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TITLE: The Function of Renal Macrophages in Lupus Nephritis

PRINCIPAL INVESTIGATOR: Anne Davidson

CONTRACTING ORGANIZATION: Feinstein Institute for Medical Research, Manhassett, NY

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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 addresses 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. We are in the process of determining how ATG14 deficiency in B cels influences disease. We also investigated the role of PGC-1α 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:** We are in the final year of this grant. Our goal was to use a systems approach to understand key features that are relevant to the diagnosis and treatment of human lupus nephritis. Renal infiltration with macrophages is one of the few histologic features associated with poor prognosis in humans – therefore our proposal focused on these cells. We proposed both a discovery and a functional component to our studies. In the discovery component we have been using single cell RNA sequencing to determine the heterogeneity of renal macrophage subsets and the changes that occur when they enter the kidneys. In collaboration with the Hacohen laboratory at the Broad Institute, 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, we are setting up systems to study the role of shared transcription factors in renal macrophages. In the functional component, we proposed to study the role of autophagy and mitochondrial dysfunction in these cells with the long term goal of understanding how targeting of dysfunctional macrophages in LN can lead to improved outcomes and a decrease in progression to chronic renal impairment. We found, unexpectedly, that Rubicon deficiency protects mice from disease and 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 have generated mice with B cell deficiency of ATG14 to determine the role of classical autophagy in B cells. We have also generated uMT Sle1 mice so that we can study the role of Rubicon deficiency specifically in B cells.

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 will be submitted by the end of the 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.

      This goal is still in progress and we are following up with studies to determine how these pathways influence autoantibody production. A manuscript describing the effect of Rubicon deficiency has been submitted.

      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

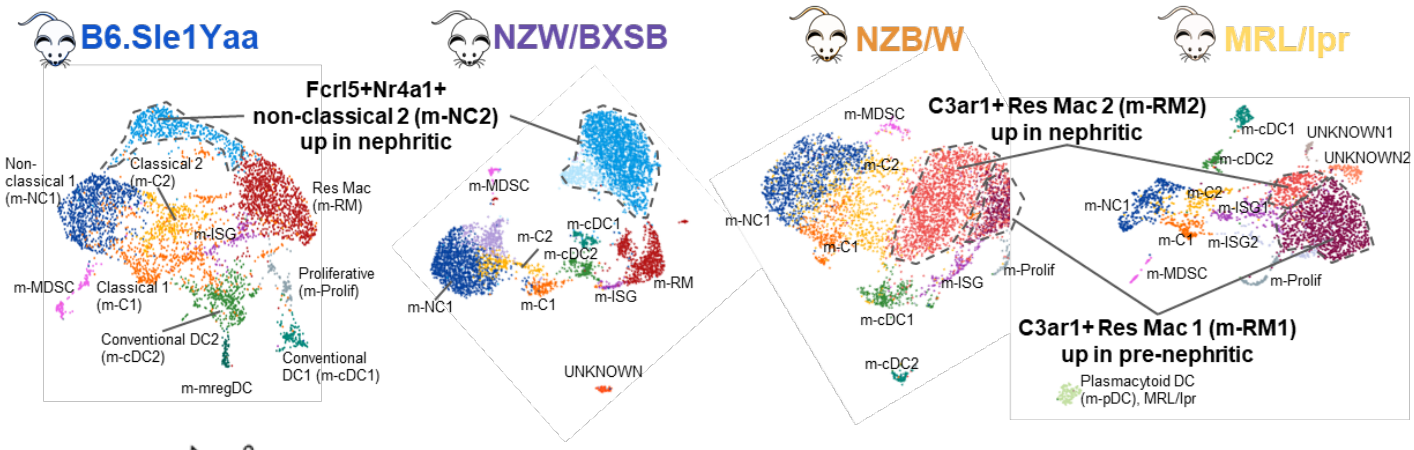
Major Tasks Specific Aim 1	Single cell RNASeq (Broad) and data processing (NYGC)
Molecular characterization of single cells	
Data analysis is finished and m*anuscript almost complete. A new DOD grant was awarded for continuation of these studies and a new RO1 is being revised and resubmitted (priority score 17)*	
Changes in macrophage function over time	The single cell experiment needs to be completed.
Major Tasks Specific Aim 2A	The mice are generated and analyses of Rubicon mice are completed. ATG14 macrophage specific KO mice have no change in renal outcome. B cell deficient ATG14 KO mice have been generated and are being monitored. New grant planned to study Rubicon deficiency
Generate Rubicon and ATG14 deficient mice and follow for nephritis onset. Accelerate disease if necessary	
Manuscript submitted (unexpected effect of Rubicon deficiency on B cells)	
Major Task 3 Specific Aim 2B	uMT knockouts generated so that chimeras can be made 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	
Completed	

What was accomplished under these goals?

