Tertiary Lymphoid Tissue in the Urinary Bladder of Aging Mice and Women

Marianne Morris Ligon

Washington University in St. Louis

Follow this and additional works at: https://openscholarship.wustl.edu/art_sci_etds

Recommended Citation
https://openscholarship.wustl.edu/art_sci_etds/2651

This Dissertation is brought to you for free and open access by the Arts & Sciences at Washington University Open Scholarship. It has been accepted for inclusion in Arts & Sciences Electronic Theses and Dissertations by an authorized administrator of Washington University Open Scholarship. For more information, please contact digital@wumail.wustl.edu.
Tertiary Lymphoid Tissue in the Urinary Bladder of Aging Mice and Women
by
Marianne Morris Ligon

A dissertation presented to
The Graduate School
of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

May 2022
St. Louis, Missouri
# Table of Contents

List of Figures ............................................................................................................................ v
List of Tables ................................................................................................................................. vii
Acknowledgements ..................................................................................................................... viii
Abstract ......................................................................................................................................... xiv

Chapter 1: Introduction .................................................................................................................. 1  
1.1 Diseases and disorders of the bladder in women ................................................................. 3  
1.2 Clinical and epidemiological considerations for UTIs ..................................................... 4  
1.3 The urinary bladder: structure and function ...................................................................... 7  
1.4 Pathogenesis of UTIs ........................................................................................................... 9  
1.5 Bladder defense mechanisms: the urothelium ............................................................... 11  
1.6 Bladder defense mechanisms: the immune system ...................................................... 14  
1.7 The aging immune system ................................................................................................. 17  
1.8 Tertiary lymphoid tissues ................................................................................................. 19  
1.9 Currents gaps in the field and key questions addressed in this thesis ......................... 23  
References ...................................................................................................................................... 25

Chapter 2: Trans-mission control in the urinary tract: local cytokine regulation  
               of monocyte proliferation to combat infection ............................................................ 37  
References ......................................................................................................................................... 45

Chapter 3: Single cell- and tissue-transcriptomic analysis of murine bladders  
               reveal age-and TNFα-dependent but microbiota-independent tertiary  
               lymphoid tissue formation ............................................................................................... 46  
3.1 Introduction ......................................................................................................................... 48  
3.2 Results .................................................................................................................................. 50  
   3.2.1 Tissue and single cell transcriptomics reveal substantial  
         effects of aging on the bladder immune system ............................................................ 50  
   3.2.2 Lymphocytes in aged mouse bladders organize into  
         tertiary lymphoid tissues ............................................................................................... 53  
   3.2.3 bTLTs contain germinal centers and promote class-
switched IgA responses ................................................................. 55
3.2.4 Age-associated bTLT form independently of microbiota
but require TNFα ................................................................. 57
3.3 Discussion ................................................................................. 59
3.4 Methods .................................................................................. 64
References ...................................................................................... 83

Chapter 4: Bladder mucosal cystitis cystica lesions are tertiary lymphoid tissues
that correlate with recurrent urinary tract infections in older/postmenopausal
women ......................................................................................... 89
4.1 Introduction .............................................................................. 91
4.2 Methods ................................................................................. 93
4.3 Results ..................................................................................... 95
  4.3.1 Factors associated with cystitis cystica .................................. 95
  4.3.2 Association between CC and time to UTI recurrence ............... 96
  4.3.3 Improvement and resolution of CC lesions on repeat cystoscopy.. 96
  4.3.4 Histopathology of cystitis cystica biopsies ............................. 97
4.4 Discussion ................................................................................ 98
References ...................................................................................... 108

Chapter 5: Conclusion and future directions ........................................ 112
5.1 Conclusions ............................................................................. 113
5.2 On-going work and future directions .......................................... 116
  5.2.1 Influence of sex on bTLT formation ....................................... 116
  5.2.2 Signals and sequence of bTLT formation I: TNFα ..................... 117
  5.2.3 Signals and sequence of bTLT formation II: LTβR ..................... 118
  5.2.4 Signals and sequence of bTLT formation III: Chemokines ....... 119
  5.2.5 Urothelial permeability: a possible trigger for age-associated
      bTLT formation? ................................................................... 121
  5.2.6 Role of bTLT during infection ............................................... 123
  5.2.7 Role of B cells in the bladder ............................................... 124
  5.2.7 Treatment and biomarkers of cystitis cystica in humans .......... 125
5.3 Concluding remarks................................................................................................. 126
5.4 Methods ................................................................................................................... 133
References .................................................................................................................... 135
Appendices .................................................................................................................... 139
  Appendix 1: Oxysterol signatures distinguish age-related macular
degeneration from physiologic aging ........................................................................... 139
  Appendix 2: A non-canonical autophagy-dependent role of the
ATG16L1\textsuperscript{T300A} variant in urothelial vesicular trafficking and
uropathogenic \textit{Escherichia. coli} persistence ...................................................... 152
  Appendix 3: The impact of methenamine hippurate treatment on bladder
barrier function and inflammation in aged mice and women with
urinary tract infections ............................................................................................... 169
Curriculum Vitae ........................................................................................................... 194
# List of Figures

## Chapter 1:
- **Figure 1.1** Structure of the urinary bladder .................................................. 7
- **Figure 1.2** Pathogenic cycle of uropathogenic *E. coli* (UPEC) in the urothelium.................................................. 10
- **Figure 1.3** Sequence of autophagy and proteins associated with each step ................. 13
- **Figure 1.4** Organization of a lymph node and germinal center .......................... 19

## Chapter 2:
- **Figure 2.1** Interleukin-6 response to acute urinary tract infection with uropathogenic *E. coli* (UPEC).................................................. 41

## Chapter 3:
- **Figure 3.1** Tissue transcriptomic map of young and aged bladders ...................... 73
- **Figure 3.2** Single cell transcriptomic map of immune cells from young and aged bladders .................................................................................. 74
- **Figure 3.3** Lymphoid infiltrates form bladder tertiary lymphoid tissues (bTLT) during aging .......................................................... 75
- **Figure 3.4** bTLT are centers for B cell recruitment, activation, germinal center reactions, and plasma cell differentiation .......................................................... 76
- **Figure 3.5** bTLT size and number are independent of microbial status and dependent on age-associated TNFα .......................................................... 77
- **Figure S3.1** Cxcl12 and Ccl21 expression are unchanged in bladders from aged mice compared to those from young .......................................................... 78
- **Figure S3.2** Isolation and initial analysis of CD45+ cells from young and aged bladders used for scRNA-seq ............................................................................. 79
- **Figure S3.3** Expression of canonical markers used to identify cell types .................. 80
- **Figure S3.4** Aged bladders contain unique macrophages and B cells that are absent in young bladders .......................................................... 81
- **Figure S3.5** Polymeric Ig receptor (pIgR) expression does not change in aged bladders .................................................................................. 82

## Chapter 4:
- **Figure 4.1** UTI-free survival of rUTI patients stratified by CC status .................. 103
- **Figure 4.2** Improvement and resolution of CC upon repeat cystoscopy .................. 104
- **Figure 4.3** *Cystitis cystica* lesion biopsies ........................................................ 105
Chapter 5:

- Figure 5.1 Effect of sex on age-associated bTLT formation ........................................ 127
- Figure 5.2 Effect of anti-TNF treatment on age-associated bTLT .................................. 128
- Figure 5.3 Lymphotxin (LT) signaling in age-associated bTLT ............................... 129
- Figure 5.4 Cytokine and chemokine expression over the lifespan in mice ......................................................... 130
- Figure 5.5 Urothelial permeability in aged mice ......................................................... 131
- Figure 5.6 UPEC infection in young and aged mice .................................................... 132
# List of Tables

Chapter 3
Table 3.1 Markers used to identify cell cluster identities from scRNA-seq............ 72

Chapter 4
Table 4.1 Clinical characteristics of biopsied patients................................. 106
Table 4.2 rUTI patient characteristics .......................................................... 107
Acknowledgements

As anyone who has pursued a PhD knows, it is a long and arduous road full of ups, downs, and long years of uncertainty. While working on my thesis, I have experienced many hard times and learned valuable life lessons. In retrospect, there are much better ways to have done this work. I find that most of this process is not necessarily about the science, but how you get there. I cannot count the intangible knowledge and skills I now have that I didn't even realize I needed to learn 4 years ago. Many wonderful people have helped me get here, and it's truly impossible to imagine what this journey would have looked like without each and every one of you.

First, none of this work would have been possible without Indira. I'm so glad I was able to find a home in your lab. You have become a mentor not just in science, but in life. You've taught me so many different perspectives, how to navigate science and academia, and to believe in myself and my work. Your unending optimism for this work was greatly needed to balance my own critiques. Indira gave me what I wanted in a PhD mentor: someone to invest in my career development and myself as a person; someone that would let me explore my own scientific interests; and someone that would make sure I had fun doing it. I greatly appreciate your efforts to bring your trainees into all parts of the scientific progress and not just as bench-workers. I am also ever grateful for the opportunities you afforded me to travel to present and develop my work, which included visits to the UK, Canada, China, and Australia among others. I never thought my science would take me so far in such little time. I know I will always be part of the family and call
on you for advice in the future. It's been a pleasure to work, live, and grow under your direction these past 4 years. Thank you for all that you do!

I would like to thank my thesis committee for (hopefully) awarding me this PhD! Each of you has aided me in developing my ideas, proposing my project, and giving input and suggestions along the way. I never dreaded my proposal or updates because I truly enjoyed the discussions we had and the support I received. When I realized that work similar to what I wanted to pursue in the bladder had been studied by Shabaana Khader and Rodney Newberry, you both spent time listening, helping, and giving suggestions on my project. I am also grateful to Rodney for giving me his remaining stock of LTβR-Ig to test in my mice. Joel Schilling was always enthusiastic about my findings, asking questions and making suggestions to link my basic and clinical projects. Raj Apte listened to my work and questions and also gave me the opportunity to collaborate on his projects, resulting in co-authorship on a paper. Jerry Lowder was essential to my understanding of the clinical implications of my work and giving me the drive to pursue a clinical study as part of my thesis. And Ali Ellebedy, for helpful discussions on B cells and also joining my committee on short notice! Again, I've enjoyed working with every one of you and hope to call you my colleagues in the future.

To my many past mentors, I would not be in this program and at such an amazing institution without your investment in me and my future. My first research experience back at Clemson University with Kim Paul, Patrick, and Sunayan taught me how fun science could be and made me pursue bench research as a career. Michael Terns, Rebecca Terns, Sonali, and Caryn spent countless patient hours with me as an undergrad, developing my skills and teaching me what it means to do actually do science. Dan Littman welcomed me into his lab at NYU for the summer,
and despite the short time, always had kind words to say, fruitful discussions, and quick responses to my emails. I am grateful for being exposed to immunology and microbiology in your lab and getting a lot of my ideas of interest during that time. Furthermore, you helped me find a position at Oxford with Kevin Maloy and his lab. To Kevin and the gang, it was an invaluable experience to contribute to your lab, be trusted with my own project, and learn how science is done across the pond. I am lucky to have spent the time learning techniques I continued to use in my PhD. It was a pleasure reuniting at MICS 2018! Finally, the process of navigating applications and scholarships and just thinking about and pursuing my future was pushed along by Jessica Hunt and Joel Oppenheimer. I know each of you will be proud to see where I made it today (and be shocked at how fast the time went!).

I'd like to thank the Center for Reproductive Health Sciences (CRePHS) and the rest of the BJCIH 10th floor who all made coming to work every day and staying late in the evening a pleasure. I would especially like to thank all the hard work Magdalena does for the center, as our in-house, knows-how-to-fix-everything expert! I'd like to thank Casey for handling some of those needs as well, and especially for bringing back chocolate chip bagels! I'd like to thank Debbie for critical input on manuscripts and grants that helped keep us grounded in our ideas and slowly develop a story about my work that actually made sense! Finally, I'd like to thank Alma for the thousands of histology slides she cut for me and our fun conversations. I will miss seeing each of you on a daily basis, but I hope to stop by to see everyone in the coming years.

I'd like to thank everyone in the MSTP office for recruiting me to the program in the first place! You have always been responsive and helpful and really care about making sure our
experience is valuable and enjoyable. Ya'll are one of the reasons I decided to come to WashU, and you haven't let me down yet!

I would like to acknowledge everyone in the Mysorekar lab (past and present) that I had the pleasure to learn from, work with, or pass on my knowledge to: Nana, Janey, Emily, Bin, Lindsay, Bisi, Amy, Paul, Chetan, Sonam, Elaine, Paula, Caihong, Kyle, Yin, Melanie, Cat, Haidy, Stacey, Jess, Laura, Priyanka, Mackenzie, and many other undergrads. Each of you made our lab a happy place. I will always remember our outings, parties, float trips, and more. Most of you know how we, as a lab, have so much input on who we ask to join the lab, so I am grateful I fit in and was welcomed into the family!

I'd like to give special thanks to a few lab mates who have really made substantial impressions on me. First, I'd like to thank Sonam and Paula for encouraging me to push through and complete my thesis work. The timing of both of you arriving and having the wisdom of freshly-minted PhDs helped me see the end of the tunnel (and more importantly how to get there). Not to mention indulging my love of immunology. You were both always so cheerful and could brighten a bad day with a smile. I'll miss our other favorites: coffee, wines, and conversations over them. Thank you for making such a difference in the short time you've been in the lab with me. Next, I'd like to thank Yin for her amazing hospitality when I visited China. I did not really know what to expect from my trip, but the few days I got to explore Nanjing with you were by far the best part of the trip! Your laugh, smile, and love of food were also always uplifting. I hope to keep in touch with each of you and see you again, somewhere in the world!

Finally, my biggest thanks to my every-day mentor, my colleague, my friend, Caihong. It's funny, I think I've told you this before, that at the beginning of my rotation I was kinda intimidated
by you. You won't believe that I am actually a shy person and it is weird to be thrown into a tight
knit group like the lab! But by the end of the summer, I was hoping to see you (and eat your food!)
again....and again. Then the next summer you lured me back with spring rolls! You have taught
me nearly all the techniques I used in the lab and helped me figure out how to troubleshoot them,
especially in the beginning. You helped teach me to write that first grant, and now grants are not
scary at all! You always reassured me and reminded me that I was good at what I was trying to do.
From being a teacher and mentor, you then became a close friend and the person I wanted to talk
to every day (to the point that you moving your desk greatly increased both of our productivity)! I
am forever thankful that I was able to share my hard times with you and you did the same with
me. While we all miss you in the lab, I am excited to see what your future holds and the amazing
work you continue to do.

I am lucky to have made such supportive friends during my time at WashU, and even
luckier that many of them were my MSTP classmates! I would especially like to thank Brett,
Michelle, and Chuner for the many, many coffees, dinners, outings, and complaining sessions! It
is truly necessary to have others who understand the trials and tribulations of this program, and I'm
lucky we have become amazing friends in the process. To all my friends back home in Clemson
and Georgia, I'm incredibly lucky that I am still in touch with so many people who are so far away.
Even if we don't see each other as often, I can always count on laughs and hugs whenever we are
both in town!

I'd like to thank my family, especially my parents, for everything they have done for me. I
never had any specific career plans as a child until midway through high school, but I always knew
I wanted to get a PhD, since my dad had one, of course! Now that I know what that entails, you

xii
maybe should have warned me! Even when I felt that life wasn't working out the way I wanted it, I always knew you would be there to support and love me wherever the journey goes. I'm sad you, Walter, and Leonard will not be able to come to my defense in person, but I'm happy you will be there virtually. Thank you all for the years of supporting me as a forever-student. One day I might be totally finished.

And to my love, my best friend, my partner, my husband, Michael. I'm so thankful to have had you by my side during this process, even if you didn't really know what I was doing most days. You believed in me from day one and was there every single day to make sure it was going well. We both struggled with hard times over the past 4 years, and we've grown closer experiencing them together. I can't thank you enough for your support and everything you do to make life a little easier on me. I can't imagine spending quarantines, worldly adventures, and snuggles with Alden, Hemingway, and Coco with anyone else. I would also like to thank all of your wonderful family members (especially Denise, Ted, Joc, Mark, and Matt) for treating me like family and helping to make St. Louis my new home. I love you forever and always, to Pluto and beyond!

I love you all and thank you for joining me on this journey!

This work was funded in part by NIH grants R01DK100644, R01AG052494, P20DK119840, and R56AG064634 to IUM, NIH training grants T32AI007172 and T32GM007200 for MML, and the Washington University Institute for Clinical and Translational Sciences.

Marianne Ligon

Washington University in St. Louis

May 2022
ABSTRACT OF THE DISSERTATION

Tertiary Lymphoid Tissue in the Urinary Bladder of Aging Mice and Women

by

Marianne Morris Ligon

Doctor of Philosophy in Biology and Biomedical Sciences
Molecular Microbiology and Microbial Pathogenesis
Washington University in St. Louis, 2022
Indira U. Mysorekar, PhD, Chair

Urinary tract infections (UTIs) are the most common infection of women, affecting roughly half of all women during their lifetime. UTIs frequently recur within 6 months to 1 year of the initial infection to cause recurrent UTIs (rUTIs). Repeated treatment of rUTIs with antibiotics may lead to antibiotic resistance, disruption of the microbiota, and adverse effects of the drugs. To avoid excess antibiotic use, there is an urgent need to understand the mechanisms of host defense against infection to identify new targets for UTI treatment and prophylaxis in women with rUTIs. Postmenopausal and older women have the highest risk of rUTIs; however, why rUTIs become more frequent after menopause and during old age is incompletely understood. Aging has substantial effects on the immune system that lead to impaired protection against pathogens yet heightened and prolonged inflammation. How the immune system and its responses to infection changes within the bladder mucosae during aging remains unknown. In this thesis, I investigated how the cellular and molecular immune landscape within the bladder was altered during aging and clinical implications of these changes.

xiv
I first found that aged mice developed bladder tertiary lymphoid tissues (bTLTs), which are absent in young mice. TLTs resemble the composition and organization of secondary lymphoid organs, such as lymph nodes and Peyer's patches, but arise ectopically in chronically inflamed tissues rather than during development. bTLTs contained large numbers of T cells and B cells and were capable of germinal center formation, class-switch recombination, and plasma cell differentiation to secrete IgA into the urine. The formation of bTLTs in mice occurred as a function of age and was independent of microbes but dependent on TNFα, as aged germ-free mice also harbored bTLTs while aged TNFα-deficient mice did not. Furthermore, bTLTs were predominantly found in female bladders and rarely in male bladders. Together, my results have identified a profound age-associated change to the immune landscape of aging female bladders that might drive the significant increase in UTI susceptibility.

To determine the relevance of age-associated bTLTs in humans, I examined biopsies of nodular lesions, known as cystitis cystica, that are frequently found by cystoscopy (bladder endoscopy) in women with rUTIs. These biopsies resembled the structure and composition of bTLTs found in aged mice. To further define the role of bTLT in rUTIs, I performed a clinical retrospective analysis of women that have had a cystoscopy. From a cohort of 138 women with culture-proven rUTIs, 38.4% of women had cystitis cystica (CC). Women with CC were significantly older than those without, had a greater number of culture-proven UTIs in the past year, and were more likely to have pelvic floor myofascial pain upon examination. Furthermore, after controlling for the number of prior UTIs, those with CC had a significantly shorter time to next UTI after cystoscopy than those without CC and a greater number of UTIs in the year following cystoscopy. These clinical data suggest that if postmenopausal bladders contain TLTs,
they are more likely to be pathogenic than protective. In concordance with these clinical findings, I showed that aged mice were also more susceptible to UTI recurrences than young mice. Together these findings demonstrate that age-associated immune dysfunction at the bladder mucosae contributes to altered immune responses and adverse UTI outcomes. My work establishes a new avenue of investigation of the bladder immune system in homeostasis, aging, and disease.
Chapter 1: Introduction
The global elderly (65+) population is expected to double in size by 2050 and exceed 20% of the population. Given this worldwide shift in age demographics, we must prepare to handle the healthcare needs of an aging population. The elderly are particularly susceptible to infectious diseases, the most common being respiratory infections, urinary tract infections (UTIs), and skin and soft tissue infections. The elderly are both more susceptible to contracting these infections as well as suffer greater morbidity and mortality due to more severe infections. The reasons for this increased susceptibility and severity in the elderly is not well understood, but may involve functional changes to the immune system with age, known as immunosenescence. Immunosenescence comprises several characteristic changes with both a reduction in protective responses against infections and excess or prolonged inflammation, resulting in chronic inflammation termed inflamm-aging. While systemic immunosenescence has been well-characterized, how aging and immunosenescence affects mucosal immunity to infections (where they most often occur) is only briefly described and focuses on immunity in the gut and lung. No such work has previously examined the impact of aging on immunity in the bladder.

Older women (50+) are highly susceptible to bladder disorders including recurrent urinary tract infections (rUTIs), overactive bladder/urge incontinence (OAB), and interstitial cystitis/bladder pain syndrome (IC/BPS), among others. These bladder diseases all have a chronic inflammatory component as well as overlapping symptoms, known as lower urinary tract symptoms (LUTS). How and why bladder disorders and bladder inflammation become more prevalent with aging is not currently understood. Since aging is characterized by chronic, low-grade systemic inflammation termed inflamm-aging, the common association of bladder disorders with both aging and chronic inflammation suggests that an underlying driver of pathology may be age-associated inflammation. Since UTIs are highly prevalent in the elderly,
this thesis will address how aging impacts the immune system in the bladder and influences poor outcomes to UTIs.

1.1 Diseases and disorders of the bladder in women

There are many diseases of the bladder, and due to differing anatomy, the array of bladder diseases is distinct between men and women. While men are more likely to develop bladder cancer, other bladder disorders in men are frequently mediated by the prostate rather than the bladder alone. In women, dysfunction and disorders of the bladder are more likely to be bladder-intrinsic but may also be secondary to changes in the vagina. Here, I will focus on homeostasis, aging, and disease of the female bladder.

The most common bladder complaint among all women is urinary tract infection (UTI), or bacterial infection of the bladder or kidneys. Urinary tract infections (UTIs) affect at least half of all women during their lifetimes. UTIs are particularly bothersome because 25-50% of infections will recur within 6 months to 1 year after an initial infection. The majority of UTIs in women are uncomplicated, meaning limited to the bladder (cystitis) without complicating factors such as anatomic abnormality, systemic symptoms such as fever, or indwelling catheter.

Other common issues of the bladder in women include: (1) interstitial cystitis/bladder pain syndrome (IC/BPS), (2) overactive bladder (OAB, also known as urgency incontinence), (3) stress incontinence, and (4) incomplete bladder emptying. IC/BPS is a little-understood, sterile inflammatory disease characterized by urothelial permeability defects, inflammation, ulceration, scarring and fibrosis of the bladder, hemorrhage upon distension, and reduced bladder capacity. It is most commonly diagnosed in women in their 40s. OAB is characterized by uninhibited detrusor contractions with or without incontinence, while stress incontinence when the
urinary sphincter and pelvic floor muscles are too weak to prevent urine leakage during high intraabdominal pressure such as coughing, laughing, or sneezing. Women may also experience mixed incontinence, with symptoms of OAB and stress incontinence\textsuperscript{13, 16, 22}. While only 3\% of women under 35 experience symptoms of incontinence, over 50\% of women over 65 report urinary incontinence. While incontinence is not usually considered an inflammatory condition, macrophage inflammatory protein (MIP)-1\(\beta\) and tumor necrosis factor (TNF)-\(\alpha\) are increased in the urine of OAB patients compared to controls. Furthermore, OAB has been postulated to result from inflamm-aging, with urine levels of nerve growth factor (NGF) and the chemokines CCL2 and CXCL1 increasing with age and severity of OAB\textsuperscript{14, 19}. Incomplete bladder emptying also increasing with aging and may be due to multiple factors, including reduced detrusor strength and reduced sensation. Treatment for these bladder diseases may involve invasive procedures or surgeries that further increase the risk of UTI in older women.

In total, bladder diseases become highly prevalent with advancing age and significantly impact women's quality of life. In this thesis, I will investigate age-associated changes to the underlying biology of the bladder that may contribute to these diseases with a focus on UTIs and the bladder immune system.

\textit{1.2 Clinical and epidemiologic considerations for UTIs}

Each year in the U.S., UTIs result in 7-13 million outpatient visits, over 1 million emergency room visits, and nearly half a million hospitalizations. Furthermore, the economic impacts UTIs is over $2.8 billion (in 2011 dollars), and UTIs account for at least 15\% of all antibiotic prescriptions\textsuperscript{23-27}. UTIs may also ascend to the kidneys, causing pyelonephritis, then the blood stream, causing bacteremia and urosepsis. Furthermore, UTIs frequently recur, resulting in
multiple "episodes" of infection over the next 6 months to 1 year following initial infection and termed recurrent UTIs (rUTIs)\textsuperscript{28, 29}. Repeated treatment of rUTIs with antibiotics may lead to antibiotic resistance, disruption of the microbiota, and adverse effects of the drugs. Elderly patients are at increased risk of toxicity from certain antibiotics due to interactions with other medications and decreased renal function. Thus, there is an urgent need to identify new targets for UTI treatment and prophylaxis in women with rUTIs.

Symptoms of uncomplicated cystitis include dysuria (pain with urination), frequency, urgency, and suprapubic pain. UTIs can be diagnosed by these symptoms alone or in conjunction with point of care (dipstick) urinalysis, urine microscopy, and urine culture. Urinalysis can detect immune cell infiltration in the urine (pyuria, measured by urinary leukocyte esterase), which is nearly always present in UTI. Microscopy can further evaluate the degree of pyuria, hematuria, and presence of bacteria or exfoliated epithelial cells in the urine. While not necessary for a diagnosis, urine culture will identify the specific organism and provide guidance of antibiotic treatment by identifying resistance to common antibiotics. In cases of rUTI, urine cultures are important to ensure that recurrences are not due to inadequate antibiotic choice (based on the organism present) and to monitor the emergence of antibiotic resistance\textsuperscript{28, 30}.

Risk factors for rUTIs vary among age demographics. The incidence of UTIs over the lifespan forms a J-shaped curve\textsuperscript{31}. Some risk factors, such as uncontrolled diabetes, can be present at any age, but the prevalence of this co-morbidity increases with age. Infant girls are at risk for UTIs primarily from urinary tract abnormalities and vesicoureteral reflux, but they become less prone to UTIs as they grow. During childhood, there is little risk of UTI. After puberty, UTI risk increases and is frequently associated with sexual intercourse in adult women. These risks plateau until menopause when UTIs become more frequent\textsuperscript{32}. Postmenopausal and elderly women are
particularly susceptible to rUTIs; in 1 study in a primary care setting, 53% of women over 55 had at least 1 UTI recurrence within a year compared to only 36% of those under 55\textsuperscript{29}. Postmenopausal vaginal atrophy and a change to the vaginal microbiota are related to reduced estrogen and increase the risk of UTIs\textsuperscript{33}. Estrogen also plays a protective role against UTIs and has direct effects on urothelial barrier function\textsuperscript{34-36}. Vaginal estrogen therapy, which has low systemic absorption, is effective in reducing UTIs in postmenopausal women and reduces inflammation in the urinary tract\textsuperscript{37, 38}. Prolapse of the bladder (cystocele) and other causes of incomplete bladder emptying promote UTIs since uropathogens can more easily grow in stagnant urine within the bladder. Older women are also more likely to have incontinence, have had urogenital surgery, or be catheterized, all of which are associated with higher rates of UTIs and rUTIs\textsuperscript{32}. It is currently unknown whether age alone, or its associated co-morbidities, may have biologic effects on the bladder that mediate the increased risk of rUTIs.

Bladder endoscopy (cystoscopy) may be used to identify sources of rUTIs, such as bladder uroliths, diverticula, or mesh erosion into the bladder\textsuperscript{30}. One common finding in women with rUTIs is \textit{cystitis cystica} (CC), which appears as multiple mucosal cysts or nodules that may be red, yellow, pink, or grey in appearance\textsuperscript{39-45}. Histologically, CC is described as invaginations of the urothelial von Brunn's nests that may become separated from the surface urothelium and fill with cystic fluid\textsuperscript{46}. However, in \textbf{Chapter 4}, I provide evidence that the commonly observed CC lesions on cystoscopy are histologically consistent with mucosal lymphoid follicles, or bladder tertiary lymphoid tissues (bTLTs). CC and other chronic inflammatory lesions of the bladder have been described since the late 1800s\textsuperscript{39, 45, 47}. Since these lesions are frequently associated with UTIs and not malignant in nature, they have not been thoroughly studied and have been considered "non-specific" chronic inflammatory responses. While CC may be difficult to distinguish from other
histological entities by cystoscopy alone, biopsies of these lesions are not frequently performed to confirm the specific inflammatory pathology. Why some patients form CC lesions while others do not and whether CC lesions affect responses to uropathogens is not known.

