated the average of the scaled expression for each human signature across mouse clusters to identify those most similar to human. We also calculated the Pearson correlation coefficient between human and mouse clusters using homologous genes with identical names between species.

Results: We mapped gene signatures from the human myeloid states: 'inflammatory' macrophages (CM0) that likely enter from blood and transition to 'phagocytic' (CM1) and 'reparative' (CM4) states; resident macrophages (CM2); dendritic cells (CM3). We identified the human CM0 signature in clusters from NZBW (and weakly in Sle1.Yaa) post-nephritic pro-inflammatory macrophage/DC clusters that infiltrate from blood, similar to CM0 cells in humans. The human CM1 signature mapped to clusters in both mouse models that resembled DCs and that were enriched for transendothelial migration. We identified in both models the CM4 signature in pre- and post-nephritic mouse clusters that resembled resident macrophages and were enriched for antigen and presentation and lysosomal processing. Like humans, the post-nephritic CM4 mouse equivalent cluster exclusively expressed Ccl8, a leukocyte chemoattractant and activator, and Gas6, a mediator of apoptotic cell clearance and was skewed towards an alternative phenotype. Finally, we mapped the human CM3 signature to mouse CD103+ DCs and identified gene programs shared with humans for immunoregulation and antigen presentation.

Conclusions: From the kidneys of Sle1.Yaa and NZBW mice we identified human myeloid gene signatures: 'inflammatory' macrophages (CM0), 'phagocytic' (CM1), 'reparative' (CM4) states; dendritic cells (CM3); we did not identify the CM2 human resident macrophage signature. We identified putative functions shared between mouse and human clusters including antigen presentation, phagocytosis, Ccl8 expression. These analyses indicate that we may be able to study in mice some of the myeloid subsets and genes that are highly relevant to human disease.

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

Paul Hoover¹, Tom Eisenhaure², David Lieb², Anne Davidson³ and Nir Hacohen^{2, 1}Brigham and Women's Hospital, Boston, MA, ²Broad Institute, Cambridge, MA, ³Feinstein 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.

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

Rakesh Mishra ¹, Celine Berthier ², Heather Geiger ³, Weijia Zhang ⁴ and **Anne Davidson**⁵, ¹Feinstein Institute for Medical Research, Manhasset, NY, ²University of Michigan, Ann Arbor, MI, ³New York Genome Center, New York, NY, ⁴Mount Sinai School of Medicine, New York, NY, ⁵Feinstein Institutes for Medical Research, Manhasset

Meeting: 2019 ACR/ARP Annual Meeting

Keywords: lupus nephritis and Macrophage

SESSION INFORMATION

Date: Monday, November 11, 2019

Session Type: ACR Abstract Session

Session Title: 4M094: SLE – Animal Models (1782–1787)

Session Time: 2:30PM-4:00PM

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.

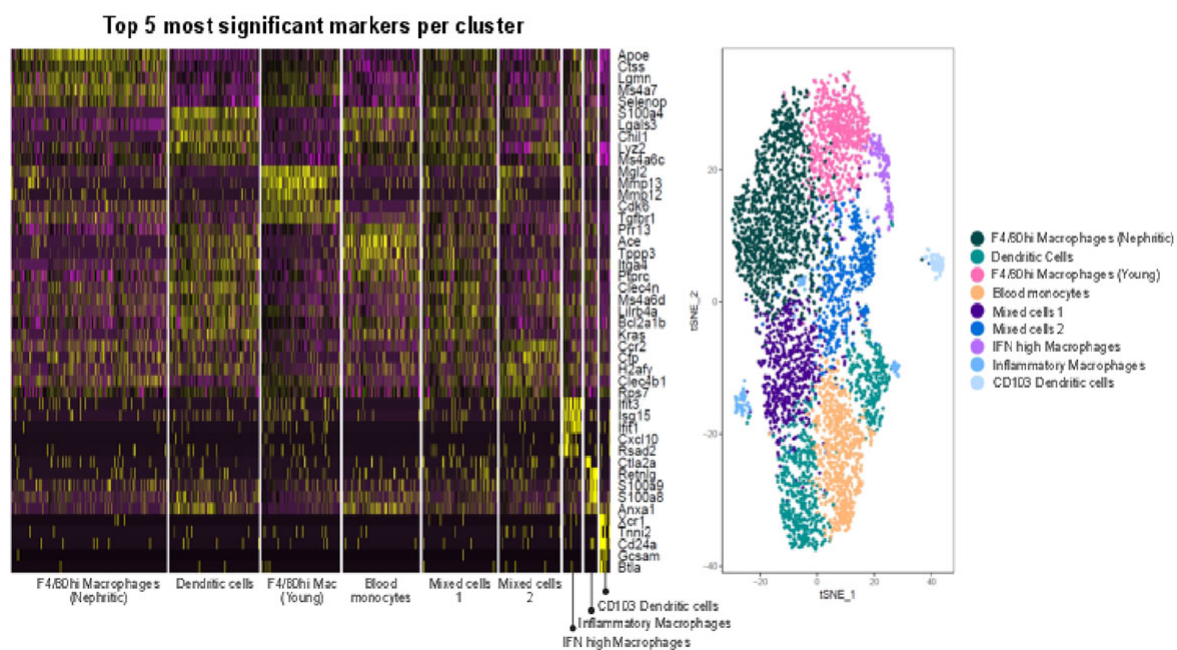
Methods: To characterize the myeloid subsets infiltrating the LN kidney we performed RNASeq of 4 subsets of isolated renal macrophages and dendritic cells from kidneys of nephritic (classical DCs, CD103 DCs, F4/80hi macrophages, F4/80lo monocytes) and pre-nephritic (F4/80hi macrophages only) NZB/W mice 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 oligonucleotides 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

Disclosure: R. Mishra, None; C. Berthier, None; H. Geiger, None; W. Zhang, None; A. Davidson, None.

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ACR Meeting Abstracts - <https://acrabstracts.org/abstract/single-cell-analysis-of-renal-myeloid-cells-from-nzb-wf1-mice-with-lupus-nephritis-reveals-multiple-subsets-with-altered-functions/>

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ABSTRACT NUMBER: 0472

An Atlas of Human and Mouse Intrarenal Immune Cells in Lupus Nephritis Reveals Homologous Immune Populations Across Common Mouse Strains and Species

Paul Hoover¹, Michael Peters², David Lieb², Runci Wang³, Garrett Dunlap⁴, Deepak Rao¹, Nir Hacohen² and Anne Davidson⁵, ¹Brigham and Women's Hospital, Boston, MA, ²Broad Institute, Cambridge, MA, ³Brigham and Women's Hospital, Boston, MA, ⁴Harvard University, Somerville, MA, ⁵Institute of Molecular Medicine, Feinstein Institutes for Medical Research, Manhasset, NY

Meeting: [ACR Convergence 2021](#)

Keywords: [genomics](#), [Lupus nephritis](#), [Mouse Models](#), [Lupus](#), [Nephritis](#), [Systemic lupus erythematosus \(SLE\)](#)

SESSION INFORMATION

Date: [Saturday, November 6, 2021](#)

Session Title: [Abstracts: SLE – Animal Models \(0470–0473\)](#)

Session Type: Abstract Session

Session Time: 11:30AM-11:45AM

Background/Purpose: We discovered 21 immune cell-types in lupus nephritis kidney biopsies as part of the Accelerating Medicines Partnership (AMP) consortium. These immune cells are the basis of new hypotheses about the drivers of disease. However, we cannot feasibly collect immune cells from human kidney biopsies for mechanistic testing. Mouse lupus models and humans share important clinical features including autoantibody development and kidney injury that progresses to failure. How mice recapitulate human lupus nephritis remains an open question. Here, we used single cell RNA sequencing to identify homologous intrarenal immune cell subsets and conserved gene programs from humans and four common lupus strains.

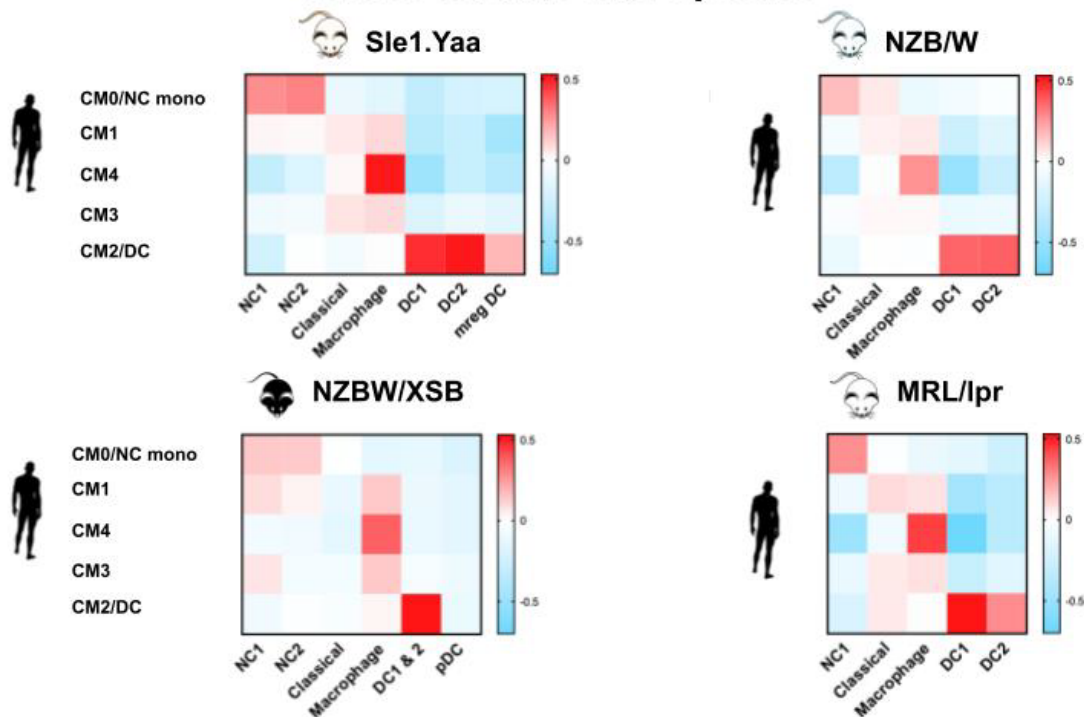
Methods: Using AMP protocols we sorted CD45+ cells from dissociated mouse kidneys with spontaneous adaptive-driven autoimmunity (NZB/W and MRL/lpr) and TLR7-overexpression innate-driven autoimmunity (Sle1.Yaa and NZW/BXSB) in early and nephritic disease. We profiled single cell transcriptomes using 10x Genomics and analyzed droplets that contained >500 genes and UMIs after doublet removal using Seurat 3.0. We analyzed the top 2000 variable genes for clustering and differential gene expression. We compared transcriptomes of intrarenal immune cells collected from mice at early and peak clinical disease to those from humans at peak clinical disease.

Results: We identified intrarenal myeloid, T-, and B-cell subsets from >75,000 cells passing QC. Many subsets were shared among strains and homologous to human subsets. In particular, the myeloid compartment in mice and humans contained: i) non-classical monocytes that expressed TNF and fate related transcription factors (Spi1, Nr4a1); ii) a novel Fcrl5+ non-classical monocyte unique to TLR7-overexpression strains that emerged in nephritic mouse kidneys and expressed Nr1h3 known to regulate lipids and inflammation; iii) resident-like macrophages that expressed genes for tissue repair (Igf1, Pdgfrb), immunomodulation (Creb5, Dab2), and phagocytosis (Mertk, Gas6); resident-like macrophages in adaptive-driven lupus strains shifted gene expression from anti-fibrotic to remodeling that correlated with proteinuria. We spatially mapped these homologous cells in kidney sections and found they localized to similar compartments in mice and in most human patients.

Conclusion: Most intrarenal myeloid, T-, and B-cell subsets in early and nephritic disease were common to mouse strains; unique subsets correlated with genetic susceptibility. TLR7-overexpression strains contained a novel and unique Fcrl5+ non-classical monocyte. Spontaneous strains contained a population of residential-like macrophages that shifted gene expression from anti-fibrotic to remodeling in nephritic mice. Both non-classical and residential-like macrophages in mice and humans shared genes critical for tissue repair, immunomodulation, and immune cell recruitment. Further, each of these cell-types localized to similar locations in kidney sections in mice and a subset of human patients. These findings support shared roles of these cells in lupus nephritis. We hope our work opens a new path using mouse models to more precisely study aspects of human disease.

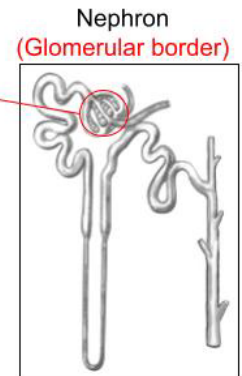
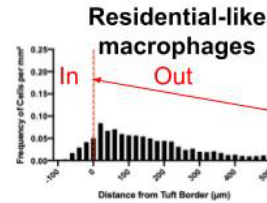
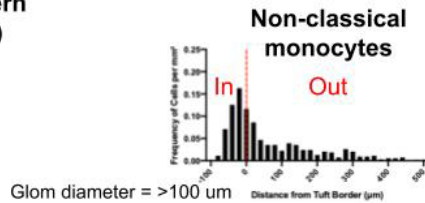
Heatmaps representing how well intrarenal mouse myeloid clusters from each lupus strain expressed gene signatures from human myeloid subsets from lupus nephritis biopsies. The warmer colors represent higher expression of human signatures in corresponding mouse clusters. Mouse NC1 and NC2 (non-classical 1 and 2) highly expressed the signature from human CM0/NC1 (non-classical); mouse macrophage expressed the signature from human CM4; mouse DC1 and DC2 (dendritic cell 1 and 2) expressed the signature from human CM2/DC2.

Non-classical, resident macrophages, and DC subsets are homologous across strains and species

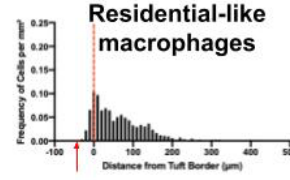
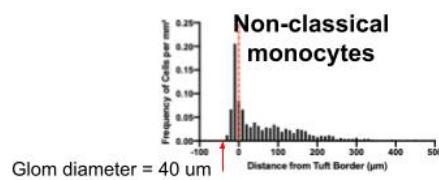


Distribution of homologous non-classical and residential-like states is similar in mice and a subset of human patients

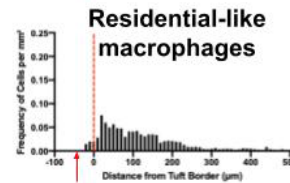
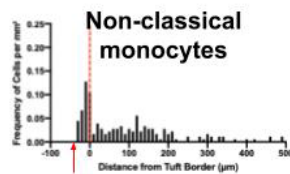
Major pattern
(8/14 pts)



Sle1.Yaa



NZB/W



Homologous subsets were spatially mapped across kidney sections and plotted as function of distance relative to the glomerular border. This location was chosen because significant histopathologic tissue changes occur here that influence disease classification. Homologous human and mouse non-classical monocytes (left panel of histograms) are both enriched inside glomerular structures and form a gradient toward the borders outside the glomerulus. Homologous human and mouse residential-like macrophages (right panel) both localize to the glomerulus to a lesser extent, and form a gradient toward the glomerular edge. This spatial phenotype is conserved in mice and in the majority but not all of the patients we interrogated. Thus, the distribution of these homologous cells is similar in mice and a subset of human patients. These data suggest homologous cells carry out conserved effector functions in the same anatomic compartments in mice and a subset of humans.

REVIEW ARTICLE

Renal Mononuclear Phagocytes in Lupus Nephritis

Anne Davidson 

Renal mononuclear phagocytes are a highly pleiotropic group of immune cells of myeloid origin that play multiple protective and pathogenic roles in tissue homeostasis, inflammation, repair, and fibrosis. Infiltration of kidneys with these cells is a hallmark of lupus nephritis and is associated with more severe disease and with increased risk of progression to end-stage renal disease. This review presents current knowledge of the diversity of these cells and their involvement in kidney inflammation and resolution and describes how they contribute to the chronic inflammation of lupus nephritis. A better understanding of the subset heterogeneity and diverse functions of mononuclear phagocytes in the lupus nephritis kidney should provide fertile ground for the development of new therapeutic approaches that promote the differentiation and survival of protective subsets while targeting pathogenic cell subsets that cause inflammation and fibrosis.

Introduction

Lupus nephritis (LN) is a common manifestation of systemic lupus erythematosus that can cause irreversible renal injury. Although the prognosis of LN has improved substantially over the past 50 years, standard immunosuppressive therapies induce complete remission in less than 50% of treated patients. The rate of progression of LN to renal failure has plateaued in the United States in the past 20 years, and approximately equal to 10% of patients develop end-stage renal disease (ESRD) within 10 years of diagnosis. This failure reflects disease complexity and heterogeneity as well as insufficient patient access to high-quality medical care (1).

Although the pathologic classification of LN is based predominantly on renal glomerular inflammation, it is increasingly recognized that interstitial inflammation and fibrosis are associated with a worse long-term prognosis. The presence of renal macrophages, and dendritic cells (DCs) (mononuclear phagocytes) in particular, correlates with more severe disease and an increased likelihood of disease progression (2,3). There has therefore been increasing interest in understanding the diversity of these cells and how they contribute to tissue injury in LN as well as in multiple other inflammatory and fibrotic diseases.

This review will describe the heterogeneity of the mononuclear phagocyte system in tissues and how this landscape changes during inflammation and resolution. It will then show how

this general paradigm applies to LN and how this knowledge may inform approaches to treat LN and prevent progression to ESRD.

Pathogenesis of LN

LN is initiated by the deposition of nucleic acid containing autoantibodies and debris in the glomeruli. Subendothelial deposits activate the glomerular endothelium and engage complement; this induces the recruitment of circulating proinflammatory cells, leading to proliferative disease and the formation of glomerular crescents. By contrast, subepithelial deposits face the urinary space and cause podocyte injury with foot process effacement, leading to proteinuria (4). Mesangial deposits in isolation are less likely to directly damage the glomerular filtration apparatus. In addition, soluble inflammatory mediators derived either from the circulation or from activated glomerular cells amplify glomerular cell activation and injury (5–10).

The glomerular filtration apparatus comprises the endothelial layer, the glomerular basement membrane, and the podocyte layer. Podocytes and glomerular endothelial cells secrete growth factors to support each other's survival, and mesangial cells provide growth factors that support the endothelium. Because of these interrelated functions, injury to any glomerular cell type can eventually damage the others (10–12). This is followed by glomerular cell dedifferentiation and loss of integrity of the whole

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glomerular tuft. Because glomerular cells have limited regenerative capacity (13,14), sequential insults to LN kidneys result in progressive glomerular loss. Compensatory glomerular hypertrophy and glomerular hypertension then compromise tubulointerstitial viability because interstitial capillaries are supplied solely by vascular runoff from the glomeruli. Disease progression is therefore associated with tubulointerstitial hypoxia and compromise of epithelial cell metabolic pathways, leading to cell death (15,16). Although renal epithelial cells have the capacity to regenerate, interstitial capillary rarefaction may be irreversible. Tissue damage and hypoxia leads to the accumulation of immune infiltrates in the interstitium; this is followed by the differentiation of multiple cell types, such as stromal cells and pericytes, into fibroblasts (1).

Changes to the renal tubulointerstitium (including tubular atrophy, fibrosis, and interstitial infiltrates) are known prognostic markers for progression to ESRD in patients with LN (17,18). Furthermore, immunohistochemical studies of kidney biopsies from patients with LN have suggested that the presence of renal macrophages, both glomerular and tubular, is associated with a worse prognosis in patients with LN, particularly at the second biopsy (2,19,20). The predictive power of the second biopsy reflects the ability of this biopsy to detect those patients in whom initial therapy has failed to control inflammatory lesions and in whom early fibrosis fails to reverse (2,18). Because mononuclear phagocytes can play a role in both inflammation and repair, newer studies are now beginning to address the heterogeneity and functions of these cells in renal injury in general, and in LN in particular,

to address whether it is possible to target pathogenic populations while retaining those that could mediate repair.

The origins and development of renal macrophages and DCs

Renal mononuclear phagocytes derive from precursors that form in the fetal yolk sac and from hematopoietic precursors in the bone marrow (Figure 1).

Yolk sac-derived macrophages. Yolk sac-derived macrophages are the source of resident macrophages in multiple organs, including the kidneys. Resident macrophages of embryonic origin are long lived and self-renewing, and they play an essential role in tissue homeostasis by monitoring the tissue for pathogens and removing any dead materials. These cells are slowly replaced throughout life by macrophages of hematopoietic origin that take on the organ-specific features of the resident cell. The rate of this replacement varies in each organ and increases during periods of tissue inflammation; if the organ is irradiated, the entire population can be replaced from bone marrow-derived cells that adopt a tissue-specific profile as they enter the tissue microenvironment (21,22).

Bone marrow-derived macrophages and DCs. Bone marrow-derived macrophages and DC are highly related subsets that derive from common progenitors. Monocytes are short-lived circulating cells that differentiate into either macrophages or

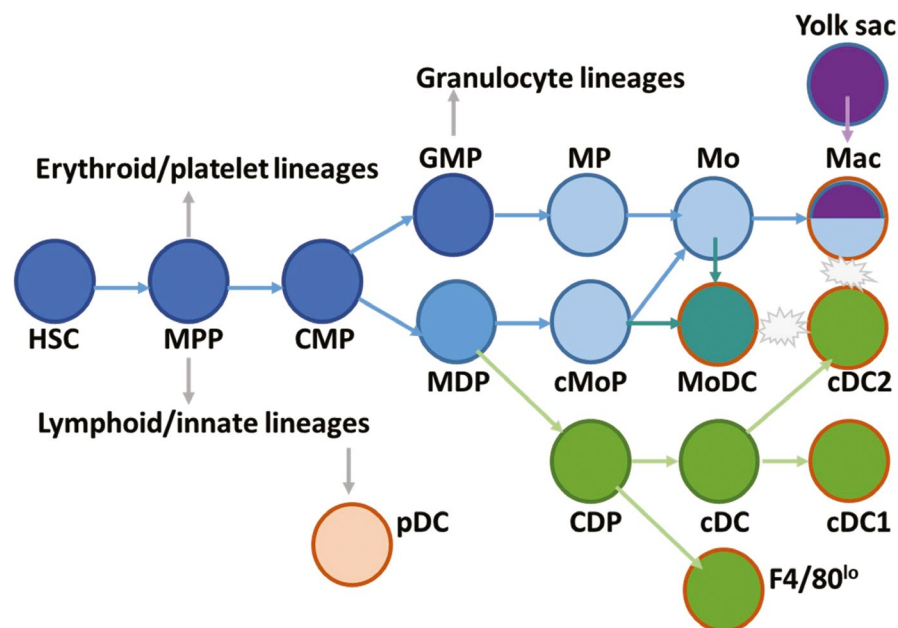


Figure 1. Development of the myeloid lineage from hematopoietic stem cells (HSC) or from yolk sac macrophages (Mac). Cells with an orange border are found in normal kidneys. The three cell types connected by grey clouds have similar phenotypes and may be difficult to distinguish in inflamed tissues by phenotyping alone. cDC, classic dendritic cells; CDP, common dendritic cell progenitors; CMoP, common monocyte progenitors; CMP, common myeloid progenitors; GMP, granulocyte macrophage progenitors; MDP, macrophage dendritic cell progenitors; Mo, monocyte; MoDC, monocyte-derived dendritic cells; MP, monocyte progenitors; MPP, multipotent progenitors; pDC, plasmacytoid dendritic cells.

DCs once they enter tissues. Macrophages are highly phagocytic cells that play both pathogenic and protective roles during tissue injury and repair. DCs engulf smaller-sized material than macrophages and are efficient antigen-presenting cells that function to activate the adaptive immune system. All these cells are highly pleiotropic, and recent studies have shown overlapping phenotypes and functions between the macrophages and DCs that infiltrate injured tissues (22).

Renal mononuclear phagocytes. Normal kidneys contain a network of long-lived tissue-resident macrophages that occupy niches around the periphery of glomeruli and adjacent to tubules and peritubular capillaries; these cells function in immune surveillance and homeostasis (23–26). Resident renal macrophages in mice are initially of yolk sac origin but are replaced by cells of hematopoietic origin as the mice become adults (27). These resident macrophages are characterized by the high expression of F4/80 and CD64 and are different from those in many other organs because there is no basement membrane that separates them from endothelial cells, allowing them to rapidly take up small immune complexes and other small particles in a manner analogous to that of splenic macrophages and Kupffer cells (25).

The normal kidney also contains smaller populations of other phagocytic cells. Renal DCs include both type 1 classic dendritic cells (cDC1) and type 2 classic dendritic cells (cDC2), which are phenotypically and functionally distinguishable subsets. cDC1 cross-present antigen to CD8 T cells via class I major histocompatibility complex and can also induce regulatory T cells. cDC2 are classic antigen-presenting cells that interact with and activate the adaptive immune system. Further subsetting of DCs has been reported, and even more phenotypes have been identified in the setting of inflammation (22,27). Under homeostatic conditions, some renal DCs migrate to lymph nodes, where they help to maintain tolerance to renal antigens. Studies in mice show that in contrast to resident macrophages, kidney cDCs turn over rapidly, suggesting that they traffic continuously into the kidneys (27,28). Plasmacytoid DCs are also present in normal kidneys. These cells derive from the lymphoid lineage and function to produce innate cytokines, including type I interferons (IFNs), on exposure to innate stimuli. Finally, there is a small renal population of cells of common DC progenitors origin that express CD11b but are low for F4/80. These cells have phenotypic differences from both macrophages and cDC2 (23,27).

The precise location of DC subsets in normal kidneys has not been fully mapped because of the lack of specificity of many myeloid cell markers. Extensive flow cytometry characterization using multiple markers and lineage tracing experiments in mice are beginning to address this issue (22,27). Using the cDC-specific *Zbtb46* as a reporter for all cDCs and *Snx22* as a reporter for cDC1, cDCs were found to be localized predominantly around blood vessels (29). Experiments using *Clec9a* as a reporter have by contrast suggested that up to 30% of renal

cells with a resident macrophage phenotype (F4/80^{hi}/CD64^{hi}) and localization are cDCs (27). The interpretation of the fate mapping studies depends on the specificity of the reporters for the various cell lineages, and further studies are needed to fully understand the role of each subset during homeostasis and disease.

Single-cell RNA sequencing analyses of immune cells from normal mouse kidneys have recently been reported (30). Single-cell analysis samples the most abundantly expressed genes in individual cells at a single time point and therefore represents only a snapshot of the overall diversity of renal mononuclear phagocytes. Nevertheless, these studies have confirmed the presence of the subsets described above and are beginning to allow for further subsetting and functional analysis of renal macrophages and DCs, especially in diseased tissues.

General alterations of renal mononuclear phagocyte function in inflammatory renal diseases

Macrophages. Macrophages are highly pleiotropic cells that can rapidly be polarized *in vitro* to multiple phenotypes and alter their phenotype and function *in vivo* in response to their microenvironment. During acute kidney injury in mouse models, proinflammatory macrophages are recruited to the glomeruli under the influence of chemokines and other chemoattractants. Both classical monocytes (CD14⁺ in humans and Ly6C^{hi} in mice) and nonclassical monocytes (CD16⁺ in humans and Ly6C^{lo} in mice) can acquire an inflammatory phenotype in tissues and contribute to tissue injury. TLR7 engagement, in particular, is associated with the recruitment of nonclassical (patrolling) monocytes to activated endothelium (31). Proinflammatory function of infiltrating macrophages is reinforced by inflammatory cytokines and other damage associated molecular patterns by tubular release of micro-RNAs and by activation of inflammasome-mediated pathways (32). These cells produce inflammatory cytokines, nitric oxide synthase, procoagulants, and matrix metalloproteinases, and their pathogenic role has been confirmed in mouse models in which their depletion during the inflammatory phase after acute injury attenuates renal injury (26,33).