Aim 1A: In this first section we used single cell PCR to define the heterogeneity of macrophage subsets in the lupus kidney in several mouse models and compare this with data from human kidneys. We successfully performed two 10X experiments with hashing at NYGC using NZB/W and Sle1.Yaa mice. This allowed us to pool 4 samples to decrease the risk of batch effects. In each experiment we used PBMC from blood, renal myeloid cells from young mice and renal myeloid cells from nephritic mice. We formed an extremely successful

collaboration with the Hacohen lab at the Broad Institute, added two more 10X experiments in NZB/W and Sle1.Yaa mice in which we analyzed all renal immune cells and extended our studies to add 2 more lupus strains. Extensive bioinformatic analyses have now been completed on all 4 strains and comparisons made with human data from AMP Phase 1 as well as initial comparative analyses with data from AMP Phase 2.

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



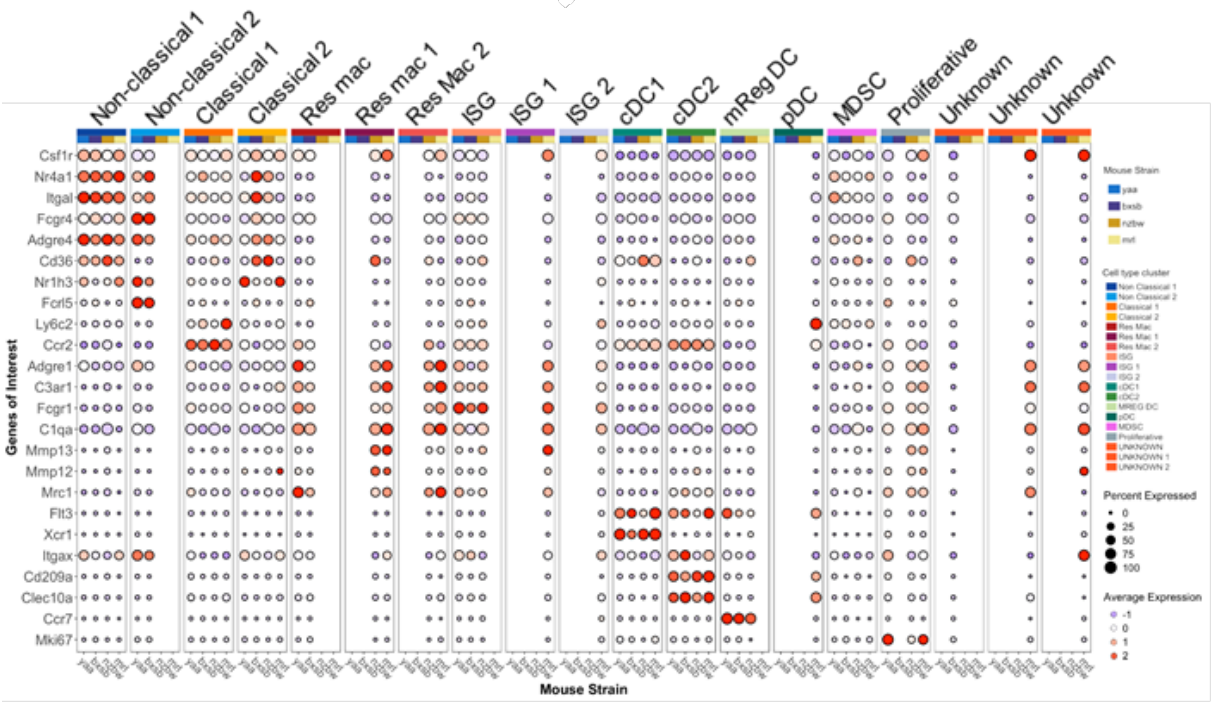


Figure 1: (above) UMAPs of kidney myeloid cell clusters from 4 different mouse strains

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

While most of the cell subsets are shared among the strains, we have found 2 major differences between the two strains overexpressing TLR7 (Sle1.Yaa and NZW/BXSB) and the two other strains (NZB/W and MRL/lpr).

1. The two TR7 over expressing strains have a novel and dominant 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.

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. Now that we have a better idea of the heterogeneity of the renal myeloid subsets, we have first established flow cytometry protocols to identify renal subsets based on the single cell data described above. Using the marker set in the figure below we can clearly identify and sort resident macrophages, classical macrophages and non-classical macrophages type 2 from Sle1.Yaa mice and are continuing to refine the multi-color flow to classify all the subsets. Examples are shown in Figure 3. This 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.