1.3 The urinary bladder: structure and function

The urinary system consists of the kidneys, ureters, bladder, and urethra (Figure 1.1). While the kidney continuously filters blood and processes wastes into urine, the urinary bladder stores urine until a socially appropriate time to void. The urinary bladder is lined by a specialized epithelium, known as the urothelium. The urothelium is a pseudostratified epithelium with three distinct layers: the lumen-facing superficial umbrella cells that provide a watertight barrier to the urine; an intermediate layer that is able to replace damaged superficial cells by rapid

Figure 1.1. Structure of the urinary bladder. (A) Organs of the urinary system. (B) Histology of the bladder after voiding. (C) Structure of the urothelium. D, detruser muscle. E, epithelium. L, lumen. LP, lamina propria. Dashed black lines separates the epithelium from the lamina propria. Dashed red lines outline superficial urothelial cells.
differentiation; and a basal layer that contains a quiescent stem cell reserve that is activated in the case of damage to the urothelium\textsuperscript{51,52}. The urothelium is unique among epithelia in several ways: it is the most impermeable epithelium in the body; it does not rapidly turnover (like the skin or intestinal epithelium) unless it is damaged\textsuperscript{51,53}; it changes shape from thick, multiple layers with large, cuboidal superficial cells when the bladder is empty to nearly a single cell layer covered by thinly stretched superficial cells as the bladder fills with urine; finally, the stretched urothelium rapidly contracts to its thick form during voiding. These specialized properties allow the bladder to store hypo- or hypertonic urine with wastes and toxins for long periods of time and accommodate different volumes of urine.

The highly-specialized superficial cells mediate the bladder's impermeability by several mechanisms. Tight junctions between superficial cells prevent the paracellular absorption of most water, ions, small molecules, and other wastes\textsuperscript{53,54}. Uroplakin proteins on the lumenal membrane of the superficial cells form hexagonal plaques that prevent water and solutes from affecting the intracellular compartment and underlying tissue. There is also a thin, lumenal layer of mucus composed of glycosaminoglycans to further protect from the urinary wastes\textsuperscript{56,55}. In the full bladder, many plaques are needed to cover the large surface area. As the bladder contracts to expel urine, the superficial cells endocytose the plaques into specialized, fusiform vesicles in order to recycle them for later use\textsuperscript{56,57}. This process mediates the dramatic change in size and shape of the superficial cells during filling and voiding.

Below the urothelium is a basement membrane, its underlying lamina propria, and an outer muscle layer covered by serosa. The lamina propria is loose connective tissue containing collagens and other extracellular matrix components, blood and lymphatic vessels, mesenchymal stromal cells, and immune cells. The composition of the immune compartment will be detailed below.
Outside the lamina propria is the thick, smooth muscle called the detrusor, which contracts to expel urine.

1.4 Pathogenesis of UTIs

UTIs are caused by different uropathogenic strains of bacteria. These include the Gram-negative bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter* species, *Citrobacter* species, *Pseudomonas aeruginosa*, and the Gram-positive bacteria group B *Streptococcus* (GBS) and other *Streptococci*, *Staphylococcus aureus* and coagulase-negative *Staphylococci*, and *Enterococcus* species, among others. The most common uropathogen is uropathogenic *E. coli* (UPEC), accounting for approximately 80% of all UTIs. Though UPEC still accounts for the majority of UTIs in older women, they are more likely to be infected with the less common uropathogens than younger women.

How UPEC causes UTIs and rUTIs is well-studied. Since *E. coli* are normal inhabitants of the gut microbiota, UPEC often originate from an intestinal reservoir. UPEC may "travel" from the intestine to the urinary tract via contamination from the anus to the urethra. UPEC may also colonize the vagina in some women, particularly postmenopausal women, which increases its chance of reaching the urinary tract. UPEC then ascend the urethra to reach the bladder, where they are able to survive and grow in the urine. Among other factors, the shorter urethra and its closer proximity to the anus in females greatly increases the risk of UTIs in women compared to men. UPEC initially colonize the bladder using type 1 pili that are tipped with the virulence factor adhesin FimH. FimH tightly binds the mannosylated uroplakins on the superficial cells to attach to the urothelium. FimH binding triggers internalization of the bacteria to invade the cell, ultimately escaping this compartment to reach the cytosol of the superficial cells.
A single, invading bacterium can then rapidly replicate to form a large, biofilm-like structure termed the intracellular bacterial community (IBC)\textsuperscript{61}. IBCs may fill nearly an entire superficial cell, and a single IBC reportedly consists of approximately $10^2$-$10^3$ bacterial cells\textsuperscript{62}. UPEC may then break out of the superficial cells, causing cell death, and infect other urothelial cells or replicate in the urine.

A subset of bacteria that infect the urothelium become sequestered intracellularly within autophagosomes (discussed below), persist without causing active infection or immune responses, and are known as quiescent intracellular reservoirs (QIRs)\textsuperscript{63, 64}. QIRs are highly resistant to antibiotics and appear to be "hidden" from the immune system within this intracellular niche\textsuperscript{65}. QIRs may become reactivated, replicate, and break out of urothelial cells to begin a recurrent

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Pathogenic cycle of uropathogenic \textit{E. coli} (UPEC) in the urothelium. During the first 24 hours post infection (hpi), UPEC attach to and invade superficial cells. Intracellular UPEC rapidly replicated to form an intracellular bacterial community (IBC). Superficial cells may die and exfoliate into the lumen. Abundant neutrophils are seen crossing the epithelium and attached the exfoliated IBC. After clearance of the infection, quiescent intracellular reservoirs (QIRs) remain within superficial cells, seen at 14 days post infection (dpi) and later.}
\end{figure}
infection. Thus, QIRs contribute to same-strain rUTIs by long-term persistence within the bladder followed by recurrence of infection. While spontaneous reactivation of QIRs is not understood, damage to the urothelium can trigger the release of UPEC into the urine where it can replicate and infect again. The urothelium can be later damaged by other, transient bacteria, such as *Gardnerella vaginalis*, or be exfoliated by physical or chemical means to stimulate reactivation of latent infection⁶³, ⁶⁶. Thus, UPEC have evolved a mechanism to live long-term in the bladder and continue to cause infections without re-inoculation from the intestinal reservoir.

1.5 Bladder defense mechanisms: the urothelium

Flow of urine can flush many, but not all, bacteria out of the body. The urothelium has cell-autonomous defense mechanisms to further prevent UTIs and limit bacterial colonization of the bladder. One early defense strategy is the expulsion of bacteria that have already invaded the cell⁶⁷-⁶⁹. Intracellular bacteria are trapped within double-membrane autophagosomes, trafficked back to the plasma membrane, and released back into the urine. How a bacterium enclosed within an autophagosome is routed to expulsion or hijacks this compart to form a QIR is not understood. Bacteria that have formed an IBC are resistant to this defense. These infected superficial cells die by apoptosis to force the bacteria back into the urine where it can be expelled from the body. Most of the superficial cells will follow suit, resulting in a burst of exfoliation of the outer layer of the urothelium in an effort to eradicate the bacteria. To protect from deeper invasion of the bladder, the intermediate cell layer rapidly differentiates into new superficial cells and the stem cells proliferate to replace the lost cells⁵¹, ⁶³, ⁷⁰.

Urothelial superficial cells utilize a network of autophagic vesicles directed by Rab GTPase proteins for membrane recycling of uroplakins⁵⁶, ⁵⁷, ⁷¹, ⁷². Autophagy is a conserved cellular process
that recycles macromolecules and organelles in response to starvation or stress\textsuperscript{73}. This complex process involving many components may also be used to capture and degrade intracellular pathogens, termed xenophagy\textsuperscript{74}. While autophagy may be a useful early defense against invading UPEC, these bacteria also utilize autophagosomes to establish QIRs that can cause recurrent infections\textsuperscript{63,75}. UPEC are attracted to autophagosomes within urothelial cells in order to scavenge iron from ferritin\textsuperscript{76}, which requires autophagic degradation (ferritinophagy) to release free iron from the ferritin cage. Mice that are hypomorphic for the essential autophagy gene \textit{Atg16L1} have faster UPEC clearance and form fewer QIRs than wildtype mice, indicating that UPEC normally benefit from the hosts' autophagy response\textsuperscript{77}. The early clearance of UPEC in these mice was found to be due to heightened inflammation via increased production of IL-1\(\beta\) by macrophages, while the reduced QIR formation was intrinsic to loss of ATG16L in the urothelium\textsuperscript{77,78}. While several pathogens have evolved to evade the autophagy machinery, escape its compartments, or prevent lysosomal degradation, UPEC appear to use autophagosomes for long-term persistence within the bladder.

Autophagy begins with identifying cargo for degradation and the nucleation of the phagophore, a vesicular membrane derived from other cellular compartments such as the ER (\textbf{Figure 1.3})\textsuperscript{79}. This first phase involves the autophagy proteins ATG14L1, Beclin1, ULK1, and others, and is marked by conversion of the autophagy marker LC3-I to LC3-II as it is conjugated to the phagophore membrane via phosphatidylethanolamine. The phagophore is then elongated by ATG7, which conjugates ATG5 to ATG12 with subsequent dimerization with ATG16L1 to form a scaffold that induces curvature of the membrane around its cargo. This final step is mediated by EPG5 and small Rab GTPases, which mediate vesicle budding, movement, and targeted fusion
with other organelles. A completed autophagosome is trafficking for fusion with a lysosome in order to degrade its cargo.

In humans, the ATG16L1 gene has a single nucleotide polymorphism (the T300A variant) that is present in 50% of Caucasians and increases the risk of the inflammatory bowel disease, Crohn's disease.\textsuperscript{80, 81} Since decreased levels of ATG16L1 in mice led to improved UPEC clearance and decreased QIR formation, we investigated how the human T300A variant impacted UPEC pathogenesis. As detailed in \textbf{Appendix 2}, we found that the T300A variant reduced the establishment of QIRs without altered the early immune response, as was the case in \emph{Atg16l1} hypomorphic mice. Reduced QIR establishment was consistent with urothelial-specific impairment of proteins involved in the elongation phase (ATG16L1 and ATG7) but not proteins involved in other phases of autophagy (EPG5 and ATG14L1). The protective effects of the T300A variant were mediated by altered expression of a subset of the Rab GTPases. This work demonstrated how UPEC utilizes a specific urothelial trafficking pathway to maintain long-term persistence in the bladder.

Autophagy becomes dysregulated during aging, and new evidence is beginning to suggest that urothelial cells may be highly affected. One study in aged rats demonstrated that an expanded endolysosomal compartment with accumulation of undigested material.\textsuperscript{82} Since UPEC utilize

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{autophagy_and_proteins_associated_with_each_step}
\caption{Sequence of autophagy and proteins associated with each step.}
\end{figure}
autophagosomes for QIR formation, the aged urothelium may be more conducive to harboring latent UPEC within QIRs. In Chapter 5, I demonstrate that aged mice indeed form larger and more numerous QIRs. The mechanisms by which altered vesicular homeostasis in aging and senescent urothelial cells is currently under investigation.

1.6 Bladder defense mechanisms: the immune system

Compared to other mucosal sites, relatively little is understood about the immune system at the bladder mucosa. During steady state, the bladder is inhabited by fewer immune cells in its thin lamina propria than at other mucosal sites. The largest group of immune cells in the bladder are tissue-resident macrophages, comprising approximately 40% of CD45+ cells. Dendritic cells (DCs) of several types make up another 20% of immune cells in the bladder. Interestingly, γδT cells, which make up a very small portion of the systemic immune population, are the third most abundant immune cell type in the bladder. The remaining immune cell population is comprised of small numbers of αβT cells, mast cells, NK cells, monocytes, and eosinophils. Recently, innate lymphoid cells were reported within the bladder, but their specific lineage and role in the bladder remain undefined. However, evidence suggests they may contribute to IL-17 production in response to UPEC infection. In Chapter 3, I identify novel sets of immune cells at steady state in young and aged mouse bladders to add to this repertoire of immune cells in the bladder.

The earliest signals of UTI likely originate in the urothelium. Urothelial cells are able to sense the presence of UPEC with the pattern recognition receptor (PRR) toll-like receptor 4 (TLR4). TLR4 binds to lipopolysaccharide (LPS) present within the outer membrane of Gram-negative bacteria such as UPEC. TLR4 triggers early signals leading to the production of protective, antimicrobial factors, activation of autophagy and bacterial exocytosis, and nuclear
translocation of the major immune-response transcription factor nuclear factor kappa-light chain enhancer of activated B cells (NFκB). NFκB promotes transcription of innate immune factors in urothelial cells including IL-6 and IL-8. UPEC also triggers the NLRP3 inflammasome, which produces mature IL-1β. These cytokines are the earliest alert to the immune system of the infection.

The earliest cytokine produced, urothelial-derived IL-6, begins the coordinated immune response and has several effects on the local immune system (see Chapter 2, Fig. 1). IL-6 promotes extravasation of immune cells into the tissue, signals macrophages to sequester iron away from invading pathogens, and stimulates proliferation of recruited monocytes. Tissue resident macrophages (F4/80+Ly6C−) also act as sentinels within the bladder tissue, detecting early breaches of the urothelium. These macrophages produce the chemokines CXCL1, CCL2, and macrophage migration inhibitory factor (MIF) that recruit neutrophils and monocytes from the blood into the infected bladder. Neutrophils are the most rapidly recruited, peaking around 6 hours post infection (hpi) in mice. Monocytes (Ly6C+) enter the infected tissue, peaking at 24 hpi, and later differentiate into macrophages. These 3 cell types coordinate the early elimination of the infection. Incoming DCs and monocytes produce TNFα, which further activates the resident macrophages to produce CXCL2. CXCL2 stimulates neutrophils to produce matrix metalloproteinase 9 (MMP9), which degrades the basement membrane to allow them to migrate through the epithelium and into the urine. Macrophages may also migrate into the urothelium to phagocytose bacteria during infection.

Neutrophils phagocytose the majority of bacteria and can also be seen attacking exfoliated superficial cells containing IBCs. However, neutrophils cause substantial collateral damage to the tissue and must be regulated to prevent excessive injury to the bladder. For example, reducing the
number of neutrophils during a UTI paradoxically reduces the bacterial burden in the bladder\textsuperscript{97}. Inhibition of inflammatory enzyme cyclooxygenase-2 (Cox-2), made by neutrophils and other cells, during a UTI can protect from developing chronic, severe infection\textsuperscript{98}. To control inflammation after infection, macrophages phagocytose dying neutrophils (a process known as efferocytosis) within the urothelium. These macrophages are attracted to the urothelium by the chemokine CX\textsubscript{3}CL1, which is upregulated by IL-6\textsuperscript{90}. IL-6 also stimulates proliferation and iron sequestration by bladder macrophages\textsuperscript{92}. After clearance of bacteriuria, usually 1 to 3 days post infection (dpi), the immune response begins to resolves and the bladder returns to homeostasis. This phase of the infection is not well understood.

Adaptive immunity in the bladder is also not well understood, and the predilection for recurrent UTIs has long suggested that eliciting effective adaptive immune response may not be common. Several studies have demonstrated that repeat infection with the same UPEC strain in mice results in lower bladder bacterial titers during the challenge infection\textsuperscript{83, 99}. However, sterilizing immunity has not been observed, and latent UPEC reservoirs (QIRs) continue to form upon challenge infections. Furthermore, mice that have previously had severe, chronic UTI are more susceptible to future UTIs than those that resolve their initial infection\textsuperscript{100}, suggesting that divergent immune responses may dictate future responses to reinfection. T cell responses contribute to antigen-specific immunity to UTIs, but one study also found that bladder macrophages may be responsible for inhibiting these responses\textsuperscript{83, 99}. Interestingly, the abundant $\gamma$\textdollar T cells in the bladder rapidly produce IL-17 upon infection, which aids in bacterial clearance\textsuperscript{101}. Whether these cells are responding in an antigen-specific manner is not known. Of note, adaptive immune responses are markedly different if infection is limited to the bladder or ascends to the kidneys. Kidney infection induces a robust, systemic, adaptive immune response, including
pathogen-specific IgG. In humans, pyelonephritis is also marked by dramatically higher urine IgA concentrations. While antibody responses to UPEC may be antigen-specific, whether they are protective has not been demonstrated. Nevertheless, UTI vaccines are currently being investigated and several are already in use outside the U.S. It is important to note that these vaccines are used as immunotherapy in patients with chronic UTIs rather than acting to prevent initial infection of the urinary tract.

1.7 The aging immune system

The elderly are more susceptible to infection and suffer disproportionate morbidity and mortality from infections than younger patients. The reasons for this increased susceptibility and severity is not well understood, but may involve functional changes to the immune system with age, known as immunosenescence. Immunosenescence comprises several characteristic changes: (1) increased myeloid output and decreased lymphoid output from the bone marrow; (2) declining naïve repertoire of adaptive immune cells (decreased diversity of T and B cell receptors) with the oligoclonal expansion of antigen-experienced cells; (3) an increased rate of autoimmune disease; (4) poor control of latent infections and increased susceptibility to new infections, and (5) chronic low-grade inflammation of unknown origin, termed inflamm-aging. While UTIs are one of the most common infections in both elderly men and women, how immunosenescence affects the bladder has not been investigated.

Inflamm-aging is characterized by low-grade, systemic inflammation and non-specific activation of the innate immune system. Pro-inflammatory mediators, such as IL-6, TNFα, IL-1β, and IL-18 are increased with advancing age. Higher serum levels of these cytokines are indicative of frailty in the elderly and increase the risk of all-cause mortality. Chronic
TNFα exposure results in dysfunctional responsive to pathogens such as *Streptococcus pneumoniae*, leading to impaired clearance, heightened inflammation, and increased risk of death from infection\textsuperscript{103, 104}. Inflamm-aging also promotes autoimmunity, metabolic dysfunction, neurologic dysfunction, arthritis, cachexia, and other age-related diseases\textsuperscript{105}. While inhibition of these cytokines may be beneficial in reducing age-related chronic diseases, they are also often required for acute defense against infection, including UTIs.

In the lymphoid compartment, production of new B and T cells from the bone marrow is reduced, while production of innate immune cells of the myeloid lineage is increased\textsuperscript{3}. Furthermore, newly generated lymphocytes are more likely to be autoreactive. Loss of self-tolerance is partly explained by the age-related involution of the thymus, which regulates T cell maturation, though other mechanisms are likely also at work. Conversely, antigen-experienced lymphocytes are oligoclonally expanded. This shift in the BCR and TCR repertoires may result in favoring adaptive immune responses to new challenges that are less-effective or less-specific to the new pathogen. Interestingly, immunoglobulin levels are increased with aging, but levels of antigen-specific antibodies in response to pathogen challenge are lower than in younger animals and may require higher antigen thresholds to stimulate a response\textsuperscript{106}. Furthermore, the appearance of age-associated B cells (ABCs), a newly characterized B cell subset, promotes inflamm-aging and autoimmunity and suppresses B cell hematopoiesis\textsuperscript{107, 108}. ABCs may represent a distinct memory B cell subset that accumulate over time after numerous antigen challenges during the lifetime. ABCs could also be antigen-naive B cells that take on an antigen-experienced phenotype due to replicative exhausted from repeated homeostatic proliferation throughout life\textsuperscript{109}. Interestingly, ABCs appear to mediate protective responses to some pathogens, particularly viruses and other intracellular pathogens\textsuperscript{110}. Whether and how ABCs may contribute to poor response to
infectious disease in the elderly is not yet determined. Furthermore, the role of bladder-localized B cells in UTI has not been studied at any life stage.

1.8 Tertiary lymphoid tissues

The immune system is made up of cells that inhabit nearly all tissues in the body and are frequently migratory between body tissues, lymphatics, and blood. The primary lymphoid organs produce new immune cells and are comprised of the bone marrow, where all hematopoietic cells are generated and B cells mature, and the thymus, which regulates T cell maturation. Secondary lymphoid organs (SLOs) include the spleen, lymph nodes, Peyer's patches, and other, organized mucosa-associated lymphoid tissues. SLOs harbor large numbers of densely packed immune cells and function to facilitate adaptive immune responses. These organs are highly structured to facilitate a multitude of intercellular interactions that must take place in order to generate adaptive immune responses (Figure 1.4)\(^1\). For example, lymph nodes (the prototypical SLOs) are

**Figure 1.4. Organization of a lymph node and germinal center.** After antigen recognition, B cells proliferate and aggregate into a germinal center within the B cell follicle. B cells undergo somatic hypermutation and are further selected by follicular dendritic cells (FDCs) displaying captured antigens. B cells may die by apoptosis or differentiation into antibody-secreting plasma cells/memory B cells.
encapsulated SLOs where naive B and T cells survey the draining tissue fluid (lymph) for pathogen-derived antigens before it returns to the blood. Naive lymphocytes enter the paracortex of the lymph node from the blood via high endothelial venules, which are specialized blood vessels within SLOs that attract naive lymphocytes and permit their homeostatic extravasation. B and T cells migrate to B cell follicles in the cortex and T cell zones in the paracortex, respectively. The lymph may contain soluble antigens, whole pathogens, or migratory immune cells, such as DCs, containing phagocytosed and processed antigens. The complex organization of the SLO optimizes the chances of B and T cells encountering their cognate antigens in the appropriate context and facilities further T cell help to activated B cells. Within the B cell follicles, antigen-activated B cells may form germinal centers where they undergo proliferation, somatic hypermutation, class-switch recombination, and differentiation into memory B cells or antibody-secreting plasma cells\textsuperscript{112}. Thus, both T and B cell responses to pathogens and other insults typically originate within the SLOs.

Tertiary lymphoid tissues (TLTs) are so named by their structural and functional similarity to SLOs. Like SLOs, TLTs are capable of generating adaptive immune responses, and these responses are thought to be tissue-specific based on their location. In contrast to SLOs, TLTs form ectopically at sites of chronic inflammation and antigenic stimulation rather than at pre-defined locations during embryogenesis\textsuperscript{113-116}. During development, SLOs form at areas known as lymph node anlagen where hematopoietic-derived lymphoid tissue inducer (LTi) cells and mesenchymal-derived lymphoid tissue organizer (LTo) cells aggregate. Retinoic acid produced by neurons stimulates stromal cell to become LTo cells and produce the chemokine CXCL13, which initiates attraction and clustering of LTi cells\textsuperscript{117}. LTi cells express membrane-bound lymphotoxin (LT\(\alpha_1\beta_2\)), which engages LT\(\beta R\) on LTo cells. LTo cells then secrete homeostatic lymphoid
chemokines (CXCL13, CXCL12, CCL19, CCL21) and growth factors (VEGF, FGF2, HGF), which induce expression of endothelial adhesion molecules including VCAM1, ICAM1, MAdCAM1, and others. Together, these chemokines and adhesion molecules attract naïve lymphocytes to the developing lymph node as well as throughout the life of the adult. Lymphocytes later become the primary source of LTα1β2 stimulation and maintain a positive feedback loop of lymphotoxin-chemokine signaling. Work in other tissues demonstrates that TLTs form by similar interactions between hematopoietic and stromal cells, but different cell types may substitute as LTi and LTo cells during TLT formation. For example, vascular smooth muscle cells in artery TLTs and synovial fibroblasts in rheumatoid arthritis-associated TLTs are able to act as LTo cells\textsuperscript{118, 119}. Similarly, diverse hematopoietic cells that accumulate during chronic inflammation may take on the role of LTi cells during TLT formation. TLTs have been found in numerous tissues such as the lung\textsuperscript{120-123}, kidneys\textsuperscript{124-126}, intestines\textsuperscript{127-130}, and meninges\textsuperscript{131}, and in varied disease states, including chronic infection, cancer, and autoimmune diseases. Interestingly, TLTs appear to be more common in the liver, intestines, and kidneys of aged mice\textsuperscript{125, 126, 132, 133}, indicating that an aspect of the aging process may stimulate the development of TLTs. Whether immune responses arising within TLTs are pathogenic (such as in autoimmune disease) or protective (as in some cancers and in some infections) depends on both the tissue location and type of insult. Why TLTs form during aging and what type of responses these TLTs mediate is not currently known.

At mucosal barriers, microbes appear to play an important role in stimulating TLT formation. Germ-free mice develop primitive isolated lymphoid follicles (ILFs, a prototypical TLT found in the small intestine), but microbiota are required to mature these structures\textsuperscript{134, 135}. Furthermore, IgA responses generated at ILFs are directed towards the luminal microbiota. In the lung, infectious agents and pattern-associated molecular patterns (such as LPS) can generate
inducible bronchus associated lymphoid tissue (iBALT, the TLTs in the lung)\textsuperscript{122}. Recently, aged germ-free mice were shown to lack significant leukocyte infiltration in the lungs that is common in conventional aged mice\textsuperscript{104}. While these infiltrates were not characterized as iBALT, their histological patterns suggest peribronchiolar organization that is characteristic of iBALT. Importantly, shifts in the composition of the intestinal microbiota led to similar infiltration when transplanted into young mice, suggesting a role for microbial stimulation in age-associated TLT formation. Interestingly, this age-associated inflammation depended on the inflamm-aging cytokine, TNF\(\alpha\) and intestinal permeability to microbial products\textsuperscript{9, 104}. Since the bladder mucosa plays an important barrier function and may be exposed to pathogenic, transient, and commensal microbes, it is possible that similar mechanisms could stimulate TLT formation in the aging bladder.

TLTs and TLT-like structures have been reported in the bladder, but little detail of their structure, function, causes, and consequences, are currently described. Mice given repeat UTIs have larger, more distinct T cell influx at 24 hpi\textsuperscript{99}. A subset of C3H/HeN mice with persistent bacteriuria, chronic cystitis, and pyelonephritis also have CD45\(^+\) aggregates\textsuperscript{88, 136}. Transgenic mice with constitutive Cox-2 expression in the urothelium develop bladder cancer and lymphoid aggregates containing B and T cells\textsuperscript{137}. Furthermore, small lymphoid aggregates were observed in one small study of aged mice\textsuperscript{138}. Finally, TLTs have been identified in a small sample of muscle-invasive bladder cancer\textsuperscript{139}. Despite these occurrences, TLTs in the bladder are largely uncharacterized and their function remains entirely unexplored. In this thesis, I will begin to address how and why TLTs form in the bladder and how their functions may affect UTI responses and outcomes in both mice and humans.
1.9 Current gaps in the field and key questions addressed in this thesis

Despite the prevalence of bladder diseases, particularly UTIs, in the growing elderly population, there are significant gaps in the biology of the aging bladder and translational aspects to guide clinical treatment:

(1) It is entirely unknown whether and how the immunological landscape in the bladder changes with age and how age affects responses to bladder infection.

(2) The composition, functionality, and impacts of TLTs in the bladder on disease outcomes has not been investigated.

(3) The prevalence and clinical factors associated with TLTs in humans are not clear.

In this thesis, I will address these key questions. In Chapter 2, I briefly review the role of IL-6 and monocytes in experimental UTI and discuss the potential implications of inflamm-aging-associated IL-6 on UTIs in older patients. In Chapter 3, I define the immunological landscape of the bladder in aged mice, finding that they contain bladder tertiary lymphoid tissues (bTLT). I further characterize the cellular and molecular components of these bTLT using a variety of techniques. I show that they function to generate B cell responses, resulting in increased urine IgA. Furthermore, I show that bTLT depend on age-associated TNFα, but are independent of microbial colonization, suggesting that age-related factors alone may stimulate formation of bTLT in mice.

In Chapter 4, I examine the clinical finding of cystitis cystica on cystoscopy, demonstrating that these lesions histologically contain lymphoid follicles akin to bTLT found in aged mice. I determine the association of risk factors, including age, with CC and demonstrate that patients with CC have more frequent and more numerous rUTIs. I demonstrate that CC is an independent
risk factor for further UTIs. In Chapter 5, I detail on-going and future work that will build on the present work and discuss conceptual hypotheses to explain these findings. Together, these studies elucidate age-associated alterations to the immune system in the bladder and its clinical implications for rUTIs.
REFERENCES


64. Mulvey MA, Schilling JD, Hultgren SJ. Establishment of a persistent Escherichia coli reservoir during the acute phase of a bladder infection. *Infection and immunity* 2001; 69(7): 4572-4579.


Bauckman KA, Mysorekar IU. Ferritinophagy drives uropathogenic Escherichia coli persistence in bladder epithelial cells. *Autophagy* 2016: 0.


92. Owusu-Boaitey N, Bauckman KA, Zhang T, Mysorekar IU. Macrophagic control of the response to uropathogenic E. coli infection by regulation of iron retention in an IL-6-dependent manner. *Immun Inflamm Dis* 2016; **4**(4): 413-426.