As renal disease transitions from the acute to the chronic phase, both infiltrating and resident renal macrophages take on an alternatively activated reparative phenotype (34,35). This could be due to a switch in cell phenotype or sequential recruitment and differentiation of different cell populations. Alternatively activated macrophages play a major role in removal of debris and collagen, regulation of inflammation, and regeneration of the tubular epithelium; their depletion during the repair phase of injury exacerbates renal fibrosis (33,36). Induction of this phenotype is regulated by local cytokines and metabolic conditions. In mice, transfer of macrophages polarized by interleukin 4, interleukin 13, and/or interleukin 10 promotes renal repair after acute injury. Conditions in local microenvironment, such as tissue hypoxia and

iron metabolism and tubular secretion of colony-stimulating factor 1, can also drive alternative macrophage polarization. Nevertheless, excessive accumulation of reparative macrophages results in the production of profibrotic mediators, such as transforming growth factor β and platelet-derived growth factor.

Studies in several human renal diseases have shown an association between alternatively activated macrophages in renal biopsies and poorer disease outcome and fibrosis. Similarly, macrophages with an alternatively activated phenotype can promote both glomerulosclerosis and interstitial fibrosis in rodent models of chronic renal injury (26). The balance between renal repair and chronic fibrosis is regulated by cross talk between fibroblast precursors, tubular epithelium, and macrophages (37,38).

The full scope of pathogenic and protective populations of macrophages in chronically inflamed kidneys and the specific roles infiltrating and resident macrophages (39,40) are still not completely delineated. Macrophages with the capacity to digest and remove collagen *in vitro* have been identified (41), but the precise molecular and functional characteristics of these cells and how to induce them *in vivo* remain to be established. Matrix metalloproteinase 13 has emerged as a key matrix metalloproteinase that promotes resolution of fibrosis in multiple organs, including the kidney (42,43). Phagocytic receptors of the TAM family may also regulate resolving functions; for example, MerTK-positive macrophages promote resolution of inflammation, whereas MerTK-negative macrophage subsets induce the differentiation of inflammatory fibroblasts (40,44). By contrast, the phagocytic TAM receptor Axl promotes tissue fibrosis by enhancing TLR signaling and metabolic reprogramming (45). Recent studies have highlighted the role of the neural guidance protein Netrin-1 as an antiinflammatory mediator that is induced in macrophages in hypoxic areas of tissue (46). Netrin-1 stimulates the production of pro-resolving lipid mediators, shortens the time required for resolution of inflammation, and promotes tissue regeneration. However, Netrin-1 macrophages can also promote fibrosis during chronic inflammation. Interestingly, the interaction of Netrin-1 with its receptor induces adrenergic nerve remodeling and release of norepinephrine, which promotes fibrogenesis (47).

Recent discoveries with respect to the regulation of immune cell function by intracellular metabolic pathways have enhanced our understanding of the mechanisms for macrophage polarization. Inflammatory macrophages are characterized by an increase in glycolysis and a decrease in mitochondrial function with production of succinate and itaconate as a result of breaks in the Krebs cycle (48). Succinate enhances production of inflammatory cytokines and reactive oxygen species. By contrast, itaconate has both an antibacterial and an antiinflammatory role, acting to restore homeostasis. Alternatively activated and pro-resolving macrophages are characterized by an increase in fatty acid oxidation and mitochondrial respiration and by the secretion of arginases that produce metabolites that suppress inflammatory responses (49). An emerging interest has been to define transcription factors

that dictate programs that regulate the metabolism, phenotype, and function of macrophage subsets (21). Resident and infiltrating macrophages are regulated by different transcription factors, and macrophage programs can also be influenced by the type of material they phagocytose in each specific niche. For example, Spi-C transcription factor regulates a macrophage program needed for iron handling, whereas Nr1h3 is induced by the accumulation of cellular cholesterol (via uptake of lipids from dying cells) and, in turn, can suppress inflammation, promote macrophage survival, and enhance antiinflammatory apoptotic cell clearance (13,50,51). Notably less is known about myeloid cell subsets and states and the core transcription factors that regulate them in the kidneys than in other major organs.

DCs. During inflammatory disease, infiltrating DCs derive from both monocytes and circulating DCs. Like macrophages, DCs in tissue are heterogeneous and can perform both inflammatory and suppressive functions. Distinction of tissue macrophages from tissue DCs can be difficult, especially in inflammatory settings in which infiltrating cells display features of both cell types (22,52). This has resulted in some confusion over nomenclature of renal myeloid cells that is still not fully resolved. Studies in mouse models of renal inflammation suggest that cDC1 are protective, whereas cDC2 are pathogenic (22). Mouse splenic cDC2 have recently been divided into two subsets that express different transcription factors and appear to employ different metabolic programs (53). Gene expression differences between these two subsets suggest that cDC2A are more likely to be involved in tissue repair, whereas cDC2B are more likely to be proinflammatory. Other subsets include CCR7 expressing DCs that have the capacity to migrate to lymph nodes and may also help to organize local lymphoid infiltrates. Transcription factors that direct DC ontogeny and function are beginning to be described (53). How each of the DC subsets contributes to renal injury and repair is currently not known.

Alterations of the renal myeloid compartment in LN

The renal mononuclear phagocyte compartment undergoes extensive remodeling as LN evolves, with an influx of new myeloid cells, the accumulation of mixed immune infiltrates, and changes in the function of resident cells.

Glomeruli. Glomerular macrophages can be abundant in LN, with heterogeneity among mouse models and patients with LN. These cells are located either within the glomerular tuft, where they are adherent to glomerular capillary walls, or within the glomerular crescents (54). Phenotypic studies in mice have shown that there are at least two subpopulations of these cells in glomeruli. In mouse models characterized by overexpression of TLR7, glomerular macrophages are located within glomerular capillaries

and are F4/80^{lo}, CD11c^{hi}, and Ly6C^{lo}, characteristic of nonclassical or patrolling macrophages. These cells express the transcription factor Nr4a1, and deletion of this transcription factor abolishes their recruitment to glomeruli and attenuates glomerular injury (55). In the NZM2328 model, infiltrating F4/80^{lo}, CD11b⁺, CD11c⁺ cells are found in the glomeruli of severely proteinuric mice; these cells are also Ly6C^{lo} and have an alternatively activated phenotype (6,56). The molecular characteristics of macrophages located in glomerular crescents have not yet been reported in mice.

In human LN, glomerular macrophages correlate with the severity of glomerular lesions, being found more frequently in diffuse proliferative disease. Studies using a limited number of cell surface markers suggest that these cells are heterogeneous and may include both inflammatory and reparative phenotypes (20).

Interstitial resident cells. Macrophages with a resident phenotype increase in number during LN. Peritubular renal macrophages are susceptible to immune complex-mediated activation because of their anatomic location near small peritubular vessels without an intervening basement membrane (25). Our phenotypic and single-cell studies in mice have confirmed that there is a major shift in the resident macrophage profile during LN, with loss of protective and homeostatic functions and acquisition of an alternatively activated/reparative phenotype (23,57). An analogous population of macrophages with an

alternatively activated profile is highly represented in human LN biopsies (58).

Immune cell infiltrates. Mixed tubulointerstitial leukocyte infiltrates, sometimes with features of lymphoid organization, are found in LN (2,17,18,59) and are associated with renal scarring and a worse prognosis. These infiltrates contain both cDC1 and cDC2, especially during advanced disease (3). In human LN biopsies, CD209 expressing cDC2 are found in periglomerular regions, within lymphoid infiltrates, and scattered in the interstitium (60). CD103 (cDC1) DCs and plasmacytoid DCs are also present in smaller numbers both in mice and humans with LN (3,23). Approximately 40% of patients who have moderate to severe infiltrates progress to ESRD over a 4-year period (17), but it is still not known how to distinguish those patients who will progress from those who will not.

Lessons learned from molecular profiling and single-cell analyses of LN kidneys

Our early studies in diverse mouse models of LN identified a myeloid cell signature that was shared among LN mouse models and human biopsies. Using flow cytometric analyses, we found both expansion and activation of the dominant CD11b⁺/F4/80^{hi} renal macrophage population during active LN as well as a large population of cDC2 during proliferative systemic lupus

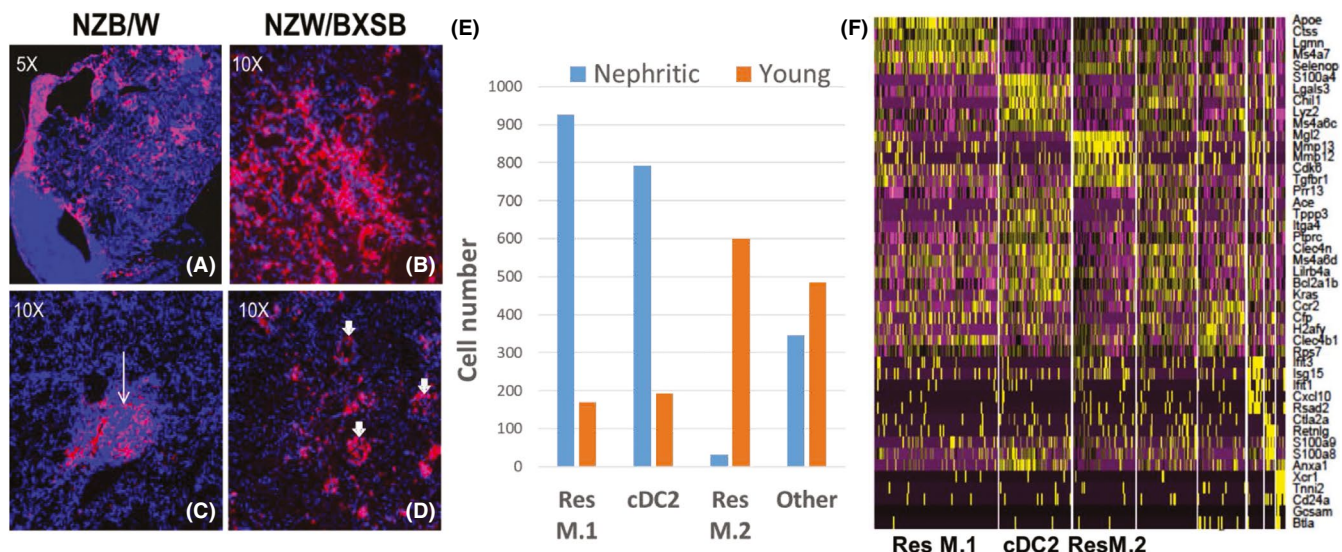


Figure 2. Diversity of mononuclear phagocytes in LN kidneys. **A**, F4/80 staining of macrophages in a kidney from a nephritic NZB/W mouse shows localization around glomeruli and in the interstitium but exclusion from lymphoid aggregates. **B**, F4/80 staining of macrophages in a kidney from a nephritic NZW/BXSB (TLR7 overexpressing) mouse shows localization around glomeruli and extensive infiltration in the interstitium. **C**, CD11c staining of classic dendritic cells (cDCs) in a kidney from a nephritic NZB/W mouse shows localization within the mixed perivascular immune infiltrate. **D**, CD11c staining in a kidney from a nephritic NZW/BXSB (TLR7 overexpressing) mouse shows localization inside glomerular tufts; these cells are most likely nonclassical (patrolling) monocytes. **E**, Single-cell RNA sequencing (RNASeq) analysis of CD11b/c cells from nephritic and prenephritic (young) mice. Bars show the number of cells in the three major clusters. Resident macrophages are in resident macrophage cluster 2 (Res M.2) in young mice and predominantly in resident macrophage cluster 1 (Res M.1) in nephritic mice. Also note the expansion of the cDC2 population in nephritic mice. **F**, RNASeq analysis of CD11b/c cells from NZB/W mice shows eight subclusters of cells. The top five genes for each cluster are shown on the right.

erythematosus nephritis that disappears during remission. We found an additional population of CD11c-positive glomerular cells in mice overexpressing TLR7 (23,61) (Figure 2).

Single-cell analyses are now beginning to reveal the complexity of the myeloid cell landscape in LN kidneys. Our studies in NZB/W mice have identified at least eight subsets of renal mononuclear phagocytes, with significant differences in the relative representation of each subset between prenephritic and nephritic mice (62) (Figure 2). We found that the three major clusters of cells in NZB/W mice are two clusters of resident macrophages and a cluster of cDC2. Resident macrophages from nephritic and prenephritic mice cluster separately because of the differences in gene expression that occur in this subset at disease onset. We also identified smaller populations of cDC1, CCR7^{hi} DCs, monocyte-derived DCs, proliferating cells, and cells expressing an inflammatory and IFN signature. Interestingly, an additional population was found in Sle1.Yaa mice corresponding to the glomerular subset of CD11c⁺ cells (62).

Our team is part of the Accelerating Medicines Partnership–Systemic Lupus Erythematosus (AMP-SLE), which is addressing the complexity of LN using single-cell RNA sequencing (scRNASeq) analysis of renal cells (58,63). Recently, the AMP-SLE group has successfully completed scRNASeq analysis of a total of 160 LN biopsies using 10x Genomics technology. This AMP-SLE phase 2 study will yield a rich set of data that can be correlated with disease outcome in a manner that has previously been unprecedented. Initial scRNASeq data of CD45⁺ immune cells isolated from the first 23 biopsies analyzed in the AMP-SLE phase 1 study have been published and include 466 myeloid cells (58). Although this sample size is rather small, we were able to identify five subpopulations of cells, of which only one was detected in normal kidneys harvested from healthy living renal donors. We found four subpopulations of macrophages cluster myeloid (CM0, CM1, CM2, and CM4), of which only CM0 was detected in the peripheral blood. Three of these populations, CM0, CM1, and CM4, are related along a developmental trajectory, starting with tumor necrosis factor–producing CD16⁺ nonclassical monocytes (CM0) that transition to a phagocytic (CM1) and then to an alternative macrophage phenotype (CM4) that also produces inflammatory chemokines. We classified the fourth population that was found in both healthy donors and LN biopsies (CM2) as resident macrophages. These cells acquired both an IFN signature and an antiinflammatory signature in LN biopsies (58). We also detected a small population of cDC2, but their number was too small to analyze them in detail. The results of the phase 2 study will be available later this year; these data include a much larger population of myeloid cells, and preliminary analyses indicate that additional myeloid cell subsets are present (A. Arazi: personal communication). Our preliminary analyses also indicate that there are substantial similarities between the populations that are present in humans and those we find in mice (64), suggesting that mechanistic studies will be possible in selected mouse models.

It is impractical to collect sequential human renal biopsies for testing causal disease mechanisms or monitoring treatment responses. It is therefore of great interest to test whether myeloid cell subsets appear in the urine and can be used as surrogate markers for renal infiltration. Preliminary analyses of the AMP-SLE phase 1 samples have shown that myeloid cells constitute the majority of immune cells in the urine, especially CM1 cells (58), suggesting that these have access to the urinary space, most likely as a result of localization in glomerular crescents. Further analyses in the AMP-SLE phase 2 observational study will address whether the inflammatory myeloid cell subsets correlate with renal infiltrates and/or responses to treatment and whether urine can be used to assess treatment responses and prognosis. Ongoing mapping studies will also determine the location of the various myeloid cell subsets.

Applications to clinical care

Many types of mononuclear phagocytes are found in the kidneys of individuals with LN. A better understanding how each infiltrating cell type contributes to renal injury is now needed so that pathogenic cells can be targeted, whereas those involved in organ protection and repair can be spared. Because the features of reparative macrophages that promote resolution and resorption of collagen and extracellular matrix components are not well defined and may be organ specific (35,65–67), it is not always possible to extrapolate studies from other inflammatory diseases and organs to LN.

There are several approaches to identifying the function of tissue-derived myeloid cell subsets. Recent studies of synovial macrophages found that the presence of MerTK⁺ synovial tissue macrophages during disease remission was associated with protection from disease flare after cessation of treatment (44). When isolated, these cells failed to induce inflammatory fibroblasts in coculture experiments. By contrast, MerTK[−] macrophages were more likely to produce inflammatory mediators and interact with stromal cells to induce the development of inflammatory fibroblasts that promote fibrosis. An alternative approach that can be used in the case of organs, like kidneys, that are difficult to access, is to compare the phenotype of organ-specific macrophages with that of macrophages induced by a broad panel of cytokines and to reproduce a similar phenotype *in vitro* that can then be used for coculture studies (68). Finally, mouse models can be particularly useful for functional and therapeutic studies if they reflect human physiology. In this context, comparison of data from mouse models with the AMP-SLE phase 2 data will be highly informative.

Conclusions

Identifying and targeting pathogenic myeloid cell subsets and prevention and treatment of renal fibrosis should improve the

treatment of LN. Therapeutic manipulation of metabolic pathways offers some new opportunities for the treatment of inflammatory diseases, including LN (69). Strategies are beginning to emerge for modulating macrophage glycolysis through the inhibition of inducing signals, such as toll like receptors and the NLRP3 inflammasome (32). Induction of reparative macrophages will require a better understanding of how fibrosis and resolution are balanced, but new inroads are being made by using mouse models of injury and repair. For example, the combination of a decoy form of soluble Axl with an antagonist of MerTK shedding was highly effective at promoting repair in a model of cardiac injury (45). Another novel approach is the use of $\alpha 1$ agonism to oppose the activity of Netrin-1; this strategy prevented fibrosis in a mouse model of early interstitial pulmonary fibrosis, although it was ineffective at reversing established fibrosis (47). Therapies can also be directed at correcting metabolic abnormalities of the tubular epithelium because these cells induce profibrotic functions in adjacent macrophages (15,70). Prevention of recruitment and/or differentiation of inflammatory macrophages and DCs is another potential approach. In this context, it is of interest that B cell activating factor has a role in directing myeloid cell function that could help account for the therapeutic efficacy of belimumab in LN (71–73). Uncovering the role of each renal mononuclear phagocyte subset in LN should provide a fruitful area for research and further therapeutic discovery.

AUTHOR CONTRIBUTIONS

Dr. Davidson drafted the article, revised it critically for important intellectual content, and approved the final version to be published.

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REVIEW



AQ2

Lupus nephritis: challenges and progress

AQ3

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

AQ7
end stage
renal disease

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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Systemic lupus erythematosus and Sjogren syndrome

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.

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

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 [■]]

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

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B cell activating factor
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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 [15²²]. A second study showed that chronicity at 6 months after induction predicted long-term renal outcome regardless of clinical or histologic remission status [31²³]; 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 [32²⁴]. 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 [34²⁵]. 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

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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 interacting protein 1
major histocompatibility complex
intergrin subunit alpha M

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that specifically target these pathways [36,37]. A polymorphism that increases the expression of the adapter Dab2 that mediates TGF β 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].

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 [44[■]]. 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 patients 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 IFN γ -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[■]].

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 antiseif/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].

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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None.

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Conflicts of interest

There are no conflicts of interest.

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kidney injury
molecule 1
neutrophil gelatinase
associated lipocalin
monocyte chemoattractant
protein 1

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Emerging areas for therapeutic discovery in SLE

Naomi I Maria and Anne Davidson

Recent advances in the field of autoimmunity have identified numerous dysfunctional pathways in Systemic Lupus Erythematosus (SLE), including aberrant clearance of nucleic-acid-containing debris and immune complexes, excessive innate immune activation leading to overactive type I IFN signalling, and abnormal B and T cell activation. On the background of genetic polymorphisms that reset thresholds for immune responses, multiple immune cells contribute to inflammatory amplification circuits. Neutrophils activated by immune complexes are a rich source of immunogenic nucleic acids. Identification of new B subsets suggests several mechanisms for induction of autoantibody producing effector cells. Disordered T cell regulation involves both CD4 and CD8 cells. An imbalance in immunometabolism in immune cells amplifies autoimmunity and inflammation. These new advances in understanding of disease pathogenesis provide fertile ground for therapeutic development.

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Introduction

Systemic lupus erythematosus (SLE) is a devastating autoimmune disease in which autoantibodies to nuclear antigens cause inflammation and organ damage. Current treatment of SLE relies on broadly immunosuppressive medications with insufficient efficacy and significant toxicities. Despite large efforts in drug development, only one modestly effective biologic, belimumab, is FDA approved for treating active SLE. It is imperative, therefore, that advances in immunologic knowledge drive improvements in the treatment and quality of life of SLE patients. In this review we will summarize newly discovered pathways of immune activation that point to new therapeutic approaches. We will not address the genetic

causes of SLE or epigenetic changes in immune cells of SLE patients as these are reviewed elsewhere [1,2]. We also refer readers to a summary of treatments currently in clinical trials [3].

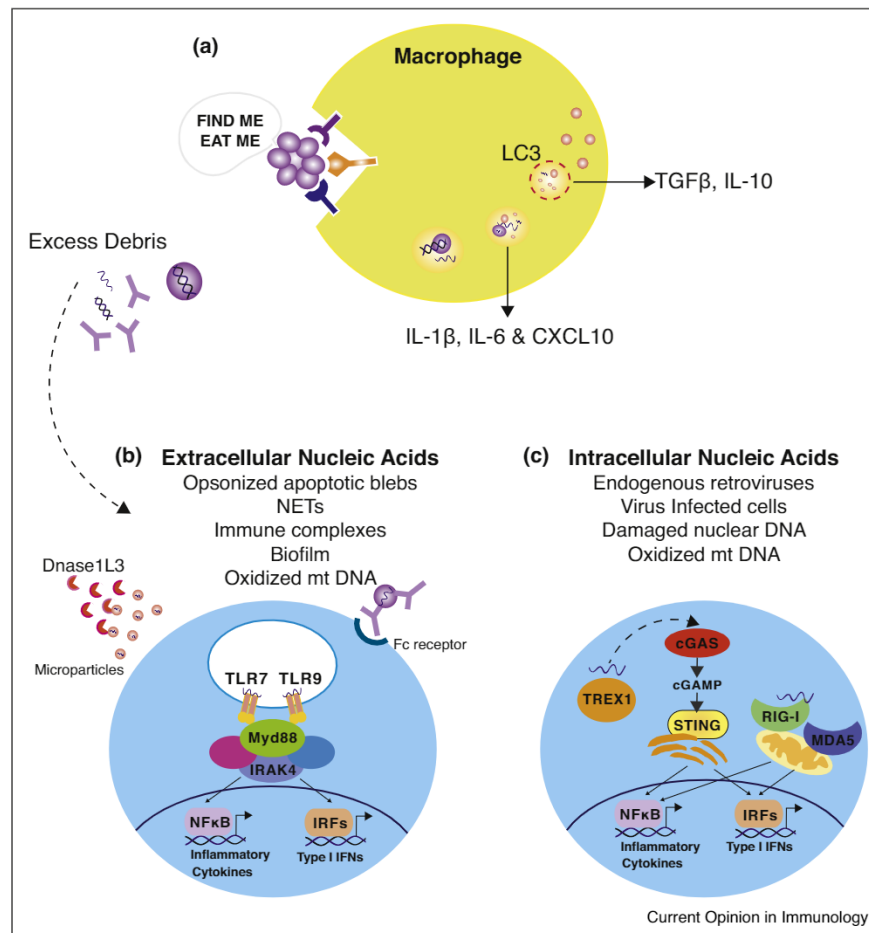
Induction of SLE by nucleic acid antigens

Following the discovery that dead cells are a continuous source of the major nuclear autoantigens in SLE, advances have been made in defining inflammatory and non-inflammatory types of cell death (reviewed [4]). Safe disposal of dead cell material is a primary function of macrophages that are located in secondary lymphoid tissues and in each organ (Figure 1a). An overload of apoptotic cells beyond the capacity of macrophage disposal, can result in progression to inflammatory forms of cell death that yield nucleic acids in immunogenic form Refs. [4,5]. Other sources of excess extracellular and intracellular nucleic acids and the sensors that recognize them are shown in Figure 1b and c.

Targeting of the innate pathways and sensors involved in induction of inflammatory cytokines and Type I interferons may form the basis for SLE therapy in appropriate patients. Ablation of cGAS in *Trex1* deficient mice abrogates SLE, confirming its role as a sensor of cytoplasmic DNA overload [6[•],7[•]]. It is not clear in how many patients this pathway is engaged; a recent report suggests that ≈15% of SLE patients have increased serum cGAMP levels [8[•]]. Surprisingly, STING deficiency exacerbates SLE in mouse models, reflecting the complex structure of this molecule that has both inflammatory and decoy forms and is also regulated by post-transcriptional modifications [9,10[•]]. MAVS aggregation has been observed in mitochondria of PBMCs from ≈30% of SLE patients, suggesting frequent activation of this pathway [11[•]]. Other targets in the nucleic acid sensing pathway include the endosomal TLRs and their downstream effectors IRAK4 and IRF5 (Figure 1b). Although IRF5 could be a valid target, IRF5 deficient mice develop accelerated atherosclerosis as a result of impaired efferocytosis by macrophages [12]. Finally, patients with high levels of Type 1 interferon may respond to interferon blockade or Jak1 inhibitors. Nevertheless, the disappointing failure of an antibody to the type 1 interferon receptor to reduce disease activity in a recent phase 3 trial, suggests that interferon blockade alone is not sufficient to address the inflammatory burden of active SLE.

The cytokine response induced by nucleic acid sensors resembles that of a chronic viral infection and is accompanied by activation of lymphocytes and, in the appropriate genetic environment, the generation of antibodies to

Figure 1



Innate immune mechanisms in SLE.

(a) An intact pathway for safe recognition and digestion of dead cells by macrophages includes a functional sensing mechanism, upregulation of scavenger receptors and activation of an alternative autophagy pathway called lysosome activated phagocytosis (LAP) that recruits part of the autophagy machinery, including LC3, to single membrane phagosomes and induces the release of regulatory cytokines such as IL-10 and TGFβ. Absence of LAP, but not of canonical autophagy in macrophages results in inefficient clearance of phagosomal material and release of inflammatory cytokines; this is associated with a mild spontaneous form of SLE in mouse models [58**].

(b) Extracellular particles containing nucleic acids within immune complexes, microparticles, biofilms and neutrophil NETs are internalized, often via receptor mediated phagocytosis, and encounter endosomal Toll like receptors, TLRs 7–9 within phagosomes. These TLRs use the adaptor molecular MyD88 and induce either inflammatory cytokines or Type 1 interferons. Absence of MyD88, IRAK4 and IRF5 all prevent SLE in mouse models. A role for DNase1L3 in clearance of DNA associated with extracellular microparticles has recently been described [59*].