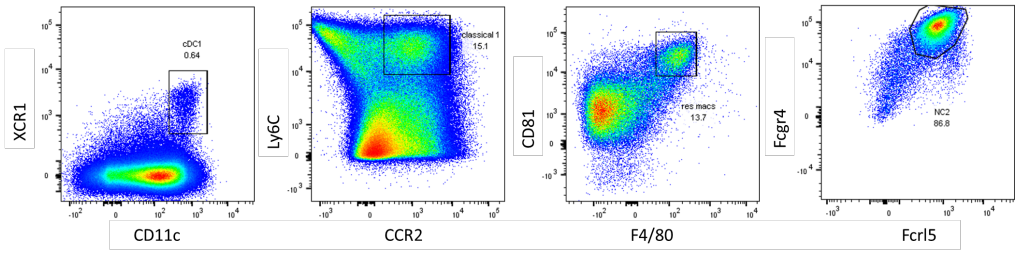


Figure 3: Flow cytometry identification of cDC1 (A), classical monocytes (B), resident macrophages (C) and non-classical monocytes 2 (D).

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 with 25 clusters identified as shown in Figure 4. Nevertheless, the initial observations that we made in Phase 1 of the AMP have been replicated and there is still substantial overlap of the human data with the mouse data. 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. 160 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, especially between non-classical monocytes and human CM0/CM1, resident macrophages and human CM4, classical monocytes and human CD14+ monocytes and correspondence of the dendritic cell subsets. Furthermore, there are several subsets that correlate with activity and chronicity scores. In particular, CM1 correlates with disease activity whereas CM4 and cDC2 correlate with disease chronicity and poorer outcomes. We have further localized CM0 and CM1 cells to glomeruli in both mice and humans and CM4 cells to the interstitium in both species.

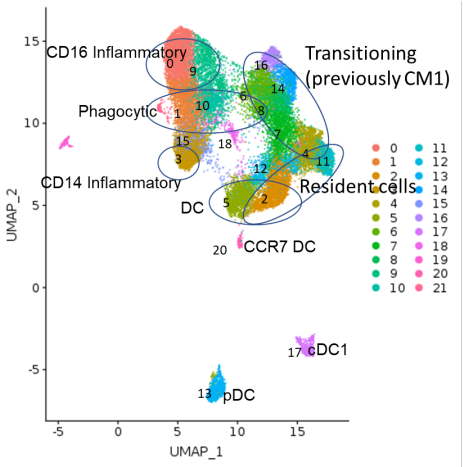


Figure 4: UMap of myeloid cell subsets identified in AMP Phase 2. Subsets overlapping with the mouse subsets are identified

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. While we initially planned to target transcription factors using CRISPR libraries to transduce Cas9-expressing Sle1.Yaa bone marrows, Dr Hacohen has discovered that all available Cas9-expressing mice have an innate immune defect that is due to insertion of the Cas9 locus. We will therefore be optimizing an

alternative system in which both Cas9 and guide RNAs are transduced simultaneously using a lentiviral system.

Table 1: Overlapping transcription factors in non-classical monocytes and resident macrophages from humans and at least 2 mouse strains

Non-classical	Sle1.Yaa	NZW.BXSB	NZB/W	Mrl	Resident	Sle1.Yaa	NZW.BXSB	NZB/W	Mrl
Cebp1	x	x			Maf	x	x	x	x
Nr4a1	x	x	x	x	Mef2c	x			x
Pou2f2		x	x	x	Zfhx3	x	x		x
Klf4	x	x	x	x	Mxd4	x			x
Klf2	x	x	x	x	Creb5	x	x		x
Plagl2	x	x	x	x					
Tcf7l2	x	x							
Ikzf1	x		x	x					
Ets1	x			x					
Nfat5	x			x					
Bcl6			x	x					

Our data has led us to several different hypotheses that we are now ready to test. 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 will proceed beyond the end of this granting cycle, they are the functional experiments that will form the basis for our new RO1 proposal.

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 have been working with the Shlomchik laboratory on these mice and the manuscript has now been submitted. We have bred the 3H9 autoreactive transgene into this mouse strain and we similarly see a marked delay in the emergence of anti-DNA antibodies. This will allow us to study the repertoire of the B cells to determine whether the defect is in germinal center entry, B cell differentiation into plasma cells, somatic mutation or some other mechanism. We are planning a new R21 grant based on these studies.

We have now generated uMT Rubicon deficient Sle1.Yaa mice. These mice will be reconstituted with Rubicon sufficient B cells and this will allow us to study the effect of Rubicon deficiency in renal macrophages once the mice develop nephritis. We will also transfer Rubicon deficient B cells into uMT deficient Sle1.Yaa mice to further analyze whether the Rubicon mediated protection is B cell intrinsic.