Chapter 2: *Trans-mission control in the urinary tract: Local cytokine regulation of monocyte proliferation to combat infection*

This chapter was published in *Journal of Leukocyte Biology*. MML wrote this chapter.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATG16L1</td>
<td>autophagy related protein 16-like 1</td>
</tr>
<tr>
<td>CCL2</td>
<td>chemokine (C-C motif) ligand 2</td>
</tr>
<tr>
<td>CCR2</td>
<td>C-C chemokine type receptor 2</td>
</tr>
<tr>
<td>CXCL2</td>
<td>chemokine (C-X-C motif) ligand 2</td>
</tr>
<tr>
<td>F4/80</td>
<td>epidermal growth factor-like module-containing mucin-like hormone receptor-like 1 (EMR1/ADGRE1)</td>
</tr>
<tr>
<td>gp130</td>
<td>glycoprotein 130</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IL-6R</td>
<td>IL-6 receptor</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Ly6C</td>
<td>leukocyte antigen 6 complex, locus 1</td>
</tr>
<tr>
<td>MMP-9</td>
<td>matrix metalloproteinase 9</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>sgp130</td>
<td>soluble gp130</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UPEC</td>
<td>uropathogenic <em>E. coli</em></td>
</tr>
<tr>
<td>UTI</td>
<td>urinary tract infection</td>
</tr>
</tbody>
</table>
Urinary tract infections (UTIs) are one of the most common bacterial infections worldwide, affecting approximately half of all women during their lifetime. The majority of infections are acute and self-limiting due to the rapid and potent host immune response. At the frontline of these host defenses is the production of the pro-inflammatory cytokine interleukin-6 (IL-6), which is important for recruiting immune cells from the periphery to the bladder mucosa. IL-6 has long been identified as a highly induced cytokine during UTIs in both mice and humans\(^1\). Bladder epithelial cells are known to produce large amounts of IL-6 in response to infection with uropathogenic *E. coli* (UPEC), the most common culprit causing UTIs. Furthermore, some UPEC strains dampen IL-6 secretion from these cells, a virulence strategy hinting at IL-6’s importance in eliminating the bugs from the bladder mucosa\(^2\). Nevertheless, the role IL-6 plays in *in vivo* host defense, particularly in the context of UTIs, continues to be elucidated. In this issue, Dixit et al.\(^3\) shed new light on IL-6 activity during UTIs in mice. They examined cytokine control of *in situ* monocyte proliferation and elucidated how the initial defenses are orchestrated. Their results demonstrate that local production of IL-6 induces proliferation of monocytes that have infiltrated the bladder mucosa. This new work adds to the growing list of functions served by the pleiotropic cytokine IL-6, as well as to our understanding of the immune response to bladder infection.

Despite the well-known and seemingly commonplace role of IL-6 in host defenses, control of IL-6 production is not well understood due to its multiple functions and signaling modalities\(^4\). Classical IL-6 signaling through membrane-expressed IL-6 receptor (IL-6R) and its signal transducer gp130 is restricted to hepatocytes, which coordinate the acute phase response, and to hematopoietic cells. However, nearly all other cells in the body express gp130 in the absence of IL-6R and respond to IL-6 via *trans*-signaling, which requires IL-6 to be bound to soluble IL-6R. Furthermore, IL-6 has both pro-inflammatory functions (e.g. stimulating granulopoiesis,
neutrophil respiratory burst, and hyperthermia) as well as anti-inflammatory functions (e.g. mediating neutrophil apoptosis, inhibiting further chemokine and cytokine expression, and resolving inflammation). These seemingly antagonistic properties have made dissecting the role of IL-6 during infections challenging; IL-6 may have drastically different functions throughout the course of infection, impact different cell types, and act via different signaling mechanisms.

Here, Dixit et al. show that locally produced IL-6 induces leukocyte antigen 6 complex, locus 1+ (commonly referred to as Ly6C+) monocyte proliferation within the inflamed bladder mucosa. In a seminal paper, the same group previously demonstrated that infiltrating Ly6C+ monocytes act as a key component in coordinating an effective immune response against UPEC. In that work, Ly6C+ monocytes were shown to produce TNFα during UTIs. Interestingly, monocyte-derived TNFα indirectly licensed neutrophil transepithelial migration by first acting on resident Ly6C- macrophages, stimulating them to secrete CXCL2. Neutrophils then responded to CXCL2 by secreting MMP-9, which degrades the uroepithelial basement membrane, allowing neutrophils access to infected superficial urothelial cells and the bladder lumen. In the current work, the group identified in situ proliferation of infiltrating, inflammatory Ly6C+ monocytes. While these monocytes are known to proliferate in the bone marrow and migrate to tissues during inflammation in a CCL2/CCR2-dependent manner, their proliferation within inflamed tissues in response to infection has not been well-described. In contrast to the infiltrating cells, Dixit et al. showed that Ly6C-F4/80+ resident macrophages demonstrated only minimal proliferation during acute infection. Importantly, they were able to replicate their results in an LPS-induced peritonitis model, indicating that in situ monocyte proliferation is not limited to the bladder and may be a feature of the innate immune response to local infection throughout the body. Furthermore, they showed that Ly6C+ monocyte proliferation was dependent on IL-6 trans-signaling rather than classical IL-6
signaling and that local IL-6 production was primarily produced by non-hematopoietic cells (likely bladder epithelial cells). However, while the work provides strong evidence for in situ monocyte proliferation during UTIs, whether blocking such proliferation affects the outcome of UTIs need to be further explored. Similarly, how local monocyte proliferation affects the duration and severity of sterile inflammation (modeled here by LPS-induced peritonitis) should be further investigated.

Figure 2.1. Interleukin-6 response to acute urinary tract infection with uropathogenic E. coli (UPEC). Model shows urothelial cells secrete IL-6 and Ly6C− resident macrophages secrete CCL2 and other chemokines. These early signals recruit inflammatory Ly6C+ inflammatory monocytes and neutrophils to the bladder mucosae. Ly6C+ monocytes respond to local IL-6 via trans-signaling to proliferate in situ.
The role of monocytes in UTIs is somewhat controversial. Five different groups studying UTIs in mice have depleted monocytes using clodronate liposomes, which deplete phagocytes such as blood monocytes and, to a lesser extent, resident tissue macrophages. Interestingly, the results are not entirely consistent among studies, indicating an incomplete understanding of the role these cells play during UTIs. In one study, depleting monocytes with clodronate liposomes decreased the bacterial burden in the bladder at 24 hours post-infection, but CCR2−/− mice, which are unable to recruit monocytes to the inflamed tissue did not show a decreased bacterial burden. These results suggest that monocytes might exacerbate UTIs rather than contribute to its resolution. In support of that hypothesis, another group observed a reduction in inflammation at 24 hours post-infection as well as a reduction in chronic cystitis at 4 weeks post-infection in clodronate-treated mice, but did not identify changes in bacterial burden in the bladder at 24 hours post-infection. In contrast, the seminal paper described above demonstrated a critical role for recruited monocytes in coordinating the action of neutrophils and resident macrophages during UTIs. When that group used clodronate liposome depletion or CCR2−/− mice, they found an increased bacterial burden in the bladder 24 hours post-infection. In support of those findings, another group showed treatment with clodronate liposomes resulted in an increased bacterial burden in the bladder, but no difference in the bacteriuria titers at 24 hours post-infection. In yet another study, in mice deficient in ATG16L1 (an essential autophagy protein), which exhibit an enhanced ability to clear UTIs, clodronate liposome treatment reversed this enhanced clearance, suggesting a role for monocytes in UPEC clearance. While these discrepancies could be due to differences in UPEC strains, mouse strains, or experimental procedures among labs, the varying results highlight the need for further investigation into the role of the monocyte/macrophage lineage during an acute
Interestingly, the current study used instillation of anti-IL-6, anti-gp130, and soluble gp130 (sgp130) directly into the bladder to investigate the role of IL-6 in the local tissue. The success of this technique indicates that locally applied antibody therapies may be effective in treating inflammatory disorders of the bladder, such as chronic/recurrent UTIs and interstitial cystitis/bladder pain syndrome. These disorders are particularly prevalent among post-menopausal women, who have decreased estrogen, a well-known repressor of IL-6 expression. We have previously shown that estrogen deficiency secondary to ovariectomy delays bacterial clearance from the bladder and results in increased inflammation, IL-6 levels, and quiescent intracellular reservoirs that can seed chronic and recurrent UTIs\textsuperscript{10}.

Anti-IL-6 therapy via bladder instillation could potentially alleviate the inflammatory component of these diseases while reducing systemic side effects. Tocilizumab, a monoclonal antibody against IL-6R, is already used clinically to treat rheumatoid arthritis (RA) and other chronic inflammatory diseases\textsuperscript{4}. Whether inhibiting monocyte proliferation in inflamed synovial joints contributes to the efficacy of tocilizumab in RA is not currently known. This work suggests that targeting specific IL-6 signaling mechanisms, in this case IL-6 \textit{trans}-signaling via sgp130, could be an effective strategy to control monocyte-driven inflammation without disrupting essential functions of classical IL-6 signaling. Clearly, more work would need to be done to establish the safety and efficacies of these potential new therapies. While many questions remain regarding the complete and dynamic role of IL-6 during bacterial infections, this new work sheds light on at least one new function of IL-6.
Authorship

MML and IUM wrote the paper.

Acknowledgements

We thank Dr. Caihong Wang and Brooke Liang for comments. This work was funded in part by NIH grants T32 AI007172 and T32 GM007200 (to MML) and R01 DK100644 (to IUM).

Conflict of Interest Disclosure

The authors declare no conflict of interest.
REFERENCES


Chapter 3: Single cell and tissue-transcriptomic analysis of murine bladders reveals age- and TNFα-dependent but microbiota-independent tertiary lymphoid tissue formation

This chapter was published in *Mucosal Immunology*. MML wrote this chapter and performed all experiments and analysis unless otherwise noted.

ABSTRACT

Aging has multifaceted effects on the immune system, but how aging affects tissue-specific immunity is not well-defined. Bladder diseases characterized by chronic inflammation are highly prevalent in older women, but mechanisms by which aging promotes these pathologies remain unknown. Tissue transcriptomics of unperturbed, young and aged bladders identified a highly altered immune landscape as a fundamental feature of the aging female bladder. Detailed mapping of immune cells using single cell RNA-sequencing revealed novel subsets of macrophages and dendritic cells and unique changes to the immune repertoire in the aged bladder. B and T cells are highly enriched in aged bladders and spontaneously form organized bladder tertiary lymphoid tissues (bTLTs). Naïve, activated, and germinal center B cells and IgA+ plasma cells are found within bTLT and associated with increased urinary IgA concentrations. bTLTs form with increasing age and their formation is dependent on TNFα. Microbiota are not required to form bTLT, as aged germfree mice harbor them. Thus, bTLTs require age-dependent TNFα but are independent of the microbiota. Our results indicate that chronic, age-associated inflammation underlies a fundamental alteration to the bladder and establishes a resource for further investigation of the bladder immune system in homeostasis, aging, and disease.
3.1 Introduction

Immune dysfunction during aging is characterized by chronic, low-grade inflammation, termed inflamm-aging\(^1\). Aging is the strongest risk-factor for many chronic diseases, including cardiovascular disease, neurodegeneration, osteoarthritis, and cancers\(^2\). While these diseases are all linked by chronic inflammation, immune responses vary by tissue, resulting in tissue-specific immune dysfunction\(^3\).

The bladder is a storage organ with a mucosal barrier that provides protection from both urinary wastes and pathogens\(^4,5\). Bladder diseases are highly prevalent among the elderly, and women are predominately affected by these diseases\(^6-9\). Older women (50+) are highly susceptible to bladder disorders including overactive bladder/urge incontinence (OAB), interstitial cystitis/bladder pain syndrome (IC/BPS), and recurrent urinary tract infections (rUTIs). These disorders all have a chronic inflammatory component as well as overlapping symptoms, known as lower urinary tract symptoms (LUTS)\(^10-12\). How and why these bladder diseases become more prevalent with aging is not currently understood\(^9\). Given the common association of bladder diseases with both aging and chronic inflammation, local immune dysfunction at the bladder mucosa may underlie mechanisms driving age-related bladder diseases.

During homeostasis, bladder immune cells consist of ~80% antigen-presenting macrophages and dendritic cells, ~10% T cells, and small numbers of NK cells, mast cells, eosinophils, and patrolling monocytes\(^13,14\). Whether aging affects immunity in the bladder mucosa is not known; however, since chronic bladder inflammation is highly prevalent in older women, age-associated disruption of immune homeostasis in the bladder may mediate inflammatory pathology and lower urinary tract symptoms.
The bladder lacks dedicated mucosal secondary lymphoid organs (SLOs) that form during development like the Peyer’s patches of the small intestine, resulting in a uniquely quiescent bladder mucosal immune system. Non-lymphoid organs may sometimes form ectopic, SLO-like structures in response to chronic inflammation and antigen exposure\textsuperscript{15-17}. Lymphoid aggregates have been reported in cases of chronic bacteriuria, muscle-invasive bladder cancer, and IC/BPS\textsuperscript{18-20}. However, lymphoid aggregates in the bladder remain largely uncharacterized and are not reported to spontaneously form in the mouse bladder. Furthermore, factors influencing their formation, composition, or persistence, have not been identified.

In this report, we sought to identify underlying effects of aging on bladder tissue and the bladder immune cell repertoire using global tissue transcriptomics and targeted, single-cell transcriptomics from young and aged mouse bladders. We find that aging fundamentally alters the bladder immune landscape on the cellular and tissue-transcriptomic levels. We demonstrate that in unperturbed aged bladders, expanded numbers of B and T cells organize into structures we term bladder tertiary lymphoid tissue (bTLT). bTLT in aged mice serve as centers for B cell recruitment, activation, and differentiation into plasma cells. Age-dependent bTLT form in aged germ-free mice, but aged TNFα\textsuperscript{-/-} mice have fewer and smaller bTLT, indicating that bTLT require age-dependent TNFα but are independent of the microbiota. Together these data reveal a profound change in the bladder mucosal immune system that is fundamental to aging in this tissue.
3.2 Results

3.2.1 Tissue and single cell transcriptomics reveal substantial effects of aging on the bladder immune system

Older age is associated with many bladder diseases, yet little is known about the fundamental changes to the bladder that occur during aging. Since women have a higher prevalence of bladder disorders and have distinct lower urinary tract anatomy, physiology, and age-related changes from men\(^2\), we chose to study how aging affects the bladder using aged female mice as a model. We first sought to characterize age-related global transcriptomic changes to the bladder by performing RNA-sequencing (RNA-seq) on whole bladder tissue from young (3 month old) and aged (18 month old) female mice. Bladders from aged mice had at least a 2-fold increase in expression of 416 genes and decrease in expression of 60 genes compared to those from young mice (Figure 3.1, A, Benjamini-Hochberg adjusted false discovery rate (FDR) \(\leq 0.05\), Supplementary Table 1). We then performed pathway analysis of all expressed genes using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database and identified 13 up-regulated pathways and 1 down-regulated pathway in the bladder transcriptome of aged mice compared to those of young mice (Figure 3.1, B). Highly up-regulated pathways included antigen presentation, B- and T-cell activation, cytokine-receptor interactions, and intestinal IgA production (Figure 3.1, B-C). Enrichment of these pathways suggested that immune cells may be recruited to and activated within the aging bladder. Using independent samples, we validated increased expression of the most highly upregulated chemokine, Cxcl13 (Figure 3.1, D), a homeostatic lymphoid chemokine that attracts naive lymphocytes to organized lymphoid tissues. We examined expression of the 3 other homeostatic lymphoid chemokines, finding that Ccl19, but not Cxcl12 or Ccl21, was also upregulated in aged bladders (Figure 3.1, D, Figure S1). In mice,
bladder tissue typically harbors a small number of immune cells compared to other mucosal barriers, and many of these cells have unknown functions in bladder homeostasis or disease\textsuperscript{4,13,22}. Using flow cytometry, we found that aged bladders contained nearly 3 times as many CD45\(^+\) cells as young bladders (\textbf{Figure 3.1, E}), further indicating that the tissue transcriptomic changes we observed were likely due to high numbers of immune cells in aged bladders compared to young bladders. Notably, \textit{Tnf} was also among the most highly upregulated cytokines in the aged bladder (\textbf{Figure 3.1, C}). TNF\(\alpha\) plays a role in driving inflamm-aging and is a predictor of frailty and mortality in humans\textsuperscript{23,24}. Along with chemokine-mediated recruitment, locally increased TNF\(\alpha\) in the bladder could be driving an age-associated inflammatory environment and activating immune cells in the aged bladder. Since the bladder mucosa is normally immunologically quiescent, these data suggested that an altered immune landscape was fundamental to the aging bladder.

To take an unbiased approach to identifying the immune cell types that were driving the immune cell accumulation and tissue transcriptomic changes in aged bladders, we performed droplet-based single-cell RNA-seq (scRNA-seq) on enriched CD45\(^+\) cells isolated from young and aged bladders. After removing urothelial and stromal cells from analysis, we identified 21 distinct immune cell clusters (\textbf{Fig. 2A, Fig. S2-3}). Using canonical cell type markers and queries against the ImmGen database of immune cell transcriptomes\textsuperscript{25,26}, we assigned identities to all of the immune cell clusters (\textbf{Table 1, Supplementary Tables 2-3}). We identified immune cells that have been previously reported in bladder tissue, including macrophages, monocytes, dendritic cells (DCs), natural killer cells, \(\gamma\delta T\) cells, and both CD4\(^+\) and CD8\(^+\) \(\alpha\beta T\) cells\textsuperscript{13,14,27,28}. In both young and aged bladders, we identified 2 distinct macrophage clusters distinguished by high expression
of Retnla in the smaller cluster (Figure S4, A). Retnla (also known as Relmα or Fizz1) is a marker of alternatively activated macrophages; thus, this macrophage cluster may have a more pronounced tissue-reparative phenotype compared to the larger macrophage cluster. Interestingly, a small, third cluster of macrophages were exclusively found in the aged bladder (Figure 3.2, B, Figure S4, B) and were distinguished by high expression of Cxcl13 (Figure 3.2, C), one of the most highly upregulated genes identified by tissue RNA-seq. While there were not surface markers to distinguish these cells, we were able to detect highly increased expression of Cxcl13 in sorted F4/80+CD64+Ly6C- macrophages from aged mice compared to those from young mice (Figure 3.2, D). Thus, macrophages are the most likely source of the high Cxcl13 expression identified in the tissue transcriptome of the aged bladder and likely contribute to immune cell recruitment during aging. We also identified a distinct cluster of type 1 classical DCs (cDC1), which most likely correlate to previously reported CD103+ DCs, and 3 clusters of type 2 cDC (cDC2), likely corresponding with previously reported CD11b+ DCs13, 29. However, we were unable to detect mast cells, which are known to play important roles in bladder inflammation and infection, or eosinophils, which have also been previously reported within mouse bladder tissue13, 29, 30. These cells may not have been isolated by our methods or the populations may too small to resolve by clustering since they are known to be minor immune cell populations within the bladder. Our transcriptional analysis of the bladder immune repertoire at single-cell resolution is generally consistent with the literature while also identifying novel subsets of cells that may play distinct roles in bladder homeostasis, aging, and disease.

The most striking difference in the immune cell repertoire between young and aged bladders was a dramatic increase in B and T cells within aged bladders (Figure 3.2, B, Figure S4, B). B cells are generally absent from young bladders, and T cells comprise only a small number
of cells in young bladders\textsuperscript{13}. The largest cluster of B cells were naive B cells marked by strong \textit{Ighd} expression (\textbf{Figure S3, Figure S4, C}). Two smaller B cell clusters expressed markers of activated B cells, such as \textit{Cila4} and \textit{Mzb1} (\textbf{Figure S4, C}). In one B cell cluster, 95\% of cells expressed a specific immunoglobulin germ-line variable gene (\textbf{Figure S3, Figure S4, C, Supplementary Table 2}), suggesting that some of these B cells may have been clonally expanded. Both B cell clusters also expressed \textit{Fcrl5} (\textbf{Figure S4, C}), a marker of dysfunctional B cells, termed age-associated B cells (ABCs), which have been implicated in inflamm-aging and autoimmune disorders. The exact function of ABCs is debated due to their similarity to a subset of atypical memory B cells\textsuperscript{31,32}. scRNA-seq also demonstrated an increase in plasma cells in aged bladders. Plasma cells expressed high levels of \textit{Jchain} (\textbf{Figure S4, C}), which forms part of the multimeric, secretory forms of IgM and IgA. Thus, a spectrum of B cell states, including naive, activated, and differentiated B cells, were present in the aged bladder and could potentially be participating in active, local immune responses.

3.2.2 Lymphocytes in aged mouse bladders organize into tertiary lymphoid tissues

Since aging resulted in a substantial increase of lymphoid cells in the bladder according to scRNA-seq analysis, and little is known about these cell types in the bladder, we further investigated these cells in aged bladders. Flow cytometry confirmed that aged bladders indeed harbored a significant increase in total T cells, CD4\(^+\) and CD8\(^+\) T cell subsets, and B cells compared to young bladders (\textbf{Figure 3.3, A}). To investigate how the influx of lymphocytes affected the bladder tissue architecture, we examined bladders histologically. Surprisingly, we found that the majority of aged bladders contained large aggregates of lymphocytes in the lamina propria that were completely absent in young bladders (\textbf{Figure 3.3, B}). Since aging is a continuous and highly
variable process, we examined bladders from mice aged 3 to 18 months (m) (Figure 3.3, C). We found that lymphoid aggregates first appeared in some bladders at 9 m, and all bladders contained these structures at 15 m. These observations indicated that lymphoid aggregates began to form in ‘middle’ age and became a common feature of mouse bladders by 18 m (approximately equivalent to 60 years old in humans). These structures led us to hypothesize that the B and T cells accumulating in the bladder during aging were accumulating within these aggregates. Indeed, bladder lymphoid aggregates in aged mice were primarily composed of CD3\(^+\) T and B220\(^+\) B cells, while these cells were not found elsewhere in the lamina propria (Figure 3.3, D). In larger aggregates, B and T cells tended to segregate into respective zones, reminiscent of the highly organized structure of lymphoid tissues, such as lymph nodes\(^{15, 16}\). Lymphoid tissues contain specialized structures that facilitate their form and function in generating B cell responses. In the aged bladder, we identified CD31\(^+\) (platelet endothelial cell adhesion molecule, PECAM) vessels that also expressed peripheral node addressin (PNA\(_d\)), a specific marker of specialized, high endothelial venules (HEVs) (Figure 3.3, E). HEVs are otherwise only found in lymphoid tissues and facilitate the circulation of naive lymphocytes into the structure. We also found CD35\(^{hi}\) (complement receptor 1, CR1) follicular dendritic cell (FDC) networks within larger aggregates (Figure 3.3, F). FDCs are stromal cells that structurally support the organization of lymphoid tissues. These findings demonstrate that aged bladders, but not young bladders, contain lymphoid aggregates with features of organized lymphoid tissue; thus, hereafter we term these structures bladder tertiary lymphoid tissues (bTLTs). TLTs typically elaborate functions that otherwise only occur within the secondary lymphoid organs, but TLTs are localized to the site of inflammation and immune response\(^{15, 16}\). Lymphoid aggregates have been reported in a few models of chronic
bacteriuria and bladder cancer but remain poorly characterized and have thus far not been reported in unperturbed bladders\textsuperscript{18, 19, 33}. Since bTLTs are specifically found in aged bladders, they could facilitate inflamm-aging within the bladder tissue and significantly alter immune responses to a variety of stimuli. To begin to understand these structures, we sought to further characterize them and determine their functional capacity during aging.

3.2.3 bTLTs contain germinal centers and promote class-switched IgA responses

Since our scRNA-seq data indicated the presence of a range of B cell states, we hypothesized that there were active B cell responses occurring within bTLTs. Like their SLO counterparts, TLTs can support germinal center (GC) reactions where B cells are activated by antigen recognition, may undergo further selection, affinity maturation, and class-switch recombination, and differentiate into plasma cells that secrete large quantities of antibodies\textsuperscript{34, 35}. We found that a large number of B cells in bTLTs were IgD\textsuperscript{+} naive B cells (Figure 3.4, A), consistent with our scRNA-seq data. Locally active GCs were identified within bTLTs by the highly-specific GC marker GL-7 (Figure 3.4, B). We confirmed the presence of increased numbers of CD138\textsuperscript{+} (Syndecan-1\textsuperscript{+}) plasma cells by flow cytometry (Figure 3.4, C) and localized them to bTLT (Figure 3.4, D), indicating that differentiated B cells were also part of these structures. While the majority of plasma cells identified by scRNA-seq expressed Ighm (Figure S2, C), the presence of GCs within bTLTs suggested that class switch recombination (CSR) may occur locally. Furthermore, we demonstrated that larger bTLTs contained GCs where CSR typically takes place. Tissue RNA-seq also indicated that multiple class-switched isotypes were enriched within the aged bladder (Figure 3.4, E). The most highly enriched, class-switched isotype was IgA, which is the major secretory isotype. This finding agreed with the enriched intestinal IgA
production pathway we previously identified in aged bladders (Figure 3.1, B), which includes processes that typically occur at Peyer's patches in the intestinal mucosa. IgA is found in urine and polymeric Ig receptor (pIgR), which transports secretory IgA across epithelial barriers, is expressed in the urothelium\textsuperscript{36, 37}. However, little is known about the local production, transport, and regulation of IgA at the bladder mucosa. To determine if increased IgA was locally produced in the aging bladder, we first measured IgA concentration in the urine. Urine from aged mice contained \textasciitilde10-fold higher concentrations of IgA compared to urine from young mice (Figure 3.4, F). In the kidney, IgA transport by pIgR is decreased during aging, which may contribute to IgA deposition at the glomerulus\textsuperscript{38}. Pigr was not identified as significantly up- or down-regulated in our tissue RNA-seq data, and independent quantification of Pigr expression in bladders from young and aged mice did not show any significant differences (Figure S5). These data suggest the increased urine IgA in aged mice is likely due to increased local production and not changes in the excretion rate of IgA. To further determine if the increased amount of IgA was locally produced, we cultured bladders ex vivo for 24 hours, finding that aged bladders consistently produced higher concentrations of IgA than young bladders (Figure 3.4, G). Furthermore, we identified IgA\textsuperscript{+}CD138\textsuperscript{+} plasma cells associated with bTLT in aged bladders (Figure 3.4, B, D), indicating that these cells were locally differentiated and likely contributed to the high amounts of IgA in the urine from aged mice. Altogether, these findings indicate that bTLT in aged mice are functionally
active in the local recruitment, activation, and differentiation of B cells into IgA-secreting plasma cells.

3.2.4 Age-associated bTLT form independently of microbiota but require TNFα

Both intrinsic and extrinsic factors influence age-associated changes in the immune system. Several lines of evidence point to the importance of microbial stimuli in TLT formation in other tissues mucosal tissues including the lung and intestine\textsuperscript{39,40}. Furthermore, the intestinal microbiota significant changes in old age and correlates with markers of inflamm-aging\textsuperscript{41}. To determine if microbes triggered age-associated bTLT formation, we examined bladders from aged germ free (GF) mice and age-matched, specific pathogen free (SPF) mice. Surprisingly, there were no differences in the number or size of bTLT in aged GF mice compared to SPF controls (Figure 3.5, A-B), demonstrating that a living microbiota is not required for age-associated TLT formation in the bladder. Tissue RNA-seq of young and aged bladders identified a number of cytokine and chemokine mediators that are likely involved in age-associated bTLT formation (Figure 3.1, C). In particular, we identified and validated increased expression of \textit{Tnf} (TNFα, Figure 3.5, C), a pro-inflammatory cytokine that increases during aging and impacts age-related pathologies\textsuperscript{23,24,39}. While TNFα is required during acute immune responses, particularly to combat bacterial infection, excess or prolonged TNFα can lead to disease. The role of age-associated TNFα in many tissues, including the bladder, remain unknown. To test whether increased TNFα with age was a key driver of bTLT formation, we aged TNFα\textsuperscript{−/−} mice to 18-24 m and examined their bladders for bTLT formation. While aged TNFα\textsuperscript{−/−} mice had small, perivascular infiltrates in the bladder (Figure 3.5, B), they had fewer and smaller bTLT than age-matched WT controls (Figure 3.5, C). Due to the
rarity of bTLT in aged TNFα−/− mice, we were unable to identify comparable structures by immunofluorescence. These data suggest that age-associated TNFα plays a significant role in the expansion and/or maturation of bTLT but may not be responsible for the initial recruitment of small numbers of lymphocytes to the aging bladder. In the context of our overall findings, age-associated TNFα is an important mediator reshaping the immune landscape in the aging bladder to promote the formation of bTLTs.
3.3 Discussion

The consequences of immune aging on mucosal tissues such as the bladder are only beginning to be unraveled\(^{21}\). To gain insight into the connection between aging, inflammation, and bladder disease, we sought to define how aging affects the bladder in an unperturbed state. At the tissue transcriptomic level, we found that nearly all highly significant changes point to an altered immune landscape being a fundamental feature of the aging bladder. The presence of lymphoid aggregates, composed of B and T cells organized into bladder tertiary lymphoid tissue (bTLT), underlie these tissue-level transcriptomic changes. Our findings suggest that age-associated inflammation, or inflamm-aging, could explain why aging is a risk factor for a plethora of bladder diseases. Bladder diseases are highly prevalent among women over 55 and continue to increase with age\(^9,^{21}\). For example, overactive bladder (OAB) affects approximately 50% of women over age 65\(^6\). Inflamm-aging is proposed as a causative or exacerbating factor of OAB, and one study found that elevated urinary CCL2, CXCL1, and nerve growth factor (NGF) correlated with age in OAB patients\(^12\). Women over age 55 are also more susceptible to recurrent urinary tract infections (rUTIs)\(^8\). Mouse models of rUTIs have demonstrated that a history of chronic bacteriuria, characterized by severe inflammation, predisposes to a more severe secondary infection after antibiotic treatment\(^42\). Interestingly, these mice develop lymphoid aggregates in the bladder, while those that resolve their infections do not\(^18\). The composition and structure of these aggregates have not been described; thus, it is unknown whether they are similar to bTLT found in aged mice. Since aged mice harbor bTLT at steady state, the impact of these structures on the bladder tissue environment could be similar in these models. Our findings suggest that an altered or dysfunctional local immune repertoire in the aging bladder could explain why aging is a risk factor for multiple distinct bladder diseases. Considering the high prevalence of bladder diseases, an expanding aged
population, and the relative lack of data on how established disease models differ with age, investigation of these bladder diseases in aged animals is warranted. Our work provides a framework establishing how the bladder differs between young and aged female mice at steady state, including the presence of bTLTs.