(c) Intracellular nucleic acids engage a different set of sensors. The intracellular DNase Trex1 clears cytoplasmic DNA; complete deficiency causes an interferonopathy called Aicardi-Goutieres syndrome. Cyclic GMP-AMP synthase (cGAS) catabolizes the formation of the cyclic dinucleotide cGAMP from excess digested cytoplasmic DNA. cGAMP binds to and activates the stimulator of IFN genes (STING), located in the endoplasmic reticulum, resulting in phosphorylation of NFκB and IRFs and the production of inflammatory cytokines. Similarly, cytoplasmic RNA sensors, RIG-I and MDA5 bind to MAVS, a mitochondrial membrane protein that phosphorylates NFκB and IRFs to induce inflammatory cytokines needed for the anti-viral response. An immunogenic role has been defined for oxidized mitochondrial DNA released from activated neutrophils that do not perform mitophagy efficiently. Both TLR9 and STING have been implicated in recognition of neutrophil derived oxidized mitochondrial DNA [15**,16**]. Mitochondrial DNA released into the cytoplasm during apoptosis may also trigger the activation of cGAS.

nucleic acids. Nucleic acid-containing immune complexes further activate innate cells that express activating Fc receptors, including plasmacytoid dendritic cells (pDCs) and neutrophils. pDCs are poised to release Type I interferons and their absence in mouse models prevents the initiation of disease [13]. Activated neutrophils

release NETs and/or oxidized mitochondrial DNA, both of which amplify the dysregulated immune response. While neutrophil NETs are immunogenic and can deposit in target organs, SLE-prone mice still develop disease in the absence of NET formation [14]. Nevertheless, interest in neutrophils is based on their ability to

release immunogenic mitochondrial DNA and on the abnormal neutrophil signature that associates with SLE activity [15^{••},16^{••},17[•],18[•]].

Innate immune mechanisms enhance autoantibody production by B cells

Innate receptors in B cells play a major role in regulating the production of lupus-related autoantibodies. In particular, endosomal TLRs and Type I IFN regulate B cell functions at multiple developmental stages. The disparate roles of TLR7 and TLR9 in B cells have recently been addressed [19^{••},20]. B cell intrinsic TLR7 expression is required for optimal germinal center formation and for the majority of the SLE phenotype observed in TLR7 overexpressing mice [21[•]]. Induction of SLE by excess TLR7 also requires a functional B cell autophagy pathway [22]. IRF5, which is downstream of TLR7, appears to regulate B cell differentiation and plasmablast differentiation [23]. On the other hand, co-engagement of TLR9 and the BCR dampens TLR7 mediated signals and induces apoptosis of naïve B cells unless they are rescued by survival or costimulatory signals [19^{••}]. This observation helps explain why TLR9 deficiency exacerbates SLE even though it is required for an anti-DNA response. Because autoreactive B cells recognize and engulf nucleic acid containing material via their BCR and TLR engagement upregulates expression of the BAFF receptor TACI, TLR7 overexpression and/or excess BAFF can preferentially drive the survival and differentiation of such cells [24] (Figure 2).

TLR8 is expressed in myeloid cells but has been difficult to study in animal models because mouse TLR8 does not recognize RNA. Mice with multiple copies of human TLR8 spontaneously develop multi-organ inflammation with lupus-related autoantibodies and glomerulonephritis [25^{••}]. A role for TLR8 has also been proposed in the placental damage caused by anti-phospholipid antibodies [26] and in the induction of T follicular helper cells (TFH) [27].

Sources of autoantibodies

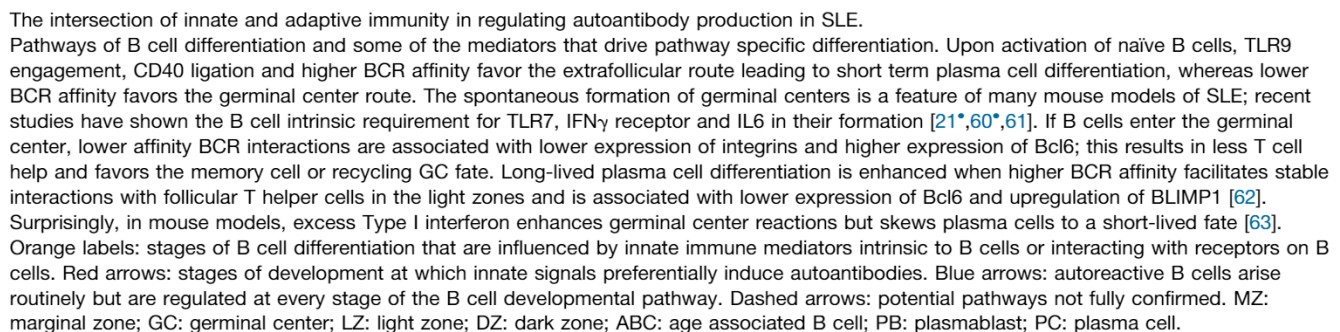
Autoantibody production is central to the pathogenesis of SLE. SLE patients have defects in naïve B cell tolerance even during periods of disease quiescence; this is associated with inappropriate responses to TLR9 ligands, decreased cell surface expression of CD19 and CD21 and decreased induction of IL10 [28[•],29]. What is not known is whether these defective naïve cells are the ones that expand into pathogenic B effector cells. Nor is it known whether the eliciting antigen is always self-antigen or whether other antigen sources are responsible. One potential source of exogenous antigen in the absence of overt infection is the gut, from where bacteria capable of eliciting autoantibody responses may leak through the colon wall [30[•]].

Activated B cells proceed along several different differentiation pathways to become either memory cells or long or short-lived plasma cells (Figure 2). Activated B cells may also differentiate into a subset termed 'age associated B cells (ABCs)'. The presence of ABCs correlates with anti-DNA antibodies and hypocomplementemia as well as renal and skin manifestations of SLE [31]. These cells have a unique phenotype, including expression of the myeloid marker CD11c and the transcription factor T-bet and they express a unique transcriptome. ABCs can derive from the extrafollicular pathway, but it is still not clear whether they also routinely arise in a germinal center. They are expanded in mice and humans with SLE, are preferentially autoreactive and are readily differentiated into plasma cells by endosomal TLR ligation together with IFN γ and IL21 produced by activated T cells [32,33]. The cooperation of T-bet with IRF5 in activated ABCs, promotes class switching to IgG2a or IgG2c, maturation into plasma cells and the transcription of genes encoding inflammatory mediators [34[•]].

SLE flares are often associated with the emergence of autoantibody secreting plasmablasts in the peripheral blood. Studies in mice have shown that whether the autoantibody response derives from short or long-lived plasma cells is strain specific and can be skewed by the cytokine milieu. The NZW/BXSB strain has been informative in this regard since the early autoreactive response is germinal center derived but during the late stages of disease, germinal centers disappear and the response becomes extrafollicular [35]. Next generation sequencing studies are beginning to address the origins of plasmablasts in human SLE. Recent studies of patients with SLE flares have shown that the repertoire of these cells is polyclonal, with dsDNA or RNA specific cells accounting for about 1–3% of the repertoire. Importantly, approximately 10–30% of these plasmablasts derive from 'naïve activated' B cells that have overlapping characteristics with ABCs, most likely through the extrafollicular route, whereas the rest derive from memory cells [36^{••}]. These two pathways of plasmablast differentiation could potentially be sensitive to different therapeutic interventions.

Abnormalities in SLE T cells

SLE T cells are abnormal in several ways. CD4 T cells from active SLE patients have altered signaling and a faster T cell calcium flux than those of healthy individuals due to lysosomal degradation of the CD3 ζ signaling component of the TCR complex and its replacement by the FcR γ common chain [37]. This recruits the adaptor molecule Syk rather than ZAP70 and activates the downstream kinase calcium/calmodulin-dependent protein kinase type IV (CaMK4) that enhances production of IL-17 and blocks production of IL-2 [38[•]]. Active SLE patients also have an increase in circulating CXCR5+ (TFH) CD4 T cells [39,40], perhaps skewed to the TFH1 (IFN γ producing) phenotype [41[•]] and they

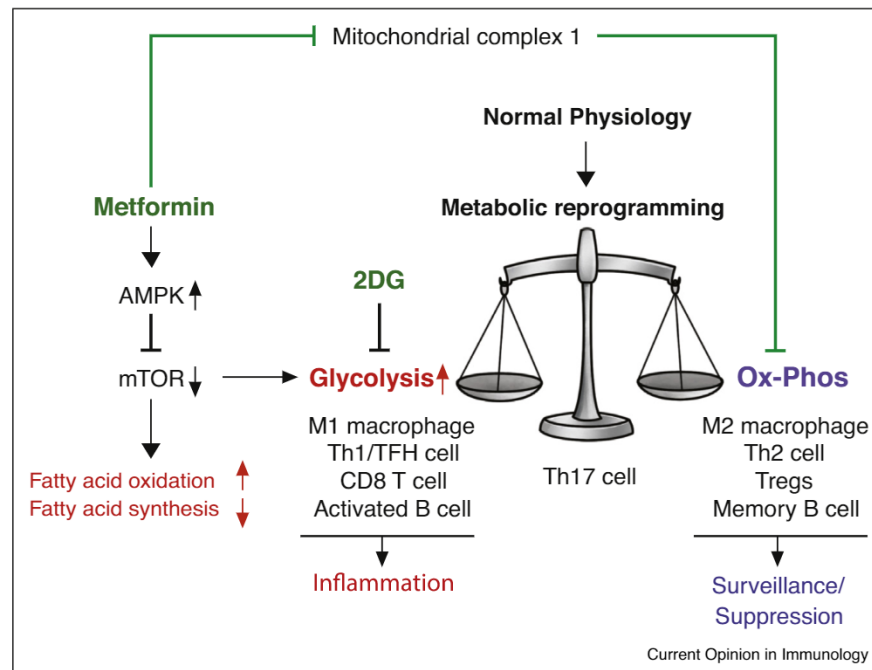


A role for CD8 T cells is being re-examined in SLE. Some SLE patients have a phenotype in which a low costimulatory profile of CD4 T cells is associated with an ‘exhausted’ phenotype of CD8 T cells. This phenotype has been associated with a lower incidence of SLE flares and an improved outcome [44[•]]. A recent study has highlighted the role of C1q in suppressing the activation and expansion of CD8 effector T cells in a SLE model, revealing a new function of this molecule in addition to its role in opsonization and non-inflammatory cell clearance [45[•]].

The activation of immune cells is associated with changes in metabolic programs that harness the requisite sources of energy for cell proliferation, protein production, the generation of anti-bacterial and anti-inflammatory

The function of other immune cells is also highly influenced by their metabolic state. Macrophage differentiation depends on metabolic switches; use of glutamine and activation of the arginosuccinate shunt promotes the

Figure 3



Metabolic pathways are fertile ground for therapeutic interventions in SLE.

Metabolic pathways influence effector function of multiple cell types. Glycolysis is typically associated with inflammatory functions of immune cells whereas oxidative phosphorylation is associated with suppressive or reparative functions. Activated CD4 T cells in SLE have enhanced glycolysis and oxidative phosphorylation. Metformin inhibits mitochondrial complex 1, thereby inhibiting oxidative phosphorylation, but also acts via the mTOR pathway to increase fatty acid oxidation and glycolysis. The latter is inhibited by 2 deoxy-glucose (2DG), explaining why the combination of metformin and 2DG can reverse active SLE in mouse models, whereas neither drug can do this alone [51**].

function of inflammatory macrophages, whereas alternatively activated macrophages use fatty acid oxidation and have an intact Krebs cycle [52]. Activated B cells are predominantly glycolytic; this pathway is enhanced by high levels of BAFF [53*]. Interestingly, long-lived plasma cells have a higher respiratory capacity and take up more glucose than their short-lived counterparts; this is converted to pyruvate which is required for their long-term survival (reviewed [54]). Potential approaches for targeting specific immune functions in human SLE using metabolic inhibitors have been recently reviewed [46,49].

Organ damage

New application of kinase inhibitors or anti-cytokine antibodies may add to the anti-inflammatory armamentarium available for the treatment of SLE [3]. It is increasingly appreciated, however, that organ damage in SLE patients involves mechanisms that may not respond well to standard immunosuppressive drugs. Neurologic damage may occur via antibody-mediated cytotoxicity of neurons by antibodies that cross a damaged blood brain barrier and/or by synaptic pruning of neurons by activated microglia. Placental damage induced by anti-phospholipid antibodies is due to trophoblast activation and complement-mediated inflammation. Premature myocardial infarctions and strokes are due to accelerated

atherosclerosis that is associated with loss of protective function of circulating HDL, endothelial dysfunction and the presence of low density granulocytes. Progression of SLE nephritis involves progressive interstitial fibrosis and metabolic dysfunction of tubular cells leading to tubular atrophy. Readers are referred to recent reviews addressing these areas [26,55–57].

Conclusion: application of new knowledge to improved patient care

Inappropriate patient selection for clinical trials not only exposes patients to potential toxicities but poses the risk for failure if the responding patient subset is diluted with non-responders. The current challenge in SLE is to find the underlying immune mechanisms that are perturbed in each patient so that one can ask whether that particular pathway can be normalized and, if so, at which disease stage this approach should be applied. Advances in therapy will require both a better understanding of disease pathogenesis and new approaches to therapy (Box 1). Finally, it is important to recognize that wide disparities exist in patient access to optimal medical care for SLE. Development of new therapies must therefore be accompanied by changes in health policy that bring the needed improvements in care to all SLE patients.

Box 1 Some current areas for investigation in SLE• Disease pathogenesis

- Which innate nucleic acid sensors are crucial in inducing or protecting from SLE?
- What are the origins of the plasma cells that produce autoantibodies and what is their relationship to protective antibody responses?
- What are the metabolic changes associated with autoimmune T and B cells?
- What other pathways for example microbiome, neurologic inputs, contribute to the dysregulated immune response?
- What are the mechanisms for tissue damage in each organ?

• Stratifying patients for clinical trials

- Can we identify the source of immunogenic nucleic acids and the relevant innate sensors in individual patients?
- Can patients with SLE be stratified based on their genetic, epigenetic or modular profiles [64*,65]?
- Are there biomarkers that distinguish SLE patients based on either immune mechanisms for tolerance loss or mechanisms of target organ damage?

• Have we exhausted therapies that target adaptive immune responses?

- Is there a way to tolerize to lupus-related autoantigens without compromising protective or anti-tumor immune responses?
- Will strategies for induction of T regulatory cells prevent or treat SLE?
- Is it possible to induce the CD8 T cell exhaustion phenotype?
- Is it possible to target metabolic pathways without compromising protective immunity or the function of vital organs?

• Targeting disease stages

- What is the role of animal models of disease in testing immune interventions?
- Can we enhance clearance of immunogenic nucleic acid containing material to prevent disease initiation or flares?
- Are there current or experimental therapies that will synergize and how should these be tested [66]?
- Can we identify and safely treat the preclinical phase of disease?
- Is it more effective and safer to target quiescent versus active disease?
- Can we design therapies specific for injury mechanisms in each organ?
- Is it possible to arrest or reverse tissue fibrosis?

Conflict of interest statement

Nothing declared.

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Promise and complexity of lupus mouse models

As a follow up to a 2010 meeting deliberating on the benefits of studying mouse models of systemic lupus erythematosus (SLE), the virtual conference “Mouse models of lupus 10 years later” convened on 10 December 2020 to address a challenging decade that saw few new therapies approved, despite leaps in knowledge.

Abnormalities in both innate and adaptive immunity characterize SLE, a systemic autoimmune disease with potentially severe consequences in patients. Treatment has traditionally been limited to broad-acting, side-effect-heavy immunosuppressants, which are also incompletely effective. Thus, the need for specific therapies targeting the pathogenic mechanisms of SLE remains unmet. Affirming the utility of mouse models in lupus research, a meeting convened in 2010 recommended that the next decade's research embrace these for their mechanistic insights; their diversity of phenotypes, which mirrors the heterogeneity observed among patients with lupus; and as a platform for clinical exploration. A decade later, hope persists; studies of murine models of lupus have uncovered putative therapies, and many were moved to clinical trials. Unfortunately, few interventions improved patient outcomes: belimumab was the sole therapy brought to market in the 2010s. The continuing difficulties in translating potential into success^{1–3} prompted a second conference, which was recorded.

On 10 December 2020, leading scientists and clinicians from all over the world, in partnership with the National Institutes of Health and the National Cancer Institute, gathered virtually. Hans-Joachim Anders (Ludwig Maximilian University of Munich) sparked much discussion with his opening address highlighting the multiple clinical trial design, diagnostic and scientific pitfalls that have seriously hindered translating success in lupus models to humans. Four scientific sessions followed, each delving into recent basic and translational advances in our understanding and treatment of SLE. In his closing keynote talk, Eric Morand (Monash University) proposed that the recent study of the interferon signature in SLE and the interventions it produced be used as a model for moving between human studies and mouse models, while stressing the scientific considerations needed to successfully utilize lupus models. Through discussions during the 2020 conference, participants aimed to revisit the utility of mouse models in the study of lupus by answering the following questions: Have animal models expanded our understanding

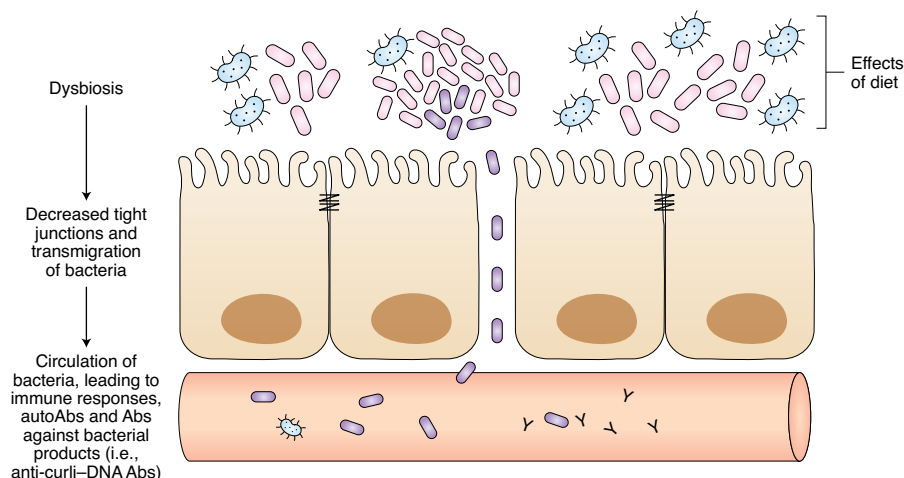


Fig. 1 | Dysbiosis and gut permeability contribute to lupus disease. Expansion of pathogenic gut microbiota can lead to increased permeability of the gut epithelial barrier, providing a route through which bacteria and bacterial antigens can enter systemic circulation. The immune response to these foreign epitopes may generate self-reactive antibodies, such as anti-curli–DNA, and contribute to lupus pathology.

of lupus pathogenesis? In what ways can we target the mediators of the lupus immune response? How has advanced technology been merged with the study of both lupus models and patients with lupus?

Infection and microbiome

Extraneous infection and the resident microbiome both act as routes through which a predisposed immune system may be hyperstimulated to precipitate loss of tolerance and trigger autoimmunity (Fig. 1). Mark Shlomchik (University of Pittsburgh) reported that experimental autoimmunity following *Salmonella* infection results from marked extrafollicular expansion of B cell populations, and the rapid maturation of B cells outside germinal centers increases autoantibody levels⁴. Interleukin (IL)-12 mediates this extrafollicular-dominant autoimmune “storm” by suppressing follicular helper T (T_{FH}) cells necessary for germinal center formation⁵, suggesting that IL-12 inhibition could treat patients with lupus who have similar immune responses.

Stefania Gallucci and Çağla Tükel (Temple University) introduced a specific microbial driver of autoantibody production. The amyloid–nucleic acid compound curli–DNA,

an Enterobacteriaceae biofilm component, is found throughout the murine intestinal tract during *Salmonella* infection. Tükel provided evidence that curli–DNA may trigger autoimmunity once it enters the blood via the intestinal epithelia or when it is shed by biofilm-bearing catheters⁶. Gallucci's group supported this hypothesis, showing that intraperitoneal injection of curli–DNA in lupus NZB×W/F1 mice increased circulating autoantibody and type I interferon (IFN) concentrations and activated macrophages and dendritic cells⁷. In addition to these hallmarks of SLE, curli–DNA appeared to induce B cell class switching independently of T cells. Finally, Gallucci described circulating anti-curli–DNA antibodies whose levels correlate with flares of active disease in patients with lupus⁸.

Supporting the hypothesis of a defective intestinal barrier exposing the immune system to microbiota and their products, Gregg Silverman (New York University) presented analyses of stool samples from patients with SLE that showed evidence of a more permeable gut. Additionally, his group characterized a new dysbiosis in patients with SLE, with skewing toward more *Ruminococcus gnavus* bacteria and

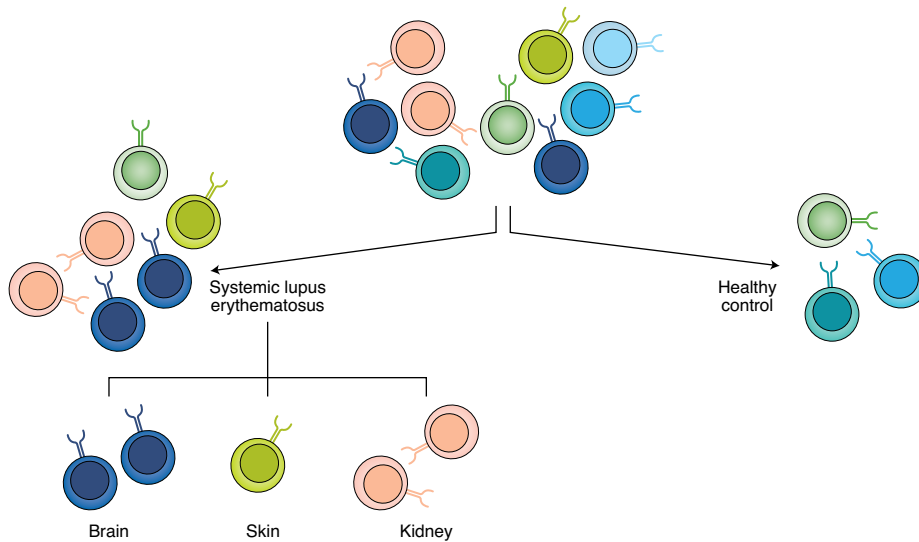


Fig. 2 | The potential application of T cell phenotyping and TCR sequence monitoring at both the organ and disease levels. T cells play a key role in organ damage caused by lupus disease. Sequencing of a T cell receptor could potentially reveal its cognate antigen, including autoantigens. TCRs can be disease and organ specific, so monitoring a patient's TCR repertoire could not only enable a lupus diagnosis but could also reveal organs potentially involved in the patient's disease.

fewer protective species. Serology from these patients showed antibodies that react to a bacterial cell wall lipoglycan, which correlated with active renal disease⁹. With this knowledge, Silverman's group colonized germ-free C57BL/6 mice with *R. gnavus* bacteria and saw levels of serum *R. gnavus* DNA rise, accompanied by elevations in gut permeability and zonulin. Administration of larazotide, a tight-junction regulator, reduced gut permeability, suggesting that *R. gnavus* induces elevated zonulin and disrupts tight junctions to increase gut permeability.

Similarly, Martin Kriegel (Yale University) presented evidence from both mice and human studies that *Enterococcus gallinarum* induces a more permeable gut, enabling its transmigration to the liver and lymph nodes. Significant immune activation, including rising type I IFN levels and anti-*E. gallinarum* antibodies, accompanies this process¹⁰. Interestingly, Kriegel's group developed a vaccine that is able to suppress transmigration of *E. gallinarum*, which could be a new treatment strategy for autoimmunity related to microbiome dysbiosis.

Xin Luo (Virginia Tech University) characterized the microbiome of diseased MRL/lpr mice; more Lachnospiraceae and fewer Lactobacillaceae were found¹¹, representing another pathogenic dysbiosis. The putatively protective Lactobacillaceae were then gavage delivered to female MRL/lpr mice, resulting in improved systemic and renal disease later in life¹². Oral administration of vancomycin lessened or worsened lupus

whether given before or after disease onset, respectively, suggesting different mechanistic effects on the immune system¹³.

Daniel Zegar-Ruiz (Memorial Sloan Kettering Cancer Center) presented additional findings that point to diet as a contributing factor in murine dysbiosis and lupus. High fiber content in the diets of Toll-like receptor 7–transgenic mice resolved the dysbiosis that contributed to increased gut permeability and a lupus phenotype¹⁴.

The roles of infection, the microbiome, gut permeability and systemic bacterial products in lupus pathogenesis are only just being appreciated; nevertheless, strong evidence points to robust and far-reaching effects of dysbiosis in mice and humans who are predisposed. While it is impractical to sterilize and then specifically reconstitute the human microbiome, researchers may longitudinally characterize and manipulate a simulated human microbiome in mice as well as evaluate its relationship with disease onset. Mouse models, therefore, are fundamental to advancing our understanding of the interaction between bacteria and the immune system in SLE.

Identifying and repurposing drug targets

Recent advances presented at the meeting identified new drug targets and repurposed therapies for use in SLE. Both Mariana Kaplan (National Institute of Arthritis and Musculoskeletal and Skin Diseases) and Laurence Morel (University of Florida) demonstrated how targeting immune

metabolism, specifically mitochondrial respiration, could improve disease burden in patients with SLE. Morel presented data for both humans and mice with SLE, showing that metformin, widely used to treat type 2 diabetes, reduced prednisone use and decreased the number of flares in SLE¹⁵. Metformin's debated mechanisms of action not only affect the AMPK–mTOR–STAT3 pathway, but also, potentially, the electron transport chain, by inhibiting complex I. As a result, metformin reduces oxidative phosphorylation in CD4⁺ T cells isolated from patients with lupus and decreases IFN- γ production. This finding was recapitulated in *Sle1Sle2Sle3* mice, but effects varied in other lupus mouse models, which is potentially indicative of strain-specific mechanisms involving mitochondrial respiration¹⁶.

Another repurposed drug, presented by Kaplan, demonstrated the benefit of targeting mitochondrial dysfunction to reduce aberrant apoptosis that occurs in SLE. Used in clinical trials for muscular dystrophies and neuropathies, idebenone acts as an antioxidant that bypasses complex I activity and enhances ATP synthesis. Without suppressing the immune system, idebenone improved renal disease in both the MRL/lpr and NZM2328 strains, decreased mitochondrial ROS production and neutrophil extracellular trap formation, and attenuated vasculopathy¹⁷. Similar findings were observed with the use of Mito-Q, another mitochondrial antioxidant, in MRL/lpr mice. These findings, along with Morel's, demonstrate the potential of targeting immunometabolism in SLE.

The prospect of using anti-hypertensive angiotensinogen-converting enzyme (ACE) inhibitors to treat neuropsychiatric lupus manifestations was presented by Betty Diamond (Feinstein Institute). Building upon increased brain parenchymal ACE expression in lupus mice, Diamond demonstrated that administration of ACE inhibitors led to decreased microglial activation, prevented dendritic loss by increasing expression of the inhibitory receptor LAIR-1 and improved cognition in lupus mice¹⁸. Furthermore, in conjunction with human evidence indicating that ACE polymorphisms are associated with SLE, these findings demonstrate that modulating microglia by ACE inhibition is a beneficial repurposed therapy for patients with neuropsychiatric lupus.

Chaim Putterman (Azrieli Faculty of Medicine) discussed the potential of targeting CD6–ALCAM (activated leukocyte cell adhesion molecule) interactions, which are involved in the costimulation and activation of T cells as well as the trafficking of effector T cells into tissues.