We have 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 have completed the autoantibody profiles in these mice and there are no differences between KO mice and wild type mice. RNASeq analysis of sorted macrophages is pending data analysis. We have generated ATG KO mice using a B cell Cre and these mice are now being followed to determine their clinical outcome.

Aim 2C: Completed. Nothing to report this year

We have obtained further funding from the DOD to continue the experiments started in Aim 1 and are revising a RO1 submission that received a 17 percentile score. We are planning a R21 to continue the experiments in Aim 2.

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 this year. He presented this work at the ACR meeting in 2020 and at an NIH meeting on mouse models of lupus in 2021.

My student Mr. Chirag Raparia was admitted to the Graduate School at Feinstein and will be working on the renal myeloid cells and the Rubicon and ATG14 models. He attended the AAI meeting in 2021.

How were the results disseminated to communities of interest?

Abstracts were presented at the ACR meeting in 2020 and 2021.

One manuscript is submitted and the other is almost complete

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

We are in the last year of this proposal

Aim 1: We will complete the manuscript described above and continue to work on the experiments outlined in Aim 1B and 1C. We are also planning new grant applications.

Aim 2A and 2B. We will continue to work on completing the studies in ATG14 and Rubicon deficient mice. New grant applications are planned for 2022.

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?

Completed data is being reported in manuscripts as described above. 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.

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:

We have not made major changes this year. The following minor changes are

a. We are continuing to extend the studies in Aim 1

b. We added B cell deficient ATG14 KO mice and generated uMT deficienct Sle1 mice in order to complete a more extensive characterization of the role of autophagy in both B cells and macrophages given the unexpected phenotype of the Rubicon KO mice.

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.

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

Promise and complexity of lupus mouse models.
Moore E, Reynolds JA, **Davidson A**, Gallucci S, Morel L, Rao DA, Young HA, Putterman C.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. Manuscript submitted

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

Nothing to report

Other publications, conference papers, and presentations.

Mouse models of SLE (NIH conference) December 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?

Haiou Tao

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

Funding Support:

Ke Lin

Project Role: *Senior technician*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *2*

Contribution to Project: *Working on Aim 1*
Funding Support: *Also partly funded by Feinstein funds to the Davidson laboratory*

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: *Also partly funded by Feinstein funds to the Davidson laboratory*

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*

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

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 abstract

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 Hoover[1], Michael Peters[2], David Lieb[2], Runci Wang[3], Garett Dunlap[4], Deepak Rao[1], Nir Hacohen[2] and Anne Davidson[5], [1]Brigham and Women's Hospital, Boston, MA, [2]Broad Institute, Cambridge, MA, [3]Brigham and Women's Hospital, Boston, MA, [4]Harvard University, Somerville, MA, [5]Institute 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