We also generated a single-cell resolution, transcriptomic map of resident immune cells within bladder tissue of both young and aged mice, identifying a plethora of immune cells and novel subsets. Prior single-cell studies have used the whole dissociated bladder, resulting in sparse immune cell analysis in this tissue\textsuperscript{43-45}. In aggregate, these studies have identified macrophages, NK cells, 2 sets of dendritic cells, T cells, and monocytes. Using scRNA-seq of enriched immune cell populations, we identified known bladder immune cell populations as well as novel sets of immune cells. In the myeloid lineage, we identified 2 distinct subsets of macrophages, 4 subsets of classical DCs, monocytes, plasmacytoid DCs, and migratory DCs from both young and aged mice in our study. Bladder macrophage subsets could be distinguished by expression of Retnla (also known as RELMα or Fizz1), a hallmark gene of an alternatively-activated macrophage (M2) phenotype. Macrophages typically play a role in tissue repair and homeostasis, but this function has not yet been investigated in the bladder. In aging, macrophages and other immune cells maintain tissue homeostasis by clearing debris as cells die, become senescent, or accumulate damaged proteins\textsuperscript{46}. Impaired clearance of these cells by macrophages contributes to age-related pathologies and inflammation; in the aged bladder, failure to clear these cells could potentially lead to bTLT formation in an effort to compensate for this deficiency. We also identified a small group of macrophages expressing Cxcl13 exclusively in the aged bladder. Since CXCL13 is a homeostatic lymphoid chemokine that attracts and organizes lymphocytes into SLOs and TLTs, these aged bladder macrophages likely play a key role in bTLT formation. While stromal cells are
frequently a source of CXCL13 in other tissues, macrophages appear to take on this role in the aged bladder, demonstrating how the bladder may be a uniquely permissive tissue for TLT formation. In the lymphoid lineage, we identified B cells, NK cells, γδT cells, CD4+ and CD8+ T cells, and ILC2. The most striking difference between young and aged bladders was the largely expanded T and B cell compartments that made up the bTLT described herein. In whole, these data demonstrate that the bladder immune system is likely much more complex than previously appreciated and dramatically altered during aging. This single-cell transcriptomic map of bladder immune cells will be a resource to further dissect the function of different immune cells in bladder health, disease, and aging.

bTLT in the aging bladder represents an unexpected and significant shift of the immune repertoire in the steady state bladder. Unlike other mucosae, the bladder does not normally harbor organized lymphoid tissue; the finding is otherwise only reported in diseased bladder tissue. Here we determined the cellular composition and structural organization of age-associated bTLT and demonstrate that they contain active germinal center (GC) reactions. A spectrum of B cell states was identified by scRNA-seq in aged bladders, including naive, activated, and atypical/dysfunctional memory B cells (or age-associated B cells), as well as a substantial increase in the number of antibody-producing plasma cells. B cell hematopoiesis is reduced in aging and results in oligoclonal expansion of existing B cells47. In humans, these observations derive primarily from peripheral blood, and in mice, most studies focus on the spleen and bone marrow. Here we show that B cells, including a large population of naive B cells, accumulate within the bladder tissue. B cell accumulation in tissues such as the bladder could be one factor contributing to the decline of B cells in the periphery. Our data showed that in aged mouse bladders, bTLT
were capable of recruiting naive B cells to undergo local activation, GC reactions, and plasma cell differentiation.

TLT are more likely to form in aged tissues in response to stimuli. For example, in the lung, aged mice more robustly form inducible bronchus-associated lymphoid tissue (iBALT) in response to cigarette smoke exposure\(^{48}\). Aged mice also spontaneously develop distinct lymphocytic infiltrates in the lung, though these structures have not been formally defined to be iBALT\(^{39}\). In contrast to our findings in the bladder, age-associated lung infiltrates are greatly reduced in aged GF mice, highlighting tissue-specific differences in potential triggers of TLT formation. In the intestine, isolated lymphoid follicles (ILFs) develop after birth and thus are considered TLTs. A greater number of both mature and immature ILFs are found in aged mice compared to young mice, and these ILFs contain altered cellular compositions\(^{49}\). Interestingly, ILFs from aged mice produce lower amounts of chemokines yet produce more IgA than their younger counter parts. ILFs and their precursors are present in GF mice\(^{50}\), but microbial colonization promotes the maturation of these TLTs\(^{40}\), and intestinal microbiota are frequently the antigenic target of IgA produced therein\(^{51}\). These studies establish that TLTs do not absolutely require a live microbiota, implying that they could target non-microbial antigens as well. GF mice are still exposed to environmental, dietary, and altered-self antigens that could drive the formation and maturation of these structures. In the urinary tract, microbial colonization also does not appear to be required to form age-associated bTLT, suggesting that in the urinary tract environment, these TLT typically target non-microbial antigens. One explanation may be an increased burden of altered proteins in the urinary environment, which is designed to store these toxic wastes. TLTs have also been found in the liver and kidneys of aged mice, and drivers of these TLTs also remain elusive\(^{52,53}\).
Urine from aged mice had higher levels of IgA, and aged bladders supported increased local IgA production. In humans, IgA is elevated in the serum during aging\textsuperscript{54, 55}, and one study found IgA enriched in the urine proteome of elderly humans compared to young and middle-aged humans\textsuperscript{56}. While total IgA appears to be elevated in both mice and humans, generation of antigen-specific IgA is impaired\textsuperscript{57}. How IgA functions in the urinary tract remains ill-defined. IgA is increased during UTIs, particularly pyelonephritis, but whether it is protective in subsequent infections or dysregulated in those with rUTIs has not been shown\textsuperscript{58}. Patients with selective IgA-deficiency are not reported to be more susceptible to UTIs, but this association has not been studied extensively and could be due to redundant or compensatory IgM\textsuperscript{37}. The target antigens of homeostatic or bTLT-derived IgA in the urinary tract is unknown, and could be a response to cumulative UTIs over the life, age-associated antigens, tissue damage from long-term urine exposure, the urinary microbiome, or age-associated dysfunctional B cell responses. Our data demonstrate that GF mice form similar numbers and sized bTLT, suggesting that non-microbial factors are likely causative. Further investigation of upstream factors influencing bTLT formation during aging and other urinary tract insults could begin to answer some of these questions.

We also identified a key, age-associated inflammatory cytokine, TNF\(\alpha\), as a mediator of bTLT formation in the aging bladder. Aged TNF\(\alpha\)-deficient mice had reduced numbers and size of lymphoid aggregates compared to aged WT mice. TNF\(\alpha\) is a mediator of inflamm-aging and has many roles in pathologic changes that arise during old age\textsuperscript{23, 24}. Increased TNF\(\alpha\) in centenarians is an independent risk factor for all-cause mortality, and chronic TNF\(\alpha\) exposure impairs beneficial responses to pneumococcal pneumonia\textsuperscript{23, 39}. However, TNF\(\alpha\) is also essential for defense against pathogens, particularly extracellular bacteria. Indeed, TNF\(\alpha\) is required for
effective immune responses during UTIs\textsuperscript{14,59}. However, excess, inappropriate, or prolonged TNFα can promote pathologic changes leading to inflammatory disease and dysfunction during aging. TNFα contributes to interstitial cystitis/bladder pain syndrome (IC/BPS), an inflammatory disorder of the bladder of unknown etiology that is more common in those over 50 years old\textsuperscript{7,11,20,60}. In a mouse model of IC/BPS, ectopic expression of TNFα in the bladder resulted in heightened pain sensitivity in response to bladder filling\textsuperscript{61}, but bTLT-like structures were not reported. These results could be due to factors such as age or sex and suggests that multiple processes are required to support these complex structures. Thus, a fine balance of protective inflammatory responses during acute insults and effective resolution of inflammation throughout life may be key to healthy aging. Considering our findings and the role of TNFα in inflamm-aging and bladder disease, clinical studies examining the relationship between age, TNFα levels, and bladder inflammation could provide further insight into these relationships.

While many questions about bTLT remain a mystery, this new finding demonstrates that aging leads to fundamental changes to the bladder characterized by a dramatically different immune landscape. Future work on the aging bladder should consider the presence of bTLT, as they underlie significant changes that likely impact multiple physiologic and homeostatic processes in the bladder. Finally, our single-cell transcriptomic map of bladder immune cells provides a new resource for studying the complexity of the bladder immune system.
3.4 Methods

Mice

All experimental procedures were approved by the animal studies committee of Washington University in St. Louis School of Medicine (Animal Welfare Assurance #A-3381-01) and McMaster University’s Animal Research Ethics Board. 2- to 24-month-old C57BL/6/J mice were obtained from the National Institute of Aging (NIA). NIA mice are bred and housed 5 mice/cage with wood shavings at Charles Rivers Laboratory on a 12 hr light-dark cycle, at 67-73 deg F and 35-55% humidity, fed sterilized NIH31 food, and provided with water at pH 7.5 with 4-6 ppm chlorine (see https://www.nia.nih.gov/research/dab/aged-rodent-colonies-handbook/barrier-environmental-information for more information). Upon receipt at Washington University, mice are housed at 5 mice/cage with autoclaved Bed-O’Cobs ¼” (Andersons Lab Bedding) on a 12 hr light-dark cycle, at 68-74 deg F, 30-70% humidity, fed sterilized Purina Lab Diet 5053, and provided with sterilized tap water. At McMaster University, mice are housed at 5 mice/cage with Teklad 7090 sani-chips on a 12 hr light-dark cycle, at 21-23 deg C, 30-55% humidity, fed Teklad Irradiated Global 14% protein maintenance diet, and provided with sterilized water and an exercise wheel. Germfree (GF) mice were housed at McMaster University Farncombe Family Axenic-Gnotobiotic Facility otherwise in the same conditions except fed Teklad S-2335 diet. Mice were maintained under specified pathogen-free conditions and monitored for mouse pathogens by ongoing sentinel testing. Tnf<sup>-/-</sup> (originally from Jackson Laboratories #005540<sup>62</sup>), WT mice (originally from Jackson Laboratories #000664), and GF mice were bred and aged to 18-24 months in house at McMaster University<sup>30</sup>. To account for colony differences, Tnf<sup>-/-</sup> mice and GF mice were compared to age-matched WT mice raised in the same facility. In all studies, mice from multiple cages, cohorts, and facilities (for data with WT only) were analyzed.
**Histological and Immunofluorescence analysis**

Bladders were aseptically removed, cut in half, and fixed in 10% neutral buffered formalin or methacarn (60% methanol, 30% chloroform, 10% acetic acid), embedded in paraffin, stained with hematoxylin and eosin (7211, Richard-Allen Scientific) and imaged on a Nanozoomer 2.0-HT system (Hamamatsu). Posterior bladder halves were compared within groups. Number and area of bTLTs were determined in 5 sections spaced 150 µm apart using NDP,view2 software (Hamamatsu). Compact aggregates >10,000 µm² were considered bTLTs. For immunofluorescence analysis, anterior bladders halves were embedded in OCT Compound (4583, Tissue-Tek) and flash frozen. 7 µm sections were fixed with 1:1 methanol-acetone, rehydrated in PBS, and blocked with Avidin/Biotin Blocking Kit (SP2001, Vector Laboratories) followed by 1% BSA in PBS. Primary antibodies against B220 (13-0452, eBioscience), CD3 (14-0031-82, eBioscience) PNAd (120803, BioLegend), CD31 (ab28364, Abcam), CD138 (142511, BioLegend), CD35 (558768, BD Biosciences), GL7 (13-5902-81, eBioscience), IgA (1040-31, Southern Biotech), and IgD (1120-01, Southern Biotech) were incubated overnight at 4° and detected with streptavidin-conjugated and species-specific secondary antibodies followed by Hoechst dye.Slides were covered slipped with Prolong Gold antifade (P36930, Invitrogen) and imaged on Zeiss Axio Imager M2 microscope with Hamamatsu Flash4.0 camera using Zeiss Zen Pro software.

**Organ culture and IgA ELISA**

Bladders were aseptically removed, bisected, rinsed with PBS, and both halves cultured together in 500 µL RPMI-1640 with 10% FBS, 1% Pen/Strep (15-140-122, Gibco), 10 mM HEPES, and
1% Glutamax (35030-061, Gibco). Supernatants were removed after 24 hrs and cleared of debris by centrifugation. IgA concentration in urines and culture media was determined by ELISA according to manufacturer protocol (88-50450-22, Invitrogen).

Flow cytometry
Bladders were aseptically removed, minced with scissors, and digested at 37° for 30 minutes in RPMI-1640 with 10mM HEPES, collagenase D (C5318, Sigma-Aldrich), and DNase (10104159001, Sigma-Aldrich). Bladders were forced through a 70 µm cell strainer (352350, Corning) and washed with 5% FBS in PBS. Single cell suspensions were stained with anti-CD45-eFluor450 (48-0451-82, eBioscience), anti-CD3-APC (17-0032-82, eBioscience), anti-CD19-PE (115511, BioLegend), anti-CD4-FITC (100405, BioLegend), anti-CD8-PE/Cy7 (100721, BioLegend), anti-CD138-BrillantViolet605 (142515, BioLegend), and 7-AAD (420404, BioLegend). Data was acquired on LSR II flow cytometer (BD) and analyzed with FlowJo software v10.0. Gates were determined with isotype antibodies in bladder suspensions from young mice.

Tissue RNA-sequencing
RNA was purified from snap frozen, homogenized bladders with RNeasy Mini Kit (74101, Qiagen) and RNase-free DNase digestion kit (79254, Qiagen). Libraries were prepared with Ribo-Zero rRNA depletion kit (Illumina) and sequenced on a HiSeq3000 (Illumina). Reads were aligned to the Ensembl GRCm38.76 top-level assembly with STAR version 2.0.4b and gene counts were derived from the number of uniquely aligned unambiguous reads by Subread:featureCount version 1.4.5. Sequencing performance was then assessed for total number of aligned reads, total number
of uniquely aligned reads, genes detected, ribosomal fraction, known junction saturation, and read distribution over known gene models with RSeQC version 2.3. All gene counts were then imported into the R/Bioconductor package EdgeR and TMM normalization size factors were calculated to adjust samples for differences in library size. Ribosomal features as well as any feature not expressed in at least 3 samples were excluded from further analysis, and TMM size factors were recalculated to create effective TMM size factors. The TMM size factors and the matrix of counts were then imported into the R/Bioconductor package Limma and weighted likelihoods based on the observed mean-variance relationship of every gene and sample were then calculated with the voomWithQualityWeights function. Generalized linear models were then created to test for gene level differential expression, and differentially expressed genes were then filtered for Benjamini-Hochberg False Discovery Rate (FDR)-adjusted p-values less than or equal to 0.05 and absolute log₂ fold-change ≥1. The log₂ fold-changes for all genes were then imported into the R/Bioconductor packages GAGE and Pathview to find pathways whose mean expression were perturbed versus background with Benjamini-Hochberg FDR-adjusted p-values ≤0.05.

**scRNA-seq and cell type identification**

Bladders were digested with Multi-tissue dissociation kit (130-110-201, Miltenyi) for 30 min at 37 deg. Bladder cells were filtered, washed, and incubated with magnetic CD45 microbeads (130-052-301, Miltenyi) and purified by 2 sequential positive selections (Fig. S4A). Cells were washed and resuspended at appropriate concentrations for loading into 10X Chromium Controller using the Chromium Single Cell 5' Library and Gel Bead Kit. Viability was determined by Trypan Blue with a hemocytometer. Reads were processed using cellranger v3.0.1. Filtered outputs were imported into the R package Seurat and combined. Default Seurat parameters were used unless
otherwise noted. Strict filters of >200 genes/cell, <5% mitochondrial reads/cell, <7500 features/cell, and <2500 genes/cell were used. RunPCA was performed on the top 3000 variable genes from FindVariableFeatures. FindNeighbors and FindClusters were used with the first 40 principle components (PCs) and visualized using Uniform Manifold Approximation and Projection (UMAP) (Fig. S2B). Three clusters drove variation attributed to PC1 (Fig. S2C). Expression of urothelial specific gene Upk3a identified urothelial cells in 2 of these clusters; expression of Dcn identified stromal cells as the other cluster; and CD45 (Ptprc) expression identified the remaining cells as immune cells (Fig. S2D). Urothelial and stromal cells were excluded from further analysis. PCA was again performed on the top 3000 variable genes of this subset. The first 2 PCs demonstrated balanced grouping of approximately 3 main groups of cell types (Fig. S2C). Find Neighbors and FindClusters were used with the first 30 PCs of this subset, resulting in 21 cell clusters. FindAllMarkers was used with min.pct=0.25 to identify cluster-specific genes (Supplemental Table 2). Cell identities were determined by expression of canonical gene markers (Table 1 and Fig. S3). The remaining cell types were identified by literature searches of top differentially expressed genes (Supplementary Table 2) and by loading all cluster markers into the Cluster Identity PRedictor (CIPR)²⁵ (Supplemental Table 3) web tool (https://aekiz.shinyapps.io/CIPR/). The dot product method was used with ImmGen as the reference database.

RT-qPCR

Bladders were flash frozen or stabilized in RNA Save (01-891-1A, Biological Industries) and RNA extracted using TRIzol reagent (15596018, Invitrogen) according to manufacturer protocol followed by gDNA digestion with TURBO DNA-free kit (AM1907, Invitrogen). cDNA was
generated using Superscript II Reverse Transcriptase (18064014, Invitrogen). qPCR was performed with SsoAdvanced Universal SYBR Green Supermix (1725275, Bio-Rad) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Fold-changes were calculated using ΔΔCt method and normalized internally to 18S expression.

Statistical analyses

Statistical tests were performed in GraphPad Prism 8. Data sets were evaluated for normality and lognormality with Anderson-Darling, D’Agostino-Pearson, Shapiro-Wilk, and Kolmogorov-Smirno tests. Lognormal distributions were log-transformed and analyzed as a parametric distribution. Unpaired t-tests (with Welch’s correction where appropriate) or two-way ANOVA with Bonferroni post-tests were used for parametric data, and Mann-Whitney U test or Kruskal-Wallis with Dunn’s multiple comparison tests were used for non-parametric data. P<0.05 was considered significant. Data points represent individual animals. Lines represent the mean for normal distributions, geometric mean for log-normal distributions, or median for non-parametric distributions. Error bars represent SEM. For RNA-seq experiments, Benjamini-Hochberg False Discovery Rate (FDR)-adjusted p-values ≤0.05 were considered significant.
Acknowledgments

We thank Drs. Deborah Frank, Jason Mills, and Paula Saz-Leal for editorial comments, the Genome Technology Access Center (GTAC) for performing and processing sequencing data, and Eric Tycksen for bioinformatic analysis and statistical explanations of tissue RNA-seq data.

Funding

This work was funded in part by NIH grants R01 AG052494, P20 DK119840, and R56 AG064634 to IUM; T32 GM007200 and T32 AI007172 to MML; CIHR #153414 to DMEB; Deutsche Forschungsgemeinschaft fellowship #SCHU3131/1-1 to CS; Ontario Early Researchers award to END; NIH Shared Instrumentation Grant S10 RR0275523 to Alafi Neuroimmaging Core; and P30 CA91842 and UL1 TR002345 to GTAC.

Author contributions

Conceptualization MML, CW, IUM. Methodology MML, CW, IUM, DMEB. Investigation MML, CW, CS, END. Data analysis MML. Resources IUM, DMEB. Supervision CW, DMEB, IUM. Funding IUM, DMEB. Visualization MML. Writing--original draft MML, IUM. Writing--review & editing MML, CW, DMEB, IUM

Competing Interests

The authors have no financial interests to disclose.

Data and materials availability

All sequencing data is available at GSE149571.
Table 3.1. Markers used to identities cell cluster identities from scRNA-seq

<table>
<thead>
<tr>
<th>Cluster Number/Name</th>
<th>Cell Identity Markers</th>
<th>Top CIPR&lt;sup&gt;25,26&lt;/sup&gt; match</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - Macrophages</td>
<td><em>Adgre1</em></td>
<td>Interstitial macrophage (lung)</td>
</tr>
<tr>
<td>1 - B cells (naïve)</td>
<td><em>Cd79a, Ms4a1, Ighd</em></td>
<td>Spleen follicular B cell</td>
</tr>
<tr>
<td>2 - cDC2 (CD209)</td>
<td><em>Mgl2</em></td>
<td>Adipose tissue CD11b- DC</td>
</tr>
<tr>
<td>3 - Macrophages (<em>Retnla&lt;sup&gt;hi&lt;/sup&gt;</em>)</td>
<td><em>Adgre</em></td>
<td>Interstitial macrophage (lung)</td>
</tr>
<tr>
<td>4 - CD8+ T cells</td>
<td><em>Cd3d, Cd3g, Cd3e, CD8b1, Gzmk</em></td>
<td>Naïve CD8+ T cell 96 hours after in vitro stimulation</td>
</tr>
<tr>
<td>5 - Monocytes</td>
<td><em>Ly6c2</em></td>
<td>Blood Ly6C+ MHCII+ monocytes</td>
</tr>
<tr>
<td>6 - B cells (activated)</td>
<td><em>Cd79a, Ms4a1, Ctl4a, Mzb1</em></td>
<td>Marginal zone B cell</td>
</tr>
<tr>
<td>7 - NK cells</td>
<td><em>Gzma, Gzmb</em></td>
<td>Splenic NK cell Ly49H+ subset</td>
</tr>
<tr>
<td>8 - cDC2 (<em>Retnla&lt;sup&gt;hi&lt;/sup&gt;</em>)</td>
<td><em>Sirpa, Mgl2</em></td>
<td>Adipose tissue CD11b+ DC</td>
</tr>
<tr>
<td>9 - cDC1</td>
<td><em>Flt3, Xcr1, Clec9a</em></td>
<td>Adipose tissue CD11b+ DC</td>
</tr>
<tr>
<td>10 - γδT cells</td>
<td><em>Tcrl, Tcrg, Tcre</em></td>
<td>Thymus B6 E15 TCRgammadelta</td>
</tr>
<tr>
<td>11 - Proliferating cells</td>
<td><em>Mki67, Top2a, Ube2c</em></td>
<td>Pre-T cell double positive blasts*</td>
</tr>
<tr>
<td>12 - ILC2</td>
<td><em>Il2ra, Gata3, Arg1&lt;sup&gt;63&lt;/sup&gt;</em></td>
<td>Innate lymphoid cells type 2 (intestine)</td>
</tr>
<tr>
<td>13 - B cells (clonally expanded)</td>
<td><em>Cd79a, Ms4a1, Ighv1-58, Igkv6-15</em></td>
<td>B cell (marginal zone)</td>
</tr>
<tr>
<td>14 - Plasma cells</td>
<td><em>Jchain</em></td>
<td>Pre-T cell double positive all</td>
</tr>
<tr>
<td>15 - CD4+ T cells</td>
<td><em>Cd3d, Cd3g, Cd3e, Cd4</em></td>
<td>CD4+ T cell 8 days after LCMV infection</td>
</tr>
<tr>
<td>16 - Migratory DCs</td>
<td><em>Flt3, Fscn1, Cacnb3, Nudt17, Socs2&lt;sup&gt;64&lt;/sup&gt;</em></td>
<td>Skin draining lymph node DC</td>
</tr>
<tr>
<td>17 - cDC2</td>
<td><em>Flt3, Sirpa, Mgl2</em></td>
<td>Thymus double negative DC</td>
</tr>
<tr>
<td>18 - Macrophages (<em>Cxcl13&lt;sup&gt;+&lt;/sup&gt;</em>)</td>
<td><em>Adgre1</em></td>
<td>F4/80hi liver macrophage</td>
</tr>
<tr>
<td>19 - Macrophages (peritoneal)</td>
<td><em>Adgre1</em></td>
<td>Peritoneal macrophage steady state</td>
</tr>
<tr>
<td>20 - pDC</td>
<td><em>Flt3, Pacsin1, Siglech, Irf8, Cox6a2, Runx2&lt;sup&gt;64&lt;/sup&gt;</em></td>
<td>Spleen CD8- plasmacytoid DC</td>
</tr>
</tbody>
</table>

*Proliferating cells most closely matched Immgen cell types that are rapidly dividing due to the strong influence of cell cycle genes*
Figure 3.1. Tissue transcriptomic map of young and aged bladders. (A) Heatmap of the global tissue transcriptome of young and aged bladders with fold change >2 and Benjamini-Hochberg FDR-adjusted P<0.05. n= 4 bladders/group. (B) Significantly enriched KEGG pathways based on fold change of all genes. (C) Heatmaps of significantly different genes from select significant KEGG pathways. (D) Gene expression validation by qRT-PCR in whole bladders (n=5-11/group). (E) Number of CD45+ cells/bladders from young and aged mice (n=8/group) determined by flow cytometry. Line at median. ***p<0.001, **p<0.01, Mann-Whitney U-test.
Figure 3.2. Single cell transcriptomic map of immune cells from young and aged bladders. (A) Clustering analysis and cell type identification of CD45+ cells from young (n= 3209) and aged bladders (n=4682). Cluster numbers are ordered by most abundant cell type from the merged data set. (B) Map of cells originating from young or aged bladders. (C) Expression of Cxcl13 in scRNA-seq macrophage clusters (numbered as in A and colored as in B). (D) RT-qPCR of sorted F4/80+CD64+Ly6C- macrophages from young and aged mice (n=3-5/group). **p<0.01, Unpaired t-test with Welch's correction. Lines at mean.
Figure 3.3. Lymphoid infiltrates form bladder tertiary lymphoid tissues (bTLT) during aging. (A) Frequency of B cells, T cells, and T cell subsets among live CD45+ cells in young and aged bladders by flow cytometry. n=5-8 per group. (B) Representative H&E images of young and aged bladders. (C) Number of bTLT over the life course of mice n=5-10/group. (D) Representative image of B cells (B220+, green) and T cells (CD3+, red) in segregated zones within bTLT in aged mice. (E) Representative image of CD31+(red) PNAd+(green) high endothelial venules (white arrowheads) within bTLT in aged mice. (F) Representative image of CD35hi follicular dendritic cell network within bTLT in aged mice. All nuclei stained with DAPI (blue). All scale bars, 50 µm.
Figure 3.4. bTLT are centers for B cell recruitment, activation, germinal center reactions, and plasma cell differentiation. (A) Representative image of naive B cells (IgD+, green) and T cells (CD3+, red) within bTLT of aged bladders. (B) Representative image of IgA+ cells within a GL7+ (green) germinal center of a bTLT. (C) Frequency of live CD45+CD138+ plasma cells in young and aged bladders by flow cytometry. n=5/group. (D) Representative image of IgA+CD138+ plasma cells within bTLT of aged bladders. (E) FDR-adjusted P values of IgM and class-switched isotypes from tissue RNA-seq of young and aged bladders. Red line, p=0.05. (F) Concentration of IgA in urine of young (n=13) and aged (n=27) mice. (G) Concentration of IgA in supernatants of young and bladders cultured ex vivo for 24 hours. n=5/group. All scale bars, 50 µm. **p<0.01, ***p<0.001. Mann-Whitney U test.
Figure 3.5. bTLT size and number are independent of microbial status and dependent on age-associated TNFα. (A) Representative H&E image of WT aged germ free (GF) bladders. (B) Number and size of bTLT found in WT specific pathogen free (SPF) and GF bladders. n=9-17/group. (C) Relative expression of Tnf and in young and aged bladders by RT-qPCR. n=10/group. (D) Representative H&E images of aged WT and TNFα−/− bladders. (E) Number and size of bTLT in aged WT and TNFα−/− bladders. Lines at median. *p<0.05, **p<0.01, Mann-Whitney U-test. Scale bars, 100 µm
Supplementary Figure 1. *Cxcl12* and *Ccl21* expression are unchanged in bladders from aged mice compared to those from young. (A) Relative *Cxcl12* in bladders from young (n=12) and aged (n=10) mice. (B) Relative *Ccl21* expression in bladders from young (n=5) and aged (n=8) mice.
Supplementary Figure 2. Isolation and initial analysis of CD45+ cells from young and aged bladders used for scRNA-seq. (A) Representative flow cytometry plot of the purity (left) and viability (right) of CD45+ cells from magnetic enrichment. (B) UMAP plot of initial clustering analysis of single cells from young and aged bladders. (C) PCA plots of cell clusters from initial analysis (left) and after exclusion of non-immune cells (right). Cluster colors correspond to initial clustering results as in B. (D) Expression of markers for urothelial cells (Upk3a, left), stromal cells (Dcn, middle), and immune cells (Ptprc, left) in the initial clustering analysis. Arrows point to relevant cell clusters.
Supplementary Figure 3. Expression of canonical markers used to identify cluster cell types.
Supplementary Figure 4. Aged bladders contain unique macrophages and B cells that are absent in young bladders. (A) Expression of Retnla in macrophage clusters numbered as in main text (Fig. 2A). (B) Cell clusters derived from young and aged bladders colored as in main text (Fig. 2A). (C) Expression of select genes differentiating B cell-lineage clusters numbered as in main text (Fig. 2A).
Supplementary Figure 5. Polymeric Ig receptor (pIgR) expression does not change in aged bladders.
REFERENCES


59. Yu L, O'Brien VP, Livny J, Dorsey D, Bandyopadhyay N, Colonna M et al. Mucosal infection rewires TNFα signaling dynamics to skew susceptibility to recurrence. *Elife* 2019; **8**.