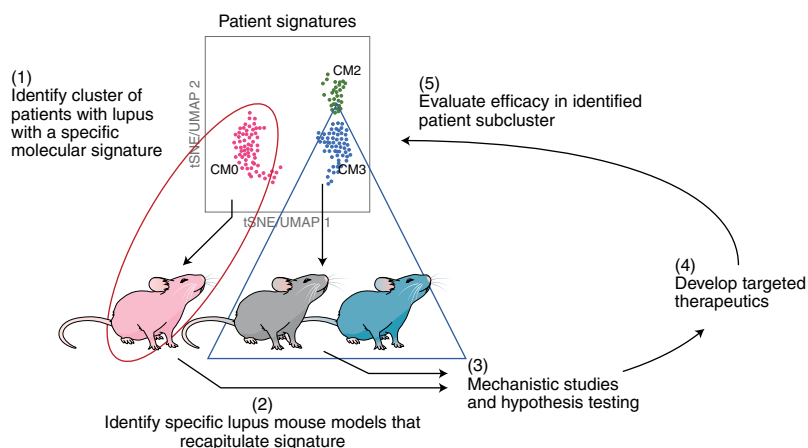


Fig. 3 | Using overlapping disease signatures in appropriate mouse models and subclusters of patients with lupus for the development of targeted therapies. With advanced technology, it is possible to select specific mouse models that recapitulate the molecular features found in subgroups of patients with lupus. The insights and therapies resulting from studying these high-fidelity models should be translated back to their matching patient subgroups, increasing the likelihood of improving patient care.

Itolizumab is a monoclonal antibody targeting CD6 that blocks CD6–ALCAM interactions without depleting T cells. In patients with SLE, urinary ALCAM concentrations are elevated, particularly in those with active lupus nephritis, and are strongly correlated with SLE disease activity index scores, glomerular filtration rate and complement concentrations, and thus could serve as a potential biomarker for stratifying patients¹⁹. In MRL/*lpr* mice, anti-CD6 treatment improved renal disease, survival and extra-renal disease, such as skin histopathology²⁰. Subsequently, a phase 1b clinical trial with itolizumab has been initiated for SLE and lupus nephritis, in which urinary ALCAM and/or CD6 will be followed to determine whether these biomarkers can predict responsiveness to itolizumab (NCT04128579).

Recent advances have not only identified new drug targets but have also elucidated potential adverse mechanisms contributing to drug-induced lupus or autoimmunity, as has been observed for tumor necrosis factor (TNF) inhibitors. Tam Quach (Feinstein Institute) highlighted the varying mechanisms leading to autoantibody production in different mouse models lacking TNF, including extrafollicular pathways, the induction of memory cells, and aberrant germinal center formation. Quach suggested that TNF may also influence negative regulatory B cell functions, and thus its deletion results in a change in the threshold for the deletion of autoreactive B cells, leading to disease acceleration and increased mortality in permissive models.

Across human and mouse SLE data, there are a number of emerging drug targets, new or repurposed, that show promise and should be further evaluated. Furthermore, additional mouse model studies may reveal select stages in lupus in which specific therapies would have the greatest beneficial effect.

New technologies

In the past 10 years, innovative techniques such as the CRISPR–Cas9 system and single-cell RNA sequencing have broadly improved the ability to test hypotheses in disease models, shed new light on established concepts in SLE, and highlight the similarities and differences between lupus mouse models and patients with SLE. Eric Meffre (Yale University) investigated the mechanisms contributing to defective central B cell tolerance by using humanized mice grafted with hematopoietic stem cells²¹. In particular, the role of the tyrosine phosphatase PTPN22, encoded by a risk allele in SLE, was evaluated for its potential contribution to the loss of tolerance. Interestingly, blockade of PTPN22 in mice humanized with SLE hematopoietic stem cells corrected the loss of central tolerance even when the risk allele was absent. Meffre suggested that increased receptor editing may account for the improvement.

In discerning the mechanisms contributing to skin manifestations in lupus, Mitra Maz (University of Michigan) demonstrated differential type I IFN–dependent immune responses in the skin of lupus mice following UVB exposure, leading to the subsequent recruitment of monocyte

and macrophage populations. This work complements previous reports that even lupus keratinocytes from non-lesional skin display a type I IFN signature²², thus highlighting the complex interplay between IFNs and both immune and stromal cells in the pathogenesis of discoid lupus.

Another emerging avenue for research includes the evaluation of altered homeostatic pathways, such as circadian rhythms, in lupus. Anne Davidson (Feinstein Institute) presented on how the kidney-specific circadian rhythm of renal homeostatic functions, such as metabolism and blood pressure regulation, are disrupted in nephritic NZB×W/F1 lupus mice. Once remission is induced, partial corrections in glycolysis, vascular remodeling and regeneration and reversal of the abnormal blood pressure dipping pattern were observed in these mice, causing their reversion to a younger, healthier phenotype²³. Davidson suggested that renal circadian rhythms, including urinary electrolyte excretion and blood pressure, measured through ambulatory blood pressure monitoring, could be tested as disease biomarkers and may also help dictate when specific therapeutics should be administered to correspond with a target organ's internal processes.

Deepak Rao (Brigham and Women's Hospital) highlighted work from the Accelerating Medicines Partnership network characterizing the adaptive immune cells in the kidneys of patients with lupus nephritis and discussed the expansion of CD4⁺ T cells among peripheral blood mononuclear cells from patients with SLE. PD-1^{hi}CXCR5⁺ peripheral helper T (T_{PH}) cells were identified and shown to mediate B cell help in a manner dependent on the transcription factor MAF²⁴. With a similar transcriptomic signature as that in T_{PH} cells, emerging evidence has identified the presence of T_{PH} cells in the pristane-induced lupus model. A benefit of mouse models highlighted by Rao is the ability to use the CRISPR–Cas9 and *Cre/loxP* systems to elucidate the various contributions similar and/or unique T_{PH} and T_{PH} cell markers make to the cell's function.

Further functional characterization of T_{PH} cells in SLE indicated the enhanced role of STAT4 in the secretion of IL-21 and IFN- γ . Jason Weinstein (Rutgers University) demonstrated that mouse lupus T_{PH} cells develop a hyper-responsiveness to STAT4 activation over time that maintains cytokine production, despite no observed increase in the expression of either IFN receptors or STAT4 itself²⁵. Similar results were observed in a cluster of circulating T_{PH}-like cells in patients with lupus, along with the association of increased STAT4 activation with disease severity.

Erica Moore (Albert Einstein College of Medicine) highlighted similarities between the mouse and human T cell receptor (TCR) repertoires in SLE. Using CDR3 sequencing, enhanced sample clonality demonstrated the directed immune response present in patients with SLE as compared to healthy controls. Additional analysis of the TCR repertoires revealed a skewed use of V genes in both humans and mice and the potential to use select TCR sequences as biomarkers for diagnostic or prognostic purposes in patients with SLE²⁶. Furthermore, select TCR sequences alongside T cell phenotyping could be used to monitor specific organ manifestations (Fig. 2), as highlighted by an identified consensus sequence in brain-infiltrating T cells in MRL/lpr mice with neuropsychiatric manifestations²⁷.

Paul Hoover (Brigham and Women's Hospital and Broad Institute) compared human SLE signatures and lupus mouse models using single-cell RNA sequencing. The characterization of myeloid cells in the kidneys of mice with lupus revealed that the main populations were residential macrophages, classical and non-classical monocytes and circulating dendritic cells in both *Sle1Yaa* and NZB/W models²⁸. Based on differentially expressed gene profiles, a number of the myeloid signatures present in humans, excluding the resident macrophage cluster, were observed in the two mouse lupus models, and some were associated with nephritis. Furthermore, Hoover showed preliminary data suggesting that the monocyte subsets occupied similar kidney compartments in both mouse and human kidney tissue. Differences between the lupus mouse strains were suggested to mirror patient-specific phenotypes as well as different aspects of disease.

Improved methodologies can more readily elucidate mechanisms in greater detail and with nuance and can identify mouse models that may best match subsets of patients with SLE. While no singular mouse model recapitulates lupus disease in humans, these recent studies highlight overlapping mechanisms between patients with SLE and mouse models and the possibility of selectively using mouse models to appropriately query facets of SLE pathogenesis and develop new treatments (Fig. 3).

Concluding remarks

The meeting gathered early career and established scientists and clinicians to

debate and discuss the usefulness of lupus mouse models. Through human and mouse data comparisons as well as lengthy discussions, the data presented showed that lupus mouse models still perform an irreplaceable function in modelling systemic autoimmunity and elucidating facets of disease pathogenesis. More specifically, experimental manipulations in these models have accelerated various avenues of research, including identifying internal triggers, via alterations in the microbiome; target-organ-specific mechanisms; and emerging drug targets, such as immunometabolism. Recognizing the heterogeneity of lupus manifestations and likely disease mechanisms, the consensus was to continue using lupus mouse models but to do so in a purposeful and directed manner, testing specific questions relevant to the particular model. Furthermore, technological advances will enhance our ability to first use findings in particular subsets of patients with SLE, which will guide the selection of mouse models and experiments in developing targeted therapies for the clinic. □

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Competing interests

C.P. is a consultant to Equilibrium and is one of the investigators on the current anti-CD6 antibody treatment trial for lupus. The other authors declare no competing interests.

REVIEW ARTICLE

Renal Mononuclear Phagocytes in Lupus Nephritis

Anne Davidson 

Renal mononuclear phagocytes are a highly pleiotropic group of immune cells of myeloid origin that play multiple protective and pathogenic roles in tissue homeostasis, inflammation, repair, and fibrosis. Infiltration of kidneys with these cells is a hallmark of lupus nephritis and is associated with more severe disease and with increased risk of progression to end-stage renal disease. This review presents current knowledge of the diversity of these cells and their involvement in kidney inflammation and resolution and describes how they contribute to the chronic inflammation of lupus nephritis. A better understanding of the subset heterogeneity and diverse functions of mononuclear phagocytes in the lupus nephritis kidney should provide fertile ground for the development of new therapeutic approaches that promote the differentiation and survival of protective subsets while targeting pathogenic cell subsets that cause inflammation and fibrosis.

Introduction

Lupus nephritis (LN) is a common manifestation of systemic lupus erythematosus that can cause irreversible renal injury. Although the prognosis of LN has improved substantially over the past 50 years, standard immunosuppressive therapies induce complete remission in less than 50% of treated patients. The rate of progression of LN to renal failure has plateaued in the United States in the past 20 years, and approximately equal to 10% of patients develop end-stage renal disease (ESRD) within 10 years of diagnosis. This failure reflects disease complexity and heterogeneity as well as insufficient patient access to high-quality medical care (1).

Although the pathologic classification of LN is based predominantly on renal glomerular inflammation, it is increasingly recognized that interstitial inflammation and fibrosis are associated with a worse long-term prognosis. The presence of renal macrophages, and dendritic cells (DCs) (mononuclear phagocytes) in particular, correlates with more severe disease and an increased likelihood of disease progression (2,3). There has therefore been increasing interest in understanding the diversity of these cells and how they contribute to tissue injury in LN as well as in multiple other inflammatory and fibrotic diseases.

This review will describe the heterogeneity of the mononuclear phagocyte system in tissues and how this landscape changes during inflammation and resolution. It will then show how

this general paradigm applies to LN and how this knowledge may inform approaches to treat LN and prevent progression to ESRD.

Pathogenesis of LN

LN is initiated by the deposition of nucleic acid containing autoantibodies and debris in the glomeruli. Subendothelial deposits activate the glomerular endothelium and engage complement; this induces the recruitment of circulating proinflammatory cells, leading to proliferative disease and the formation of glomerular crescents. By contrast, subepithelial deposits face the urinary space and cause podocyte injury with foot process effacement, leading to proteinuria (4). Mesangial deposits in isolation are less likely to directly damage the glomerular filtration apparatus. In addition, soluble inflammatory mediators derived either from the circulation or from activated glomerular cells amplify glomerular cell activation and injury (5–10).

The glomerular filtration apparatus comprises the endothelial layer, the glomerular basement membrane, and the podocyte layer. Podocytes and glomerular endothelial cells secrete growth factors to support each other's survival, and mesangial cells provide growth factors that support the endothelium. Because of these interrelated functions, injury to any glomerular cell type can eventually damage the others (10–12). This is followed by glomerular cell dedifferentiation and loss of integrity of the whole

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Changes to the renal tubulointerstitium (including tubular atrophy, fibrosis, and interstitial infiltrates) are known prognostic markers for progression to ESRD in patients with LN (17,18). Furthermore, immunohistochemical studies of kidney biopsies from patients with LN have suggested that the presence of renal macrophages, both glomerular and tubular, is associated with a worse prognosis in patients with LN, particularly at the second biopsy (2,19,20). The predictive power of the second biopsy reflects the ability of this biopsy to detect those patients in whom initial therapy has failed to control inflammatory lesions and in whom early fibrosis fails to reverse (2,18). Because mononuclear phagocytes can play a role in both inflammation and repair, newer studies are now beginning to address the heterogeneity and functions of these cells in renal injury in general, and in LN in particular,

The origins and development of renal macrophages and DCs

Yolk sac-derived macrophages. Yolk sac-derived macrophages are the source of resident macrophages in multiple organs, including the kidneys. Resident macrophages of embryonic origin are long lived and self-renewing, and they play an essential role in tissue homeostasis by monitoring the tissue for pathogens and removing any dead materials. These cells are slowly replaced throughout life by macrophages of hematopoietic origin that take on the organ-specific features of the resident cell. The rate of this replacement varies in each organ and increases during periods of tissue inflammation; if the organ is irradiated, the entire population can be replaced from bone marrow-derived cells that adopt a tissue-specific profile as they enter the tissue microenvironment (21,22).

Bone marrow-derived macrophages and DCs. Bone marrow-derived macrophages and DC are highly related subsets that derive from common progenitors. Monocytes are short-lived circulating cells that differentiate into either macrophages or

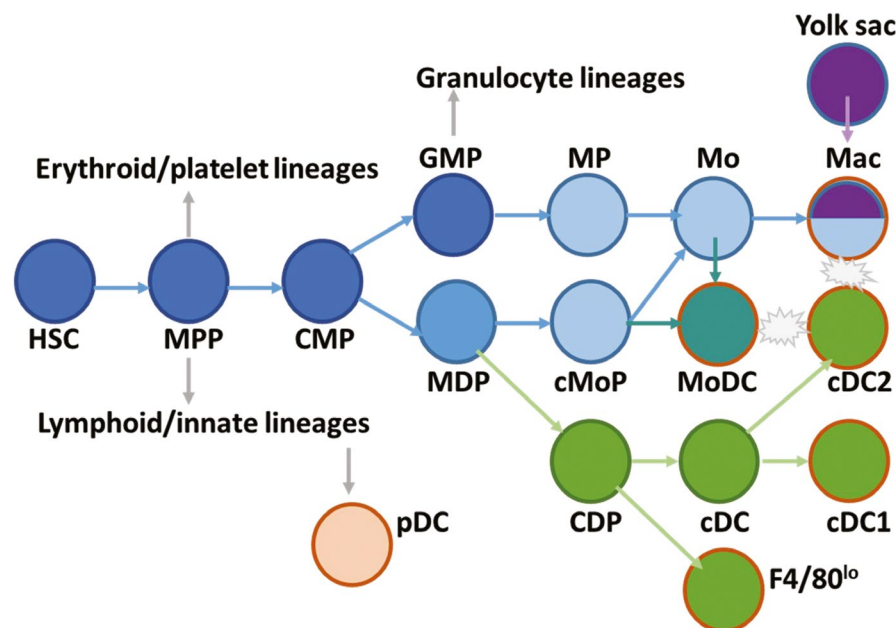


Figure 1. Development of the myeloid lineage from hematopoietic stem cells (HSC) or from yolk sac macrophages (Mac). Cells with an orange border are found in normal kidneys. The three cell types connected by grey clouds have similar phenotypes and may be difficult to distinguish in inflamed tissues by phenotyping alone. cDC, classic dendritic cells; CDP, common dendritic cell progenitors; CMoP, common monocyte progenitors; CMP, common myeloid progenitors; GMP, granulocyte macrophage progenitors; MDP, macrophage dendritic cell progenitors; Mo, monocyte; MoDC, monocyte-derived dendritic cells; MP, monocyte progenitors; MPP, multipotent progenitors; pDC, plasmacytoid dendritic cells.

DCs once they enter tissues. Macrophages are highly phagocytic cells that play both pathogenic and protective roles during tissue injury and repair. DCs engulf smaller-sized material than macrophages and are efficient antigen-presenting cells that function to activate the adaptive immune system. All these cells are highly pleiotropic, and recent studies have shown overlapping phenotypes and functions between the macrophages and DCs that infiltrate injured tissues (22).

Renal mononuclear phagocytes. Normal kidneys contain a network of long-lived tissue-resident macrophages that occupy niches around the periphery of glomeruli and adjacent to tubules and peritubular capillaries; these cells function in immune surveillance and homeostasis (23–26). Resident renal macrophages in mice are initially of yolk sac origin but are replaced by cells of hematopoietic origin as the mice become adults (27). These resident macrophages are characterized by the high expression of F4/80 and CD64 and are different from those in many other organs because there is no basement membrane that separates them from endothelial cells, allowing them to rapidly take up small immune complexes and other small particles in a manner analogous to that of splenic macrophages and Kupffer cells (25).

The normal kidney also contains smaller populations of other phagocytic cells. Renal DCs include both type 1 classic dendritic cells (cDC1) and type 2 classic dendritic cells (cDC2), which are phenotypically and functionally distinguishable subsets. cDC1 cross-present antigen to CD8 T cells via class I major histocompatibility complex and can also induce regulatory T cells. cDC2 are classic antigen-presenting cells that interact with and activate the adaptive immune system. Further subsetting of DCs has been reported, and even more phenotypes have been identified in the setting of inflammation (22,27). Under homeostatic conditions, some renal DCs migrate to lymph nodes, where they help to maintain tolerance to renal antigens. Studies in mice show that in contrast to resident macrophages, kidney cDCs turn over rapidly, suggesting that they traffic continuously into the kidneys (27,28). Plasmacytoid DCs are also present in normal kidneys. These cells derive from the lymphoid lineage and function to produce innate cytokines, including type I interferons (IFNs), on exposure to innate stimuli. Finally, there is a small renal population of cells of common DC progenitors origin that express CD11b but are low for F4/80. These cells have phenotypic differences from both macrophages and cDC2 (23,27).

The precise location of DC subsets in normal kidneys has not been fully mapped because of the lack of specificity of many myeloid cell markers. Extensive flow cytometry characterization using multiple markers and lineage tracing experiments in mice are beginning to address this issue (22,27). Using the cDC-specific *Zbtb46* as a reporter for all cDCs and *Snx22* as a reporter for cDC1, cDCs were found to be localized predominantly around blood vessels (29). Experiments using *Clec9a* as a reporter have by contrast suggested that up to 30% of renal

cells with a resident macrophage phenotype (F4/80^{hi}/CD64^{hi}) and localization are cDCs (27). The interpretation of the fate mapping studies depends on the specificity of the reporters for the various cell lineages, and further studies are needed to fully understand the role of each subset during homeostasis and disease.

Single-cell RNA sequencing analyses of immune cells from normal mouse kidneys have recently been reported (30). Single-cell analysis samples the most abundantly expressed genes in individual cells at a single time point and therefore represents only a snapshot of the overall diversity of renal mononuclear phagocytes. Nevertheless, these studies have confirmed the presence of the subsets described above and are beginning to allow for further subsetting and functional analysis of renal macrophages and DCs, especially in diseased tissues.

General alterations of renal mononuclear phagocyte function in inflammatory renal diseases

Macrophages. Macrophages are highly pleiotropic cells that can rapidly be polarized *in vitro* to multiple phenotypes and alter their phenotype and function *in vivo* in response to their microenvironment. During acute kidney injury in mouse models, proinflammatory macrophages are recruited to the glomeruli under the influence of chemokines and other chemoattractants. Both classical monocytes (CD14⁺ in humans and Ly6C^{hi} in mice) and nonclassical monocytes (CD16⁺ in humans and Ly6C^{lo} in mice) can acquire an inflammatory phenotype in tissues and contribute to tissue injury. TLR7 engagement, in particular, is associated with the recruitment of nonclassical (patrolling) monocytes to activated endothelium (31). Proinflammatory function of infiltrating macrophages is reinforced by inflammatory cytokines and other damage associated molecular patterns by tubular release of micro-RNAs and by activation of inflammasome-mediated pathways (32). These cells produce inflammatory cytokines, nitric oxide synthase, procoagulants, and matrix metalloproteinases, and their pathogenic role has been confirmed in mouse models in which their depletion during the inflammatory phase after acute injury attenuates renal injury (26,33).

As renal disease transitions from the acute to the chronic phase, both infiltrating and resident renal macrophages take on an alternatively activated reparative phenotype (34,35). This could be due to a switch in cell phenotype or sequential recruitment and differentiation of different cell populations. Alternatively activated macrophages play a major role in removal of debris and collagen, regulation of inflammation, and regeneration of the tubular epithelium; their depletion during the repair phase of injury exacerbates renal fibrosis (33,36). Induction of this phenotype is regulated by local cytokines and metabolic conditions. In mice, transfer of macrophages polarized by interleukin 4, interleukin 13, and/or interleukin 10 promotes renal repair after acute injury. Conditions in local microenvironment, such as tissue hypoxia and

iron metabolism and tubular secretion of colony-stimulating factor 1, can also drive alternative macrophage polarization. Nevertheless, excessive accumulation of reparative macrophages results in the production of profibrotic mediators, such as transforming growth factor β and platelet-derived growth factor.

Studies in several human renal diseases have shown an association between alternatively activated macrophages in renal biopsies and poorer disease outcome and fibrosis. Similarly, macrophages with an alternatively activated phenotype can promote both glomerulosclerosis and interstitial fibrosis in rodent models of chronic renal injury (26). The balance between renal repair and chronic fibrosis is regulated by cross talk between fibroblast precursors, tubular epithelium, and macrophages (37,38).

The full scope of pathogenic and protective populations of macrophages in chronically inflamed kidneys and the specific roles infiltrating and resident macrophages (39,40) are still not completely delineated. Macrophages with the capacity to digest and remove collagen *in vitro* have been identified (41), but the precise molecular and functional characteristics of these cells and how to induce them *in vivo* remain to be established. Matrix metalloproteinase 13 has emerged as a key matrix metalloproteinase that promotes resolution of fibrosis in multiple organs, including the kidney (42,43). Phagocytic receptors of the TAM family may also regulate resolving functions; for example, MerTK-positive macrophages promote resolution of inflammation, whereas MerTK-negative macrophage subsets induce the differentiation of inflammatory fibroblasts (40,44). By contrast, the phagocytic TAM receptor Axl promotes tissue fibrosis by enhancing TLR signaling and metabolic reprogramming (45). Recent studies have highlighted the role of the neural guidance protein Netrin-1 as an antiinflammatory mediator that is induced in macrophages in hypoxic areas of tissue (46). Netrin-1 stimulates the production of pro-resolving lipid mediators, shortens the time required for resolution of inflammation, and promotes tissue regeneration. However, Netrin-1 macrophages can also promote fibrosis during chronic inflammation. Interestingly, the interaction of Netrin-1 with its receptor induces adrenergic nerve remodeling and release of norepinephrine, which promotes fibrogenesis (47).

Recent discoveries with respect to the regulation of immune cell function by intracellular metabolic pathways have enhanced our understanding of the mechanisms for macrophage polarization. Inflammatory macrophages are characterized by an increase in glycolysis and a decrease in mitochondrial function with production of succinate and itaconate as a result of breaks in the Krebs cycle (48). Succinate enhances production of inflammatory cytokines and reactive oxygen species. By contrast, itaconate has both an antibacterial and an antiinflammatory role, acting to restore homeostasis. Alternatively activated and pro-resolving macrophages are characterized by an increase in fatty acid oxidation and mitochondrial respiration and by the secretion of arginases that produce metabolites that suppress inflammatory responses (49). An emerging interest has been to define transcription factors

that dictate programs that regulate the metabolism, phenotype, and function of macrophage subsets (21). Resident and infiltrating macrophages are regulated by different transcription factors, and macrophage programs can also be influenced by the type of material they phagocytose in each specific niche. For example, Spi-C transcription factor regulates a macrophage program needed for iron handling, whereas Nr1h3 is induced by the accumulation of cellular cholesterol (via uptake of lipids from dying cells) and, in turn, can suppress inflammation, promote macrophage survival, and enhance antiinflammatory apoptotic cell clearance (13,50,51). Notably less is known about myeloid cell subsets and states and the core transcription factors that regulate them in the kidneys than in other major organs.

DCs. During inflammatory disease, infiltrating DCs derive from both monocytes and circulating DCs. Like macrophages, DCs in tissue are heterogeneous and can perform both inflammatory and suppressive functions. Distinction of tissue macrophages from tissue DCs can be difficult, especially in inflammatory settings in which infiltrating cells display features of both cell types (22,52). This has resulted in some confusion over nomenclature of renal myeloid cells that is still not fully resolved. Studies in mouse models of renal inflammation suggest that cDC1 are protective, whereas cDC2 are pathogenic (22). Mouse splenic cDC2 have recently been divided into two subsets that express different transcription factors and appear to employ different metabolic programs (53). Gene expression differences between these two subsets suggest that cDC2A are more likely to be involved in tissue repair, whereas cDC2B are more likely to be proinflammatory. Other subsets include CCR7 expressing DCs that have the capacity to migrate to lymph nodes and may also help to organize local lymphoid infiltrates. Transcription factors that direct DC ontogeny and function are beginning to be described (53). How each of the DC subsets contributes to renal injury and repair is currently not known.

Alterations of the renal myeloid compartment in LN

The renal mononuclear phagocyte compartment undergoes extensive remodeling as LN evolves, with an influx of new myeloid cells, the accumulation of mixed immune infiltrates, and changes in the function of resident cells.

Glomeruli. Glomerular macrophages can be abundant in LN, with heterogeneity among mouse models and patients with LN. These cells are located either within the glomerular tuft, where they are adherent to glomerular capillary walls, or within the glomerular crescents (54). Phenotypic studies in mice have shown that there are at least two subpopulations of these cells in glomeruli. In mouse models characterized by overexpression of TLR7, glomerular macrophages are located within glomerular capillaries

and are F4/80^{lo}, CD11c^{hi}, and Ly6C^{lo}, characteristic of nonclassical or patrolling macrophages. These cells express the transcription factor Nr4a1, and deletion of this transcription factor abolishes their recruitment to glomeruli and attenuates glomerular injury (55). In the NZM2328 model, infiltrating F4/80^{lo}, CD11b⁺, CD11c⁺ cells are found in the glomeruli of severely proteinuric mice; these cells are also Ly6C^{lo} and have an alternatively activated phenotype (6,56). The molecular characteristics of macrophages located in glomerular crescents have not yet been reported in mice.

In human LN, glomerular macrophages correlate with the severity of glomerular lesions, being found more frequently in diffuse proliferative disease. Studies using a limited number of cell surface markers suggest that these cells are heterogeneous and may include both inflammatory and reparative phenotypes (20).

Interstitial resident cells. Macrophages with a resident phenotype increase in number during LN. Peritubular renal macrophages are susceptible to immune complex-mediated activation because of their anatomic location near small peritubular vessels without an intervening basement membrane (25). Our phenotypic and single-cell studies in mice have confirmed that there is a major shift in the resident macrophage profile during LN, with loss of protective and homeostatic functions and acquisition of an alternatively activated/reparative phenotype (23,57). An analogous population of macrophages with an

alternatively activated profile is highly represented in human LN biopsies (58).