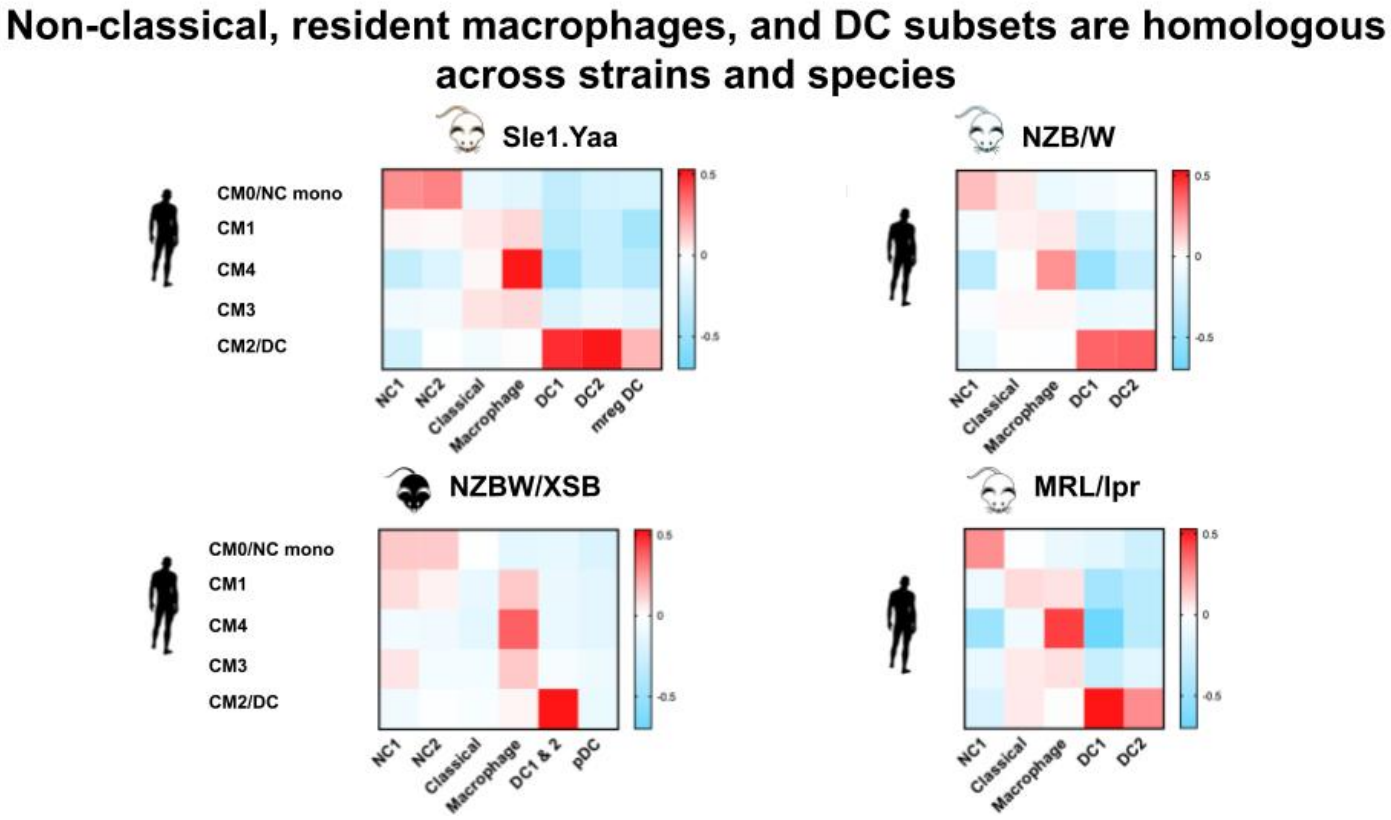
Background/Purpose: We discovered 21 immune cell-types in lupus nephritis kidney biopsies as part of the Accelerating Medicines Partnership (AMP) consortium. These immune cells are the basis of new hypotheses about the drivers of disease. However, we cannot feasibly collect immune cells from human kidney 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.

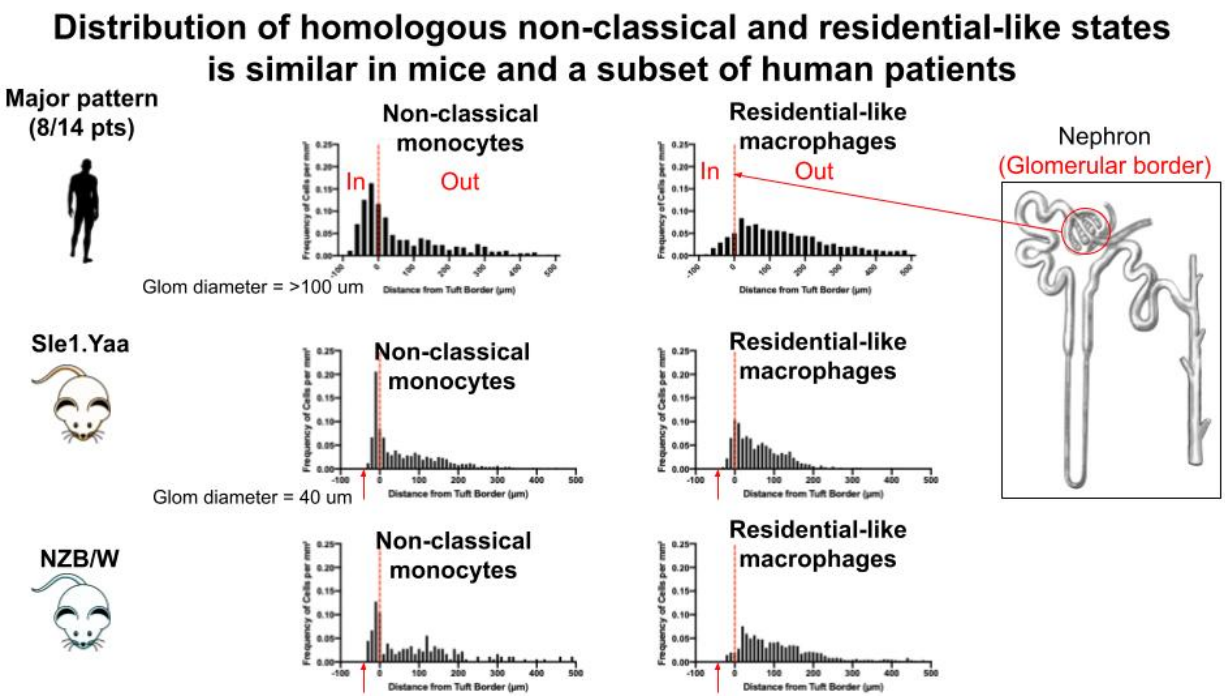
Methods: Using AMP protocols we sorted CD45+ cells from dissociated mouse kidneys with spontaneous adaptive-driven autoimmunity (NZB/W and MRL/lpr) and TLR7-overexpression innate-driven autoimmunity (Sle1.Yaa and NZW/BXSB) in early and nephritic disease. We profiled single cell transcriptomes using 10x 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.