Chapter 4: Bladder mucosal *cystitis cystica* lesions are tertiary lymphoid tissues that correlate with recurrent urinary tract infections in older/postmenopausal women.

This chapter was submitted for publication in a peer-reviewed journal. MML wrote this chapter and performed all experiments and analysis unless otherwise noted.

ABSTRACT

Objective: To identify correlates and clinical outcomes of *cystitis cystica* (CC), a poorly-understood chronic inflammatory change of the bladder, in women with recurrent urinary tract infections (rUTIs).

Methods: A retrospective, observational cohort of women with rUTIs that underwent cystoscopy (n=138) from 2015 to 2018 were identified using electronic medical records. CC status was abstracted from the cystoscopy report and correlations identified by logistic regression. UTI-free survival time associated with CC was evaluated by Cox proportional hazards regression. Multinomial logistic regression was used to identify factors associated with changes to CC lesions on repeat cystoscopy. Biopsies of CC lesions were examined by routine histology and immunofluorescence.

Results: 53 patients (38%) had CC on cystoscopy. In multivariable-adjusted analyses, CC was associated with postmenopausal status (odds ratio [OR] [95% confidence interval [CI]]: 5.53 [1.39-37.21]), pelvic floor myofascial pain (PFMP; 6.82 [1.78-45.04]), having ≥4 UTIs in the past year (2.28 [1.04-5.09]), and a shorter time to next UTI (hazards ratio: 1.54 (1.01-2.35). 42 patients (82%) demonstrated improvement or resolution of lesions, most commonly those who took ≥1 week of antibiotics since their first cystoscopy (p<0.01). 13/14 (93%) biopsied CC lesions contained organized tertiary lymphoid tissues with germinal centers.

Conclusions: CC lesions are associated with postmenopausal status, PFMP, and number of UTIs in the prior year. CC predicts worse rUTI outcomes. CC lesions are tertiary lymphoid tissue that may improve or resolve over time with treatment. Identifying CC in rUTI patients may be useful in stratifying future UTI risk and tailoring appropriate treatment strategies.
4.1 Introduction

Urinary tract infections (UTIs) are the most common infection in women, affecting at least half of all women during their lifetime. UTIs result in an estimated 13 million outpatient visits and cost $2 billion per year in the US alone\textsuperscript{1}. UTIs are particularly bothersome because they frequently recur within 6 to 12 months after an initial infection, causing recurrent UTIs (rUTIs)\textsuperscript{2-4}. Postmenopausal women are particularly susceptible to UTIs and rUTIs\textsuperscript{3,5}. Whereas UTIs recur in approximately 25-35\% of younger UTI patients, over 50\% of UTIs in women over 55 become recurrent\textsuperscript{2}. Furthermore, UTIs may lead to pyelonephritis, sepsis, and death, particularly in elderly patients. While the pathogenesis of the most common uropathogens, such as \textit{E. coli}, has been well-studied, less is known about the immune response to UTIs, clinical factors that influence this response, and how the immune response influences susceptibility to future infections.

Immune responses to a prior UTI likely play a significant role in promoting recurrent infections. Mouse models of UTIs demonstrate that severe bladder inflammation increases binding of some bacteria to the urothelium and predisposes to more severe and chronic infections from a variety of uropathogens, even after antibiotic treatment\textsuperscript{6-10}. Adaptive immune responses to UTIs are not well understood, and in humans, few studies have examined immunologic factors that may influence risk of recurrent infection. Furthermore, aging and menopause are known to impact the immune system such that it is less effective at eliminating pathogens while simultaneously promoting excess and prolonged inflammation\textsuperscript{11-13}. The higher UTI recurrence rate in older, postmenopausal women could therefore be related to dysregulated immune responses to infection.

Another factor that contributes to the high risk of UTIs in older, postmenopausal women is estrogen, which has been shown to play a protective role against UTIs\textsuperscript{14-16}. Thus, lower estrogen levels during menopause increase the risk of UTIs. Vaginal estrogen therapy, which has low
systemic absorption, is effective at reducing UTIs in postmenopausal women and reduces inflammation in the urinary tract\textsuperscript{4,17,18}. Additional factors that contribute to greater UTI risk in older, postmenopausal women include common, comorbid conditions, including incomplete bladder emptying, incontinence, catheterization, advanced pelvic organ prolapse, and pelvic surgical procedures\textsuperscript{19}.

Cystoscopy is used to identify sources of lower urinary tract symptoms and potential niduses for rUTIs. One common cystoscopy finding in women with rUTIs is cystitis cystica (CC), which appears as multiple mucosal cysts or nodules that may be red, yellow, pink, or grey in appearance\textsuperscript{20}. Grossly, lesions described as cystitis cystica, cystitis glandularis, and follicular cystitis may appear similar, but biopsies of these lesions are not frequently performed to confirm the specific inflammatory pathology. Furthermore, lesions containing all 3 pathologic changes have been reported within the same specimen.\textsuperscript{21} It is currently unknown why some patients develop CC while others do not, whether the presence of CC impacts the pathogenic cycle of rUTIs, and what specific immune responses occur within these lesions.

We utilized a series of retrospectively-assembled clinical studies and samples from the Washington University Women’s Genitourinary Tract Specimen Consortium (WGUTSC) biobank to address the following objectives: 1) identify clinical factors associated with the presence of CC on cystoscopy; 2) determine whether CC is associated with rUTI outcomes; 3) evaluate whether CC lesions resolve over time; and 4) begin to explore pathologic mechanisms of CC by characterizing immune infiltrates in biopsies of CC lesions.
4.2 Methods

Patient population

All studies were approved by the Washington University in St. Louis Institutional Review Board (#201712113, #201901033, and #20190534).

Eligible patient records for the retrospective cohort were identified using Washington University in St. Louis billing records for "cystourethoscopy" (hereafter referred to as "cystoscopy") from 2015 to 2018 and manually reviewed by 3 investigators. Data were recorded in a REDCap database. Information on patient demographics, medical history, cystoscopy findings, urine cultures, and rUTI treatment and prevention strategies were recorded. Urine cultures from 12 months prior to and 12 months following initial cystoscopy were considered. Inclusion criteria were as follows: female, ≥18 years old, and culture-proven rUTIs, defined as at least 2 culture-proven UTIs within 6 months or 3 culture-proven UTIs within 12 months prior to initial cystoscopy. Patients were considered to have pelvic floor myofascial pain (PFMP) based on standardized exam and the physician's assessment and plan note. For the repeat cystoscopy study, a subset of patients with ≥2 cystoscopy records were included. Days between cystoscopies, UTI prophylaxis therapies and antibiotic treatments ≥7 days used between cystoscopies were recorded.

Women’s genitourinary tract specimen consortium (WGUTSC) participants were recruited from the Washington University in St. Louis Female Pelvic Medicine and Reconstructive Surgery (FPMRS) outpatient clinic. All FPMRS patients were eligible, but those with rUTIs were preferentially recruited. WGUTSC participants provided deidentified biologic specimens with linked clinical data (as above) that were abstracted to a REDCap database.
Bladder biopsy samples

WGUTSC participants that had CC during on office cystoscopy and were undergoing clinically-indicated gynecologic surgery or exam under anesthesia consented to provide bladder biopsies. Pinch biopsies were obtained, fixed in formalin, and submitted to pathology for diagnosis and to the WGUTSC biobank. Pathology reports documented by board-certified pathologists were obtained from the electronic medical record.

Histology

Biopsies were embedded in paraffin and sectioned at 5 µm thickness. Sections were deparaffinized in xylene, rehydrated, and microwaved in 10 mM sodium citrate for antigen retrieval. Sections were permeabilized with 0.1% Triton X-100, blocked in 1% BSA in PBS, stained with anti-CD3, anti-CD20, and anti-CD21 antibodies overnight at 4°C, and detected with species-specific AlexaFluor-conjugated secondary antibodies and Hoescht dye. Images were acquired using Zeiss Axio Imager M2 microscope with a Hamamatsu Flash4.0 camera and Zeiss Zen Pro software.

Statistics

All statistical analyses and visualization were performed in R 3.6.1, Graphpad Prism v8, or SAS version 9.4. R packages used for data analysis included tidyverse, glm, survival, gtsummary, and ggfortify. For the first objective (to identify clinical correlates of CC), univariate analyses were conducted using Mann-Whitney U-test, Chi-square, and Fisher’s exact tests, and multivariable
analyses were conducted using logistic regression. Restricted analyses were also performed among post-menopausal women to distinguish the effect of age from menopausal status. For the second objective (to identify rUTI outcomes related to CC), survival analyses were conducted using log-rank tests and Cox proportional hazards regression. Participants were followed from the date of the baseline cystoscopy to the date of their first symptomatic, culture-positive UTI, last known contact date with the FPMRS clinic in the medical record, or 365 days after cystoscopy, whichever came first. For the third objective, we used Kruskal-Wallis, Chi-square, and Fisher’s exact tests, as well as multinomial logistic regression to identify factors associated with improvement and resolution of CC. P<0.05 was considered significant.

4.3 Results

4.3.1 Factors associated with cystitis cystica

Over the 3-year study period, 464 women age ≥18 had at least one cystoscopy procedure. A total of 138 (29.7%) patients met criteria for documented rUTIs prior to cystoscopy. Cystoscopy procedure notes described bladder changes consistent with CC in 53 (38.4%) of these 138 rUTI patients.

In univariate analyses, women with CC were significantly older (median [interquartile range, IQR]: 71 [63, 75] vs 66 [55, 76], p=0.035), had a higher number of UTIs in the prior 12 months (median [IQR], 4 [3, 5] vs 3 [1, 4]; p=0.042), were more likely to be post-menopausal (96% vs. 82%; p=0.046), and were more likely to have PFMP on examination (94% vs. 78%; p=0.006; Table 4.1). Other known risk factors for rUTIs, including diabetes, self-catheterization, incontinence, sexual activity, incomplete bladder emptying (as determined by post-void residual), prior urogenital surgery, prolapse, and systemic immunosuppression, were not significantly
associated with CC. Within the subset of post-menopausal women (n=119), age was not significantly associated with CC, but the associations of CC with number of prior UTIs and PFMP persisted. In multivariable-adjusted analyses of all and post-menopausal women, respectively, postmenopausal status (OR [95% CI]: 5.53 [1.39-37.21]), PFMP (6.82 [1.78-45.04] and 6.68 [1.73-44.32]), and having ≥4 UTIs in the past 12 months (2.28 [1.04-5.09] and 2.36 [1.05-5.42]) remained significantly associated with CC.

4.3.2 Association between CC and time to UTI recurrence

To determine whether patients with and without CC have different clinical outcomes, we tracked culture-positive UTIs in the 12 months following cystoscopy. Patients with CC had a significantly greater number of culture-positive UTIs (median [IQR]: 1 [0-4]) than patients without CC (1[0-2], p<0.01), and a shorter median UTI-free survival time (74 versus 185 days, p=0.03; Figure 4.1) In adjusted analyses, CC (HR: 1.54 [1.01-2.35] and number of UTIs in the prior year (HR: 1.20 [1.07-1.34]) were independently predictive of reduced UTI-free survival times. No other variables were significant predictors of UTI-free survival time. In these analyses, the postmenopausal subgroup did not differ from the entire cohort, and postmenopausal status was not predictive of a shorter UTI-free survival time.

4.3.3 Improvement and resolution of CC lesions on repeat cystoscopy

Whether CC lesions are reversible is not clearly established. Therefore, we investigated how these lesions change over time and whether they respond to antibiotic or prophylactic treatments. Fifty-one participants with CC had at least 1 repeat cystoscopy and were included in the analysis. Based on procedure notes, 9 (17.7%) participants had no change in CC status on
repeat cystoscopy, 23 (45.1%) had improvement in their CC, and 19 (37.3%) resolved their CC (Figure 4.2). Time between cystoscopies was longest for those who resolved (median=84 days, IQR: 28-229), intermediate for those who improved (41 [24-77]) and shortest for those without change (27 [20-37] p=0.048). This finding suggests that resolution of CC lesions may take approximately 3 months. Participants who were treated with ≥1 week of antibiotics for an acute UTI between cystoscopies were more likely to have improved or resolved CC lesions on repeat cystoscopy (52.4% and 38.1%, respectively) than those who did not receive antibiotics (11.1% and 33.3%, p<0.01). This finding was independent of time between cystoscopies. No differences were observed for other therapies, such as vaginal estrogen therapy, D-mannose, and methenamine, although use of these therapies was heterogenous.

4.3.4 Histopathology of cystitis cystica biopsies

To characterize the immunologic and pathologic changes to the bladder that occur with CC, we obtained biopsies of CC lesions from 14 women during clinically-indicated gynecologic surgery or exam under anesthesia who were identified on prior office cystoscopy (clinical characteristics presented in Table 4.2). Separate biopsies taken simultaneously were evaluated by clinical pathologists. Two biopsies had insufficient tissue for pathologic evaluation. The remaining 12 biopsies (86%) were reported to have chronic inflammation with no malignant changes. Seven of the 12 (58%) biopsies contained distinct lymphoid follicles in the lamina propria, consistent with a pathologic diagnosis of follicular cystitis (Figure 4.3, A-B). Further analysis of biopsies from 13 of the 14 patients (93%) using immunofluorescence identified distinct B and T cell zones with follicular dendritic cell networks organized into lymphoid follicles with distinct germinal
centers (Figure 4.3, C-D). These findings indicate that CC lesions contain a distinct inflammatory infiltrate known as tertiary lymphoid tissue (TLT).

4.4 Discussion

*Cystitis cystica* (CC) has been generally regarded as a benign, insignificant, and non-specific finding of chronic inflammation and thus rarely reported in the literature. Its prevalence, pathogenesis, and impact on UTIs has not been previously well-described in this population. We report an analysis of the frequency of CC in rUTI patients who underwent cystoscopy and clinical factors associated with this finding. We found that postmenopausal status, number of UTIs in the prior year, and PFMP were highly associated with finding CC on cystoscopy. Compared to those without CC, patients with CC were also more likely to have a greater number of UTIs in the year following cystoscopy and a shorter time to their next UTI. We also observed that resolution of CC lesions typically occurs after approximately 3 months. Improvement or resolution of lesions was associated with at least 1 week of antibiotic use.

CC has also been associated with rUTIs in prepubertal girls. While some cases are associated with urinary tract anomalies, one study examined CC in patients without a clear explanation for their rUTIs. That study found correlations between the number of CC lesions observed on cystoscopy with number of UTIs in the prior year and bladder wall thickness measured by ultrasound. In contrast to our work, another study found no changes in the number of lesions after patients were given extended antibiotic treatment and were UTI-free for at least 6 months. These findings led the authors to suggest that CC may promote infection, forming a positive feedback loop of cyclical infection and inflammation. However, another group observed that some pediatric patients with CC could resolve their years but required years of antibiotic therapy. Our
study in primarily postmenopausal women showed that antibiotics were also associated with CC resolution, but on a much shorter timescale. In our study, patients were also taking other UTI prophylactic agents, such as vaginal estrogen, D-mannose, and methenamine, though the regimens were heterogeneous. Given these studies in prepubertal girls, our finding that CC and postmenopausal status are associated, and the known protective effects of estrogen in the bladder, forming CC lesions is likely influenced by estrogen or other sex hormones.

Our histologic studies demonstrate that CC lesions contain tertiary lymphoid tissues (TLTs) that are better described in pathologic terms as follicular cystitis. TLTs are organized lymphoid tissues that arise in non-lymphoid organs and are capable of generating local, adaptive immune responses. TLTs in organs other than the bladder have been associated with chronic inflammation in response to chronic infection, autoimmune disease, cancer, or other persistent stimuli\(^{29-34}\). Although localized, adaptive immune responses would intuitively be protective against infection, TLTs have been associated with both protective and pathogenic responses to different chronic infections. For example, TLTs in the lung are associated with control and latency of *Mycobacterium tuberculosis* and are thus thought to protect against reactivation of latent *M. tuberculosis*\(^{35-37}\). In contrast, TLTs associated with chronic hepatitis C virus infection result in greater inflammatory liver pathology and autoimmune complications such as cryoglobulinemia\(^{38}\). In chronic *Helicobacter pylori* gastroenteritis, TLTs have the potential to become mucosa-associated lymphoid tissue lymphomas\(^{39,40}\). Interestingly, these gastric TLTs, both benign and malignant, frequently regress after eradication of *H. pylori* with antibiotics, akin to resolution of CC that we observe with antibiotic and rUTI prophylaxis treatments. Given that patients with CC had a shorter time to their next UTI and a greater number of UTIs in the following year, TLTs may
potentially play a pathogenic role in the bladder, generating ineffective immune responses that promote further inflammation rather than resolution of rUTIs.

The strengths of this paper include a well-characterized clinical cohort of women with culture-proven rUTIs, cystoscopically-confirmed CC, and associated biospecimens that formed the basis of this study. In addition, a subset of women underwent repeat cystoscopy by the same FPMRS attending physician that performed the baseline cystoscopy that allowed visual confirmation of the status of CC post-intervention. Limitations of this study include the overall small sample size and a smaller subset of women that underwent repeat cystoscopy. There is also not a validated method to cystoscopically assess severity of CC. Thus, this retrospective study relied on documented descriptions reflecting each physician's impression. Developing a validated severity scale would improve monitoring CC lesions over time.

In summary, we find that CC in women with rUTI is associated with post-menopausal status, PFMP, a higher number of prior UTIs, and shorter UTI-free survival time after cystoscopy. CC lesions are composed of tertiary lymphoid tissues with germinal centers. Lesions appear to improve or resolved over time, most commonly associated with ≥7 days of antibiotic treatment. Given these findings, we suggest that CC is a specific inflammatory response to rUTIs that promote further susceptibility to future UTIs, and this cycle may be interrupted by extended, pathogen-directed antibiotic treatment.
CONTRIBUTIONS

MML, IUM, and JLL conceived the studies. MML, BL, and SML designed the studies. MML, BL, PP, and SML extracted clinical data. MML analyzed clinical and biopsy data. SS provided statistical expertise. JLL and IUM supervised the study. MML, JLL, and IUM wrote the paper. All authors approved of the final manuscript.
ACKNOWLEDGEMENTS

We thank Zoe Jennings and Women’s Genitourinary Tract Specimen Consortium (WGUTSC), for consenting patients, collecting, and cataloging samples; Jessica Sawhill and Dr. Melanie Meister for collecting biopsies. This work was funded in part by NIH grants R01AG052494, R56AG064634, and P20DK119840 (to IUM), T32 GM007200 and T32AI007172 (MML), and, NIH CTSA Grant Number UL1TR002345, Washington University Institute for Clinical and Translation Sciences (ICTS) grant (to JLL). ICTS JIT655 (to MML).
Figure 4.1. UTI-free survival of rUTI patients stratified by CC status. Time until the first UTI since diagnostic cystoscopy in rUTI patients. + indicates last known contact dates of patients lost to follow-up. p<0.03, log-rank test.
Figure 4.2. Improvement and resolution of CC upon repeat cystoscopy. Representative initial and repeat cystoscopy images of a patient with resolution of CC lesions.
Figure 4.3. Cystitis cystica lesion biopsies. (A) Representative cystoscopic view of CC lesions, encircled in black. (B) Representative H&E of lymphoid follicle with germinal center in CC biopsy. Scale bar, 200 µm (C) Representative immunofluorescent image of B cells (CD20, green) and T cells (CD3, red) within lymphoid follicle. Hoescht, blue. Scale, 50 µm (D) Representative immunofluorescent imagine of follicular dendritic cell network (CD21, red) within lymphoid follicle. Hoescht, blue. Scale, 50 µm.
Table 4.1 rUTI Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>All patients (n=138)</th>
<th>Post-menopausal patients (n=119)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no CC = 85</td>
<td>CC = 53</td>
</tr>
<tr>
<td>Age¹</td>
<td>66 [55,76]</td>
<td>71 [63,75]</td>
</tr>
<tr>
<td>Race (% white)</td>
<td>94% (80)</td>
<td>86% (44)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>82% (67)</td>
<td>96% (50)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>78% (66)</td>
<td>74% (39)</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>20% (17)</td>
<td>7.7% (4)</td>
</tr>
<tr>
<td>Neurogenic Bladder</td>
<td>6% (5)</td>
<td>1.9% (1)</td>
</tr>
<tr>
<td>Intermittent or indwelling catheter</td>
<td>18% (15)</td>
<td>15% (8)</td>
</tr>
<tr>
<td>Pelvic floor myofascial pain</td>
<td>78% (62)</td>
<td>94% (45)</td>
</tr>
<tr>
<td>Urinary incontinence</td>
<td>48% (41)</td>
<td>56% (29)</td>
</tr>
<tr>
<td>PVR¹</td>
<td>60 [25,170]</td>
<td>70 [40,174]</td>
</tr>
<tr>
<td>Prolapse (all types)</td>
<td>23% (19)</td>
<td>37% (19)</td>
</tr>
<tr>
<td>Anterior prolapse</td>
<td>12% (1)</td>
<td>21% (11)</td>
</tr>
<tr>
<td>Apical prolapse</td>
<td>6% (5)</td>
<td>17% (9)</td>
</tr>
<tr>
<td>Posterior prolapse</td>
<td>1.2% (1)</td>
<td>1.9% (1)</td>
</tr>
<tr>
<td>Prolapse stage¹,²</td>
<td>0 [0, 0]</td>
<td>0 [0, 2]</td>
</tr>
<tr>
<td>Prolapse stage¹,³</td>
<td>2 [1.5, 2.5]</td>
<td>2 [2, 3]</td>
</tr>
<tr>
<td>Fecal incontinence</td>
<td>12% (10)</td>
<td>5.7% (3)</td>
</tr>
<tr>
<td>Sexually active</td>
<td>41% (33)</td>
<td>37% (19)</td>
</tr>
<tr>
<td>Dyspareunia</td>
<td>45% (15)</td>
<td>21% (4)</td>
</tr>
<tr>
<td>Cancer</td>
<td>28% (24)</td>
<td>36% (19)</td>
</tr>
<tr>
<td>Hysterectomy</td>
<td>50% (42)</td>
<td>56% (29)</td>
</tr>
<tr>
<td>Oophorectomy (any)</td>
<td>23% (19)</td>
<td>31% (15)</td>
</tr>
</tbody>
</table>

Fisher exact tests.

¹Mann-Whitney U-test. ²Including those without prolapse as stage 0. ³Omitting those without prolapse.
### Table 4.2 Patient Characteristics of CC Biopsies

<table>
<thead>
<tr>
<th>Age</th>
<th>Menopause</th>
<th>Organ(s)</th>
<th>Cystoscopy description</th>
<th>Pathology description</th>
<th>Follicles on IF</th>
<th>UTT1</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>post</td>
<td>E. coli</td>
<td>cobblestone appearance</td>
<td>chronic cystitis</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>post</td>
<td>E. coli</td>
<td>raised lesions</td>
<td>acute &amp; chronic inflammation</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>post</td>
<td>K. pneumonia</td>
<td>cobblestone appearance</td>
<td>chronic cystitis</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>post</td>
<td>K. pneumonia</td>
<td>raised lesions</td>
<td>acute &amp; chronic inflammation</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>post</td>
<td>K. pneumonia</td>
<td>diffuse cystic raised</td>
<td>plaque-like raised lesions</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>post</td>
<td>K. pneumonia</td>
<td>cystic lesions</td>
<td>acute &amp; chronic inflammation</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>post</td>
<td>K. pneumonia</td>
<td>diffuse cystic raised</td>
<td>plaque-like raised lesions</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>post</td>
<td>K. pneumonia</td>
<td>cystic lesions</td>
<td>acute &amp; chronic inflammation</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>post</td>
<td>K. pneumonia</td>
<td>diffuse cystic raised</td>
<td>plaque-like raised lesions</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>post</td>
<td>K. pneumonia</td>
<td>cystic lesions</td>
<td>acute &amp; chronic inflammation</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes:
- UTT1: UTI type 1
- Y: Yes
- N: No
- n/a: Not applicable
REFERENCES


Chapter 5: Conclusions and Future Directions
5.1 Conclusions

In this thesis, I embarked on asking key questions regarding drivers of increased bladder disease with age in females. My work addresses (1) immunological changes to the aging bladder, (2) age-associated mechanisms of bladder tertiary lymphoid tissue (bTLT) formation, and (3) risk factors and outcomes of bTLT (clinically known as cystitis cystica [CC]) in women with recurrent urinary tract infections (rUTIs). This thesis is the first to describe the following key factors involved in bTLT formation and function in mice and women:

(1) Aging—In Chapter 3, I showed that aged mouse bladders were immunologically distinct from young bladders and harbored bladder tertiary lymphoid tissues (bTLTs). Age-associated bTLTs were composed of highly organized B and T cells and capable of forming germinal centers, plasma cells, and secretory IgA. I further find in Chapter 4 that increased age is a risk factor for developing bTLT in women with rUTIs.

(2) Sex—This work focused on aging and disease in the female bladder. In Chapter 3, I showed that age-associated bTLT in mice begin to appear around 9 mo of age and become more common thereafter. Mice generally stop breeding (known as reproductive senescence) around 9-12 mo of age, suggesting that sex, hormones, or reproductive organs may be involved in bTLT formation. In Chapter 5 (below), I demonstrate that aged male mice rarely have bTLT, further supporting a role for sex in age-associated bTLT formation.

(3) Inflamm-aging—In Chapter 3, I determined that TNFα, a prototypical inflammatory cytokine that mediates inflamm-aging, was required to form bTLT. Aged mice deficient in TNFα formed
fewer, smaller, and likely less mature bTLT than their wildtype (WT) counterparts. Thus, both aging and inflammation play a role in forming these structures.

(4) **Human health**—In Chapter 4, I demonstrated that CC lesions found on cystoscopy in women with rUTIs were lymphoid follicles analogous to bTLTs in aged mice. Using retrospective clinical data, I found that age, but not the number of prior UTIs, was a significant risk factor in rUTI-associated CC. Those with CC had less time until their next UTI and a greater number of UTIs in the following year than those without CC. I provided evidence that CC lesions may regress over time and that resolution was associated with extended antibiotic use.

(4) **Microbes**—In mice, microbial colonization was not required for age-associated bTLT formation, as shown in Chapter 3 by the presence of bTLT in aged, germ free mice. However, in Chapter 4, I found that women with CC most often had UTIs, and that these structures promoted poor rUTI outcomes. Furthermore, antibiotic use was associated with resolution of CC lesions. Thus, while microbes are not exclusively required for age-associated bTLT formation, uropathogens likely have substantial interactions with these structures. Since age was also a risk factor for CC in humans, there is likely an interplay of age-associated susceptibility to bTLT that may be triggered or exacerbated by infection.