Immune cell infiltrates. Mixed tubulointerstitial leukocyte infiltrates, sometimes with features of lymphoid organization, are found in LN (2,17,18,59) and are associated with renal scarring and a worse prognosis. These infiltrates contain both cDC1 and cDC2, especially during advanced disease (3). In human LN biopsies, CD209 expressing cDC2 are found in periglomerular regions, within lymphoid infiltrates, and scattered in the interstitium (60). CD103 (cDC1) DCs and plasmacytoid DCs are also present in smaller numbers both in mice and humans with LN (3,23). Approximately 40% of patients who have moderate to severe infiltrates progress to ESRD over a 4-year period (17), but it is still not known how to distinguish those patients who will progress from those who will not.

Lessons learned from molecular profiling and single-cell analyses of LN kidneys

Our early studies in diverse mouse models of LN identified a myeloid cell signature that was shared among LN mouse models and human biopsies. Using flow cytometric analyses, we found both expansion and activation of the dominant CD11b⁺/F4/80^{hi} renal macrophage population during active LN as well as a large population of cDC2 during proliferative systemic lupus

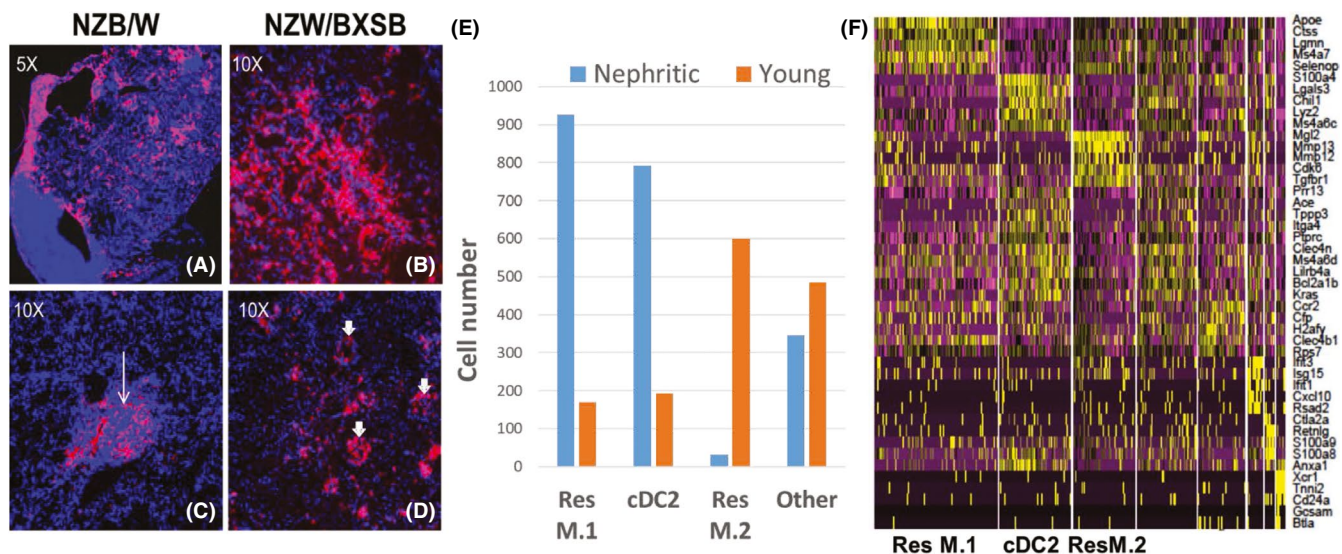


Figure 2. Diversity of mononuclear phagocytes in LN kidneys. **A**, F4/80 staining of macrophages in a kidney from a nephritic NZB/W mouse shows localization around glomeruli and in the interstitium but exclusion from lymphoid aggregates. **B**, F4/80 staining of macrophages in a kidney from a nephritic NZW/BXSB (TLR7 overexpressing) mouse shows localization around glomeruli and extensive infiltration in the interstitium. **C**, CD11c staining of classic dendritic cells (cDCs) in a kidney from a nephritic NZB/W mouse shows localization within the mixed perivascular immune infiltrate. **D**, CD11c staining in a kidney from a nephritic NZW/BXSB (TLR7 overexpressing) mouse shows localization inside glomerular tufts; these cells are most likely nonclassical (patrolling) monocytes. **E**, Single-cell RNA sequencing (RNASeq) analysis of CD11b/c cells from nephritic and prenephritic (young) mice. Bars show the number of cells in the three major clusters. Resident macrophages are in resident macrophage cluster 2 (Res M.2) in young mice and predominantly in resident macrophage cluster 1 (Res M.1) in nephritic mice. Also note the expansion of the cDC2 population in nephritic mice. **F**, RNASeq analysis of CD11b/c cells from NZB/W mice shows eight subclusters of cells. The top five genes for each cluster are shown on the right.

erythematosus nephritis that disappears during remission. We found an additional population of CD11c-positive glomerular cells in mice overexpressing TLR7 (23,61) (Figure 2).

Single-cell analyses are now beginning to reveal the complexity of the myeloid cell landscape in LN kidneys. Our studies in NZB/W mice have identified at least eight subsets of renal mononuclear phagocytes, with significant differences in the relative representation of each subset between prenephritic and nephritic mice (62) (Figure 2). We found that the three major clusters of cells in NZB/W mice are two clusters of resident macrophages and a cluster of cDC2. Resident macrophages from nephritic and prenephritic mice cluster separately because of the differences in gene expression that occur in this subset at disease onset. We also identified smaller populations of cDC1, CCR7^{hi} DCs, monocyte-derived DCs, proliferating cells, and cells expressing an inflammatory and IFN signature. Interestingly, an additional population was found in Sle1.Yaa mice corresponding to the glomerular subset of CD11c⁺ cells (62).

Our team is part of the Accelerating Medicines Partnership–Systemic Lupus Erythematosus (AMP-SLE), which is addressing the complexity of LN using single-cell RNA sequencing (scRNASeq) analysis of renal cells (58,63). Recently, the AMP-SLE group has successfully completed scRNASeq analysis of a total of 160 LN biopsies using 10x Genomics technology. This AMP-SLE phase 2 study will yield a rich set of data that can be correlated with disease outcome in a manner that has previously been unprecedented. Initial scRNASeq data of CD45⁺ immune cells isolated from the first 23 biopsies analyzed in the AMP-SLE phase 1 study have been published and include 466 myeloid cells (58). Although this sample size is rather small, we were able to identify five subpopulations of cells, of which only one was detected in normal kidneys harvested from healthy living renal donors. We found four subpopulations of macrophages cluster myeloid (CM0, CM1, CM2, and CM4), of which only CM0 was detected in the peripheral blood. Three of these populations, CM0, CM1, and CM4, are related along a developmental trajectory, starting with tumor necrosis factor–producing CD16⁺ nonclassical monocytes (CM0) that transition to a phagocytic (CM1) and then to an alternative macrophage phenotype (CM4) that also produces inflammatory chemokines. We classified the fourth population that was found in both healthy donors and LN biopsies (CM2) as resident macrophages. These cells acquired both an IFN signature and an antiinflammatory signature in LN biopsies (58). We also detected a small population of cDC2, but their number was too small to analyze them in detail. The results of the phase 2 study will be available later this year; these data include a much larger population of myeloid cells, and preliminary analyses indicate that additional myeloid cell subsets are present (A. Arazi: personal communication). Our preliminary analyses also indicate that there are substantial similarities between the populations that are present in humans and those we find in mice (64), suggesting that mechanistic studies will be possible in selected mouse models.

It is impractical to collect sequential human renal biopsies for testing causal disease mechanisms or monitoring treatment responses. It is therefore of great interest to test whether myeloid cell subsets appear in the urine and can be used as surrogate markers for renal infiltration. Preliminary analyses of the AMP-SLE phase 1 samples have shown that myeloid cells constitute the majority of immune cells in the urine, especially CM1 cells (58), suggesting that these have access to the urinary space, most likely as a result of localization in glomerular crescents. Further analyses in the AMP-SLE phase 2 observational study will address whether the inflammatory myeloid cell subsets correlate with renal infiltrates and/or responses to treatment and whether urine can be used to assess treatment responses and prognosis. Ongoing mapping studies will also determine the location of the various myeloid cell subsets.

Applications to clinical care

Many types of mononuclear phagocytes are found in the kidneys of individuals with LN. A better understanding how each infiltrating cell type contributes to renal injury is now needed so that pathogenic cells can be targeted, whereas those involved in organ protection and repair can be spared. Because the features of reparative macrophages that promote resolution and resorption of collagen and extracellular matrix components are not well defined and may be organ specific (35,65–67), it is not always possible to extrapolate studies from other inflammatory diseases and organs to LN.

There are several approaches to identifying the function of tissue-derived myeloid cell subsets. Recent studies of synovial macrophages found that the presence of MerTK⁺ synovial tissue macrophages during disease remission was associated with protection from disease flare after cessation of treatment (44). When isolated, these cells failed to induce inflammatory fibroblasts in coculture experiments. By contrast, MerTK[−] macrophages were more likely to produce inflammatory mediators and interact with stromal cells to induce the development of inflammatory fibroblasts that promote fibrosis. An alternative approach that can be used in the case of organs, like kidneys, that are difficult to access, is to compare the phenotype of organ-specific macrophages with that of macrophages induced by a broad panel of cytokines and to reproduce a similar phenotype *in vitro* that can then be used for coculture studies (68). Finally, mouse models can be particularly useful for functional and therapeutic studies if they reflect human physiology. In this context, comparison of data from mouse models with the AMP-SLE phase 2 data will be highly informative.

Conclusions

Identifying and targeting pathogenic myeloid cell subsets and prevention and treatment of renal fibrosis should improve the

treatment of LN. Therapeutic manipulation of metabolic pathways offers some new opportunities for the treatment of inflammatory diseases, including LN (69). Strategies are beginning to emerge for modulating macrophage glycolysis through the inhibition of inducing signals, such as toll like receptors and the NLRP3 inflammasome (32). Induction of reparative macrophages will require a better understanding of how fibrosis and resolution are balanced, but new inroads are being made by using mouse models of injury and repair. For example, the combination of a decoy form of soluble Axl with an antagonist of MerTK shedding was highly effective at promoting repair in a model of cardiac injury (45). Another novel approach is the use of $\alpha 1$ agonism to oppose the activity of Netrin-1; this strategy prevented fibrosis in a mouse model of early interstitial pulmonary fibrosis, although it was ineffective at reversing established fibrosis (47). Therapies can also be directed at correcting metabolic abnormalities of the tubular epithelium because these cells induce profibrotic functions in adjacent macrophages (15,70). Prevention of recruitment and/or differentiation of inflammatory macrophages and DCs is another potential approach. In this context, it is of interest that B cell activating factor has a role in directing myeloid cell function that could help account for the therapeutic efficacy of belimumab in LN (71–73). Uncovering the role of each renal mononuclear phagocyte subset in LN should provide a fruitful area for research and further therapeutic discovery.

AUTHOR CONTRIBUTIONS

Dr. Davidson drafted the article, revised it critically for important intellectual content, and approved the final version to be published.

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Protecting the kidney in systemic lupus erythematosus: from diagnosis to therapy

Naomi I. Maria and Anne Davidson

Abstract | Lupus nephritis (LN) is a common manifestation of systemic lupus erythematosus that can lead to irreversible renal impairment. Although the prognosis of LN has improved substantially over the past 50 years, outcomes have plateaued in the USA in the past 20 years as immunosuppressive therapies have failed to reverse disease in more than half of treated patients. This failure might reflect disease complexity and heterogeneity, as well as social and economic barriers to health-care access that can delay intervention until after damage has already occurred. LN progression is still poorly understood and involves multiple cell types and both immune and non-immune mechanisms. Single-cell analysis of intrinsic renal cells and infiltrating cells from patients with LN is a new approach that will help to define the pathways of renal injury at a cellular level. Although many new immune-modulating therapies are being tested in the clinic, the development of therapies to improve regeneration of the injured kidney and to prevent fibrosis requires a better understanding of the mechanisms of LN progression. This mechanistic understanding, together with the development of clinical measures to evaluate risk and detect early disease and better access to expert health-care providers, should improve outcomes for patients with LN.

Capillary rarefaction
A loss of capillary structure leading to reduced density of microvascular networks.

Lupus nephritis (LN) affects up to 40% of adults and 80% of children with systemic lupus erythematosus (SLE) and is a major cause of morbidity and mortality^{1,2}. LN occurs most frequently and is most severe in adolescents, in patients of non-European ancestry and in patients of lower socioeconomic status³. Current standard-of-care therapy comprises induction therapy with high-dose immunosuppressants and glucocorticoids followed by a maintenance phase that lasts for several years and then the gradual withdrawal of therapy^{4,5}. However, even within the setting of clinical trials, remission is achieved in only 30–50% of patients, and 10–20% of patients develop end-stage renal disease (ESRD) within 10 years of diagnosis^{3,6}. Although reported improvements in LN outcomes have been attributed to earlier diagnosis and optimal management in European patients over the past decade⁷, the risk of ESRD has not improved in the USA since the late 2000s^{3,8}. This lack of improvement is partly due to the substantial barriers imposed by the poor access faced by many patients with LN in the USA to high-quality health care and incomplete adherence to treatment regimens^{9,10}.

In general, improvement in outcomes for patients with LN will require both new knowledge and new strategies for conducting clinical trials. Rapid progress in our understanding of the immune mechanisms

involved in LN is leading to the design of new immunosuppressive drugs with defined targets and improved safety profiles. Similarly, a better understanding of the non-immune mechanisms of renal injury and repair could yield new ways of preventing the progression of chronic kidney disease (CKD). Substantial heterogeneity exists among patients, a difficulty that could be addressed by the improved use of biomarkers in diagnosis and by selecting patients for clinical trials on the basis of which pathogenic mechanisms are involved in promoting their disease. In this Review, we provide an overview of mechanisms of renal damage in LN, summarize the role of novel technologies in providing new data and address how such information might be exploited to achieve diagnostic and therapeutic advances for patients with LN.

The pathogenesis of lupus nephritis

LN is initiated by the deposition of nucleic acid-containing material in the glomeruli, which triggers the engagement of complement, the activation of renal stromal cells and the recruitment of circulating pro-inflammatory cells¹¹. Disease progression is associated with tubulointerstitial hypoxia, metabolic dysfunction of the tubular epithelium, tubulointerstitial capillary rarefaction, accumulation of mixed lymphoid

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Key points

- Lupus nephritis (LN) is a heterogeneous complication of systemic lupus erythematosus that remains a considerable unmet medical need.
- Genetic and epigenetic factors confer risks of LN incidence and progression.
- Single-cell analyses and enhanced microscopic analyses of renal tissues are yielding new information about LN pathogenesis and the progression of chronic kidney disease.
- Improvements in risk assessment using genetic or transcriptomic biomarkers could enable the design of clinical trials to prevent LN onset and progression.
- Trials might need to be tailored according to the genetic profile of the patient, a biomarker-based evaluation of their renal tissue and/or the mechanism of action of each new drug.
- Developments in the understanding of tubulointerstitial injury and repair are yielding new strategies for preserving renal function and preventing fibrosis.

infiltrates and fibrosis (FIG. 1). Distinguishing features of LN include the high degree of associated systemic inflammation, the deposition of immune complexes that contain ligands for endosomal Toll-like receptors (TLRs), the activation of inflammasome-mediated and type I interferon-mediated pathways that contribute to endothelial dysfunction^{12,13}, the production of pathogenic antibodies to complement protein C1q that amplify complement-mediated injury¹⁴ and an additional tendency towards thrombosis.

Genetic risk of lupus nephritis

Genetic polymorphisms contribute to both the loss of immune tolerance that precedes the development of pathogenic autoantibodies and the risk of development and progression of LN (FIG. 1a). More than 100 genetic polymorphisms are associated with the risk of developing SLE, many of which are linked to myeloid cell and B cell activation pathways and the type I interferon pathway^{15,16}. Several of these genetic risk alleles, including HLA alleles and the newly identified *BAFF* variant¹⁷, correlate with the early onset of both SLE and LN, suggesting that some SLE risk polymorphisms also predispose to LN¹⁸. By contrast, some genetic polymorphisms, such as *PDGFRA*¹⁹, are associated with LN risk but not with SLE risk per se; in this context, differences have been observed between individuals of different ethnic backgrounds²⁰. Finally, some gene polymorphisms are associated with the risk of progression to CKD in patients with underlying renal disease of any cause^{21,22}. Of these, the *APOL1* polymorphism that is associated with CKD progression in African Americans might partially be responsible for poorer LN outcomes in African-American patients as it is associated with both an increased risk of LN and a more rapid disease progression²³. Functional analysis of LN risk genes has revealed various possible pathogenic mechanisms, including induction of a pro-inflammatory state (*ITGAM*, *FCGR3A*, *TNIP1*, *TNFSF4*, *IRF5* and *NEATC*), altered immune complex clearance (*FCGR2A*) and altered intrinsic response to renal injury (*APOL1*, *DAB2*, *PDGFRA*, *KLK* and *HAS2*)²⁴. At the individual level, each polymorphism contributes only a small increase to the odds ratio for LN, making it difficult to reliably predict LN risk on the basis of genetic testing.

Glomerular injury

Initial immune complex-mediated glomerular damage varies according to the site of immune complex deposition (FIG. 1b). Subendothelial deposits cause the recruitment of pro-inflammatory cells from the blood, leading to proliferative disease and glomerular crescents, whereas subepithelial deposits that contact only the urinary space cause membranous disease, characterized by podocyte injury with foot process effacement and consequent proteinuria. Podocytes, endothelial cells and mesangial cells within the glomerulus interact with and support each other: podocytes produce vascular endothelial growth factor (VEGF) and other angiogenic factors required for endothelial cell survival^{25,26}; endothelial cells make platelet-derived growth factor (PDGF) that is needed for mesangial cell survival; and mesangial cells sequester latent transforming growth factor- β (TGF β), thereby protecting the endothelium from apoptosis²⁷. Therefore, progressive injury to one cell type can eventually lead to damage of the other cell types.

Activation, dedifferentiation or proliferation of glomerular cells causes loss of structural integrity to the glomerular tuft and eventual nephron death. Glomerular endothelial cells are also damaged by circulating pro-inflammatory mediators and by TLR ligand-mediated activation, which induces the release of cytokines that cause glomerular cell death and of chemokines that enhance the recruitment of circulating immune cells^{28,29}. Injured glomerular cells amplify damage and inflammation by a variety of mechanisms. Damaged podocytes and endothelial cells both secrete endothelin 1, which causes vasoconstriction and mitochondrial dysfunction²⁵. Stressed endothelial cells also release pro-inflammatory and pro-coagulant mediators and increase their expression of adhesion molecules such as VCAM1 and ICAM1, which aid the recruitment of circulating immune cells²⁵. Podocytes and mesangial cells further amplify inflammation by producing pro-inflammatory cytokines, such as IL-6 and IL-1, chemokines and growth factors, including macrophage colony-stimulating factor (M-CSF)^{27,30–32}.

Both mesangial cells and podocytes have limited regenerative capacity, and their loss is associated with glomerulosclerosis^{33,34}. Therefore, methods for the early detection of glomerular injury are needed so that therapies that preserve glomerular structure and function in patients with LN can be used. For example, in lupus-prone MRL/lpr mice, increased podocyte expression of calcium/calmodulin-dependent protein kinase type IV (CAMK4), a protein that regulates podocyte integrity, precedes the onset of proteinuria. In these mice, podocyte-targeted delivery of an inhibitor of CAMK4 during the time window between increased expression and proteinuria onset protected podocytes from toxic injury and foot process effacement³⁵. However, this type of intervention depends on disease stage and might not be effective once podocytes have been lost. An important challenge for the treatment of human LN will be to identify targets that are early mediators of injury and to understand at what stages of disease early-acting therapies should be instituted to prevent irreversible renal damage.

Glomerular crescents

A response to severe injury in which crescent-shaped glomerular lesions that consist of epithelial cells, fibroblasts, immune cells and matrix form adjacent to the Bowman's capsule.

Foot process effacement

A podocyte reaction to injury or damage in which the epithelial foot processes become flattened and lose their barrier function, resulting in proteinuria.

Glomerular tuft

A network of small blood vessels and supporting cells that forms the initial structural component of the nephron.

Glomerulosclerosis

Scarring of the glomeruli that leads to loss of function.

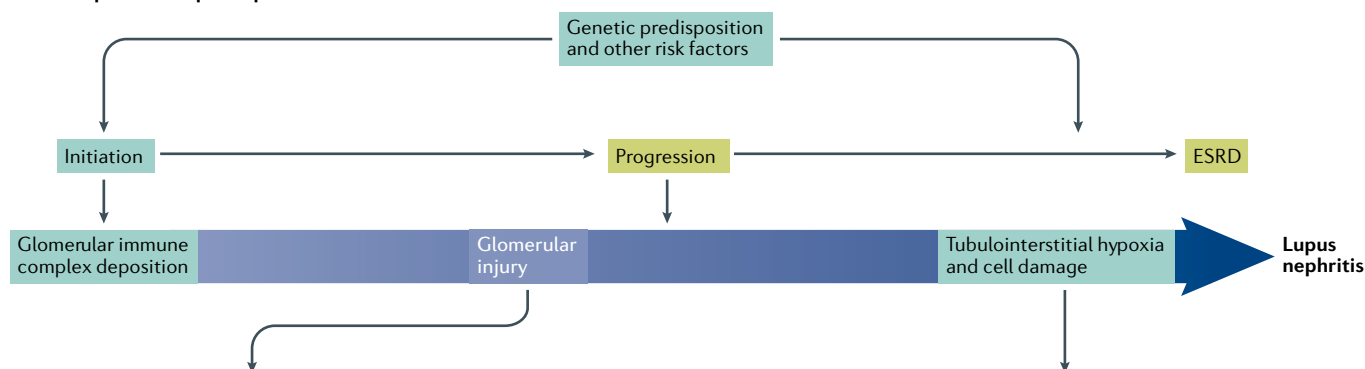
Although glomerular injury in LN is classically initiated by glomerular immune complex deposition, a rare form of LN that also injures the glomeruli is thrombotic microangiopathy, in which complement-mediated endothelial injury causes glomerular microthrombi that are associated with proteinuria, haemolytic anaemia, thrombocytopenia, hypertension and rapidly declining renal function. Thrombotic microangiopathy is related to other complement-mediated thrombotic renal diseases such as haemolytic uraemic syndrome and can

be successfully treated with complement protein C5 inhibition³⁶.

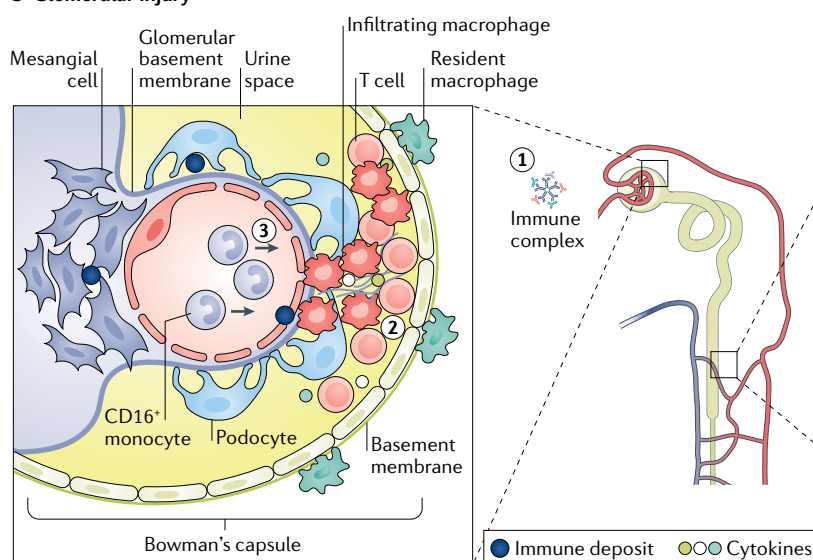
Tubulointerstitial injury

The blood supply to the renal tubulointerstitium is provided by run-off from the glomeruli; therefore, glomerular loss compromises tubulointerstitial viability. Changes to the renal tubulointerstitium caused by this loss in viability, such as tubular atrophy, fibrosis and interstitial infiltrates (FIG. 1c), are known prognostic

a Development of lupus nephritis



b Glomerular injury



c Tubulointerstitial damage

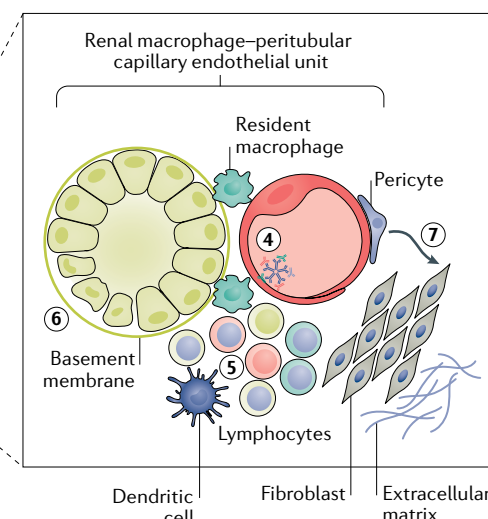


Fig. 1 | Glomerular injury and tubulointerstitial damage in lupus nephritis. **a** | Schematic timeline representing the development and progression of lupus nephritis. Genetic polymorphisms and other risk factors contribute to both the initiation phase of glomerular injury and the risk of subsequent tubulointerstitial damage during the development of lupus nephritis. **b** | During glomerular injury, circulating pro-inflammatory cytokines and the subendothelial deposition of immune complexes (1) contribute to endothelial dysfunction and the recruitment of pro-inflammatory CD16⁺ macrophages and T cells into crescents (2) that might also contain proliferating epithelial cells from the parietal layer of the Bowman's capsule. CD16⁺ monocytes are recruited from the blood into the crescents (3), and changes in their gene expression profiles occur as they begin to infiltrate into the tissue parenchyma and differentiate into macrophages. Resident renal macrophages are located around the outside of the Bowman's capsule, where new lymphoid tissue often accumulates during chronic inflammation. Subendothelial and mesangial deposition of immune complexes causes damage to podocytes and mesangial cells,

respectively, but pro-inflammatory cell recruitment to these sites is limited during glomerular injury because the cells have little access to the intravascular space. **c** | Resident renal macrophages are located next to tubules and tubular capillaries in the 'renal macrophage-peritubular capillary endothelial unit'. Renal macrophages can be activated by small immune complexes that transit from the adjacent endothelium to resident macrophages owing to the lack of a basement membrane (4). Recruited tubulointerstitial pro-inflammatory immune cell infiltrates include myeloid dendritic cells, plasmacytoid dendritic cells and various lymphocytes and are sites at which antigen presentation to T cells and T cell-B cell interactions can promote the differentiation of B cells into plasma cells that secrete antibodies to renal antigens (5). Inflammation and tubulointerstitial hypoxia induce metabolic dysfunction and atrophy of tubular cells (6) with inadequate repair. Growth factors such as transforming growth factor- β induce fibroblast differentiation from mesenchymal stromal cells such as pericytes, resulting in renal fibrosis and irreversible damage (7). ESRD, end-stage renal disease.