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.

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DOI 10.1002/acr2.11269

American College of Rheumatology
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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

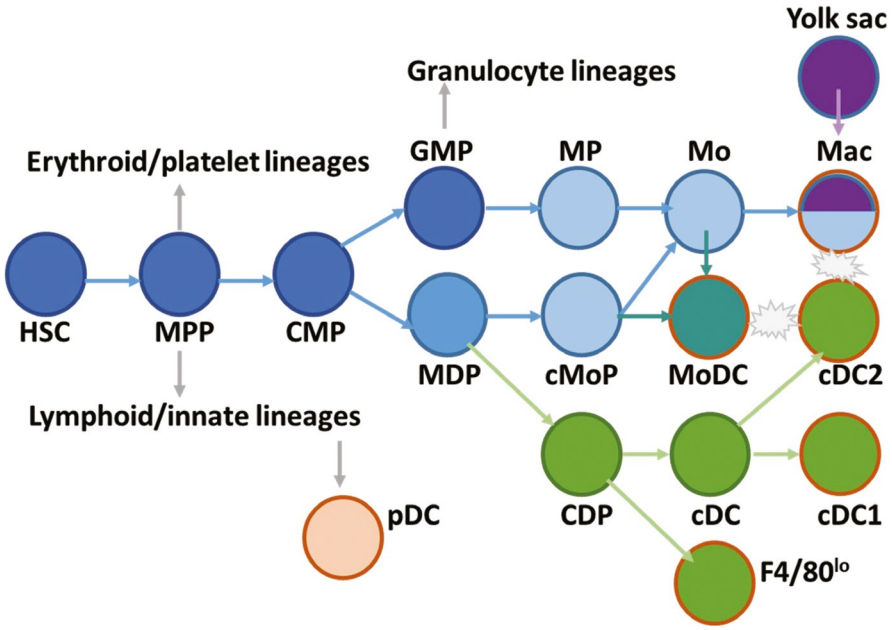

Figure 1. Development of the myeloid lineage from hematopoietic stem cells (HSC) or from yolk sac macrophages (Mac). Cells with an orange border are found in normal kidneys. The three cell types connected by grey clouds have similar phenotypes and may be difficult to distinguish in inflamed tissues by 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