My work thus provides a new framework for studying TLTs within the urinary bladder and the immunology of the bladder during old age that can be translated to clinical outcomes and potentially therapies for women with rUTIs. This new concept of the interplay between aging, infection, and local immune responses within the bladder generates many new important questions.
for the field. First and foremost, how do bTLT respond to infection on the cellular and molecular level? By what mechanisms are they protective or pathogenic with regard to UTIs? What is the sequence and key signals that initiate bTLT formation? What stimulates bTLT formation during aging in mice in the absence of microbes? And, how can we apply this knowledge to the management of UTIs in humans? In the final chapter of this thesis, I will discuss on-going work that begins to answer some of these questions, other hypotheses to be tested, and the future impact of this work.
5.2 On-going work and future directions

5.2.1 Influence of sex on bTLT formation

Since most UTIs occur in women and age-related bladder diseases are distinct between men and women, this thesis focused on using aged female mice to model the aging bladder and examined clinical factors affecting *cystitis cystica* (CC) and rUTIs in women. While mice do not have as dramatic a reduction in estrogen levels as human do during menopause, they do have decreased estrogen levels and lose their reproductive ability as they age\(^1\), \(^2\). Interestingly, reproductive senescence occurs around 9 to 12 months in mice, which coincides with the early appearance of bTLTs (*Chapter 3, Figure 3.3, C*). Since the urothelium is estrogen-responsive, processes occurring during reproductive senescence and atrophy of the ovaries and uterus may trigger changes leading to bTLT formation. Reduced estrogen, particularly its local effects in the genitourinary tract, is known to increase the risk of UTIs in women. Furthermore, mouse and human studies have demonstrated that estrogen is protective during UTIs by reducing inflammation and promoting urothelial repair following infection\(^3\)-\(^6\). However, the interaction between age, sex, hormone levels, and reproductive senescence on the bladder is complex.

To test whether sex was important to bTLT formation, we compared the bladders of age-matched male and female mice raised in the same facility. We found that male mice do not form bTLT as robustly as female mice. Male mice had significantly fewer bTLT than female mice (*Figure 5.1, A*). Since there were few bTLT in male mice, it was inconclusive whether these bTLT were smaller than those in female mice (*Figure 5.1, B*). We also observed that the male mice that did contain bTLTs tended to be older; however, our sample size was limited. These findings suggest that in the bladder, the aging process is significantly different in males than in females.
Female aging is thought to be more inflammatory, which could influence bTLT formation. However, levels of specific cytokines and other genes have not been tested in male bladders.

*Future directions:* To test the interplay of sex, hormones, and age, male and female mice with and without gonadectomy and with and without hormone replacement could be aged and examined for bTLT. Several studies have used these techniques to determine the effects of sex and hormones on UTIs in mice, finding that male mice are less likely to clear experimental UTI than female mice and that these effects are at least partially mediated by testosterone\(^7\,^9\). Testosterone was also recently found to impact the immune response to UTI; this finding suggests that sex and hormones may indeed have a substantial impact on the aging immune system within the bladder. However, this hypothesis remains to be formerly tested. With regard to B cells, age-associated B cells (ABCs) accumulate earlier and in greater quantities in female mice compared to male mice\(^\text{10, 11}\). Furthermore, ABCs are found in models of autoimmune disease, which again is more common in females than males. These findings agree with the model that females are more inflammatory than males and in conjunction, and the aging process in females is also more inflammatory than in males.

5.2.2 *Signals and sequence of bTLT formation I: TNFα*

In Chapter 3, I showed that TNFα was required to form age-associated bTLT. Mice deficient in TNFα formed fewer, smaller, and likely less mature age-associated bTLT. We also tested whether TNFα was required for the maintenance of bTLTs once they had formed. We treated aged, female wild type (WT) mice with anti-TNFα or isotype control antibody every other day for 4 weeks. However, we saw no changes in the size or number of bTLT in anti-TNFα treated
mice compared to controls (Figure 5.2). This experiment suggests that while TNFα may be required for initiation or growth of bTLT, it is likely not required for maintenance of bTLT.

**Future directions:** The effect of anti-TNFα treatment on the composition, organization, or functionality of bTLT remains to be determined. We could also test whether anti-TNFα treatment at an earlier age, such as 9 or 12 months when bTLT begin to form, could prevent the growth of bTLT and formation of new ones over a more extended period of time. These future studies would help further elucidate the mechanisms by which age-associated TNFα promotes bTLT formation in female mice. Identifying the cellular source of TNFα in the bladder during aging would also provide insight into mechanisms driving bTLT formation. Preliminary analyses of scRNA-seq suggests that TNFα may be made by DCs (data not shown), but more data is needed to confirm this finding.

5.2.3 Signals and sequence of bTLT formation II: LTβR

TLTs are complex structures with many cellular and molecular interactions occurring during the formation and maintenance of the structures. One interaction of interest, lymphotoxin (LT)-signaling, plays an important role in the development of the secondary lymphoid organs (SLOs) and the formation other TLTs. Membrane-expressed, heterotrimeric LTα1β2 binds its receptor, LTβR to stimulate chemokine and growth factor secretion that builds and maintains the structures\(^\text{12, 13}\). As described in Chapter 1, LTα1β2 is usually expressed on a hematopoietic, lymphoid tissue inducer (LTi) or LTi-like cells, and LTβR is usually expressed on stromal lymphoid tissue organizer (LTo) cells. Ltb was one of the most highly expressed genes in aged
bladders compared to young bladders in our tissue-RNA-seq data (Chapter 3, Figure 3.1, C). We validated the increased expression of both Lta and Ltb in aged bladders with RT-qPCR (Figure 5.3, A), finding that both were roughly 10-fold higher in aged bladders compared to young bladders. We also tested whether LTβR-signaling was required for bTLT maintenance by treating aged WT mice with the decoy receptor LTβR-Ig fusion protein, which blocks this interaction, or isotype control antibody and examined the bladders after 1 week. We found fewer bTLT in LTβR-Ig treated mice compared to control mice but the difference was not quite statistically significant (Figure 5.3, B, p=0.06), and there was no difference in the size of individual bTLT (Figure 5.3, B). This experiment suggested that LTβR-signaling might be required for bTLT maintenance, but more evidence is needed to confirm these findings. Given the high amount of variation between aged animals, we were unable to repeat the experiment with a sufficient sample size to definitively conclude either way. Due to the limited quantity of LTβR-Ig, we also only gave a single 100 µg dose. Other studies frequently use 3 doses over 3 weeks to fully block LT-signaling. Despite these pitfalls, this line of investigation remains promising.

5.2.4 Signals and sequence of bTLT formation III: Chemokines

Chemokines attract cells to structures such as the bTLTs. Four chemokines, known as the homeostatic lymphoid chemokines, promote recruitment and organization of SLOs and TLTs: CCL19, CCL21, CXCL12, and CXCL13^{14-18}. In particular, CXCL13 is highly specific for organized lymphoid tissue and is involved in the organization of the B cell follicles and germinal centers. In Chapter 3, I demonstrated that a small number of macrophages expressed CXCL13 in aged mice but not in young mice (Chapter 3, Figure 3.2, C-D). This finding differs from most other models of lymphoid neogenesis, in which a stromal cell secretes CXCL13 to maintain the
organization of the TLT. For example, the LTo cells that initiate lymph node development secrete CXCL13. There are a few other reports of macrophages producing CXCL13, such as subsets of peritoneal macrophages, M2-like macrophages during resolution of inflammation, within atherosclerotic plaques (that often contain artery TLTs), and in macrophages from patients with idiopathic pulmonary fibrosis (IPF)\textsuperscript{19-23}. Recently, CXCL13\textsuperscript{+}CX3CR1\textsuperscript{+} resident intestinal macrophages were shown to induce IgA-producing TLT in response to Salmonella infection\textsuperscript{24}. Thus, our work and others may be changing the paradigm of which cells typically produce CXCL13 to promote TLT formation. Why aged bladder macrophages begin to express CXCL13 remains to be determined. In aging, tissue macrophages maintain tissue homeostasis by clearing debris as cells die, become senescent, or accumulate damaged proteins\textsuperscript{25}. Impaired clearance of these cells by macrophages contributes to age-related pathologies and inflammation. One possibility is that CXCL13 production by macrophages could increase due to phagocytosis of these senescent cells and subsequently lead to bTLT formation. If senescent cells contribute to macrophage-derived CXCL13, that mechanism may explain why multiple organs form TLTs during aging and why germ-free mice still form TLTs.

How, when, and why age-associated bTLT formation begins remains unknown. Expression of several genes were examined over the life course of mice from 2 to 18 months (\textbf{Figure 5.4. A-B}). \textit{Ccl8} and \textit{Ccl19} expression in bladder tissue began to as early as 6 months, accelerating between 9 and 12 months. \textit{Tnf} did not increase until 12 months (\textbf{Figure 5.4, C}), and \textit{Cxcl13} did not increase until 18 months (\textbf{Figure 5.4, D}). Again, there is significant variation between individual mice, but these data suggest that CCL8 and CCL19 may initiate bTLT formation (likely by initially recruiting B and T lymphocytes), followed by TNF\textalpha and CXCL13 that may promote enlargement and maturation of bTLT. These genes were selected among the top differentially-expressed genes
between young and aged bladders (Chapter 3, Figure 3.1, C). There is likely a role for several others identified in this dataset, and yet other distinct signals acting at different ages. Since we find that the earliest bTLT appear to form between 6 and 9 months of age, future work could focus on this time period to determine the earliest events in bTLT formation. It will also be imperative to identify which cells are producing different cytokines and chemokines at different life stages in order to pinpoint the precise timing, sequence, and mechanism of bTLT formation.

5.2.5 Urothelial permeability: a possible trigger for age-associated bTLT formation?

Why do mice develop bTLT during aging? Our initial hypothesis that aging-induced disturbance of the microbiota may lead to their formation was refuted by our findings that germ free mice also have age-associated bTLTs (Chapter 3, Figure 3.5). Another hypothesis is that urinary contents may stimulate bTLT formation. Kidney function declines with age and the composition of the urine could thus be substantially altered26. Furthermore, urine contains toxic wastes that are being excreted by the body. If urothelial impermeability is compromised with age, then the urinary contents could damage the underlying tissue and stimulate chronic inflammation27-32. To test this hypothesis, we instilled fluorescein (FITC)-dextran into the bladders of young and aged mice for 90 minutes. Histological examination of the bladders demonstrated that young bladders are able to limit the diffusion of FITC-dextran to the outer superficial cell layer of the urothelium (Figure 5.5, A). In contrast, FITC-dextran is observed penetrating all layers of the urothelium in aged mice. Furthermore, quantification of fluorescence in the stroma is also higher in aged mice than in young mice (Figure 5.5, B), indicating penetrance past the basement membrane and into the underlying tissue. TNFα, which is locally increased in the bladder tissue in aged mice (Chapter 3, Figure 3.5, C) and a systemic mediator of inflamm-aging, causes
increased permeability of the intestinal epithelium by altering the tight junction protein zonula occludens-1 (ZO-1) via myosin light chain kinase (MLCK)\textsuperscript{33, 34}. Aged TNFα\textsuperscript{-/-} mice are protected from age-associated intestinal permeability and intestinal dysbiosis\textsuperscript{35}. However, in the bladder, TNFα\textsuperscript{-/-} mice had FITC-dextran permeability similar to age-matched WT mice (Figure 5.5, C). This finding could mean that in the bladder, urothelial permeability might precede inflammation and that TNFα may be an inflammatory response rather than causative agent of urothelial permeability. Another possibility is that our assay, which is primarily qualitative, could not detect a subtler difference between WT and TNFα\textsuperscript{-/-} mice. We also found that FITC-dextran drained to the bTLT and accumulated in vesicle-appearing structures (Figure 5.5, D), another observation supporting the hypothesis that urinary contents may stimulate bTLT formation. The cells that were accumulating FITC-dextran were likely to be antigen-presenting cells, such as DCs and macrophages, processing antigen for presentation within the bTLT. Further identification of these cells by flow cytometry or immunofluorescence is needed.

**Future directions:** One way to determine whether antigen processing and presentation was occurring within bTLT would be to use a well-studied model antigen, such as ova (OT-I or OT-II for CD8 and CD4 T cells, respectively) or 4-hydroxy-3-nitrophenyl-acetyl (NP antigen for B cells) that would stimulate antigen-specific cell proliferation or generation of cytokines and antibodies. Those antigens could be instilled into the urethra and carboxyfluorescein succinimidyl ester (CFSE)-labeled antigen-specific cells injected i.v. If antigen presentation were occurring within bTLT, these cells should be attracted to the bTLT and could be identified by histology. In that experiment, comparing the draining lymph node and the bladder would indicate whether local antigens are being directed to the bTLT instead of the draining lymph node. Another way to test
the permeability hypothesis would be to use an *in vitro* system. There are no well-differentiated urothelial cell lines that act like superficial cells and form a tight barrier similar to *in vivo*. However, urothelial stem cells can be differentiated in trans-wells to form stratified, urothelial-like cultures that better recapitulate the *in vivo* urothelium than traditional cell culture. Comparing cultures derived from young and aged mice or young and older humans could be used to identify mechanisms of urothelial permeability. Furthermore, organoid models, similarly derived from urothelial stem cells but grown in extracellular matrix proteins that promote a spherical, 3D structure, could also be used to test permeability. We have developed bladder organoid culture in our laboratory and are poised to address some of the questions raised above in this system.

5.2.6 Role of bTLT during infection

Ultimately, the most important question is whether and how age-associated bTLT impact the course and outcome of a UTI. Our clinical data suggests that the presence of bTLT (CC lesions) do indeed predispose to more UTI recurrences. To do determine if this was the case in our mouse model, we infected young and aged mice with a recurrent cystitis strain of uropathogenic *E. coli* (UPEC), the most common uropathogen. While early colonization at 6 hours post infection (hpi) was similar between the 2 groups, aged mice actually had a lower urine bacterial titer at 24 hpi (*Figure 5.6, A*). Overall acute clearance of bacteriuria was also similar between the young and aged mice, but a greater number of aged mice had recurrences after initial clearance of the infection (*Figure 5.6, B*). This time course parallels what we observe in women with CC: they have more frequent recurrences than those without CC (see *Chapter 4*). In our aged mice, there are likely multifactorial differences, as identified in this thesis, between young and aged mice that could contribute to these observations. One possibility is the chronic inflammatory state of aging and
hyper-inflammatory responses in aged mice. Aged mice had higher pyuria scores than young mice at 24 hpi (Figure 5.6, C) and higher levels of urine IL-6 at 24 hpi (Figure 5.6, D), which may explain the lower bacterial titer at that time point. However, continued inflammation could trigger recurrences in aged mice. In the laboratory setting, UTI recurrences originate from dormant E.coli that latently reside within the urothelium, known as quiescent intracellular reservoirs (QIRs). Inflammation and other triggers can release these bacteria to seed recurrent infections\textsuperscript{36, 37}. Examining the bladders of young and aged mice after 14 days of infection, we found that aged mice had larger and more frequent QIRs than young mice (Figure 5.6, E-F). Together, these data suggest that our mouse model could provide insight into differences between the young and aged immune system. The interplay between bTLT, early inflammatory responses, the role of autophagy, and QIRs in aged mice will need to be further investigated to fully understand their specific roles in the response to UTIs.

5.2.7 Role of B cells in the bladder

The role of B cells in the bladder is almost entirely unknown. bTLTs have been found in association with advanced, muscle invasive bladder cancer\textsuperscript{38}. However, whether these structures predict better outcomes and responses to immunotherapies as in some other cancers is not known. Analysis of the B cell receptor sequences and resulting antibodies found within bTLT may provide insight into the antigens that stimulate these structures. Germline B cell receptor (BCR) sequence usage identified in Chapter 3 (Chapter 3, Figure S4, C) suggests that there are clonal bursts of B cells, indicating that these cells are activated by and selected for a specific antigen. Single cell BCR sequencing would provide insight into the dynamics of local B cell activation, expansion, and possibly somatic hypermutation within the bladder, and these experiments are currently on-
going. Furthermore, if B cells within bTLT are similar to age-associated B cells (ABCs), they may be producing cytokines with adverse effects on the tissue environment or promote an autoimmune-like state\textsuperscript{39, 40}. More work on the role of B cells in the bladder could also guide development of vacciens and immunotherapies for patients with rUTIs.

5.2.8 Treatment and biomarkers of cystitis cystica in humans

Clinical data presented in Chapter 4 suggest that cystitis cystica (CC) is a risk factor for continued rUTIs. This study would be strengthened by a prospective cohort in which prevention strategies, treatments, number of prior UTIs, and patient characteristics were similar in those with CC and those without. Ideally, randomized controlled trials could be used to find the most effective rUTI prophylaxis regimen in those with and without CC. More rigorous follow-up and ensuring adherence to regimens would be necessary in this type of study. Of particular interest would be evaluating the 3 main UTI prevention strategies currently used in clinical practice: vaginal estrogen therapy (VET), D-mannose, and methenamine\textsuperscript{41}. Generally, in clinical practice, rUTI patients will often start on VET, then add D-mannose and later methenamine if they continue to have culture-positive UTIs. However, since CC patients are more likely to continue to have rUTIs, retrospective data is confounded by patients with more UTIs using more prevention therapies. These patients may also begin these therapies after subsequent UTIs; thus, precisely tracking initiation times of the different therapies will be key to a high-quality study.

Another point of interest would be to further characterize the cellular and molecular components of bTLT in humans (CC lesions) and compare them to what we find in aged mice. These studies could be done with several methods: more immunostaining of fixed and frozen biopsy tissues, tissue or single cell-RNA-sequencing, or blood and urine biomarkers. While our
study found strong associations between CC, age, and myofascial pain, the predictive values of these characteristics remains low. Biomarkers of the molecular determinants of CC lesions could further facilitate identifying patients likely to have CC in a non-invasive manner. Since mouse studies point to several important cytokines and chemokines, such as TNFα and CXCL13, these would be prime candidates to test as biomarkers of CC. Serum CXCL13 correlates with disease severity in some autoimmune diseases\textsuperscript{21, 22, 42, 43}. Since lymphoid follicles promote B and T cell responses, measuring urinary antibodies, such as IgA, and T-cell associated cytokines, such as IFNγ or IL-17, could also be indicative of CC. Another goal of biomarker development would be to non-invasively track resolution of CC and rUTIs and determine if resolution of lesions results in sustained prevention of infection.

5.3 Concluding remarks

rUTIs will continue to be a burden on our aging population. Considering the increasing prevalence of antibiotic resistance among uropathogens, developing non-antibiotic interventions to treat and prevent UTIs is essential. Knowledge of host factors that prevent or promote infection can lead to targeted treatment and prevention regimens. In particular, this thesis identifies unique host factors to consider in the older, female host. Future work to further dissect the impact of age on UTIs must consider the differential presence of or susceptibility to bTLT in the aged bladder. This thesis lays a foundation for improving rUTI treatment and prevention by dissecting the age-associated alteration of the bladder mucosal immune system.
Figure 5.1. Effect of sex on age-associated bTLT formation. (A) Number of bTLT in age-matched, male and female mice. Line at median. (B) Area of individual bTLT from mice in (A). Line at mean ± SEM. n=7-10 mice/group. Mann-Whitney U-tests. **p<0.01
Figure 5.2. Effect of anti-TNF treatment on age-associated bTLT. (A) Number of bTLT in aged, female mice treated with 500 µg anti-TNF or isotype antibody every 2 days for 28 days. Line at median. (B) Area of individual bTLT from mice in (A). Line at mean ± SEM. n=8-11 mice/group from n=2 experiments. Mann-Whitney U-tests.
Figure 5.3. Lymphotoxin (LT) signaling in age-associated bTLT. (A) Relative gene expression in young and aged bladders. n=10-12/group. Lines at geometric mean. ***p<0.001, ****p<0.0001. t-test of log transformation. (B) Number and area of individual bTLT from mice treated with 100 ug LTbR-Ig or isotype control. n=9-10 mice/group, n=2 experiments. Lines at median. Mann-Whitney U-tests.
Figure 5.4. Cytokine and chemokine expression in the lifespan over the life of mice. (A-D) Gene expression relative to 2 mo old mouse bladders. n=2-5/group.
Figure 5.5. Urothelial permeability in aged mice. (A) Representative image of FITC-dextran permeability in young and aged urothelium. White arrowheads identify superficial umbrella cells with intracellular FITC-dextran. Red arrows identify basal and intermediate cell layers. Dashed line separates urothelium from lamina propria. (B) Relative quantification of FITC-dextran fluorescence in the stroma in mouse bladders. n=4/group from n=2 experiments. (C) Representative image of FITC-dextran permeability in urothelium of young and TNFα−/− mice as in (A). (D) FITC-dextran accumulation within bTLT in aged WT bladder. Red arrow head identifies cells with vesicular accumulation of FITC-dextran.
Figure 5.6. UPEC infection in young and aged mice. (A) Urine bacterial titer in young and aged mice at 6 (n=1 experiment) and 24 (n=6 experiments) hours post infection (hpi). n=8-40/group. Dotted lines, limit of detection. Lines at median. (B) Proportion of mice with bacteriuria over 14 days post infection (dpi). n=19-42/timepoint/group. (C) Semi-quantitatively scoring of polymorphonuclear (PMN) cells in urine cytology at 24 hpi. n=11-15/group from n=3 experiments. (D) Urine IL-6 at 24 hpi. n=5/group. (E) Number of quiescent intracellular reservoirs (QIRs) at 14 dpi per 8 sections determined by immunofluorescence. n=15-20/group from n=3 experiments. (F) Representative image of a QIR in an aged mouse bladder. *p<0.05, **p<0.01, Mann-Whitney U-test (A, C-E) and Fisher's exact tests (B).
5.4 Methods

Methods used in this chapter that are not described here can be found in Chapter 3.

Urothelial permeability assay

50 µL of 10 mg/mL 10 kD FITC-dextran (D1821, Invitrogen) was transurethrally inoculated into mice as previously described (Shin 2011). After 90 minutes, bladders were embedded in OCT Compound (4583, Tissue-Tek) and flash frozen. 7 µm sections were briefly dipped in 1:1 methanol-acetone and PBS, then cover-slipped with Prolong Diamond Anti-fade with DAPI (P36971, Invitrogen). Images were acquired using a fixed exposure set to detect fluorescence in young WT bladders. Stromal fluorescence was quantified by averaging the mean gray value for FITC channel in 6 random squares from 2 separate images for each mouse using ImageJ software. Values were combined for each mouse and used for statistical analysis.

Mouse Urinary Tract Infection

UTI89, a clinical UPEC isolate from a patient with recurrent cystitis was grown statically for 17 h in Luria-Bertani broth (Tryptone 10 g/L, Yeast extract. 5 g/L. and NaCl 10g/L) at 37°C prior to infection. Mice were anesthetized and inoculated via transurethral catheterization with 10⁷ colony forming units (CFUs) of UTI89 in phosphate-buffered saline (PBS; Sigma-Aldrich, D8537). Urines were collected at indicated time points and spotted onto LB-agar plates to measure bacterial titers.

QIR Quantification

At 14 dpi, bladders were fixed in methacarn, cut in half, and embedded in paraffin. Four sections of 5 mm thickness were cut spaced 50 mm apart and stained with antibodies against E. coli and E-
cadherin and Hoescht dye. QIRs were identified as single or multiple E. coli rosettes within superficial cells and totaled from the 8 sections of each bladder.

_Urine cytology_

10 µL of urine was spun onto slide using a Cytospin 3 centrifuge, briefly heat fixed, and stained with PROTOCOL Hema 3. Slides were semi-quantitatively scored based on the number of polymorphonuclear cells (PMNs) per high powered field.

_Acknowledgements_

Caihong Wang and Bisiayo Fashemi contributed to experiments in Fig. 5.
REFERENCES


Appendix 1: Oxysterol signatures distinguish age-related macular degeneration from physiologic aging

This chapter was published in *EBioMedicine*. MML performed flow cytometry experiments in this paper.

Oxysterol Signatures Distinguish Age-Related Macular Degeneration from Physiologic Aging

Jonathan B. Lin a,b,1, Abdoulaye Sene a,d, Andrea Santeford a, Hideji Fujiwara c,d, Rohini Sidhu c,d, Marianne M. Ligon e, Vikram A. Shankar a, Norimitsu Ban a, Indira U. Mysorekar c,d, Daniel S. Ory c,d,1, Rajendra S. Apte a,c,d,g,1

a Department of Ophthalmology & Visual Sciences, Washington University School of Medicine, St. Louis, MO, USA
b Neuroscience Graduate Program, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, MO, USA
c Diabetic Cardiovascular Disease Center, Washington University School of Medicine, St. Louis, MO, USA
d Department of Medicine, Washington University School of Medicine, St. Louis, MO, USA
e Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, USA
f Center for Reproductive Health Sciences, Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, MO, USA
g Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO, USA

1 These authors contributed equally to this work.

A R T I C L E   I N F O

Article info
Received 11 May 2018
Received in revised form 26 May 2018
Accepted 29 May 2018
Available online 11 June 2018

Keywords:
Age-related macular degeneration
Aging
Lipids
Cholesterol

A B S T R A C T

Macrophage aging is pathogenic in numerous diseases, including age-related macular degeneration (AMD), a leading cause of blindness in older adults. Although prior studies have explored the functional consequences of macrophage aging, less is known about its cellular basis or what defines the transition from physiologic aging to disease. Here, we show that despite their frequent self-renewal, macrophages from old mice exhibited numerous signs of aging, such as impaired oxidative respiration. Transcriptomic profiling of aged murine macrophages revealed dysregulation of diverse cellular pathways, especially in cholesterol homeostasis, that manifested in altered oxysterol signatures. Although the levels of numerous oxysterols in human peripheral blood mononuclear cells and plasma exhibited age-associated changes, plasma 24-hydroxycholesterol levels were specifically associated with AMD. These novel findings demonstrate that oxysterol levels can discriminate disease from physiologic aging. Furthermore, modulation of cholesterol homeostasis may be a novel strategy for treating age-associated diseases in which macrophage aging is pathogenic.

© 2018 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
in the pathogenesis of wet AMD [9–12]. Moreover, it has become increasingly clear that the ability of macrophages to polarize to different activation states is an important factor affecting whether macrophages promote health or disease [13]. Depending on dynamic tissue signals and the surrounding micro-environment, macrophages can polarize to a classical pro-inflammatory (M1-like) phenotype, an alternative anti-inflammatory (M2-like) phenotype, or some intermediate between these two extremes [14]. To further complicate matters, the identity of the specific activators that cause macrophage polarization may also affect the macrophage phenotype [15].

Previously, we reported that aged macrophages tend to skew to the anti-inflammatory M2-like phenotype and are less able to inhibit abnormal angiogenesis [16]. Furthermore, aged macrophages exhibit both impairments in cholesterol efflux [17] and abnormalities in IL-10 and downstream STAT3 signaling pathways that contribute to this age-associated drift towards M2-like polarization [18]. These age-associated impairments in cholesterol efflux and other lipid-related pathways may have mechanistic consequences in disease pathogenesis [19]. This possibility is supported by the fact that polymorphisms in lipid-related genes, such as hepatic lipase (LIPC), ATP-binding cassette transporters (ABCA1, ABCG1), and cholesterol ester transfer protein (CETP), are associated with advanced AMD [20]. Moreover, drusen, a clinical feature of early AMD, are lipid-rich, further supporting our hypothesis that dysregulated lipid homeostasis contributes to AMD. Despite these advances, the global programmatic changes that occur during macrophage aging need further elucidation. It is also unclear what subset of these changes are associated with physiologic aging or are pathologic and contribute to age-associated disease.

In this study, we sought to delineate the cellular pathways involved in macrophage aging and identify potential markers that may distinguish age-associated changes that are physiologic versus those that promote age-associated disease. Our results suggest that impaired cholesterol homeostasis in macrophages is a central process perturbed during aging and that these changes lead to alterations in oxysterol signatures that can distinguish AMD from physiologic aging. These findings may allow physicians to monitor progression of disease with quantifiable serum markers and may potentially lead to novel therapeutic strategies not only for AMD but also for other age-associated diseases in which alternatively-activated macrophages are pathogenic.

2. Materials and Methods

2.1. Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the Washington University School of Medicine Animal Care and Use guidelines. We obtained old (i.e., ~18-month-old), female wild-type C57BL/6j mice from the National Institute on Aging (Bethesda, MD) and compared them to strain-matched young (i.e., ~3-month-old), female wild-type C57BL/6j controls. We harvested peritoneal macrophages five days after elicitation with a 2-ml intraperitoneal injection of 4% thioglycolate broth (Sigma-Aldrich, St. Louis, MO). We harvested splenic macrophages by performing positive magnetic cell separation with the PE selection kit (Stem Cell Technologies) and PE anti-F4/80 monoclonal antibody (clone: BM8; eBioscience, Waltham, MA), following manufacturer’s instructions. We cultured peritoneal and splenic macrophages in Gibco™ RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin-streptomycin (Thermo Fisher Scientific). When indicated, we treated macrophages with 25 or 50 µg/ml of oxidized LDL (oxLDL; Alfa Aesar, Haverhill, MA) for 24 h prior to further analysis.