Fate mapping

A technique used in developmental biology to study the embryonic origin of adult cells, tissues and structures.

markers for CKD progression in patients with LN³⁷. Tubular epithelial cell injury is an important cause of renal fibrosis³⁸, although fate mapping studies have cast doubt on the ability of tubular epithelial cells themselves to directly transition to myofibroblasts^{39,40}. One potential mechanism is the secretion, by tubular epithelial cells, of pro-fibrotic factors that activate tubulointerstitial pericytes. These cells are embedded into the basement membrane of small peritubular vessels; pericytes can differentiate into myofibroblasts in injured kidneys^{39,41} and can mediate pro-inflammatory signalling via a MyD88-dependent mechanism³⁹. Other mesenchymal stromal cells can also differentiate into fibroblasts, often via the transcription factor MYC, which is important in promoting this process⁴². Detachment of activated pericytes from the endothelium leads to capillary rarefaction, which can be irreversible. Capillary loss also results from attenuated production of VEGF by hypoxic tubular epithelial cells⁴³.

Small immune complexes that are cleared through interstitial capillaries are taken up by adjacent interstitial resident macrophages; engagement of the Fc receptor FcγRIV and endosomal TLR pathways synergistically activate these cells when they encounter immune complexes containing nucleic acids⁴⁴. Resident macrophages, together with activated fibroblasts, contribute to renal injury by secreting pro-inflammatory mediators that attract immune cells to the interstitium³⁹, a feature that is associated with worse outcomes in patients with LN⁴⁵.

Immune cells in lupus nephritis

Glomerular infiltrates in LN consist mainly of macrophages, with T cells present in the more severe crescentic forms^{46,47}. Glomerular macrophages are recruited from the pool of circulating monocytes and have been extensively studied in mouse models of LN. Endothelial cells that are activated via nucleic acid-sensing TLRs such as TLR7 preferentially recruit patrolling monocytes²⁸, a CD11c⁺Ly6C^{lo} population characterized by the transcription factor Nr4a1. Glomerular CD11c⁺ cells have been uniformly observed in mouse models of LN in which TLR7 is overexpressed^{48–50}, and the human equivalent (CD16⁺ monocytes), rather than the pro-inflammatory CD14⁺ monocyte population, are preferentially recruited to human LN tissue⁵¹. Excessive intrinsic endosomal TLR signalling in monocytes results in glomerular recruitment of patrolling monocytes, leading to the subsequent recruitment of neutrophils, which cause glomerular damage even in the absence of serum autoantibodies⁵². Taken together, these studies^{48–50} identify the activation of TLRs in both the renal endothelium and circulating monocytes as important factors in recruiting pathogenic Ly6C^{lo} monocytes to glomeruli in LN. However, this mechanism might not be the only way in which glomerular macrophages are recruited, as a glomerular population of F4/80^{lo}CD11c^{lo} alternatively activated macrophages has also been described in NZM2328 mice⁵³. Classical inflammatory Ly6C^{hi} macrophages have been reported in only a few mouse models of lupus, although they are a prominent feature of ischaemic and anti-glomerular basement membrane-mediated glomerular injury.

Mixed tubulointerstitial leukocyte infiltrates, sometimes with features of lymphoid organization, are found in many forms of CKD, including LN^{45,54–56}. The immune responses that occur in situ during progressive LN have been examined using single-cell analyses (see the Single-cell analysis section below) and by mapping cellular position and shape within a tissue to identify cognate interactions. B cell and T cell clones are present in LN tissue, and T helper cells that express high amounts of inducible T cell costimulator (ICOS) and IL-21 are located next to B cells in the renal infiltrates^{57–59}. The presence of antigen-presenting cells, such as myeloid dendritic cells (DCs) and plasmacytoid DCs^{59,60}, is associated with more advanced disease in LN⁶¹. Renal B cell responses are dominated by reactivity to the intrinsic renal antigen vimentin, an intermediate filament protein that is aberrantly released from injured cells⁶². Autoantibodies to vimentin can occur following any renal transplantation and are linked to allograft injury, but the clinical utility of measuring these antibodies in LN is not known⁶³.

In addition to infiltrating cells, the kidneys have a network of tissue-resident macrophages located around glomeruli and in the tubular interstitium that are involved in immune surveillance^{64,65}. Peritubular renal macrophages are particularly susceptible to immune complex-mediated activation owing to their anatomical location near to small peritubular vessels that lack an intervening basement membrane⁴⁴ (FIG. 1c). Interestingly, an increase in the number of tissue-resident macrophages that express genes related to both pro-inflammatory and pro-reparative features has been reported in mouse models of LN^{43,66}, suggesting either a dysregulated repair process or the presence of more than one cell subpopulation.

Overall, many types of immune cells are found in the kidneys of individuals with LN. A better understanding of how each infiltrating cell type contributes to renal injury is now needed so that pathogenic cells can be targeted, whereas those involved in organ protection and repair can be spared. In particular, the role of macrophages with a reparative phenotype is not well defined. These cells are required to prevent fibrosis after acute renal inflammation, but can become dysregulated and promote tissue injury during chronic inflammation⁶⁷.

Diagnosis and monitoring

A diagnosis of LN is currently made when there is a change in the clinical status of a patient, such as haematuria, proteinuria or a decline in renal function, which prompts a confirmatory renal biopsy. The tissue sample is graded histopathologically according to the International Society of Nephrology–Renal Pathology Society (ISN–RPS) classification, which includes indices for active inflammation and chronicity⁶⁸. The presence of tubulointerstitial inflammatory cell infiltrates and a high chronicity index are both associated with a worse prognosis, independent of glomerular changes^{37,45}. Nevertheless, analysis of renal tissue is not always an accurate indicator of renal outcome, and samples taken by biopsy from patients in full clinical remission can show ongoing inflammation⁶⁹. Controversy exists as to

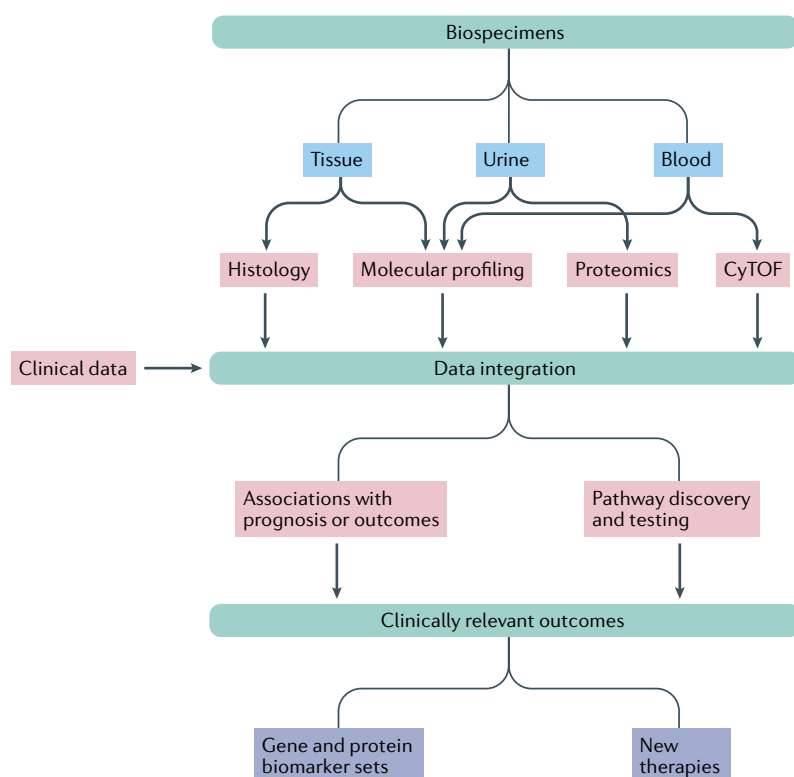


Fig. 2 | Data integration for lupus nephritis diagnosis and therapy. Clinical data from patients with lupus nephritis can be integrated with phenotypic, molecular and proteomic data obtained from biospecimens to predict the prognosis and response to treatment of these patients and to discover new pathogenic pathways for downstream testing. Big data analysis will benefit from the further development of machine learning and bioinformatics algorithms. These data-driven results should produce clinically relevant outcomes such as biomarker sets (both gene and protein) and new patient-specific therapies. CyTOF, cytometry by time-of-flight.

whether escalating therapy in patients with evidence of residual inflammation in such a follow-up tissue sample will improve renal outcome^{69,70} and, to date, a decrease in proteinuria to <0.7–0.8 g/dl by 1 year after diagnosis is the best predictor of long-term outcome^{71,72}.

Biomarker analyses

Given that renal histology might not be predictive of LN outcome, meeting the need for effective therapies in LN requires biomarkers for disease risk, as well as for response to therapy. Such biomarkers could include changes in circulating cells, concentrations of inflammatory mediators, specific urinary proteins or molecular signatures that can be assessed in renal tissue. New discovery-based approaches should lead to better diagnostic and therapeutic strategies for LN (FIG. 2).

Serum and urine biomarkers. A number of urine biomarkers can be used to differentiate patients with active LN from those with inactive disease, and multiplexed approaches have been used to identify panels of biomarkers that are associated with LN in cross-sectional studies^{73–75}. However, no new biomarkers have yet been shown to outperform the estimated glomerular filtration rate or proteinuria as measures for diagnosis, and none is currently used in clinical practice. Few studies have

as yet addressed whether longitudinal biomarker analyses can predict LN risk, detect LN before renal injury becomes clinically evident or identify those patients at highest risk of subsequent renal decline after an initial flare⁷³. An increase in plasma concentrations of soluble urokinase-type plasminogen activator receptor (uPAR) and a decrease in the urinary epidermal growth factor (EGF)–creatinine ratio are independent predictors of progression to CKD in patients with glomerular disease of multiple aetiologies^{76,77}. However, these biomarkers have not yet been systematically applied to the longitudinal study of patients with LN. The application of new proteomic technologies for unbiased and sensitive multiplexing of multiple markers will address whether it is possible to differentiate between ISN–RPS histological classes, identify treatment responders or predict long-term outcomes from serum or urine alone⁷⁸.

Modular signatures. Molecular approaches have been used to identify peripheral blood cell signatures that are associated with an increased risk of LN or SLE flares^{79,80}. One approach to simplifying the analysis of large data sets such as the whole-blood transcriptome is modular repertoire analysis, a computer-based algorithmic approach that uses a modular transcriptional analysis framework for transcriptomic studies. Modules are defined on the basis of a co-expression matrix (a module corresponds to a group of genes that are consistently co-expressed across several data sets). This data-reduction approach can be used to identify distinct, disease-specific modular gene-specific and cell-specific signatures within cross-sectional data sets and to analyse longitudinal data sets in large patient cohorts⁸⁰. Simplified modules based on co-clustered gene sets⁸¹ have been used to probe functional pathways that are abnormally expressed in patients. These studies have demonstrated molecular complexity and heterogeneity among patients with SLE^{80,82,83}. Analysis of a large longitudinal data set from a paediatric cohort of patients with SLE identified seven distinct patient clusters distinguished by their molecular profiles and clinical traits. In particular, a 20-gene neutrophil signature was associated with LN in these patients⁸⁰ and with either present or past LN in adults with SLE^{79,83,84}. However, these studies are complicated by the increase in the number of neutrophils induced by moderate-to-high glucocorticoid doses of >20 mg daily. Furthermore, the application of a nine-gene neutrophil score to longitudinal data failed to show an association with histological disease severity or class, or with the risk of subsequent flare⁸⁴. Reanalysis of the paediatric data, together with a data set from adult patients, identified three molecular clusters that did not change with either disease activity or treatment, of which only one, characterized by a lymphocyte rather than a neutrophil signature, had a significantly decreased risk for LN ($P < 0.05$ compared with the other two clusters)⁷⁹. Further work by the same group suggests that responsiveness of the gene signature to different treatments might also be cluster specific⁸⁵. In a third study of adult patients with SLE, peripheral blood signatures associated with active disease included oxidative phosphorylation, ribosome, proteasome, cell cycle and pyrimidine

Box 1 | Stratifying patients with lupus nephritis for clinical trials

Several strategies exist for stratifying patients with lupus nephritis into subgroups for clinical trials, including stratification on the following bases.

Immune mechanism

- Extrafollicular B cell activation is promoted by innate immune mechanisms, whereas germinal centre B cell activation is promoted by T cells. These mechanisms could be targeted in patients with strong innate immune or T cell signatures.
- Enhancing the CD8⁺ T cell exhaustion signature might help to prevent disease flares.
- Single-cell analyses and cross-species comparisons will foster investigation of new mechanisms and testing of new therapeutic strategies in mouse models chosen to reflect aspects of human disease.

Genetic profile

- Correcting the immune defect conferred by polymorphisms affecting different cell types might be an individualized strategy to maintain disease quiescence.
- Addressing polymorphisms that increase the risk of chronic kidney disease progression might require non-immune-based strategies to protect the kidney.

Biomarkers

- Modular profiling might identify patients at highest risk of lupus nephritis flare who can be enrolled in disease prevention studies.
- Unbiased approaches and modular analyses of peripheral blood could define patient subgroups that can be tested for responsiveness to particular therapies.

Disease stage and histological findings

- Treatment of flare and prevention of flare might each require different therapies, perhaps administered sequentially.
- The study of molecular patterns in renal tissue might yield new predictors of response to therapy and outcome that inform patient selection for trials.
- Tissue samples taken once treatment is underway might identify patients for whom treatment withdrawal is safe.
- The presence of fibrosis might require an adjuvant anti-fibrotic approach.

metabolism pathways, with the addition of a plasmablast signature in patients with LN⁸⁶. By contrast, the plasmablast signature was associated with overall disease activity, but not specifically with LN in paediatric patients with SLE⁸¹.

A different molecular signature identified by analysis of isolated peripheral blood T cells is a combination of a low co-stimulation signature in CD4⁺ T cells with an exhaustion signature in CD8⁺ T cells. This signature is associated with a poor response to infection or immunization, but also confers a decreased risk of flares in patients with SLE or anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis^{87,88}. The limited data available suggest that assignment of patients to the CD8⁺ T cell exhaustion phenotype, and even to some of the modular phenotypes described above, is mostly independent of either disease activity or treatment, so that it might be possible to use this type of data to stratify patients for LN risk or to use the LN-associated modules and cluster assignments to direct individualized therapy (BOX 1). However, although it is clear that there is much heterogeneity among patients with SLE, more longitudinal molecular and clinical data will be needed to address these issues.

Epigenetic profiling. Epigenetic profiling measures biochemically reversible changes in chromatin structure that influence whether the chromatin is accessible for gene transcription and expression of its associated

protein. Changes in DNA methylation and in histone methylation and acetylation can be mapped using biochemical methods⁸⁹. For example, epigenome-wide association studies examine the association between gene expression and disease risk by profiling the methylation and acetylation status of DNA and/or histones within peripheral blood mononuclear cells to identify specific genes that are repressed or accessible⁸⁹. Alternatively, assay for transposase-accessible sequencing (ATAC-seq) can be used to identify areas of open chromatin that are potentially available for gene transcription. Epigenetic modifications can be induced by external factors, such as smoking, oxidative stress or medications, but also vary by ethnicity^{90,91}.

Hypomethylation of type I interferon response genes has been observed in immune cells of most lineages from patients with SLE compared with immune cells from healthy individuals, although this hypomethylation is also found in other autoimmune diseases, such as rheumatoid arthritis and systemic sclerosis⁹². Cross-sectional studies have revealed differences in the methylation status of CD4⁺ T cells and total peripheral blood mononuclear cells from patients with SLE and LN compared with patients with SLE and no LN, but it is not yet known whether clinically useful data can be derived from such studies⁸⁹. In lupus-prone mice, DNA hypomethylation in T cells caused increased expression of pro-inflammatory genes and induced autoimmunity⁹³. However, the targeting of a DNA methylation inhibitor specifically to T cells ameliorated disease in the MRL/lpr mouse model of lupus⁹⁴, indicating that the relationship between methylation differences and disease expression is complex and that the result of therapeutic targeting cannot be easily predicted.

Omics analyses of the kidneys

New technologies are now enabling molecular, biochemical and cellular analyses to be performed on blood, urine and the small amounts of renal tissue that can be obtained as part of diagnostic tissue sampling in patients with LN⁷⁸. The discussion below looks at how these omics technologies can be used to examine whole organs or single cells.

Whole-organ analyses. Molecular profiling of fibrotic kidney tissue from patients with CKD of multiple aetiologies, including LN, has revealed inflammation and tubular metabolic dysfunction as the most important dysregulated pathways in a disease state⁹⁵. Further analysis showed that kidney tubular epithelial cells depend on fatty acid oxidation and mitochondrial oxidative phosphorylation as their main energy sources, and that these metabolic functions are compromised in progressive CKD, as shown by downregulation of the transcription factor PPARC1α (an important regulator of mitochondrial biogenesis)⁹⁵ and a concomitant switch from oxidative phosphorylation to glycolysis⁴². Proteome screening subsequently indicated that the transcription factors PPARα and PPARγ induce the production of lipid-metabolizing enzymes and suppress that of glycolytic enzymes in the proximal tubules of healthy kidneys⁹⁶. Similarly, screening for microRNA (miRNA)

Exhaustion signature

A cell state or phenotype with progressive loss of effector cytokine or cytotoxic function owing to prolonged antigen stimulation, often characterized by the increased expression of immune checkpoint inhibitory receptors, alterations in metabolic function and a distinct transcriptional profile that differs from that of anergic cells.

expression in both human and mouse fibrotic kidneys revealed a role for miR-21 in regulating genes involved in mitochondrial biogenesis and fatty acid oxidation, thus favouring glycolysis in the injured tissues⁹⁷.

Tubular epithelial cells that are arrested at the G2–M transition point of the cell cycle fail to proliferate and undergo repair^{38,98}. Transcriptome analysis of renal tissue from patients with renal disease of multiple aetiologies, including LN, revealed a panel of 72 genes, the expression of which correlated with the estimated glomerular filtration rate⁷⁷. This panel contained genes involved in tissue remodelling and fibrosis, including *EGF*, which is regulated by PPAR γ and has been linked to the regenerative capacity of renal tubules^{77,96}. Studies over the past decade have revealed several other pathways that determine whether tubules will undergo regeneration or senescence⁹⁹, including a role for the transcription factor FOXO3 in regulating autophagy, which provides lipids for mitochondrial oxidation¹⁰⁰. Because tubular damage induces renal fibrosis, the translation of these studies to therapeutic approaches is urgently needed.

Mice and humans with LN share common renal molecular signatures⁴³. Longitudinal transcriptomic studies of kidneys from NZB/W F1 mice showed an increase in the expression of multiple pro-inflammatory genes at proteinuria onset, followed by the downregulation of a set of PPARGC1 α -regulated genes, indicative of metabolic and mitochondrial dysfunction as the mice progressed towards renal failure¹⁰¹. This signature can be reversed with remission induction therapy, but metabolic dysfunction recurred before clinical relapse, suggesting that, once initially injured, the kidney becomes more susceptible to damage during subsequent disease flares¹⁰¹. These studies further showed that the decreased expression of VEGF at proteinuria onset does not reverse with remission induction, suggesting that the small renal vessels might be compromised, even during remission^{43,101}. Preclinical translation of human-relevant findings into therapeutic approaches is beginning. Notably, overexpression of PPARGC1 α in kidney tubule cells protects against tubule injury and fibrosis in several mouse models of acute renal injury⁹⁵. Similarly, systemic miR-21 depletion protects against acute renal injury in mice, whereas PPAR γ inhibition causes tubulointerstitial fibrosis⁹⁶.

Single-cell analyses. Single-cell RNA sequencing is an important technical advance that has enabled the molecular profiling of individual renal cells and immune cells and that should advance our knowledge of the pathogenic mechanisms in LN^{102,103}. The advantage of this approach is that rare cell types can be identified and differences in gene expression profiles between cells of each renal and infiltrating cell type from healthy individuals and patients with LN can be evaluated. The Accelerating Medicines Partnership (AMP) is currently undertaking a large-scale project in LN; enrolment is completed and analysis is planned for 160–200 tissue samples from patients with LN that have associated clinical metadata, peripheral blood analyses and urine proteomic data⁷⁸. Molecular and proteomic analyses of renal tissue and urine will be correlated with disease outcomes at 12 months

and have the potential to reveal additional biomarkers for treatment response and outcome⁷⁸.

Initial results of single-cell data from the first phase of the AMP LN studies were published in 2019 (REFS^{51,104}). Preliminary analyses of tubular epithelial cells from 21 patients with LN suggested that patients with proliferative disease or who were unresponsive to 6 months of treatment had a higher baseline interferon signature¹⁰⁴. Treatment non-response was also associated with a tissue remodelling or fibrosis signature that was independent of histologically evident fibrosis¹⁰⁴. Single-cell RNA sequencing analysis of immune cells sorted from kidney samples from 24 patients with LN and ten living transplant donors revealed multiple immune cell types in the LN tissues, including ten clusters of natural killer cells and T cells, four clusters of B cells, six clusters of macrophages and DCs and one mixed cluster of dividing cells⁵¹. By contrast, the immune cell populations in healthy kidney were less diverse and dominated by memory CD4⁺ T cells and resident macrophages. Apart from the confirmation of *in situ* B cell activation and differentiation, several novel observations have come from this study⁵¹, including the identification of an interferon signature in most cell types; the presence of proliferating cells consisting mostly of natural killer cells and CD8⁺ T cells; the identification of novel CD8⁺ T cell subsets; the absence of exhausted CD8⁺ T cells; and a lack of clear skewing of CD4⁺ T cells to either a T helper 1 cell or T helper 17 cell phenotype. Renally derived leukocytes could also be detected in the urine, suggesting the possibility of non-invasive profiling⁸⁵.

Focusing on myeloid cells, the infiltrates in the kidneys of patients with LN included both classic and plasmacytoid DCs, as well as four subpopulations of macrophages or monocytes, only one of which could be detected in the peripheral blood⁵¹. One of these subsets was also detected in healthy kidney, and therefore probably represents a tissue-resident renal macrophage population; however, the transcriptome of this macrophage population is modulated in LN, whereby it acquires a mixed interferon signature and an anti-inflammatory signature. The other three macrophage or monocyte subpopulations are related along a developmental trajectory that starts with a population that most resembles pro-inflammatory CD16⁺ monocytes and progresses to a phagocytic macrophage phenotype and then to an alternatively activated phenotype that is also a major cellular source of chemokines⁵¹. The presence of CD16⁺ monocytes in the kidneys of patients with LN is consistent with previous histological data^{105,106} and with results in mouse models of LN in which Ly6c^{lo} monocytes are recruited to the kidneys⁴⁸. The reason for the preferential recruitment of the CD16⁺ monocyte population over the CD14⁺ population in LN could reflect the important role of TLR activation by nucleic acid-containing immune complexes and debris in the recruitment of patrolling monocytes.

Overall, these studies^{51,104} have established the feasibility of applying a single-cell analysis approach to LN and have highlighted the complexity and heterogeneity of the intra-renal immune response that contributes to disease progression. Data from the phase II AMP studies

that are currently in progress will enable the robust correlation of molecular patterns with outcomes, as well as the discovery of new pathogenic mechanisms that might inform us about how best to therapeutically target pathogenic immune cell populations and modulate stromal cell dysfunction in LN. Given that changes in the tubular epithelium are associated with progression and fibrosis, it will be of great interest to determine whether signatures of tubular regenerative capacity and metabolic function can be inferred from the molecular data and correlated with disease outcome.

Future perspectives for therapy

Standard immunosuppressive treatments for LN have a high non-response rate and currently, only one biologic drug, belimumab, is approved for the treatment of SLE, and none for the treatment of LN. However, this poor outlook could be about to change.

Improving clinical trial design

The failure of clinical trials to yield an effective new treatment strategy for LN has partly been attributed to the complexities of clinical trial design^{107,108}. LN is a heterogeneous disease, and the global recruitment of patients to clinical trials adds additional geographical diversity in ethnicity, approaches to standard of care and the risk of adverse events. All new drugs are tested against a background of standard-of-care therapy that confers a response rate higher than that of a typical placebo response, necessitating large cohorts to observe meaningful differences. Another difficult issue in LN clinical trial design is how to mitigate the confounding effects of glucocorticoids and standard-of-care therapies, the potential interactions of which with any new drug are mostly unknown. Comparisons between completed trials are also challenging because of differences in design and outcome measures.

Lessons from failed clinical trials in LN¹⁰⁹ have spurred efforts to define the most informative outcome measures and to consider the addition of molecular phenotyping. In this regard, a proteinuria measurement of <0.7–0.8 g/dl at 12 months needs to be tested as a surrogate biomarker for long-term outcome in patients with LN. Because the immune disturbances underlying disease initiation are heterogeneous, individualized approaches might be required to target the inciting cells or pathways; strategies for this approach are outlined in BOX 1. Accurate evaluation of risk in individual patients is needed so that smaller and shorter preventive trials in high-risk patients can be performed without unnecessary exposure of low-risk patients to potentially toxic regimens. In addition, reliable and inexpensive biomarkers for the early stages of LN or of LN recurrence need to be identified so that all patients can be monitored more closely and treated earlier. Finally, because patient compliance with polypharmacy is low, methods for monitoring and encouraging compliance need to be built into clinical trials and clinical practice. The establishment of LN registries that contain both clinical data and biospecimens and the universal access of patients with LN to specialized care and clinical trials will accelerate progress in all areas.

New therapeutic approaches

New strategies to prevent and treat LN continue to be reported in mouse models (TABLE 1), but these studies often use a single model of disease and do not have standardized intervention times or outcome measures. The failure to translate many therapies that are effective in mouse models of LN into successful clinical trials in patients with LN possibly reflects not only interspecies differences but also the relatively late detection of human disease and the heterogeneity of renal injury mechanisms in genetically diverse human populations. Despite these setbacks, ongoing discovery has fuelled continued efforts to repurpose and combine currently available immunosuppressive drugs and to test new immune-modulating therapies.

New successes in immune modulation for the treatment of SLE, as well as LN, seem to be on the horizon. The first of these, a phase III study of belimumab for LN, has achieved its primary end point and all major secondary end points¹¹⁰ and is on track for regulatory submission during the first half of 2020. Several new late-phase trials in SLE have achieved their primary end point, the most notable of which are a phase II study of the IL-12–IL-23 inhibitor ustekinumab¹¹¹ and one of two phase III studies of the type I interferon receptor antagonist anifrolumab^{112,113}. A phase II study of anifrolumab for LN is in progress¹¹⁴, with an estimated completion date of January 2021. In patients with LN, the combination of mycophenolate mofetil (MMF) with the calcineurin antagonist tacrolimus¹¹⁵ showed improved rates of remission induction over either drug alone in several Asian cohorts¹¹⁶, although the potential toxicity of this combination¹¹⁷ has relegated its use in the USA to patients in whom therapy with MMF alone fails. Two successful phase II trials of voclosporin, a potent and safer alternative calcineurin antagonist, in combination with MMF and a rapid steroid taper have also been completed in patients with LN^{117,118}, and a phase III study has completed recruitment¹¹⁹. The phase II trials showed a substantially better response rate to combination treatment than to MMF alone, but complete response rates were still <50%, and adverse events were more common in those receiving combination therapy^{117,118}. Finally, the potent B cell-depleting agent obinutuzumab has achieved FDA breakthrough status for expeditious development and review¹²⁰ on the basis of a successful phase II study in LN¹²¹. Other clinical trials in progress for LN that focus on immune modulation as a strategy are listed in TABLE 2.

In addition to the immune-modulating therapies that are currently being tested, a better understanding of the pathways that are associated with renal repair versus progression to CKD is needed so that the non-immune response to renal injury can be modulated, intrinsic renal cells and structure preserved and fibrosis prevented. Although there are, as yet, no approved treatments that halt CKD progression, headway has been made in understanding how to preserve renal tubular cells or how to promote their reparative programmes. Potential future strategies include the modulation of mitochondrial function^{39,102}, the modulation of miRNAs^{97,102}, the reversal of tubular cell senescence^{102,122},

Polypharmacy

The use of multiple medications to treat complex medical conditions.