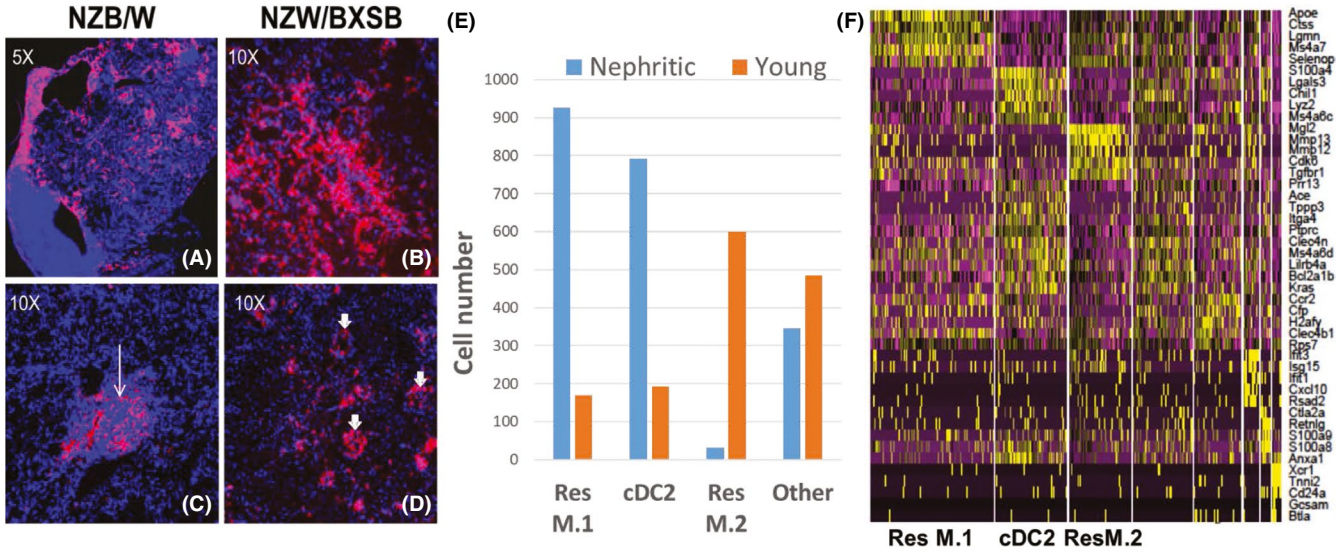

Figure 2. Diversity of mononuclear phagocytes in LN kidneys. **A**, F4/80 staining of macrophages in a kidney from a nephritic NZB/W mouse shows localization around glomeruli and in the interstitium but exclusion from lymphoid aggregates. **B**, F4/80 staining of macrophages in a kidney from a nephritic 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 α1 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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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 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?

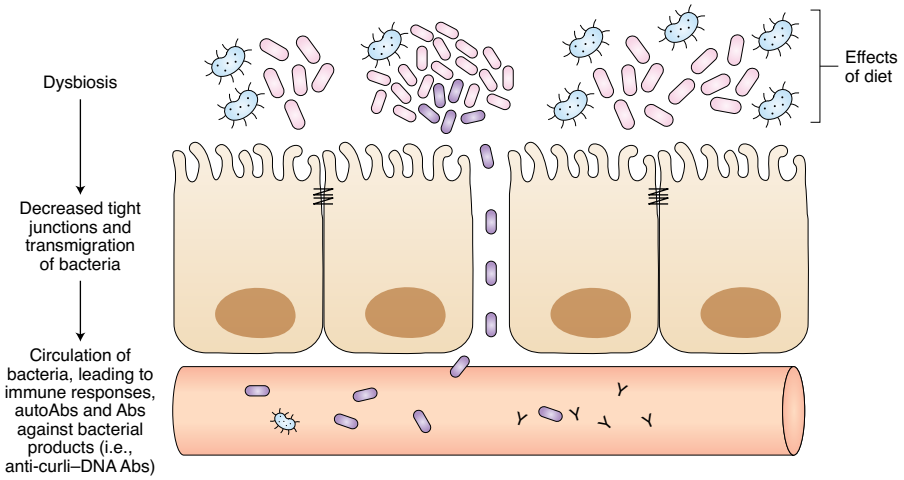

Fig. 1 | Dysbiosis and gut permeability contribute to lupus disease. Expansion of pathogenic gut microbiota can lead to increased permeability of the gut epithelial barrier, providing a route through which bacteria and bacterial antigens can enter systemic circulation. The immune response to these foreign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

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 levels[4]. Interleukin (IL)-12 mediates this extrafollicular-dominant autoimmune "storm" by suppressing follicular helper T (T_{FH}) cells necessary for germinal center formation[5], 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 catheters[6]. 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 cells[7]. 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 lupus[8].

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

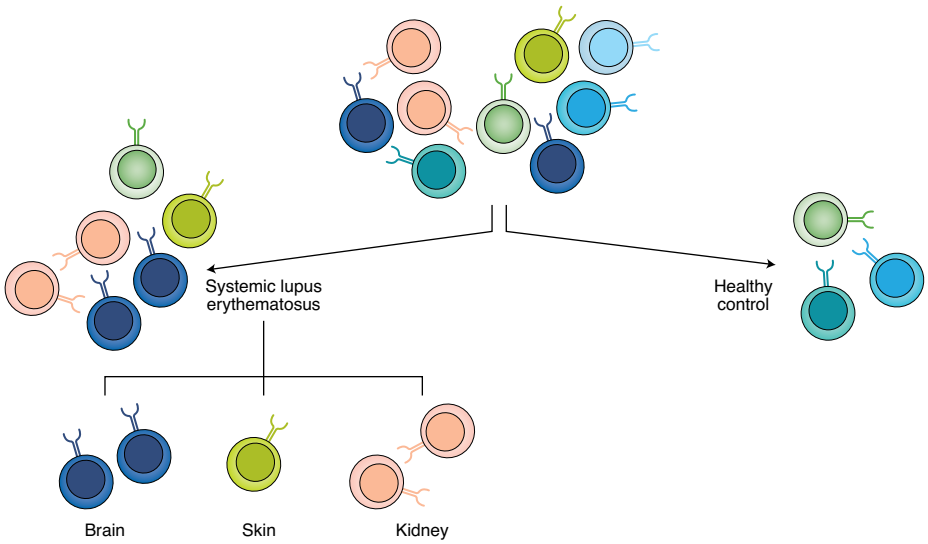

Fig. 2 | The potential application of T cell phenotyping and TCR sequence monitoring at both the organ and disease levels. T cells play a key role in organ damage caused by lupus disease. Sequencing of a T cell receptor could potentially reveal its cognate antigen, including autoantigens. TCRs can be 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 disease[9]. 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 process[10]. 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 found[11], 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 life[12]. Oral administration of vancomycin lessened or worsened lupus whether given before or after disease onset, respectively, suggesting different mechanistic effects on the immune system[13].