2.2. OCR Measurements

To perform metabolic characterization, we measured the oxygen consumption rate (OCR) of peritoneal macrophages as a surrogate marker for oxidative respiration with the XF96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA). In short, we plated peritoneal macrophages in Seahorse XF96 cell culture microplates (Seahorse Bioscience) at 100,000 cells per well. On the morning of the experiment, we washed the cells and replaced the medium with Seahorse assay medium (Seahorse Bioscience) supplemented with 25 mM glucose (Sigma-Aldrich, St. Louis, MO) and 1 mM sodium pyruvate (Thermo Fisher Scientific) and adjusted the pH to 7.4. After incubation in a non-CO₂ incubator at 37 °C for 1 h, we measured OCR at baseline and after sequential treatment with the following chemicals from the Mito Stress Test kit (Seahorse Bioscience): 5 µM oligomycin, 5 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 1 µM rotenone/antimycin A (rot/AA). Each cycle consisted of 2 min of mixing and a 1-min pause, followed by a 5-min measurement period; we repeated each cycle 3–4 times. We normalized the background of all measurements by subtracting the average OCR of each sample after treatment with rot/AA.

2.3. Gene Expression Analysis

We extracted total RNA from peritoneal macrophages with the RNAasy Mini kit (Qiagen) and prepared cDNA with the High-Capacity Reverse Transcription kit (Applied Biosystems), following manufacturer’s instructions. We performed quantitative PCR amplification of cDNA using either the TaqMan® probe-based gene expression assay for p16INK4a (Mm00494449_m1; Applied Biosystems) or custom TaqMan® Array Plates (Applied Biosystems) for lipid-related genes with the assays indicated in Supplemental Table S1. In all cases, we used the ΔACT method, normalizing to Actb, 18sRNA, Gapdh, or the geometric mean of a combination of these endogenous controls.

2.4. Transcriptomic Profiling

We isolated total RNA from peritoneal macrophages with the mirVana kit (Ambion), performed cDNA amplification with the Ovation® Pico kit (NuGEN), and performed target labeling with the Encore® Biotin kit (NuGEN), according to manufacturer’s instructions. We then performed whole transcriptome profiling of young and aged peritoneal macrophages using Mouse Gene (MoGene) 1.0 ST arrays processed with Affymetrix Expression Console (v1.3.1.187) at standard settings (RNA background correction, median polish summarization, and quantile normalization) to generate intensity values. We assigned each probe set of the MoGene 1.0 array a detection call of “present” if the ratio of the signal to background intensity was greater than 2 × SD of the negative controls. We performed data quality control to identify potential outliers by principal component analysis (PCA) plot and hierarchical clustering, as well as by quality control (QC) metrics (all probeset RLE means > 0.25) in Expression Console. From this QC, we omitted one sample in the young group. We then filtered data by probeset type (“main” in MoGene 1.0) and by detection call (any probeset without a “detected” call in any of the samples was removed). Any probe set without a gene symbol in the MoGene 1.0 data was also removed. 18,066 MoGene 1.0 probesets (from the total of 35,556) were kept for further analysis. We analyzed the data using the R package “limma” and generated gene lists based on P-values and false detection rate (FDR) q-values. We performed gene ontology (GO), pathway map, and interactome analyses with MetaCore™ (Clarivate Analytics, Philadelphia, PA). The microarray data are available at the Gene Expression Omnibus (GEO) at NCBI under accession number GSE111382.
2.5. Flow Cytometry

We plated peritoneal macrophages on untreated Petri dishes and allowed them to adhere overnight. The next morning, we lifted cells by incubating for 5 min in ice-cold Dulbecco's phosphate-buffered saline (DPBS; Thermo Fisher Scientific) without calcium or magnesium and scraping gently. We next filtered cells through a 40 μm cell strainer, washed them with DPBS, and resuspended them in DPBS containing 5% FBS, 10 mM HEPES, 1 mM EDTA, and TruStain FcX™ antibody (BioLegend, San Diego, CA). We stained 10^6 to 10^7 cells with APC anti-mouse CD36 (clone: HM36; BioLegend), PE/Cy7 anti-mouse CD64 (clone: 54–7/7.1; BioLegend), and PE anti-mouse F4/80 (clone: BMB; eBioscience) antibodies for 20 min on ice. We then washed and suspended cells in DPBS and acquired data on a BD X-20 or BD LSR II flow cytometer (BD Biosciences, San Jose, CA). We analyzed and visualized data with FlowJo v10.

2.6. Human Subjects

This study was approved by the Human Research Protection Office of Washington University School of Medicine and adhered to the Declaration of Helsinki. We obtained informed consent from all subjects prior to blood collection. To purify peripheral blood mononuclear cells (PBMCs) and plasma, we performed density gradient centrifugation with BD Vacutainer CPT™ Cell Preparation Tubes (Franklin Lakes, NJ), following manufacturer's instructions. We classified patients as no AMD, early AMD, or wet AMD based on established clinical criteria [21]. Early AMD patients had either moderate drusen (>63 μm) or pigment changes in at least one eye but no CNV or GA in either eye at the time of sample collection. Wet AMD patients had CNV in at least one eye at the time of sample collection. We stored all samples at −80 °C until further analysis. To determine the appropriate sample size, we performed an a priori power analysis with G^*Power 3.1 [22]. Estimating an effect size d of 0.6 based on pilot experiments, we calculated that we needed 94 subjects with an allocation ratio of 1:5:1 to detect a significant difference between the groups at the two-sided α = 0.05 level with 80% power. For the subanalysis, we performed chart review to identify the closest total plasma cholesterol measurement obtained within 3.5 years of the date of sample collection.

2.7. Oxysterol Profiling by LC–MS/MS

We extracted and quantified oxysterol levels from murine peritoneal macrophage pellets, the supernatant of murine peritoneal macrophages, human PBMC pellets, and human plasma samples as previously described [23]. Briefly, we added deuterated oxysterols to the samples as internal standards and then extracted oxysterols with methanol. We derivatized the extracted oxysterols and their internal standards with N,N-dimethylglycinate (DMG) to increase MS sensitivity. We performed oxysterol analysis with a Shimadzu 20 CE HPLC system (Kyoto, Japan) and a LeapPAL autosampler coupled to a tandem mass spectrometer (API 4000; Applied Biosystems) operated in MRM mode. We used positive ion ESI mode for detection of the derivatized oxysterols, injecting study samples in duplicate for data averaging. We conducted data processing with Analyst 1.5.1 (Applied Biosystems) and determined relative levels of each oxysterol by comparing its measurement with that of its corresponding deuterated internal standard. We normalized the murine macrophage oxysterol levels to cell number, murine supernatant levels to volume, human PBMC oxysterol levels to protein content, and human plasma oxysterol levels to volume. To normalize human samples processed on different days, we used one of the patient samples as an internal control.

2.8. Statistics

We performed statistical analysis as indicated in the Figure legends with Prism 5 (GraphPad Software) or SPSS Statistics Version 23 (IBM, Armonk, NY). We assessed the normality of our data graphically and with the Kolmogorov-Smirnov test and used appropriate non-parametric alternatives when necessary. We considered P < .05 to be statistically significant.
significant. To model the relationship between PBMC and plasma oxysterol signatures and AMD, we generated binary logistic regression models. Our model included the following predictor variables: age at the time of sample collection, gender, and PBMC/plasma oxysterol levels. For the subanalysis, we generated an additional binary logistic regression model, which included the following predictor variables: age at the time of sample collection, gender, plasma 24-HC levels, and total plasma cholesterol. For all regression models, we assessed fit with the Hosmer-Lemeshow lack-of-fit test and performed diagnostics by examining Cook’s distances, leverages, and residual deviances. The final reported models had seven omitted cases based on sensitivity analysis. We checked for problems with collinearity by examining variance inflation factors (VIF). We used an unadjusted alpha of 0.05 for the binary logistic regression.

3. Results

3.1. Peritoneal Macrophages from Old Mice Exhibit Features of Aging

Under steady-state conditions, tissue-resident macrophages are maintained through constant replacement or self-renewal. In many tissues, including the peritoneal cavity, there is extensive replacement of macrophages as quickly as every 3 weeks [24]. Therefore, we sought to determine whether macrophages isolated from old (i.e., 18-month-old) wild-type mice exhibited features of cellular aging when compared to macrophages isolated from young (i.e., 3-month-old) wild-type mice despite this short replenishment cycle.

Mitochondrial dysfunction is a well-established hallmark of aging, and in many tissues, there is an age-dependent decrease in mitochondrial oxygen consumption [25]. To determine whether peritoneal macrophages from old mice exhibit mitochondrial dysfunction, we measured the oxygen consumption rate (OCR) of peritoneal macrophages isolated from 3-month-old and 18-month-old mice. The baseline OCR of peritoneal macrophages from old mice was significantly lower than that of peritoneal macrophages from young mice (Fig. 1a), indicating reduced basal oxidative respiration. Although both young and aged peritoneal macrophages appropriately exhibited a reduction in OCR after treatment with the ATP synthase inhibitor oligomycin, young peritoneal macrophages maintained a somewhat higher residual OCR (Fig. 1a). In contrast, both young and aged peritoneal macrophages demonstrated similar maximal oxidative respiration in response to the uncoupling agent FCCP (Fig. 1a). The difference between the baseline OCR and the OCR after oligomycin treatment represents ATP-linked respiration. Aged peritoneal macrophages exhibited significantly reduced ATP-linked respiration (Fig. 1b), indicating mitochondrial dysfunction.

Fig. 2. Transcriptomic profiling of aged peritoneal macrophages. (a) Aged peritoneal macrophages display numerous transcriptomic changes, which suggest perturbations in various gene ontology (GO) processes (b) and pathway maps (c). (d) Interactome analysis revealed numerous overconnected transcription factors (TFs) whose known gene targets were overrepresented in the genes we identified as dysregulated in aged versus young macrophages.
Moreover, previous studies report that expressing macrophages to lipopolysaccharide (LPS) considerably alters their bioenergetic profile [26]. Furthermore, such a metabolic switch is required for macrophage activation and affects subsequent inflammatory status [26–28]. We observed distinct mitochondrial respiratory profiles when comparing young and aged, LPS-treated peritoneal macrophages: aged peritoneal macrophages exhibited a reduced OCR at baseline, despite maintaining similar responses to oligomycin and FCCP (Fig. 1c). Again, aged peritoneal macrophages exhibited significantly reduced ATP-linked respiration (Fig. 1d). Cumulatively, these data demonstrate that mitochondrial oxidative metabolism is considerably impaired in peritoneal macrophages isolated from old mice both at baseline and in response to LPS.

Furthermore, we evaluated the expression of the aging marker p16INK4a, which is a known senescence marker in bone marrow-derived macrophages [29] and has been shown to accumulate in rodents and human tissues during aging [30–32]. We found that p16INK4a mRNA expression was significantly elevated in aged peritoneal macrophages compared to young peritoneal macrophages (Fig. 1e). Collectively, these results confirm that despite their constant renewal, peritoneal macrophages from old mice exhibit multiple quantifiable signs of aging, including mitochondrial dysfunction and increased expression of the senescence marker, p16INK4a.

### 3.2. Aged Macrophages Exhibit Impaired Cholesterol Homeostasis

To determine the specific cellular processes that are perturbed in aged macrophages, we profiled the transcriptomes of young and aged macrophages with the GeneChip Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, CA). We found that 1080 probe sets were significantly differentially expressed in aged versus young macrophages [Fig. 2a; P < 0.05, FDR < 0.20]. The twenty protein-coding genes that were up- or downregulated with the highest fold change in aged versus young macrophages are presented in Table 1. To determine whether the identities of the dysregulated genes were associated with defects in particular pathways or cellular processes, we filtered for genes with a 1.5-fold change cutoff (22.1%, N = 239) and then performed gene ontology (GO) and pathway map analysis with MetaCore™ (Clarivate Analytics, Philadelphia, PA). Of interest, the first, second, and tenth most significant GO processes implicated were sterol biosynthesis, cholesterol biosynthesis, and cholesterol metabolism, respectively (Fig. 2b).

Consistently, cholesterol biosynthesis was the most significant pathway map implicated by the altered transcriptomic profile of aged macrophages (Fig. 2c). As expected, the transcriptomic profile of aged macrophages also suggested disruptions in numerous other immune response pathways (Fig. 2c). Overall, these results clearly indicate that cholesterol homeostasis is significantly perturbed during aging.

Given that numerous genes were dysregulated in aged peritoneal macrophages, we sought to identify candidate transcription factors (TFs) that may regulate the aging process. Therefore, we performed interactome analysis to identify over-connected TFs, which may regulate the observed transcriptomic changes in aged macrophages. Of interest, two of the top five most significantly overconnected TFs were sterol regulatory element-binding protein 1 and 2 (SREBP1/ SREBP2; Fig. 3d), which are known master regulators of cholesterol and lipid homeostasis. These two TFs had connectivity ratios of 14.73 and 15.15, respectively, indicating ~15-fold overrepresentation of their known targets. These findings support our assertion that global lipid homeostatic mechanisms are impaired in aged macrophages.

To determine the specific aspects of cholesterol homeostasis that are impaired in aged macrophages, we analyzed the expression profile of 113 lipid-related genes in young and aged macrophages with a custom quantitative PCR array (Applied Biosystems). We were able to detect expression of 86.7% (N = 98) of the genes we tested and found that 30 of these 98 cholesterol-related genes (30.6%) had significantly different expression in aged versus young macrophages (Table 2). When we subtyped these lipid-related genes broadly by their cellular function, we observed that they encompassed diverse biological processes, including cholesterol/lipid biosynthesis, elimination, transport, and uptake, among other processes. These findings once again confirmed that aged peritoneal macrophages exhibit global impairments in their ability to maintain cholesterol and lipid homeostasis.

---

**Table 1**

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Top ten up- and down-regulated genes in aged versus young peritoneal macrophages.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probeset ID</td>
<td>Gene symbol</td>
</tr>
<tr>
<td>10463355</td>
<td>Scd2</td>
</tr>
<tr>
<td>10506371</td>
<td>Dtna2d</td>
</tr>
<tr>
<td>10347748</td>
<td>Acel3</td>
</tr>
<tr>
<td>10560702</td>
<td>Ceacam19</td>
</tr>
<tr>
<td>10403413</td>
<td>Lbp</td>
</tr>
<tr>
<td>10544273</td>
<td>Ccc5a</td>
</tr>
<tr>
<td>10482762</td>
<td>Mfi1</td>
</tr>
<tr>
<td>10420068</td>
<td>Merl1a</td>
</tr>
<tr>
<td>10424549</td>
<td>Sple</td>
</tr>
<tr>
<td>10527920</td>
<td>Cyp51l</td>
</tr>
<tr>
<td>10552879</td>
<td>Cyp1</td>
</tr>
<tr>
<td>10538126</td>
<td>Gimpp4</td>
</tr>
<tr>
<td>10551025</td>
<td>Cd79a</td>
</tr>
<tr>
<td>10429520</td>
<td>Lyp</td>
</tr>
<tr>
<td>10466172</td>
<td>Hmna1</td>
</tr>
<tr>
<td>10526142</td>
<td>Cyp7b1</td>
</tr>
<tr>
<td>10458278</td>
<td>Mob1</td>
</tr>
<tr>
<td>10523359</td>
<td>Ccl12</td>
</tr>
<tr>
<td>10531724</td>
<td>Plac8</td>
</tr>
<tr>
<td>10429564</td>
<td>Lyp</td>
</tr>
</tbody>
</table>

* Aged versus young peritoneal macrophages; negative fold-change reflects decreased expression in aged macrophages relative to young macrophages.

**Table 2**

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Expression profiling of lipid-related genes in aged and young macrophages.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular function</td>
<td>Gene symbol</td>
</tr>
<tr>
<td>Biosynthesis</td>
<td>Fed2L</td>
</tr>
<tr>
<td></td>
<td>Fed2L</td>
</tr>
<tr>
<td></td>
<td>Fed3L</td>
</tr>
<tr>
<td></td>
<td>Fedp</td>
</tr>
<tr>
<td></td>
<td>Hmgcs2</td>
</tr>
<tr>
<td></td>
<td>Prha2</td>
</tr>
<tr>
<td></td>
<td>Scd1</td>
</tr>
<tr>
<td></td>
<td>Cypr1</td>
</tr>
<tr>
<td></td>
<td>Lpl</td>
</tr>
<tr>
<td></td>
<td>Tbias1</td>
</tr>
<tr>
<td></td>
<td>Lipa</td>
</tr>
<tr>
<td></td>
<td>Atps1</td>
</tr>
<tr>
<td></td>
<td>Apol</td>
</tr>
<tr>
<td></td>
<td>Sk27a1</td>
</tr>
<tr>
<td></td>
<td>Sk27a3</td>
</tr>
<tr>
<td></td>
<td>Sreb1d4</td>
</tr>
<tr>
<td></td>
<td>Uptake</td>
</tr>
<tr>
<td></td>
<td>Olf</td>
</tr>
<tr>
<td></td>
<td>Fosl2</td>
</tr>
<tr>
<td></td>
<td>Sreb2</td>
</tr>
<tr>
<td>Other or multiple functions</td>
<td>AAFP</td>
</tr>
<tr>
<td></td>
<td>Ali2a5</td>
</tr>
<tr>
<td></td>
<td>Fdhp4</td>
</tr>
<tr>
<td></td>
<td>Newb</td>
</tr>
<tr>
<td></td>
<td>Nfat2a</td>
</tr>
</tbody>
</table>

* Aged versus young peritoneal macrophages; negative fold-change reflects decreased expression in aged macrophages relative to young macrophages.

** Significant by 2-tailed Mann-Whitney U test.
3.3. Aged Macrophages have Altered Intracellular Oxysterol Content

We previously reported that aged macrophages have higher levels of intracellular cholesterol related to impaired efflux [17], likely related to the global changes in cholesterol homeostatic mechanisms described above. The oxidation of cholesterol to generate oxidized derivatives of cholesterol or oxysterols serves a crucial purpose to facilitate elimination of excess cholesterol. However, oxysterols themselves also play important signaling roles in regulating cholesterol homeostasis [33] and inflammation [34] and may therefore promote disease. In addition, aberrant oxysterol production can be a sign of increased oxidative stress, which is known to be pathogenic in AMD [35]. Since our transcriptomic profiling demonstrated that impaired cholesterol homeostasis is a hallmark feature of aged macrophages, we sought to explore whether these changes were associated with altered oxysterol signatures, which would not only provide mechanistic insight into why there is impaired cholesterol homeostasis but also identify a potential approach for detecting defective cholesterol homeostasis.

Fig. 3. Aged peritoneal macrophages have abnormal oxysterol content. (a-c) Aged peritoneal macrophages contained significantly more intracellular 4β-hydroxycholesterol (4β-HC) and 7-ketocholesterol (7-KC) than their young counterparts at baseline and after treatment with 25 or 50 μg/ml oxidized LDL (oxLDL) (N = 5/group; 2-way ANOVA) and significantly more intracellular cholestane-3β,5α,6β-triol (C-triol) after treatment with 50 μg/ml oxLDL (N = 5/group; 2-way ANOVA with Bonferroni post-hoc test). (d-f) Although some comparisons were statistically significant due to low within-group variance, the supernatants of young and aged peritoneal macrophages contained qualitatively similar levels of 4β-HC, 7-KC, and C-triol both at baseline and after treatment with oxLDL (N = 5/group; 2-way ANOVA with Bonferroni post-hoc test). (g) Representative flow cytometry plot from young and aged peritoneal macrophages showing gating on macrophage markers CD64 and F4/80. (h-i) Young and aged peritoneal macrophages exhibited similar CD36 surface expression. Isotype staining (iso) was identical between groups (N = 5/group; 2-tailed, unpaired t-test). Open circles depict individual data points; horizontal lines depict mean ± SEM (a-f, i) (*** P < .001).
Using liquid chromatography tandem mass spectrometry (LC-MS/MS), we measured the most abundant oxysterols, including 4β-hydroxycholesterol (4β-HC), 7-ketocholesterol (7-KC), and cholesterol-3β,5α,6α-triol (C-triol) in young and aged peritoneal macrophages. We found that aged peritoneal macrophages contained more 4β-HC and 7-KC compared to young peritoneal macrophages, both at baseline and after treatment with 25 or 50 μg/ml oxidized LDL (oxLDL) (Fig. 3a-b). Aged peritoneal macrophages also had increased C-triol content at baseline and after treatment with oxLDL, but the difference was statistically significant only after treatment with 50 μg/ml oxLDL (Fig. 3c). To account for the possibility that these increases in intracellular oxysterols may have been influenced by environmental factors, we also tested a separate cohort of young and aged mice that were housed at the same animal facility. Consistent with our original findings, these aged peritoneal macrophages also had increased levels of 4β-HC, 7-KC, and C-triol after treatment with 50 μg/ml oxLDL (Fig. S1a-c). These findings demonstrate that aged peritoneal macrophages have increased intracellular oxysterols upon challenge with oxLDL and that this difference is likely an effect of age rather than environmental factors.

We also measured the oxysterol content in the supernatant to determine whether increased intracellular oxysterol content was associated with increased oxysterol secretion. Although some comparisons were statistically significant due to low within-group variance, the levels of 4β-HC, 7-KC, and C-triol were qualitatively similar in the supernatant of both young and aged peritoneal macrophages both at baseline and after treatment with oxLDL (Fig. 3d-e; Fig. S1d-f). Of note, the levels of 4β-HC and 7-KC detected in equivalent dilutions of oxLDL were similar to those detected in the supernatants of both young and aged peritoneal macrophages (Fig. 3d-e; Fig. S1d-e), suggesting that any differences in the secretion of these two oxysterols were likely masked by the oxysterols present in the oxLDL itself.

Our finding of increased intracellular oxysterols in the absence of changes in extracellular levels suggested that there is increased oxysterol synthesis associated with aging rather than changes in uptake. In support of this hypothesis, we previously reported that young and aged macrophages have similar capacity to in vitro uptake (4β-HC and 7-KC) of oxLDL (β-HC and 7-KC) compared to young peritoneal macrophages (Fig. 3d-e; Fig. S1d-e), suggesting that any differences in the secretion of these two oxysterols were likely masked by the oxysterols present in the oxLDL itself.

In addition to measuring the same oxysterols we measured in murine peritoneal macrophages (i.e., 4β-HC, 7-KC, and C-triol), we were also able to quantify levels of two additional enzymatically generated oxysterols, 24-hydroxycholesterol (24-HC) and 27-hydroxycholesterol (27-HC), which were below the limit of detection in the murine samples. Of interest, we observed a statistically significant negative correlation between age and PBMC 7-KC levels (Fig. 4a; Spearman R = 0.2964, P = .0204), PBMC C-triol levels (Fig. 4a; Pearson R = 0.3068, P = .0171), and PBMC 24-HC levels (Fig. 4b; Pearson R = 0.3058, P = .0165). There was no association between age and PBMC 4β-HC levels (Fig. 4a; Spearman R = -0.0356, P = .7853) or PBMC 27-HC levels (Fig. 4b; Pearson R = -0.0012, P = .9930). Additionally, we observed a significant positive correlation between age and plasma C-triol levels (Fig. 4c; Pearson R = 0.2818, P = .0278). Although not statistically significant, there was a trend (0.05 < P < .10) towards a positive correlation between age and plasma 4β-HC levels (Fig. 4c; Spearman R = 0.2170, P = .0930). There was no statistically significant correlation between age and plasma 7-KC levels (Fig. 4c; Spearman R = 0.1172, P = .3683), plasma 24-HC levels (Fig. 4d; Spearman R = 0.0594, P = .7631), or plasma 27-HC levels (Fig. 4d; Pearson R = 0.1601, P = .2177). Overall, these findings suggest that healthy humans also exhibit age-associated alterations in oxysterol signatures, likely reflecting changes in cholesterol homeostasis.

3.5. Plasma 24-hydroxycholesterol Distinguishes AMD from Physiologic Aging

To further characterize the relationship between age-associated oxysterol signatures and age-related disease, we recruited human patients with early or advanced neovascular (watery) AMD and measured their PBMC and plasma oxysterol levels (Fig. S5-a-j). While AMD patients were significantly older than non-AMD control subjects, there was no difference between the groups on the basis of gender (Table 3). On average, AMD patients had decreased PBMC 7-KC levels compared to non-AMD controls (Fig. 5b) but no differences in the other PBMC oxysterol levels (Fig. S5-a-c-e). Moreover, AMD patients had elevated plasma 4β-HC levels (Fig. 5f), elevated plasma C-triol levels (Fig. 5h), elevated plasma 24-HC levels (Fig. 5i), and a trend (0.05 < P < .10) towards elevated plasma 27-HC levels (Fig. 5j) compared to non-AMD controls. There was no difference in plasma 7-KC levels (Fig. 5g).

Given the known association between AMD and age and the significant age difference between the two groups, we performed binary logistic regression to precisely model the relationship between PBMC or plasma oxysterol levels and AMD after controlling for age and gender. We selected PBMC 7-KC, plasma 4β-HC, plasma C-triol, and plasma 24-HC levels as candidates for further analysis since these were the oxysterols that were significantly different between AMD patients and controls. The binary logistic regression model for a PBMC oxysterol AMD signature included age, gender, and untransformed PBMC 7-KC (Table 4). The overall model was statistically significant (LR X² = 34.0, df = 3, P = .001) and had good fit (NX = 9.6, df = 8, P = .294).

Table 3
<table>
<thead>
<tr>
<th>Demographic parameter</th>
<th>Control</th>
<th>AMD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range)</td>
<td>64.09</td>
<td>74.11</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Sex, N</td>
<td>Male</td>
<td>37</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>25</td>
<td>22</td>
</tr>
<tr>
<td>ADMI status, N</td>
<td>No AMD</td>
<td>62</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Early AMD</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Advanced neovascular AMD</td>
<td>0</td>
<td>24</td>
</tr>
</tbody>
</table>

* Significant by 2-tailed Mann-Whitney U test.

Table 3 Demographic and clinical characteristics of human subjects.

3.4. PBMC and Plasma Oxysterol Signatures are Altered with Age in Humans

We have previously demonstrated that aged murine macrophages exhibit functional and phenotypic changes that are associated with their tendency to promote AMD [9, 17, 18]. However, it remains unclear which subset of the age-associated changes described above promote disease rather than being a part of physiologic aging. To discriminate between these physiologic versus pathologic changes, we assessed whether PBMCs and plasma samples from healthy human subjects also exhibited changes in oxysterol signatures with age and whether these changes were different in AMD patients. We chose to process the human samples minimally without cell sorting or cultures to explore how these oxysterol signatures could be used clinically. Demographic information of the human subjects is shown in Table 3.
expected, age was significantly associated with AMD ($P < .001$) with a beta coefficient of $0.098$, indicating that each additional year of age was associated with increased odds of having AMD ($aOR = 1.10; 95\% CI = 1.05$ to $1.16$). However, after controlling for age and gender, PBMC 7-KC levels were not associated with AMD ($P = .140$).

The binary logistic regression model for a plasma oxysterol AMD signature included forced entry of age, gender, and any combination of the candidate plasma oxysterol species through automated forward selection (Table 5). The final model was statistically significant ($LR \chi^2 = 44.9, df = 3, P < .001$), had good fit ($\chi^2 = 14.2, df = 8, P = .078$), and included age, gender, and plasma 24-HC levels. As expected, age was significantly associated with AMD ($P < .001$) with a beta coefficient of $0.107$, indicating that each additional year of age was associated with increased odds of having AMD ($aOR = 1.113; 95\% CI: 1.06$ to $1.17$). Of significant interest, even after controlling for age and gender, 24-HC levels were highly associated with AMD ($P < .001$) with a beta coefficient of $11.327$, indicating that each additional 0.1-unit increase in relative plasma 24-HC levels was associated with a 3.10-fold increase in odds of having AMD ($95\% CI: 1.66$ to $5.79$).