Table 1 | New therapeutic strategies for lupus nephritis tested in mouse models of disease

Targets	Results	Take-home message	Refs
Effector cytokines			
IL-17	MRL/lpr IL-17A KO mice and NZB/W mice treated with anti-IL-17A and anti-IFN γ mAbs; only anti-IFN γ mAb improved outcomes in NZB/W mice	IL-17A is not a promising target for LN	130
IL-34	LN and systemic illness is suppressed in IL-34-deficient MRL/lpr mice	Intra-renal and systemic IL-34 promotes LN in MRL/lpr mice	131
CD40	CD40 antagonist restores glomerular morphology in NZB/W and MRL/lpr mice	CD40 blockade induces very robust prevention and treatment of LN	132
JAK	The JAK inhibitor tofacitinib ameliorates LN and inhibits vascular dysfunction in MRL/lpr mice	Tofacitinib shows preventive and therapeutic efficacy	133
SYK	Tyrosine protein kinase SYK inhibitor delays LN in NZB/W mice	SYK inhibition shows dose-dependent preventive and therapeutic efficacy	134–136
BTK	BTK-specific inhibition prevents and treats LN in NZB/W and MRL/lpr mice, and in the IFN α -induced model	BTK inhibition shows dose-dependent preventive and therapeutic efficacy	137,138
ADAM17	Pharmacological blockade of either TNF or EGFR signalling protected <i>Fcgr2b</i> ^{-/-} mice from severe renal damage	Inactive rhomboid protein 2–ADAM17-dependent TNF and EGFR signalling promotes LN	139
Inflammasome	A PIM1 inhibitor suppressed NLRP3 inflammasome activation and reduced LN; by contrast, NLRP3 and ASC deficiency worsen disease in C57BL/6 ^{lpr} mice ¹⁴⁰	The inflammasome is a potential target but has both pathogenic and protective properties	141
	Piperine ^a ameliorated LN in pristane-injected mice through NLRP3 inflammasome inhibition		142
Metabolism			
mTOR	Baicalin ^b ameliorated LN in MRL/lpr mice through mTOR axis inhibition	Targeting cell metabolism by inhibition of mTOR might be beneficial for LN	143
	Mangiferin ^c ameliorated LN in FasL-deficient B6/ <i>gld</i> mice by inducing regulatory T cells via mTOR pathway inhibition		144
	The mTOR inhibitor rapamycin is cytoprotective in podocyte injury ^d		145
	Rapamycin reduces renal fibrosis in NZB/W mice		146
Oxidative phosphorylation and glycolysis	Combined oxidative phosphorylation and glycolysis inhibition by metformin and 2DG reduced disease severity and reversed LN in NZB/W mice	Each therapy individually prevented disease, but both were needed to reverse established disease	147
Kidney cells			
Podocyte CAMK4	Podocyte-targeted CAMK4 inhibition preserved ultrastructure, averted immune complex deposition and crescent formation, and suppressed proteinuria in lupus-prone mice	Targeted CAMK4 inhibition preserves podocyte structure and function when used as preventive therapy	148
Cholinesterase	The cholinesterase inhibitor galantamine attenuates hypertension, glomerulosclerosis and fibrosis in nephritic NZB/W mice	Anti-inflammatory, antihypertensive and renoprotective effects are mediated through the cholinergic anti-inflammatory pathway	149
Other			
DNA methylation	The demethylation inhibitor 5-azacytidine targeting either CD4 ⁺ or CD8 ⁺ T cells prevents disease in MRL/lpr mice by expanding regulatory T cells and inhibiting the expansion of double-negative T cells	Illustrates the complexity of targeting DNA methylation in vivo, as regulatory elements might be hypermethylated	94
Immune cell modulation	MSCs prevent disease in MRL/lpr mice in a CCL2-dependent manner	This study is one of many reports of the efficacy of MSCs, which function via various mechanisms	150

2DG, 2-deoxy-D-glucose; ADAM17, disintegrin and metalloproteinase domain-containing protein 17; ASC, apoptosis-associated speck-like protein containing a CARD; BTK, Bruton tyrosine kinase; CAMK4, calcium/calmodulin-dependent protein kinase type IV; CCL2, CC chemokine ligand 2; EGFR, epidermal growth factor receptor; FasL, Fas ligand; JAK, Janus kinase; KO, knockout; LN, lupus nephritis; mAbs, monoclonal antibodies; MSCs, mesenchymal stem cells; mTOR, mechanistic target of rapamycin; NLRP3, NOD-, LRR- and pyrin domain-containing 3; PIM1, serine/threonine-protein kinase pim-1. ^aPiperine is a natural compound in black pepper and related herbs. ^bBaicalin is extracted from an anti-inflammatory traditional Chinese herbal medicine. ^cMangiferin is extracted from natural herbs, including *Mangifera indica*. ^dOnly tested in vitro.

protection of the renal vasculature from injury^{25,26,123} and the use of inhibitors of prolyl hydroxylase (currently being tested for CKD-associated anaemia) that enhance hypoxia-inducible factor (HIF) and FOXO3 activity^{99,100} by altering their prolyl hydroxylation and degradation. Pro-fibrotic cytokines such as TGF β and connective tissue growth factor (CTGF) are also logical therapeutic targets for late-stage disease³⁹. The multiple roles of TGF β in promoting renal fibrosis have been well described, but inhibition of the cytokine itself has not been successful;

alternative approaches to targeting TGF β are reviewed elsewhere¹²⁴. Given the role of activated fibroblasts in producing pro-inflammatory cytokines and chemokines, targeting of effector cytokines is being considered to prevent amplification of renal damage in fibrotic tissues³⁹. For example, a role has been identified for IL-1 in promoting fibrosis via the induction of the transcription factor MYC in stromal mesenchymal cells⁴², suggesting that IL-1 inhibitors could be tested for therapeutic efficacy in LN in the future. In addition, drugs that block

Table 2 | Therapies currently in clinical trials for lupus nephritis

Drug or therapy name	Targets	Trial phase	Drug mechanism	Results of previous studies	Refs
Mesenchymal stem cell therapy	Immune cell modulation	II	Multiple immune-modulating effects reported	Efficacy has been demonstrated in animal models and in uncontrolled studies in humans	151–154
Tacrolimus (FK506) and MMF	Immune cell suppression	IV	Additive or synergistic effect of the combination of a calcineurin inhibitor (tacrolimus) and an IMP inhibitor (MMF)	Improved remission rate, but increased adverse events	115
Voclosporin	Calcineurin	II	A calcineurin inhibitor that is more potent and less toxic than other members of this class	Phase III studies are pending following the success of phase II trials showing the benefit of adding voclosporin to MMF for remission induction	117,118,155
CFZ533 (iscalimab)	CD40	II	Non-depleting non-agonist anti-CD40 antibodies that inhibit T cell-dependent B cell responses	Previous studies of agents targeting CD40L were terminated owing to adverse thrombotic events; safety of CFZ533 has been demonstrated in other inflammatory diseases and in transplant recipients	132,156
BI 655064	CD40	II		Safety has been demonstrated in healthy volunteers and in patients with RA	132,157,158
KZR-616	Immunoproteasome	I	A small-molecule selective inhibitor that halts pro-inflammatory cytokine production without affecting normal T cell-dependent responses	Safety and tolerability reported in a phase Ib dose-escalation trial in patients with SLE	159,160
Obinutuzumab	CD20	II	A human anti-CD20 antibody that produces a more robust B cell depletion than rituximab	Phase II study showed improved remission rate when added to standard-of-care therapy; has received FDA breakthrough therapy designation for progression to phase III trials	121,161,162
BMS-986165	TYK2	II	An inhibitor of JAK family member TYK2 that inhibits IL-12, IL-23 and type I interferon signalling	Efficacy has been demonstrated in a phase II study of psoriasis	163,164
Anifrolumab	Type I interferon	II	An anti-IFNAR monoclonal antibody that blocks the binding of type I interferons to their receptor	Positive results reported in one of two phase III trials for general SLE	112,113,165
Belimumab	BAFF	III	B cell depletion and modulation	Has achieved primary and all major secondary end points in a phase III trial of LN	110,166
Eculizumab	Complement protein C5	NA	Anti-C5 antibody blocks the terminal complement pathway and C5 cleavage	Off-label use reported in refractory LN and thrombotic microangiopathy	36
Mizoribine	Nucleotide metabolism	III	IMP and GMP inhibitor	This drug is used extensively for LN in Japan but, as yet, there has not been a large-scale randomized controlled clinical trial	167,168
Iguratimod	NF- κ B	II	NF- κ B inhibitor	This drug is approved for treating RA in East Asia; testing in human LN is based on success in animal models and preliminary observations in patients with refractory LN	169–171
Secukinumab	IL-17	II	IL-17 inhibitor	Testing in LN is based on successful use of this class of drugs in other inflammatory diseases and the reported presence of T _H 17 cells in the kidneys of patients with LN	172

BAFF, B cell-activating factor; CD40L, CD40 ligand; GMP, guanosine monophosphate; IFNAR, IFN α receptor; IMP, inosine monophosphate; JAK, Janus kinase; LN, lupus nephritis; MMF, mycophenolate mofetil; NA, not applicable; NF- κ B, nuclear factor- κ B; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; T_H17, T helper 17; TYK2, tyrosine kinase 2.

PDGF receptors are currently being used to treat lung fibrosis and could also be considered for the treatment of fibrosis of the kidneys¹²⁵. Finally, targeting of fibroblasts using chimeric antigen receptor (CAR) T cells has been applied in a mouse model of cardiac fibrosis and represents a new technology that could delay or reverse damage of fibrotic organs¹²⁶.

Importantly, patients with SLE have a high risk of premature cardiovascular disease¹²⁷, and CKD is an additional cause of endothelial dysfunction and increased cardiovascular risk¹²⁸. A multi-targeted approach using lifestyle modification, angiotensin-converting enzyme inhibitors, statins and hypertension control can decrease the cardiovascular mortality associated with CKD and should be tested in patients with LN¹²⁹.

Conclusions

LN remains a challenging clinical problem. Some progress has been made in the past few years towards improving both risk assessment and monitoring in patients with LN, including improvements in genetic risk profiling, the identification of biomarkers for flare^{73–75} and the development of patient stratification⁶⁰ and hazard index tools⁷¹. More work is required to address whether it is possible to detect preclinical LN and to design interventional studies that test strategies to prevent renal flares and/or CKD progression. In the

USA, where non-white patient ethnicity is associated with poor outcomes for LN^{3,20}, there is an urgent need to understand the mechanisms underlying genetic risk of ESRD and to address socioeconomic disparities that might affect lifestyle choices and medication adherence.

The kidney is a highly complex organ with multiple cell types that interact with and support each other and that, with the exception of the tubular cells, has a limited capacity for regeneration. Interstitial inflammatory infiltrates are associated with both CKD and a poor prognosis of LN⁴⁵. Our understanding of the various intrinsic and infiltrating cell types involved in renal injury is being advanced by single-cell analyses, with the goal of yielding new diagnostic and therapeutic strategies to improve the outcome of LN. As data become available from such studies, new technologies will enable a spatial mapping of the involved cell types and a closer analysis of the cell–cell interactions that contribute to renal damage. Although current therapies are highly focused on immune-modulating interventions, new developments in the general approach to CKD might be translatable to LN and could help to delay or prevent the terminal phases of the disease that are associated with tubular senescence, vascular impairment and fibrosis.

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Rubicon promotes rather than restricts murine lupus and is not required for LC3-associated phagocytosis

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One Sentence Summary: Rubicon promotes clinical and immunological manifestations of SLE in murine models.

Abstract

NADPH oxidase deficiency drives lupus in murine models and in patients. The mechanism by which NADPH oxidase prevents autoimmunity remains unknown. NADPH oxidase could suppress autoimmunity by facilitating dead cell clearance by macrophages via LC3-associated phagocytosis (LAP). Genetic dissection of LAP revealed CYBB, a critical component of the NADPH oxidase complex, and RUBICON as requisite for this process. The absence of LAP results in an autoinflammatory syndrome in aged, non-autoimmune mice. To test the hypothesis that NADPH oxidase deficiency drives SLE by a defect in LAP, we genetically deleted *Rubicon* in the B6.Sle1.*Yaa* and MRL.Fas^{lpr} lupus models. If NADPH oxidase deficiency exacerbates SLE by failure to perform LAP, *Rubicon*^{-/-} should phenocopy *Cybb*^{-/-} mice. Strikingly, we observe the opposite—RUBICON deficiency results in reduced mortality and renal disease in murine lupus along with reduced autoantibodies to RNA-associated autoantigens. Given that our data contradicts the published role for LAP in autoimmunity, we assessed whether CYBB and RUBICON are requisite for LAP. We show that LAP is not dependent on either of these two pathways. Our data do not support a role for LAP in lupus but highlights RUBICON as a novel regulator of SLE, possibly by a B cell intrinsic mechanism.

Introduction

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by loss of tolerance, rampant immune activation, and end-organ damage (1). Loss of tolerance to nuclear antigens and the formation of autoantibodies to nucleic acids and nucleoproteins are hallmarks of SLE pathogenesis. While the sources of autoantigens in lupus remain enigmatic, a failure to adequately dispose of dead cells and resulting debris by macrophages is a leading possibility (2). Indeed, macrophages from a subset of lupus patients exhibit an impaired ability to phagocytose, a finding also observed in murine models (3-6). Moreover, there are several lines of evidence that link dead cell clearance pathways to the development of systemic autoimmunity. Loss of function of C1q, TIM4, and MFG-E8 results in lupus-like syndromes in humans and in mice. (7-13). Taken together, these studies emphasize that inadequate clearance of dead cells can result in an immune response to self and subsequent end-organ damage.

The NADPH oxidase complex, a group of transmembrane and cytosolic enzymes responsible for the respiratory burst critical for microbial killing (14-16), is important for the clearance and degradation of dead cells by macrophages (17-22). Loss of function mutations in essential components of the NADPH oxidase including CYBA, CYBB, NCF1, and NCF2 result in chronic granulomatous disease (CGD). A link between CGD and systemic autoimmunity is well established. Male patients with X-linked CGD, characterized by loss of function mutations in *CYBB*, are at greater risk of developing a lupus-like disease (23, 24). Moreover, carrier mothers of affected males are more likely to develop SLE, indicating that heterozygous dosing of the *CYBB* allele is sufficient to drive lupus (25, 26). Recently, loss of function polymorphisms in *NCF1* and

NCF2 are reported to confer increased SLE susceptibility across multiple ethnicities (27-29). Multiple mouse models of CGD recapitulate increased autoimmunity susceptibility observed in human patients (30-35). Collectively, these studies show that the NADPH complex is critical for the regulation of autoimmune pathology in mice and humans.

Although the mechanism by which NADPH oxidase regulates the anti-self response is unknown, a compelling hypothesis is that it suppresses autoimmunity by promoting dead cell clearance by myeloid cells (17-20, 36). Recently, LC3-associated phagocytosis (LAP), a process that partially overlaps with autophagy (22, 37, 38), has been implicated in the non-inflammatory degradation of dead cell debris by macrophages (38). LAP occurs when certain types of phagocytosed particles that can stimulate aspects of innate immunity cause the recruitment of autophagy machinery to the phagosome, facilitating maturation and the degradation of the engulfed contents (22, 37, 38). Genetic dissection of LAP identified LAP-specific components (e.g. *Cybb* and *Rubicon*) as well as genes required for both LAP and autophagy (e.g. *Beclin1*, *Atg5*, and *Atg7*) (22). Martinez and colleagues reported that genetic deletion of components implicated in LAP only (*Rubicon* and *Cybb*) or both LAP and canonical autophagy (*Beclin1*, *Atg5*, and *Atg7*) led to an autoinflammatory syndrome in aged, non-autoimmune C57BL/6 mice, which spontaneously developed low-titer autoantibodies and mild renal disease (35). Strikingly, mice deficient in components implicated in canonical autophagy only (ULK1 and FIP200) did not develop systemic autoimmunity with age, suggesting that LAP but not canonical autophagy is required to prevent autoimmunity (35). Interestingly, increased levels of proinflammatory cytokines were present in the serum of these LAP-deficient mice but anti-inflammatory cytokines, such as IL-10, were reduced (35). The authors postulated that in the absence of LAP, dead cells are not cleared in an immunologically

silent way and that the inability to generate IL-10 downstream of LAP is a driver of the disease phenotype (35).

While evidence suggests that blocking LAP can drive an anti-self response in a non-autoimmune setting, the role of LAP, and the genes that promote it, remain unclear in the context of clinical SLE. We previously showed that deletion of *Cybb* in lupus-prone MRL.Fas^{lpr} mice led to markedly exacerbated disease (31). As NADPH oxidase is required for LAP, it is possible that the exacerbated disease observed in the context of *Cybb* deficiency is due to inhibition of LAP. If *Cybb* deficiency exacerbates SLE by prevention of dead cell clearance due to a defect in LAP and subsequent inhibition of anti-inflammatory cytokine production, then deleting another LAP-specific gene should have a similar phenotype of exacerbated disease in lupus-prone murine models.

We addressed this by genetically deleting another requisite component for LAP, *Rubicon*, in both the MRL.Fas^{lpr} and B6.Sle1.*Yaa* lupus models and studied effects on disease in both *Cybb*-deficient and wild type genetic backgrounds. We chose the MRL.Fas^{lpr} model as it is a leading spontaneous, polygenic system to study SLE, recapitulating nearly all features of the human disease (39). Additionally, it has been used to study the role of *Cybb* deficiency in SLE, allowing for a direct comparison of results. Furthermore, studies in MRL.Fas^{lpr} mice have accurately predicted responses in human translational studies, validating the use of this model in pre-clinical investigation (39-45). The *Sle1* locus is on the C57BL/6 genetic background and includes lupus susceptibility polymorphisms involving Slam family members (46-49). Combining this locus with

the *Yaa* locus in males confers an extra copy of *Tlr7* that drives additional autoreactivity to RNA antigens resulting in a lupus-like disease characterized by nephritis and early mortality (50).

We found that *Rubicon*-deficient SLE prone mice did not phenocopy exacerbated lupus observed in *Cybb*-knockouts. In fact, the absence of RUBICON conferred a survival advantage in SLE prone mice, including *Cybb*-deficient MRL.Fas^{lpr} mice, and protected them from renal disease. Since our results did not support the published role for LAP in autoimmunity, and in fact showed opposite roles for two proteins that are both thought to be critical for LAP, we re-assessed whether CYBB and RUBICON are indeed requisite for LAP in macrophages. Surprisingly, we show that LAP is, in fact, not dependent on either of these two proteins. Taken together, our data do not support a role for LAP in lupus and, most importantly, highlight RUBICON as a novel regulator of SLE pathogenesis.

Results

Rubicon deficiency does not reduce survival in murine SLE

To determine whether *Cybb* deficiency exacerbates SLE by prevention of dead cell clearance due to a defect in LAP, we genetically deleted another requisite component for LAP, *Rubicon*, in the B6.Sle1.*Yaa* and MRL.Fas^{lpr} lupus models. *Rubicon* was genetically deleted directly on the MRL.Fas^{lpr} background using CRISPR-Cas9 (Supplemental Figure 1A and 1B). To determine whether CYBB and RUBICON act in the same pathway (i.e. LAP), we analyzed *Cybb* and *Rubicon* single and double knockout mice in the setting of lupus. SLE pathology was analyzed at 16-18 weeks of age in MRL.Fas^{lpr} mice and at 8-21 months of age in the B6.Sle1.*Yaa* model unless otherwise indicated.

Surprisingly, *Rubicon*-deficiency actually protected from, rather than exacerbated disease, as the absence of RUBICON conferred a survival advantage in B6.Sle1.*Yaa* mice (Figure 1A, left panel). Complete deletion of *Rubicon* is required for this protection (Figure 1A, right panel). *Rubicon*^{+/-} B6.Sle1.*Yaa* mice have no differences in mortality compared to wild-type B6.Sle1.*Yaa* controls (Supplemental Figure 2). Consistent with the prior literature (31), male and female *Cybb*-deficient MRL.Fas^{lpr} mice had a reduced lifespan compared to *Cybb*-sufficient controls (31, 32, 34) as 50% or more of the experimental *Cybb*^{-/-} cohort did not survive until the experimental endpoint of 17 weeks (Figure 1B and 1C, left panels). Similar to the B6.Sle1.*Yaa* SLE model, no *Rubicon*-deficient MRL.Fas^{lpr} mice died in the analyzed cohorts (Figure 1B and 1C, left panels). Strikingly,

Rubicon deficiency increased survival in male *Cybb*^{-/-} mice (Figure 1B, right panel). However, this protection was not observed in female *Cybb*^{-/-} *Rubicon*^{-/-} MRL.Fas^{lpr} mice (and 1C, right panel).

Rubicon deficiency reduces SLE renal disease

Both *Rubicon*-deficient B6.Sle1.*Yaa* and MRL.Fas^{lpr} mice had reduced urine protein compared to wild-type controls (Figure 2A, top and middle panels). *Rubicon*-deficient B6.Sle1.*Yaa* mice were protected from glomerulonephritis (Figure 2B, top panel); however, such protection was not observed in MRL.Fas^{lpr} mice (Figure 2B, middle panel). Further, interstitial nephritis was ameliorated in *Rubicon*^{-/-} male MRL.Fas^{lpr} and B6.Sle1.*Yaa* mice, with a trend towards reduction in MRL.Fas^{lpr} females (p=0.0844) (Figure 2C, top and middle panels). Strikingly, protection from nephritis was observed in an older cohort of *Rubicon*-knockout B6.Sle1.*Yaa* mice aged to 19-21 months, nearly twice the age of the wild-type counterparts used in this study (Figures 2B and 2C, top panels).

Genetic deletion of *Rubicon* in *Cybb*-deficient MRL.Fas^{lpr} SLE prone mice reduced proteinuria in male mice (Figure 2A, bottom panel). A similar reduction in females was observed, nearly reaching significance (p=0.07) (Figure 2A, bottom panel). Concordant with the proteinuria data, *Rubicon*-deficiency reduced glomerulonephritis in *Cybb*-knockouts (Figure 2B, bottom panel). Interstitial nephritis was reduced in female but not male double knockouts (Figure 2C, bottom panel). *Rubicon*^{-/-}*Cybb*^{-/-} male and *Rubicon*^{-/-}*Cybb*^{-/-} female SLE prone mice had decreased composite disease scores compared to *Cybb*-knockout counterparts (Supplemental Figure 3A). Hence, despite differences not reaching significance in some cases, overall nephritis as assessed

by proteinuria, glomerulonephritis and interstitial nephritis were all reduced by *Rubicon* deficiency, in both murine lupus models and in the context of concomitant *Cybb* deficiency in MRL.Fas^{lpr} mice.

Rubicon deficiency reduces splenomegaly and lymphadenopathy

Spleen weights were decreased in *Rubicon*^{-/-} male and female MRL.Fas^{lpr} mice (Supplemental Figure 3B, top panel). Concordant with these data, total spleen cell counts were reduced in *Rubicon*-deficient B6.Sle1.*Yaa* mice compared to wild-type controls (Supplemental Figure 3C). Axillary lymph node weights were decreased in male mice and trended towards lower weights in female MRL.Fas^{lpr} *Rubicon*^{-/-} mice (p=0.07; Supplemental Figure 3D, top panel). *Rubicon* deficiency reduced spleen weight in male *Cybb*^{-/-} MRL.Fas^{lpr} mice but did not affect spleen weight in females nor LN weight in either gender (Supplemental Figure 3B and 3D, bottom panels).

Rubicon regulates the autoantibody response to RNA, ribonuclear proteins, and cardiolipin

Rubicon deficiency reduced anti-RNA titers in both male B6.Sle1.*Yaa* (Figure 3A left panel) and in both male and female MRL.Fas^{lpr} mice (Figure 3A middle panel). A similar trend was observed in male *Rubicon*-deficient, *Cybb*-deficient MRL.Fas^{lpr} mice (Figure 3A, right panel). Differences may have been more significant were it not for the early deaths of the presumably sickest *Cybb*-knockout mice (Figure 1B and C), as serum collection was only performed at the experimental endpoint.

Anti-Sm titers were reduced in B6. Sle1.*Yaa* male *Rubicon*^{-/-} mice at 3, 6, and 9 months of age (Figure 3B, left panel) and in female B6.Sle1 *Rubicon*^{-/-} mice at 9 months of age (Supplemental Figure 4A). Strikingly, anti-Sm autoantibodies were absent from male *Rubicon*^{-/-} MRL.Fas^{lpr} mice and only one out of 19 female *Rubicon*^{-/-} MRL.Fas^{lpr} mice had a positive anti-Sm titer (Figure 3B, middle panel). Similarly, *Rubicon* deficiency abolishes the anti-Sm response in male *Cybb*-deficient lupus prone mice, and only one out of seven female *Rubicon*^{-/-}*Cybb*^{-/-} female MRL.Fas^{lpr} mice had positive anti-Sm titers (Figure 3B, right panel). Similarly, B6. Sle1.*Yaa* male and B6. Sle1 female *Rubicon*^{-/-} mice had reduced anti-cardiolipin antibody titers at 3, 6, and 9 months of age compared to *Rubicon*-sufficient controls (Supplemental Figure 4B). Intriguingly, no differences in anti-chromatin titers were identified between any of the groups in MRL.Fas^{lpr} or B6.Sle1.*Yaa* mice (Figure 3C). By contrast, anti-chromatin titers were markedly attenuated in female B6.Sle *Rubicon* knockout mice (Supplemental Figure 4C).

Rubicon promotes autoreactive germinal center (GC) reactions

GC formation is associated with lupus progression in B6.Sle1.*Yaa* mice, although it is challenging to determine if GCs are autoreactive in this context. To address whether *Rubicon* affects autoreactive GC evolution, we generated conventional bone marrow chimeras using male CD45 congenic B6.Sle1.*Yaa* mice (Figure 4A, top panel) in which the dominant autoantibody specificity is anti-RNA. To further examine the anti-chromatin response in females, we generated a bone marrow chimera system using the 3H9 transgenic locus (Figure 4A, bottom panel). The 3H9 heavy chain pairs with specific light chains to confer affinity to ssDNA, dsDNA, and cardiolipin but not RNA antigens (51-56). In non-autoimmune mice, tolerance mechanisms prevent the enrichment

of these B cells (56-58). However, in autoimmune mouse strains, this is not the case, resulting in a population of autoreactive B cells that can enter GCs (57-60). Here, we crossed 3H9.Sle1 transgenic mice to either *Rubicon*-sufficient or -deficient B6.Sle1 SLE prone mice, generating donors in which a large fraction of B cells were autoreactive. Mixed bone marrow chimeras were made using the resulting 3H9^{+/+}-Sle1.CD45.2 *Rubicon*-sufficient or -deficient and B6.Sle1 CD45.1 mice (Figure 4 A, bottom panel). At 6 months of age, the B cell compartment in both chimera systems were assessed by FACS (Figure 4B), revealing that *Rubicon*^{-/-} B cells were less able to enter the GC compared to wild-type controls in both chimera systems (Figure 4C).