Daniel Zegarra-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 phenotype[14].

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 SLE[15]. 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 respiration[16].

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 vasculopathy[17]. 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 mice[18]. 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.

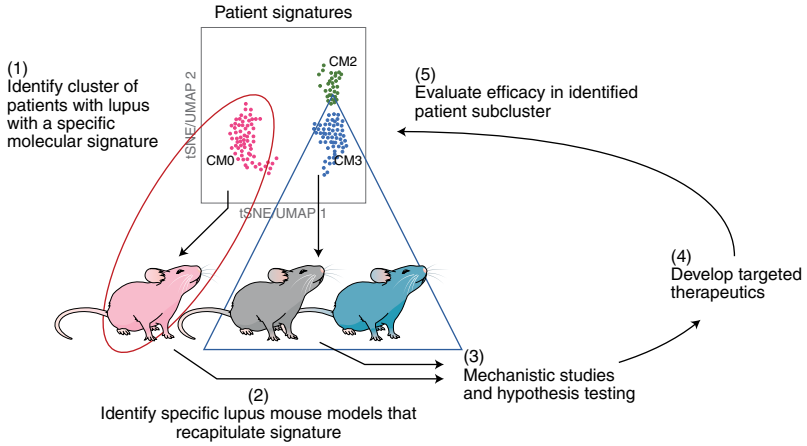

Fig. 3 | Using overlapping disease signatures in appropriate mouse models and subclusters of patients with lupus for the development of targeted therapies. With advanced technology, it is possible to select specific mouse models that recapitulate the molecular features found in subgroups of patients 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 patients[19]. In MRL/*lpr* mice, anti-CD6 treatment improved renal disease, survival and extra-renal disease, such as skin histopathology[20]. 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 cells[21]. 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 signature[22], 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 phenotype[23]. 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 MAF[24]. With a similar transcriptomic signature as that in T_{FH} 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_{FH} and T_{PH} cell markers make to the cell's function.

Further functional characterization of T_{FH} 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_{FH} 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 itself[25]. Similar results were observed in a cluster of circulating T_{FH}-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 SLE[26]. 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 manifestations[27].

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 models[28]. 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 Equillium and is one of the investigators on the current anti-CD6 antibody treatment trial for lupus. The other authors declare no competing interests.

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*$^{-/y}$ 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*$^{-/y}$ 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, $CD19^{low\text{-}int}$ $CD44^{+}$ $CD138^{+}$ 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 $CD19^{+}$ $CD93^{-}$

$CD23^{low\text{-}int}$ $CD21/35^{+}$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% ±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% (± 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 LC3β (LC3β-II), a key molecular event in LAP (22, 38). As expected, zymosan-containing particles caused robust LC3β-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 LC3β-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, LC3β-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 LC3β-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 LC3β-I to form LC3β -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 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 LC3β-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 LC3β-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 Dmit15, 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: LC3β (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*$^{-/y}$ males n=20; MRL.Fas^{lpr} *Cybb*$^{-/-}$ females n=10; MRL.Fas^{lpr} *Rubicon*$^{-/-}$*Cybb*$^{-/y}$ 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*$^{-/y}$ males n=11; MRL.Fas^{lpr} *Cybb*$^{-/-}$ females n=5; MRL.Fas^{lpr} *Rubicon*$^{-/-}$*Cybb*$^{-/y}$ 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 ± 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*$^{-/y}$ males n=11; MRL.Fas^{lpr} *Cybb*$^{-/-}$ females n=5; MRL.Fas^{lpr} *Rubicon*$^{-/-}$*Cybb*$^{-/y}$ 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*$^{-/y}$ males n=11; MRL.Fas^{lpr} *Cybb*$^{-/-}$ females n=5; MRL.Fas^{lpr} *Rubicon*$^{-/-}$*Cybb*$^{-/y}$ males n=15; MRL.Fas^{lpr} *Rubicon*$^{-/-}$ *Cybb*$^{-/-}$ females n=9 mice per group). Bars represent the median ± 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 $TCR\beta^-$ $CD44^+$ $CD138^+$ 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 ± 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-assocated 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 ± 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).