Rubicon deficiency alters the plasmablast response in SLE mice

Strikingly, CD19^{low-int} CD44⁺ CD138⁺ intracellular κ ^{high} antibody forming cells (AFCs) were reduced in *Rubicon*-deficient male and female MRL.Fas^{lpr} cohorts compared to controls (Figure 4D). There was a trend towards decreased AFCs in double knockout males (p=0.056) but not in females (Supplemental tables 1 and 2). Failure to reach statistical significance in these cohorts is likely due to premature death of *Cybb*-single knockout mice, which had worse disease and thus would likely have had more plasmablasts. *Rubicon* deficiency did not alter the percentage of CD19⁺ total B cells or CD19⁺ CD93⁻ CD23⁺ CD21/35⁻ follicular zone B cells (Supplemental tables 1 and 2). *Rubicon*-deficient female mice had an increased percentage of or CD19⁺ CD93⁻

CD23^{low-int} CD21/35⁺ marginal zone B cells, but a similar difference was not observed in other groups (Supplemental tables 1 and 2).

Rubicon effects on myeloid expansion in female Cybb-deficient SLE prone mice

Others and we have previously reported that global NADPH oxidase deficiency leads to an expansion of the myeloid compartment in both autoimmune and non-autoimmune mouse strains (31, 34, 61). In line with previous data, we observed an increased percentage of splenic macrophages in female *Cybb*^{-/-} mice, compared to controls (Supplemental table 2). Interestingly, *Rubicon* deficiency reduced the percentage of macrophages in female *Rubicon*^{-/-}*Cybb*^{-/-} lupus mice (Supplemental tables 2). *Rubicon* deficiency did not substantially alter the neutrophil, DC, or T cell compartments (Supplemental tables 1 and 2).

Myeloid IL-10 deficiency does not affect murine lupus

LAP is postulated to be immuno-protective via the induction of the anti-inflammatory cytokine IL-10. To directly test this in the context of lupus, we generated and examined *IL-10*^{fl/fl} *LysM* *Cre*^{+/-} and control *IL-10*^{fl/fl} mice on the MRL.Fas^{lpr} background. Such mice would specifically lack IL-10 in the macrophages that conduct LAP.

While the hypothesis that LAP induces IL-10 to suppress lupus would predict that *IL-10*^{fl/fl} *LysM* *Cre*^{+/-} mice would have worse disease, in fact, we did not observe this for any parameter measured. No differences in urine protein, glomerulonephritis, or interstitial nephritis were detected in in SLE prone mice with a myeloid *IL-10* defect (Figure 5A, top panel and Figure 5 B). The incidence of

dermatitis was not different across the groups (Figure 5A, bottom panel). No statistically significant differences in splenomegaly and lymphadenopathy were observed (Figure 5C).

IL-10 was efficiently deleted in splenic neutrophils of *IL-10^{fl/fl} LysM-Cre^{+/-}* mice (77.67% $\pm$ 0.67; Supplemental table 4). Paralleling our prior observation and that of the literature (62), *IL-10* deletion in *IL-10^{fl/fl} LysM-Cre^{+/-}* CD11b⁺F4-80⁺ splenic macrophages was 43.48% ($\pm$ 2.62; Supplemental table 3). As expected, *IL-10* deletion in CD19⁺ B was below the limit of detection (Supplemental table 3). There was no correlation between neutrophil or macrophage IL-10 deletion efficiency and either proteinuria score, glomerulonephritis score, or interstitial nephritis score (Supplemental Figure 5).

Myeloid IL-10 genotype did not substantially alter the autoantibody response, with one notable exception. We did not detect any differences in anti-RNA antibody (Figure 5D, left panel) or anti-nucleosome antibody (Figure 5D, right panel) titers. Female *IL-10^{fl/fl} LysM Cre^{+/-} MRL.Fas^{lpr}* had lower anti-Sm titers than *IL-10*-sufficient controls (Figure 5D, middle panel). Myeloid IL-10 deficiency did not impact immune composition in the setting of SLE as no differences in splenic B cell, T cell, macrophage, neutrophil, and DC subsets were identified across all groups (Supplemental table 4).

Neither Rubicon nor Cybb is required for LC3-associated phagocytosis

The finding that RUBICON and CYBB have opposite effects on lupus-like disease was unexpected, since both proteins are thought to be required for LAP, and the absence of LAP is

thought to promote lupus (22, 35). This caused us to revisit the requirement of each of these proteins for LAP itself. We investigated these roles in both non-autoimmune (B6) and autoimmune (B6.Sle1.*Yaa* and MRL.Fas^{lpr}) genetic backgrounds. To this end, bone-marrow derived macrophages (BMDMs) or peritoneal macrophages were produced from each of the genetic backgrounds and incubated with zymosan-containing particles to stimulate LAP. We chose 60-90 minute time points as LAP is reported to peak in zymosan stimulated macrophages within this period (37, 38). Cell lysates were then assayed by western blot for the presence of lipidated LC3 β (LC3 β -II), a key molecular event in LAP (22, 38). As expected, zymosan-containing particles caused robust LC3 β -II accumulation in wild type B6 and B6.Sle1.*Yaa* mice (Figures 6A and B; Supplemental Figure 6). However, unexpectedly, it also caused similar accumulation in mice deficient in either RUBICON (Figure 6A and Supplemental Figure 6) or CYBB (Figure 6B). Similar findings were obtained with BMDMs derived from MRL.Fas^{lpr} mice lacking RUBICON or CYBB (Figure 6C and Supplemental Figure 7). Robust LC3 β -II accumulation was sustained in RUBICON- and CYBB-deficient BMDMs in both autoimmune and non-autoimmune strains at 180 minutes post stimulation (Supplemental Figure 8). To address whether the SLE microenvironment affected whether RUBICON or CYBB were required to mediate LAP, we isolated peritoneal macrophages from 18 week-old, diseased MRL.Fas^{lpr} mice. Again, LC3 β -II was induced in wild-type and both *Cybb*- and *Rubicon*-knockouts (Supplemental Figure 9). Hence, in contrast to prior reports, we could find no evidence that either CYBB or RUBICON was required for zymosan-induced LC3 β -II accumulation in macrophages, a central assay for the detection of LAP.

Discussion

NADPH oxidase-deficient SLE prone mice develop more severe SLE than NADPH-oxidase sufficient counterparts and die prematurely (31, 32, 34). Here, we sought to determine whether exacerbated disease in the setting of NADPH oxidase deficiency is due to a failure in LAP, which was reported to require NADPH oxidase (22) and is thought to normally protect from lupus via effective and immunologically silent degradation of dead cells (35). To address this question, we genetically deleted *Rubicon*, another gene that was reported as a required mediator of LAP, in two SLE mouse models (22). If the LAP hypothesis were correct, genetic deletion of *Rubicon* should phenocopy *Cybb*-deficiency in SLE prone mice and exacerbate the lupus phenotype. In addition, deletion of both genes in the same pathway should have given a similar phenotype to each of the single phenotypes. Our actual findings stood in marked contrast to this model: *Rubicon*-deficient B6.Sle1.*Yaa* and MRL.Fas^{lpr} mice did not develop worsened clinical or immunological manifestations of SLE, as is the case for *Cybb*-deficient MRL.Fas^{lpr} mice. Rather, *Rubicon* deletion increased survival, reduced nephritis, and decreased autoantibody production in B6.Sle1.*Yaa* lupus mice. Moreover, *Rubicon* deficiency increased survival and ameliorated both glomerulonephritis and interstitial nephritis in *Cybb*^{-/-} SLE prone mice. Thus, we have established RUBICON as a new regulatory molecule in SLE pathogenesis.

These unexpected results led us to probe whether RUBICON and CYBB are essential for LAP, as had been reported by Martinez and colleagues (22). To test if *Rubicon*^{-/-} and *Cybb*^{-/-} MRL.Fas^{lpr} macrophages were deficient in LAP, we stimulated macrophages with the canonical LAP inducer, zymosan bioparticles. To our surprise, zymosan bioparticles induced the lipidation of LC3 β -I to form LC3 β -II, a molecular readout for the induction of LAP, in macrophages from both young

pre-diseased and aged *Rubicon*^{-/-} and *Cybb*^{-/-} MRL.Fas^{lpr} mice and in macrophages from *Cybb*^{-/-} and *Rubicon*^{-/-} C57BL/6 mice. Taken together, while we confirm the phenomenon of LAP, our studies highlight a potential flaw with the current view of the proteins required for this process. Importantly, our work indicates that RUBICON may be working through a LAP-independent mechanism to augment SLE pathogenesis.

As our data indicate that CYBB and RUBICON are not required for LAP, the role of LAP in SLE remains ambiguous. Production of IL-10 downstream of dead cell engulfment is thought to be a primary mechanism by which LAP protects against the immune response to self. In fact, our group identified macrophages and T cells, but not B cells, as major producers of IL-10 in murine lupus (63). To determine the role of myeloid IL-10, and by extension LAP, in the *in vivo* disease setting, we genetically deleted *IL-10* in neutrophils and macrophages in MRL.Fas^{lpr} mice by a Cre-lox approach, utilizing *LysM-Cre*. *LysM-Cre* mediated IL-10 deletion did not alter SLE pathogenesis. Because LysM-Cre is not efficient in all macrophage populations (62), it is possible that partial deletion of *IL-10* in the myeloid compartment is not sufficient to modulate SLE. However, we believe this explanation to be less likely for two reasons. First, both *IL10*^{-/-} and *IL10*^{+/-} MRL.Fas^{lpr} mice developed more severe renal disease and dermatitis compared to their *IL-10* intact counterparts, indicating that a 50% reduction in global IL-10 gene dose exacerbates SLE (64). Second, Martinez and colleagues (22, 35) used *LysM-Cre* to target multiple LAP genes in macrophages, which was sufficient to demonstrate both reduced LAP and an autoinflammatory phenotype in these animals. Considering these points, we conclude that myeloid IL-10 production, downstream of LAP or other processes, is not a major regulatory mechanism augmenting systemic autoimmunity in our IL-10 sensitive model (64). Moreover, as our prior (63) and current work do

not show a role for myeloid and B cell-derived IL-10 in mediating lupus pathogenesis, by elimination it is likely that T cell-derived IL-10 is the source of IL-10 regulating disease in MRL.Fas^{lpr} mice.

Strikingly, RUBICON deficiency regulates the formation of antibodies towards RNA, ribonucleoproteins, and cardiolipin autoantigens in the context of SLE. We have previously reported that *Cybb*-deficient MRL.Fas^{lpr} mice develop elevated anti-RNA and anti-Sm titers (31). Further, *Rubicon* deletion abrogates the anti-Sm response in both *Cybb*-sufficient and -deficient SLE prone mice. These findings may provide a clue as to how RUBICON deficiency constrains autoimmunity. The autoantibody profile in *Rubicon*-deficient SLE prone mice resembles the autoantibody response observed in *Tlr7*^{-/-} MRL.Fas^{lpr} mice, which lack anti-RNA and anti-ribonucleoprotein but make antibodies to DNA and chromatin (65, 66). Similarly to *Rubicon*-deficient SLE prone mice, *Tlr7*^{-/-} MRL.Fas^{lpr} mice are protected from renal manifestations of SLE (65, 66). It is thus possible that RUBICON and TLR7 promote disease by similar or interrelated mechanisms. TLR7 and RUBICON both localize to the endosome and it is plausible that RUBICON could regulate TLR7 trafficking or signaling in B cells (22, 67-69). Furthermore, RNA must traffic to the endosome to be detected by endosomal TLR receptors, such as TLR7. One mechanism by which RUBICON could modulate SLE pathogenesis is through trafficking of RNA cargo. Indeed, ATG5, an E3 ligase required for both autophagy and LAP, has been implicated in RNA trafficking to TLR containing endosomes in DCs (70). A similar mechanism could deliver RNA cargo to endosomal TLR7s in B cells, thus affecting the development of TLR7-dependent antibodies in SLE. Supporting this idea, B-cell specific deletion of *Atg5*, improves both survival and renal disease in the TLR7 transgenic model (TLR7.1 Tg) of SLE (71). Interplay between

RUBICON, RNA trafficking, and TLR7 signaling in lupus is thus an intriguing possibility suggested by our data.

B6.Sle1.*Yaa Rubicon*^{-/-} mice transgenic for the 3H9 Vh region that encodes lupus related autoantibody specificities show a defect in selection of 3H9 B cells into the GC compartment. These findings suggest that RUBICON enables activation of at least some types of self-reactive B cells. However, in our global RUBICON-deficient mice, is not clear in which cell types RUBICON is acting. Raso *et al.* connected the loss of integrin α_v in B cells with a loss of RUBICON-dependent non-canonical autophagy in B cells (72); however, these studies did not look at global *Rubicon*^{-/-} phenotypes in the setting of autoimmunity and failed to convincingly demonstrate that RUBICON was necessary for LC3 β -II formation in B cells (Fig. 5 of ref. 60).

Importantly, our data implicate RUBICON as a novel mediator of systemic autoimmunity, an intriguing finding that may have therapeutic implications for SLE patients. RUBICON and CYBB instead function antagonistically to each other, with CYBB restraining and RUBICON promoting disease, contrary to the original hypothesis that the two molecules work in concert to mediate LAP (22). Finally, and of substantial importance to the field, we find that LC3 β -II formation, a lynchpin of LAP, does not depend on either CYBB or RUBICON, which should lead to a reevaluation of our fundamental understanding of this process.

Materials and Methods

Detailed supplemental methods are available online.

Mice

Rubicon knockout mice on the C57BL/6 background were a kind gift from Dr Douglas Green (St Jude Children's Research Hospital, Memphis, TN). *Rubicon*^{-/-} mice were crossed to the B6.Sle1.*Yaa* strain and genotyped for Dm15, 17 and 47 (<https://www.jax.org/strain/021569>). Serum was obtained monthly for measurement of autoantibodies and urine was obtained monthly for measurement of proteinuria by dipstick (Multistix, Fisher). Groups of mice were euthanized at 8-11 or 19-21 months of age to assess SLE pathology.

Rubicon-deficient MRL.Fas^{lpr} mice were generated by *in vitro* fertilization and CRISPR-Cas9 technology as previously described by replacing Asp188 with a premature stop codon (22). To generate mice for experimental cohorts, we intercrossed: (1) *Rubicon*^{-/+}Cybb^{y/-} X *Rubicon*^{-/+}Cybb^{+/-}, (2) *Rubicon*^{-/+}Cybb^{y/-} X *Rubicon*^{-/+}Cybb^{+/-}, and (3) *Rubicon*^{-/+} X *Rubicon*^{-/+}. This breeding produced littermate controls for each group. SLE pathology was assessed at 16-18 weeks of age.

IL-10^{fl/fl} C57BL/6 mice (73) were backcrossed to the MRL.Fas^{lpr} strain for at least 9 generations (63). To generate mice for experimental cohorts, we intercrossed *LysM-Cre*^{+/-} *IL-10*^{fl/fl} to *IL-10*^{fl/fl}. This breeding allowed us to use littermate controls for each group. SLE pathology was assessed at 18 weeks of age.

All mice were housed under specific-pathogen-free (SPF) conditions.

Evaluation of SLE pathology

MRL.Fas^{lpr} and B6.Sle1.Yaa SLE cohorts were analyzed as previously described (31, 74-77).

Bone Marrow chimeras

CD45.1 Sle1.Yaa male and female mice were irradiated and male recipients were reconstituted with mixed bone marrow from CD45.2 Sle1.Yaa. *Rubicon*^{-/-} and CD45.1 Sle1.Yaa donors in a 1:1 ratio. Female recipients received mixed bone marrow from CD45.1 Sle1 and CD45.2 3H9.Sle1. *Rubicon*^{-/-} donors in a 2:1 ratio. Recipients were monitored for >6 months and were euthanized once anti-chromatin antibodies appeared in the serum.

Induction of LAP

To induce LAP, peritoneal macrophages or BMDMs were stimulated with zymosan bioparticles (ThermoFisher) 8:1 (particles/cell) at indicated time points. Inert BSA conjugated polystyrene beads or unstimulated conditions were used as negative controls.

Immunoblotting

Lysates were analyzed by SDS-PAGE. Immunodetection was achieved using the following antibodies: LC3 β (Cell Signaling; D11, 1:1000), RUBICON (Cell Signaling; D9F7, 1:1000), β -Actin HRP (Cell signaling; 8H10D10, 1:10,000), Anti-Rabbit IgG HRP (Cell signaling, 1:10,000). Proteins were visualized by an ECL chemiluminescence reagent and imaged by a Protein Simple imager.

Statistics

Statistical analysis was performed using Prism 8.0 (Graphpad). A log-rank test was used to determine statistical significance between Kaplan Meier curves. Linear regression was used to determine correlation between disease parameter and deletion efficiency in indicated cell type. A two-tailed Mann-Whitney U test, two tailed Student T test, two-tailed Welch's t test, and a Fishers Exact test were performed where indicated and appropriate. A p value <0.05 was considered statistically significant.

Study Approval

Animal studies were approved by the University of Pittsburgh Institutional and Feinstein Institutes for Medical Research Institutional Animal Care Use Committees.

Author Contributions

RG, CG, CR, AM, WH, RC, and JT performed experiments and analyzed data. SB performed histopathological evaluation of the kidneys. RG, AD, and MS designed experiments and wrote the manuscript. KM provided intellectual support.

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Figure Legends

Figure 1. The absence of RUBICON confers a survival advantage in SLE prone mice. (A-C)

Kaplan Meier survival curves for (A) B6.Sle1.*Yaa*, (B) male MRL.Fas^{lpr}, and (C) female MRL.Fas^{lpr} SLE mice of indicated genotypes. A log-rank test was used to determine statistical significance between Kaplan Meier curves (*p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001 and B6.Sle1.*Yaa* wild-type males n=8; B6.Sle1.*Yaa* *Rubicon*^{-/+} males n=9; B6.Sle1.*Yaa* *Rubicon*^{-/-} males n=28; MRL.Fas^{lpr} wild-type males n=28; MRL.Fas^{lpr} wild-type females n=16; MRL.Fas^{lpr} *Rubicon*^{-/-} males n=29; MRL.Fas^{lpr} *Rubicon*^{-/-} females n=27; MRL.Fas^{lpr} *Cybb*^{-/-} males n=20; MRL.Fas^{lpr} *Cybb*^{-/-} females n=10; MRL.Fas^{lpr} *Rubicon*^{-/-}*Cybb*^{-/-} males n=16; MRL.Fas^{lpr} *Rubicon*^{-/-}*Cybb*^{-/-} females n=11 mice per group).

Figure 2. *Rubicon* deficiency protects MRL.Fas^{lpr} and B6.Sle1.*Yaa* SLE mice from renal disease. (A)

Kaplan Meier plot depicting onset of proteinuria in B6.Sle1.*Yaa* mice (top panel). Proteinuria scores in MRL.Fas^{lpr} mice (middle and bottom panels) (B6.Sle1.*Yaa* wild-type and *Rubicon*^{+/-} males n= 17; B6.Sle1.*Yaa* *Rubicon*^{-/-} males n=28; MRL.Fas^{lpr} wild-type males n=27; MRL.Fas^{lpr} wild-type females n=16; MRL.Fas^{lpr} *Rubicon*^{-/-} males n=29; MRL.Fas^{lpr} *Rubicon*^{-/-} females n=27; MRL.Fas^{lpr} *Cybb*^{-/-} males n=11; MRL.Fas^{lpr} *Cybb*^{-/-} females n=5; MRL.Fas^{lpr} *Rubicon*^{-/-}*Cybb*^{-/-} males n=18; MRL.Fas^{lpr} *Rubicon*^{-/-}*Cybb*^{-/-} females n=9 mice per group). **(B)** Glomerulonephritis scores. **(C)** Interstitial nephritis scores. Scores are represented as a function of *Rubicon* and *Cybb* genotype. Renal pathology was evaluated in B6.Sle1.*Yaa* wild-type and B6.Sle1.*Yaa* *Rubicon*^{-/-} mice at 8-11 and 19-21 months of age respectively (☒ denotes mice > 19 months of age). Proteinuria, GN, and IN were evaluated at 16-18 weeks of age in MRL.Fas^{lpr}

unless otherwise indicated. Bars represent the median $\pm$ interquartile range (IQR). A log-rank test was used to determine statistical significance between Kaplan Meier curves. A Mann-Whitney U test was performed to determine statistical significance within each gender. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and B6.Sle1.*Yaa* wild-type and *Rubicon*^{+/-} males $n = 5$; B6.Sle1.*Yaa* *Rubicon*^{-/-} males $n = 11$; MRL.Fas^{lpr} wild-type males $n = 21$; MRL.Fas^{lpr} wild-type females $n = 8$; MRL.Fas^{lpr} *Rubicon*^{-/-} males $n = 19$; MRL.Fas^{lpr} *Rubicon*^{-/-} females $n = 20$; MRL.Fas^{lpr} *Cybb*^{-/-} males $n = 11$; MRL.Fas^{lpr} *Cybb*^{-/-} females $n = 5$; MRL.Fas^{lpr} *Rubicon*^{-/-}*Cybb*^{-/-} males $n = 15$; MRL.Fas^{lpr} *Rubicon*^{-/-}*Cybb*^{-/-} females $n = 9$ mice per group unless otherwise indicated).

Figure 3. RUBICON regulates the autoantibody response to RNA-associated autoantigens (A-C) Serum anti-RNA (A), anti-Sm (B), and anti-chromatin (B6.Sle1.*Yaa*) or anti-nucleosome (MRL.Fas^{lpr}) titers (C) in B6.Sle1.*Yaa* (left panel) and MRL.Fas^{lpr} (middle and right panels) mice. B6.Sle1.*Yaa* titers are represented as a function of *Rubicon* genotype at 3 (B6.Sle1.*Yaa* wild-type and *Rubicon*^{+/-} males $n = 9-12$; B6.Sle1.*Yaa* *Rubicon*^{-/-} males $n = 14-18$), 6 (B6.Sle1.*Yaa* wild-type and *Rubicon*^{+/-} males $n = 20-25$; B6.Sle1.*Yaa* *Rubicon*^{-/-} males $n = 20-26$), 9 (B6.Sle1.*Yaa* wild-type and *Rubicon*^{+/-} males $n = 6-9$; B6.Sle1.*Yaa* *Rubicon*^{-/-} males $n = 10-15$), and 12 (B6.Sle1.*Yaa* wild-type and *Rubicon*^{+/-} males $n = 3-7$; B6.Sle1.*Yaa* *Rubicon*^{-/-} males $n = 12$) months of age. MRL.Fas^{lpr} antibody titers are represented as a function of *Rubicon* and *Cybb* genotypes at 16-18 weeks of age (MRL.Fas^{lpr} wild-type males $n = 21$; MRL.Fas^{lpr} wild-type females $n = 8$; MRL.Fas^{lpr} *Rubicon*^{-/-} males $n = 19$; MRL.Fas^{lpr} *Rubicon*^{-/-} females $n = 20$; MRL.Fas^{lpr} *Cybb*^{-/-} males $n = 11$; MRL.Fas^{lpr} *Cybb*^{-/-} females $n = 5$; MRL.Fas^{lpr} *Rubicon*^{-/-}*Cybb*^{-/-} males $n = 15$; MRL.Fas^{lpr} *Rubicon*^{-/-}*Cybb*^{-/-} females $n = 9$ mice per group). Bars represent the median $\pm$ IQR. A Mann-Whitney U test was performed to determine statistical significance within each gender unless otherwise indicated.

Dashed lines represent the limit of detection of the Anti-Sm ELISA. A Fisher's Exact test was performed to determine statistical significance for anti-Sm titers in MRL.Fas^{lpr} mice (*p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001).

Figure 4. RUBICON is necessary for the germinal center reaction

(A, top panel) Mixed bone marrow chimeras were generated with male B6.Sle1.Yaa CD45.2 wild-type or *Rubicon*-knockout and CD45.1 B6.Sle1.Yaa wild-type donors. CD45.1 B6.Sle1.Yaa irradiated recipients were reconstituted with the aforementioned donors at a 50/50 ratio. Mice were aged for > 6 months until the presence of anti-chromatin antibodies were detected by ELISA at which time the mice were euthanized. Reconstitution of the splenic B cell and germinal center (GC) compartments were analyzed by FACS. **(A, bottom panel)** Mixed bone marrow chimeras were generated by reconstituting female irradiated CD45.1 B6.Sle1 recipients with CD45.2 3H9 B6.Sle1 *Rubicon*-sufficient or -deficient and CD45.1 B6.Sle1 wild-type donors at a ratio of 1:2. Mice were analyzed as in A (top panel). **(B)** FACS gating strategy for total splenic B cell (CD19⁺) and GCs (CD19⁺CD95⁺GL7⁺). CD45.1 and CD45.2 congenic markers were used to differentiate each donor. **(C)** Ratio of the fraction of CD45.2 GC B cells to the fraction of CD45.2 total B cells in the conventional (strategy I) and 3H9 (strategy II) mixed bone marrow chimeras (n=6 per group). **(D)** Percentages of live cells that are TCRβ⁻ CD44⁺ CD138⁺ intracellular k⁺ antibody forming cells (AFCs) in spleens of wild-type or *Rubicon*-knockout MRL.Fas^{lpr} mice (wild-type males n=20; MRL.Fas^{lpr} wild-type females n=8; MRL.Fas^{lpr} *Rubicon*^{-/-} males n=19; MRL.Fas^{lpr} *Rubicon*^{-/-} females n=18 mice per groups). Bars represent the mean ± SEM. A Student T test was performed to determine statistical significance (*p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001).

Figure 5. Myeloid IL-10 deficiency does not impact clinical or immunological manifestations in MRL.Fas^{lpr} mice. (A) Proteinuria (top panel) and dermatitis scores (bottom panel). (B) Glomerulonephritis (top panel) and interstitial nephritis (bottom panel) scores. (C) Spleen (top panel) and axillary lymph node (bottom panel) weights. (D) Anti-RNA (left panel), anti-SM (middle panel), and anti-nucleosome (right panel) antibody titers. Bars represent the median $\pm$ IQR. Data are represented as a function of *IL-10^{fl/fl} LysM Cre* genotype at 16-18 weeks of age (*IL-10^{fl/fl}* males n=23-25; *IL-10^{fl/fl} LysM^{cre/-}* males n= 34; *IL-10^{fl/fl}* females n=26; *IL-10^{fl/fl} LysM^{cre/-}* females n= 13). A Mann-Whitney U test was performed to determine statistical significance within each gender unless otherwise indicated. A Fisher's Exact test was performed to determine statistical significance for anti-Sm titers in MRL.Fas^{lpr} mice (*p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001).

Figure 6. Rubicon and Cybb-deficient macrophages can undergo LC3-associated phagocytosis (A and B) Bone marrow derived macrophages (BMDMs) generated from (A) *Rubicon*- sufficient and -deficient B6.Sle1.*Yaa* or (B) *Cybb*- sufficient or -deficient C57BL/6 mice were left untreated, stimulated with inert polystyrene-BSA beads (8 beads: 1 cell), or stimulated with zymosan bioparticles (8 particles: 1 cell) for 90 minutes. LC3b-I (top band) and LC3b-II (bottom band) were analyzed by immunoblot. LC3b-II bands were quantitated by densitometry and normalized to b-actin loading controls. These ratios were then normalized to the unstimulated condition within each genotype, given a value of 1. Bars represent the mean $\pm$ SEM. A Student T test was performed to determine statistical significance (n=3 per group). (C) BMDMs generated from 6-8 week old wild-type, *Cybb*^{-/-}, and *Rubicon*^{-/-} MRL.Fas^{lpr} mice were stimulated and analyzed as in A. Bars

represent normalized densitometry measurements for an individual experiment. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).