![Fig. 4. Age affects human peripheral blood mononuclear cell (PBMC) and plasma oxysterol signatures. (a-b) There was a significant negative correlation between age and PBMC 7-KC levels, PBMC C-triol levels, and PBMC 24-HC levels in healthy human subjects. (c-d) There was a significant positive correlation between age and plasma C-triol levels and a trend towards a positive correlation between age and plasma 4β-HC levels. Open circles depict individual data points; lines depict the best-fitting linear regression line (a-d, $r$ = Pearson product-moment correlation coefficient; $r_s$ = Spearman rank-order correlation coefficient).]
Furthermore, we performed conjunctive analysis to evaluate the clinical utility of using plasma 24-HC to discriminate between AMD patients and non-AMD subjects across varying ages. We divided patients into tertiles by plasma 24-HC levels and by age (i.e., above versus below median), tabulating the AMD prevalence in each conjoined cell (Table 6). This analysis demonstrated a clear stepwise increase in prevalence of AMD in subjects above the median age going from the lowest to the highest tertile of plasma 24-HC (i.e., from 47.1% to 66.7% to 76.2%; Fig. 6a). We observed a similar increase in AMD prevalence in subjects below the median age based on plasma 24-HC tertile (i.e., from 10.5% to 19.0% to 38.5%). These findings support the notion that plasma 24-HC levels can distinguish between the changes in cholesterol homeostasis expected during physiologic aging versus those that suggest risk of AMD. To determine the efficacy of plasma 24-HC levels as a potential marker for AMD, we generated a receiver operating characteristic (ROC) curve and found that the area under the ROC curve (AUC) was 0.866 (95% CI: 0.793 to 0.939), indicating good discrimination (Fig. 6b).

To determine the clinical utility of plasma 24-HC levels compared to existing clinical measures of lipid homeostasis, we next analyzed whether plasma 24-HC levels were correlated with total plasma cholesterol levels in the subjects for whom this information was available. In these subjects (N = 37), plasma 24-HC was indeed correlated with total plasma cholesterol (R = 0.659, P < .001). We therefore performed a subanalysis by generating a binary logistic regression for the outcome of AMD with forced entry of plasma 24-HC levels, total plasma cholesterol, age, and gender. This model was statistically significant (LR X²...

![Fig. 5. Age-related macular degeneration (AMD) patients have altered peripheral blood mononuclear cell (PBMC) and plasma oxysterol signatures. (a-j) We measured PBMC and plasma levels of 4β-HC, 7-KC, C-triol, 24-HC, and 27-HC. AMD patients (N = 44–45) had decreased PBMC 7-KC levels (b; 2-tailed Mann-Whitney U test), elevated plasma 4β-HC levels (f; 2-tailed Mann-Whitney U test), elevated plasma C-triol levels (h; 2-tailed Mann-Whitney U test), elevated plasma 24-HC levels (i; 2-tailed Mann-Whitney U test), and a trend towards elevated plasma 27-HC levels (j; 2-tailed, unpaired t-test) compared to non-AMD controls (N = 61). Open circles depict individual data points; horizontal lines depict mean ± 95% confidence intervals (a-j) (* P < .05; ** P < .01; *** P < .001).](148)
The presence of AMD features suggests that high-dose statins may indeed reduce some high-risk features of AMD. Drugs used for cardiovascular disease, may affect the development or progression of AMD. Polymorphisms in lipid-related genes such as LIPC, C and total serum cholesterol, affect risk of AMD development and progression. The regulation and upregulation of different genes having similar pathways, including biosynthesis, elimination, transport, and regulation. Interestingly, aged macrophages exhibited simultaneous downregulation and upregulation of different genes having similar functions in cholesterol-related pathways (Table 2), indicating broad and complex dysregulation of cholesterol homeostasis.

Our hypothesis that impaired cholesterol metabolism in aged macrophages contributes to AMD pathogenesis is consistent with numerous epidemiological studies that have established that parameters related to lipid status, such as baseline high-density lipoprotein-cholesterol (HDL-C) and total serum cholesterol, affect risk of AMD development and progression [36–38]. Moreover, one past genome-wide association study reported that polymorphisms in lipid-related genes such as LIPC, ABCA1, and CETP are associated with advanced AMD [20]. These findings have led to the hypothesis that statins, cholesterol-lowering drugs used for cardiovascular disease, may affect the development or progression of AMD [39]. The findings of one non-randomized study suggest that high-dose statins may indeed reduce some high-risk features of AMD [40], although large randomized studies are necessary to confirm these findings [41] given that numerous other studies report conflicting results [42]. Cumulatively, these conflicting findings highlight that the relationship between aging, impaired cholesterol homeostasis, and AMD is complex and warrants further investigation.

In this study, we found that in aged murine macrophages, a transcriptomic profile suggesting impaired cholesterol homeostasis was associated with aberrant intracellular oxysterol levels, especially when the macrophages were treated with oxDL. In many immune cells, oxysterols can directly regulate liver X receptor (LXR) transcriptional activity, which can modulate cellular lipid metabolism and the immune response, especially in inflammation-associated diseases [33]. For example, 27-HC, the most prevalent oxysterol in atherosclerotic lesions, has been shown to promote atherosclerosis by inducing inflammation [43]. Therefore, in addition to being a surrogate marker for impaired cholesterol homeostasis, altered oxysterol production may play a pathogenic role in promoting cholesterol dysregulation. Further studies are necessary to uncover the mechanisms underlying altered oxysterol content, which may be related to altered uptake or synthesis. However, our results broadly suggest that altered oxysterols in macrophages are markers of cholesterol dyshomeostasis.

We found that, similar to murine macrophages, human PBMC and plasma samples demonstrated age-dependent and disease-dependent changes in oxysterols. Although the directionality of these changes did not correspond perfectly between mice and humans, our data suggest that broad dysregulation of cholesterol homeostasis in both aging and disease is associated with altered oxysterol signatures. These findings build on recent reports that monocytes isolated from patients with neovascular AMD do indeed exhibit an altered immune-related transcriptomic signature [44] and that these cells, when activated into macrophages, demonstrate proangiogenic characteristics that may contribute to disease pathogenesis [45]. Past studies have also shown that in the outer retina, accumulation of cholesterol oxidation products such as 7-KC can disrupt the immune environment and transform resident macrophages into disease-promoting cells [46]. Specifically, uptake of 7-KC in microglial cells results in decreased production of neurotrophic growth factors and increased expression of angiogenic mediators that promote pathologic CNV [46]. Although there are likely some differences between the mechanisms of altered oxysterols in mice and humans, our work support the broad idea that oxysterols can be used to identify features of cholesterol dyshomeostasis.

How to distinguish whether changes in oxysterol signatures define aging, disease, or both is of great interest, as defects in lipid metabolism are a shared feature of multiple diseases. As an example, certain oxysterols and their metabolites have been shown to be specific biomarkers for Niemann-Pick type C (NPC) disease, an inherited lysosomal storage disease [47, 48]. In this study, we found that after controlling for age and gender, plasma 24-HC was significantly associated with AMD. Despite our modest sample size (N = 107), these findings suggest that 24-HC is a strong candidate for an oxysterol that distinguishes patients with AMD from physiologic aging. Although limited by sample size and therefore a relatively wide confidence interval, our subanalysis revealed that plasma 24-HC remained associated with AMD even after controlling for total plasma cholesterol, highlighting its diagnostic value. Larger prospective studies are necessary to validate these findings and identify additional markers of risk.

### Table 5
Beta coefficients from plasma binary logistic regression model.

<table>
<thead>
<tr>
<th>Predictor variable</th>
<th>βOR (eβ)</th>
<th>95% CI of eβ</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.113</td>
<td>1.055 to 1.174</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female gender</td>
<td>0.390</td>
<td>0.119 to 1.282</td>
<td>0.121</td>
</tr>
<tr>
<td>Plasma 24-HC levels, 0.1-unit</td>
<td>3.104</td>
<td>1.66 to 5.79</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

### Table 6
AMD as a function of age and plasma 24-HC levels.

<table>
<thead>
<tr>
<th>Age</th>
<th>Below median</th>
<th>Above median</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma 24-HC Lowest tertile</td>
<td>2/19 (10.5%)</td>
<td>8/17 (47.1%)</td>
<td>10/36 (27.8%)</td>
</tr>
<tr>
<td>Middle tertile</td>
<td>4/21 (19.0%)</td>
<td>10/15 (66.7%)</td>
<td>14/36 (38.9%)</td>
</tr>
<tr>
<td>Highest tertile</td>
<td>5/13 (38.5%)</td>
<td>16/21 (76.2%)</td>
<td>21/34 (61.8%)</td>
</tr>
<tr>
<td>Total</td>
<td>11/53 (20.8%)</td>
<td>34/53 (64.2%)</td>
<td>45/106 (42.5%)</td>
</tr>
</tbody>
</table>
Cumulatively, our findings highlight that impaired cholesterol homeostasis is a key pathway perturbed in aged macrophages and that oxysterol signatures in patient samples can distinguish AMD from physiologic aging. Ultimately, these findings may not only improve our ability to diagnose disease but also identify targets in cholesterol homeostasis pathways for novel therapeutic approaches.

Funding Sources
This work was supported by NIH Grants R01 EY019287 (R.S.A.), R01 NS081985 (D.S.O.), R01 AG052494 (L.U.M.), P30 EY02687 (Vision Core Grant), and P30 DK020579 (Diabetes Research Center Metabolomics Core); the Starr Foundation (R.S.A.); the Carl Marshall Reeves and Mildred Almen Reeves Family Gift for retinal research (R.S.A.); a Physician-Scientist Award and a Nelson Trust Award from Research to Prevent Blindness (R.S.A.); the Jeffrey Fort Innovation Fund (R.S.A.); and the Thome Foundation (R.S.A.). Additional funding comes from an unrestricted grant to the Department of Ophthalmology and Visual Sciences of Washington University School of Medicine from Research to Prevent Blindness. J.B.L. was supported by the Washington University in St. Louis Medical Scientist Training Program (NIH Grant T32 GM007200), the Washington University in St. Louis Institute of Clinical and Translational Sciences (NIH Grants UL1 TR000448, TL1 TR002344), and the VitreoRetinal Surgery Foundation. M.M.L. was supported by NIH Grants T32 GM007200 and T32 AI007172. The funders had no involvement in any aspect of this study or manuscript preparation. R.S.A. had full access to all of the data in the study and had final responsibility for the decision to submit for publication.

Declaration of Interests
The authors declare that no conflict of interest exists.

Author Contributions

Acknowledgements
The authors thank the Genome Technology Access Center in the Department of Genetics at Washington University School of Medicine for help with genomic analysis (NIH Grants P30 CA91842 and UL1 TR000448). We also thank David Scherrer for technical assistance and Stephanie Schultz for helpful discussions.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.05.035.

References


Appendix 2: A non-canonical autophagy-dependent role of ATG16L1^{T300A} variant in urothelial vesicular trafficking and uropathogenic *Escherichia coli* persistence

This chapter was published in *Autophagy*. MML performed ELISAs and contributed to the writing and revision of this paper.

A non-canonical autophagy-dependent role of the ATG16L1T300A variant in urothelial vesicular trafficking and uropathogenic Escherichia coli persistence

Caihong Wanga, Kyle A. Bauckmana, Adam S. B. Rossa, Jane W. Symingtona, Marianne M. Ligotac, Gael Scholtesa, Akhil Kumara, Hao-Wei Chang, Joy Twentyman, Bisaiyo E. Fashemia, Ramnik J. Xaviera, and Indira U. Mysorekara,b

aDepartment of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, MO, USA; bPathology and Immunology, Washington University School of Medicine, St. Louis, MO, USA; cGastrointestinal Unit and Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

ABSTRACT

50% of Caucasians carry a Thr300Ala variant (T300A) in the protein encoded by the macroautophagy/autophagy gene ATG16L1. Here, we show that the T300A variant confers protection against urinary tract infections (UTIs), the most common infectious disease in women. Using knockin mice carrying the human T300A variant, we show that the variant limits the UTI-causing bacteria, uropathogenic Escherichia coli (UPEC), from establishing persistent intracellular reservoirs, which can seed UTI recurrence. We further show that mice with the T300A variant exhibit urothelial cellular abnormalities, including vesicular congestion and aberrant accumulation of UPK (uroplakin) proteins. Importantly, presence of the T300A variant in humans is associated with similar urothelial architectural abnormalities, indicating an evolutionarily conserved impact. Mechanistically, we show that the reduced bacterial persistence is independent of basal autophagic flux or proinflammatory cytokine responses and does not involve Atg14 or Epg5. However, the T300A variant is associated with increased expression of the small GTPase Rab33b. Rab33b interacts with ATG16L1, as well as other secretory RABs, RAB27B and RAB11A, important for UPEC exocytosis from the urothelium. Finally, inhibition of secretory RABs in bladder epithelial cells increases intracellular UPEC load. Together, our results reveal that UPEC selectively utilize genes important for autophagosome formation to persist in the urothelium, and that the presence of the T300A variant in ATG16L1 is associated with changes in urothelial vesicle trafficking, which disrupts the ability of UPEC to persist, thereby limiting the risk of recurrent UTIs.

Abbreviations: 3-PEHPC: 3-pyridinyl ethyldiene hydroxy phosphonocarboxylate; ATG: autophagy; ATG16L1: autophagy related 16 like 1; BECs: bladder epithelial cells; dpi: days post infection; hpi: hours post infection; IF: immunofluorescence; IL1B: interleukin 1 beta; IL6: interleukin 6; MAP1LC3B/LC3: microtubule-associated protein 1 light chain 3 beta; MVB: multivesicular bodies; T300A: Thr300Ala; TNF: tumor necrosis factor; QIR(s): quiescent intracellular reservoir(s); siRNA: short interfering RNA; UPEC: uropathogenic Escherichia coli; UTI(s): urinary tract infection(s); TEM: transmission electron microscopy; WT: wild type

Introduction

Autophagy is a housekeeping process that maintains cellular homeostasis through recycling of nutrients and degradation of damaged or aged cytoplasmic contents. This process involves a series of dynamic membrane rearrangements that are mediated by a core set of autophagy-related (ATG) proteins. Autophagosome formation starts with the stepwise engulfment of cytoplasmic material by the phagophore, which later matures into a double-membrane-bound vesicle [1–5]. Vesicle nucleation requires the activation of the class III phosphatidylinositol 3-kinase complex, whose activation depends on the formation of a complex that includes ATG14. The vesicle elongation process involves 2 ubiquitin-like conjugation systems. The first involves the covalent conjugation of ATG12 to ATG5, which then interacts non-covalently with ATG16L1 (autophagy related 16 like 1). The second involves the conjugation of phosphatidylinethanolamine to MAP1LC3B/LC3 (microtubule-associated protein 1 light chain 3 beta) by the sequential action of ATG4, ATG7, and ATG3. Autophagosomes then undergo maturation by fusion with lysosomes to create autolysosomes, a process which requires various proteins including EPG5 [6,7].

Autophagy genes control bacterial infection and inflammation. Depletion or mutation of autophagy genes is associated with inflammatory diseases in humans. For example, a single nucleotide polymorphism (rs2241880, corresponding to the T300A variant) in ATG16L1 is present in 50% of Caucasians and is associated with a higher risk of developing Crohn disease, a form of inflammatory bowel disease [8,9].

CONTACT Indira U. Mysorekar imysorekar@wustl.edu

Department of Obstetrics and Gynecology & Pathology and Immunology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, USA

© 2018 Informa UK Limited, trading as Taylor & Francis Group

Supplementary data for this article can be accessed here.
ATG16L1T300A variant does not affect basal autophagy but affects unconventional autophagy [10] and xenophagy (a specific form of autophagy targeting pathogens [11]) and the inflammatory response to certain infections or metabolic stress signals [12–17].

Autophagy is also involved in the pathogenesis of urinary tract infections (UTIs), one of the most common, recurrent infectious diseases worldwide. UTIs afflict 150 million women per year and recur in up to 40% of cases [18]. The majority of infections are caused by uropathogenic Escherichia coli (UPEC), which invade into superficial bladder epithelial cells, replicate, and persist in a small number of urothelial cells as quiescent intracellular bacterial reservoirs (QIRs) [19]. QIRs are refractory to antibiotic therapies and do not elicit an active immune response but can seed recurrent infections. UPEC are recognized by autophagy machinery and reside in autophagosomal and/or lysosomal compartments [20,21]; mice homozygous for ATG16L1 have reduced UPEC persistence [21,22]. Furthermore, UPEC co-opt ferroptophagy, a selective form of autophagy involved in iron release from ferritin stores, to survive and persist in bladder epithelial cells [20]. However, whether ATG16L1 deficiency-mediated protection from UTIs depends on the canonical autophagy machinery or is specific to some other role for ATG16L1 is unclear. Additionally, whether other autophagy pathway genes play a role in orchestrating the response to UTIs remains to be elucidated. Further, given the important role of ATG16L1 in urothelial intracellular membrane trafficking and UPEC persistence, it remains unknown whether the T300A variant in ATG16L1 plays a role in pathogenesis of UTIs.

Superficial urothelial cells contain unique cytoplasmic organelles including fusiform vesicles, which deliver preassembled UPK (uroplakin) proteins to the apical cell surface during bladder filling. Recent studies have demonstrated that specific small RAB GTPases (RAB27B and RAB11A) are highly enriched in superficial urothelial cells and are important for vesicle trafficking. UPK recycling [23–28], and exosome-mediated expulsion of intracellular UPEC [29–31]. Interestingly, network mapping of autophagy pathways has identified a Golgi-resident small GTPase, RAB33B, which interacts directly with ATG16L1 and thereby modulates autophagosome formation [32]. Whether the T300A variant is associated with changes to RAB33B, RAB27B or RAB11A, which are implicated in altered vesicular trafficking, remains to be determined.

Here, we report that UPEC selectively utilize autophagy proteins important for autophagosome formation to persist in the urothelium. T300A mice carrying the human mutant allele at the Atg16l1 gene locus exhibit altered urothelial architecture and harbor significantly fewer persistent bacterial reservoirs in the urothelium. Importantly, this feature is conserved in humans with the rs2241880 GG genotype (homozygous for the T300A variant in human ATG16L1). We demonstrate that these effects are independent of the canonical autophagy pathway and bladder mucosal immunity, but appear to be associated with increased expression of small RAB GTPases that are known to be important for UPK recycling and UPEC expulsion. Together, our findings suggest that the T300A variant protects the bladder mucosa from persistent UTIs.

Results

Presence of the ATG16L1T300A variant confers protection from UPEC persistence in urothelium

We first sought to determine whether the T300A variant of ATG16L1 was sufficient to confer protection from UPEC persistence in the bladder epithelium, a phenotype observed in mice with reduced Atg16l1 expression (Atg16l1HMO mice) [22]. We induced UTIs in adult female wild-type (WT) and mice carrying the human mutant allele at the Atg16l1 gene locus (hereafter referred to as T300A mice) and quantified the number of QIRs at 14 d post-infection (dpi) (Figure 1(a)). Urothelial cells of T300A mice harbored significantly fewer QIRs than did those of WT mice (Figure 1(d–e)). To confirm that the reduced QIR formation was not due to reduced UPEC invasion or defects in early colonization, we measured bacterial titers in the urine and bladder at 24 h post-infection (hpi) and found comparable bacterial titers between WT and T300A mice (Figure 1(b–c)). Thus, the T300A variant specifically confers increased resistance to UPEC persistence within the urothelium but does not affect UPEC invasion or early colonization, as was noted in Atg16l1HMO mice [22].

The T300A variant is sufficient to induce cellular architectural alterations in superficial urothelial cells

In a healthy bladder, the plasma membranes of superficial epithelial cells contain hexagonal arrays of UPK proteins, which are transported by discoidal storage vesicles and multivesicular bodies (MVBs) and are critical for normal epithelial flexibility and barrier function [33,34]. Previous work has demonstrated a role for ATG16L1 in superficial cell vesicular trafficking in mice globally hypomorphic for Atg16l1 [22]. We next sought to determine whether the T300A variant was sufficient to confer the same vesicular phenotype associated with decreased UPEC persistence. Indeed, we found substantially more MVBs and lysosomes in T300A mice compared to those of WT mice (Figure 2(a–b)). Blinded analysis of sections revealed that 90% of T300A mice had vesicle congestion in superficial epithelial cells, whereas only 7% of WT mice had this phenotype (Figure 2(c)). Transmission electron microscopy (TEM) and immunofluorescence (IF) analysis confirmed that the density of MVBs, lysosomes, and UPK expression in urothelial cells was significantly higher in T300A mice than in WT mice (Figure 2(d–i)). These observations indicate that the human T300A variant is sufficient to disrupt urothelial cellular architecture in mice.

UPEC hijack a specific set of autophagy genes important for normal tissue architecture to persist intracellularly in the urothelium

We next examined if the T300A variant-conferred restriction of UPEC persistence and urothelial architectural changes were unique to ATG16L1 or were a shared feature with other canonical autophagy related proteins. First, we depleted key genes for each step of the autophagy pathway via short interfering RNA (siRNA)-mediated gene-silencing in human
bladder epithelial cells (BECs). RT-qPCR and immunoblotting validated the knockdown of mRNA levels of ATG16L1, ATG7, ATG14 and EPG5, and protein levels of ATG16L1 and ATG7 (Figure S1). We confirmed that silencing of ATG16L1 led to a significant reduction in the intracellular bacterial load in BECs at 3 and 6 hpi (Figure 3(a)). The effects were phenocopied in ATG7-silenced cells (Figure 3(b)). In contrast, knockdown of ATG14 or EPG5 did not decrease intracellular UPEC load (Figure 3(c-d)). We did not observe significant differences in bacterial load at earlier time points (0.5 and 1 hpi) in cells depleted of ATG16L1 or ATG7, indicating that these genes are not required for early invasion and colonization of BECs. Crystal violet assays did not reveal significant changes in cell growth in ATG16L1- or ATG7-silenced cells, indicating that the observed reduction in the intracellular load of UPEC was not likely due to decreased cell viability (Figure S2).

To extend the in vitro findings, we systematically examined whether loss of autophagy genes specifically in the urothelium affects UPEC persistence. To generate urothelial-cell-specific knockouts, we crossed Atg16l1^{fl/fl}, Atg7^{fl/fl}, and Atg14^{fl/fl} mice to Upk3a-GFP-Cre^{ERT2} mice, which drive deletion exclusively in superficial urothelial cells [35]. We also examined bladders of mice globally deficient in Epg5 (epg5^{−/−}), a gene important for autophagosome-lysosome fusion. Next, we induced UTIs in Atg16l1^{fl/fl}/Upk3a Cre^{+}, Atg7^{fl/fl}/Upk3a Cre^{+}, Atg14^{fl/fl}/Upk3a Cre^{+}, and epg5^{−/−} mice and examined urine bacterial loads in these mice at 24 hpi and quantified the number of bacterial QIRs at 14dpi. We

---

**Figure 1.** Mice carrying the ATG16L1^{T300A} variant exhibit reduced UPEC persistence. (a) Schematic of the experimental setup. (b) Colony forming unit (CFU) counts of bacteriuria at 24 hpi plotted as mean of the Log_{10} value. n = 19 for WT; n = 45 for T300A; n = 6 experiments. ns, not significant by Mann-Whitney test. (c) CFU counts of the bladder plotted as mean ± SD of the Log_{10} value at 24 hpi. n = 5 for WT; n = 4 for T300A mice. ns, not significant by Mann-Whitney test. (d) Quantification of QIRs in WT and T300A bladders at 14 dpi. n = 8 sections/bladder, n = 17 mice for T300A, n = 45 mice for WT. *P < 0.05 by Mann-Whitney test. (e) IF image of a bacterial QIR. Bar: 10 µm.
Figure 2. T300A mice exhibit superficial cell architectural changes. (a and b) IF imaging analysis of urothelium from WT (a) and T300A (b) mice. Green, vesicles; magenta, CDH1; blue, DAPI; red arrowhead, vesicular congestion. Bar: 10 µm. (c) Percentage of WT and T300A mice that showed vesicular congestion. (d and e) Representative TEM of superficial cell ultrastructure in WT (d) and T300A (e) mice. Red arrowhead, multi-vesicular bodies; blue arrow, lysosomes. Bar: 1 µm. (f and g) Quantification of MVBs (f) and lysosomes (g) in TEM images of urothelium from WT and T300A mice. Bars represent mean ± SEM. n = 3 mice/genotype. *P < 0.05 by unpaired two-tailed T test. (h and i) IF of UPK (green) in bladders of WT (g) and T300A (h) mice. Blue, DAPI. Bar: 10 µm.
found that loss of each of the genes had no effect on bacteriuria at 24 hpi (Figure 4(a-d)). However, at 14 dpi, Atg16l1/fl/fl Upk3a Cre+ and Atg7/fl/fl Upk3a Cre+ mice harbored significantly fewer QIRs in the bladder than their Cre− littermates (Figure 4(e-f)), an effect not seen following loss of Atg14 in urothelial cells or global deletion of Epg5 (Figure 4(g-h)).

Together, these data indicate that UPEC co-opts proteins of the elongation phase of the autophagy pathway important for autophagosome formation for persistence in QIRs but does not require proteins governing the initiation or autophagosome-lysosome fusion phases.

To determine if depletion of Atg16l1 specifically in the urothelium was sufficient to alter urothelial architecture, we examined bladders from Atg16l1/fl/fl/Upk3a Cre+ mice by TEM and IF. These mice also display the vesicular congestion phenotype, characterized by accumulation of MVBs and lysosomes (Figure 5(a)), which has been documented in Atg16l1HM mice [22] and in T300A mice, as shown above. Previously, it has been shown that loss of Atg16l1 in the myeloid compartment or in the adaptive immune compartment does not alter urothelial cellular architecture, supporting the idea that the structural changes were epithelial intrinsic [22]. To further examine if other autophagy proteins were also involved, we examined urothelial architecture in Atg2/fl/fl/Upk3a Cre+, Atg14/fl/fl/Upk3a Cre+, and epg5−/− mice by IF and TEM. We found that only mice with urothelial cells lacking Atg7 showed similar vesicular congestion as seen with loss of Atg16l1 (Figure 5(b)). Blinded quantification revealed that whereas 34% of Atg16l1/fl/fl/Upk3a Cre+ mice and 55% of Atg2/fl/fl/Upk3a Cre+ mice had vesicle congestion in superficial cells, only 7% of Atg16l1/fl/fl/Upk3a Cre− and 22% of Atg2/fl/fl/Upk3a Cre− littermate controls showed this phenotype. Quantification of MVBs and lysosomes by TEM further corroborated these findings (Figures 5(a-b) and S3). Vesicular congestion was not seen in superficial cells from Atg14/fl/fl/Upk3a Cre− or epg5−/− mice (Figures 5(c-d) and S3). Together, our data reveal a role for ATG16L1 and ATG7 but not ATG14 or EPG5 in regulating both UPEC persistence in bladder epithelial cells as well as cellular membrane trafficking that is known to be important for bacterial persistence.
Decreased UPEC persistence in ATG16L1 T300A urothelium is independent of innate immune responses in the bladder mucosa

Recent studies in the intestinal mucosa have shown that the T300A variant sensitizes mice to cellular stress induced by intestinal infections with *Salmonella enterica* serovar *Typhimurium* and *Yersinia enterocolitica*, in large part due to increased ATG16L1 cleavage by the apoptotic proteases, CASP3 (caspase 3) and CASP7 [13, 14]. Here, we found that levels of CASP3 were equivalent in urothelium of WT and T300A mice before infection (Figure 6(a)) and at 3 and 6 hpi (Figure 6(b-c)). Next, we determined whether T300A variant-mediated resistance to UPEC persistence was associated with infiltration of innate immune cells into the bladder. We found that the urine of infected T300A mice harbored similar numbers of neutrophils and monocytes upon infection as did the urine of WT mice (Figure 6(d)). Previous studies have shown that depletion of ATG16L1 in macrophages amplifies inflammatory IL1B signaling in response to bacterial lipopolysaccharide or UPEC [36, 37]. However, we found that bone-marrow-derived macrophages from T300A mice produced similar levels of IL1B, IL6, and TNF (Figure 6(e-g)) in response to UPEC infection as those from WT mice.

Further analysis of mRNA levels of IL1b, Il6, and Tnf from bladders at 24 hpi did not show a significant difference between WT and T300A mice (Figure 6(h-j)). Together, these data suggest that the T300A variant does not elicit an elevated inflammatory cellular influx or inflammatory cytokine response upon UPEC infection.

The ATG16L1 T300A variant does not affect bulk autophagy in the bladder

We next reasoned that, if ATG16L1 is critical for bacterial persistence in the bladder, then the T300A variant may cause decreased persistence by affecting ATG16L1 protein expression or autophagic activity in the bladder. However, we found that levels of ATG16L1 protein in WT and T300A mice before infection (Figure 7(a)) and after infection (Figure 7(b)), were indistinguishable, indicating that the stability of ATG16L1 is not decreased in T300A mice. Next, to examine autophagic activity, we measured levels of LC3-II before and after starvation WT and T300A mice for 24 h. Although the LC3-II/LC3-I ratio increased upon starvation in both WT and T300A mice, we detected no differences in LC3-II levels between the 2 genotypes (Figure 7(c)). To examine whether the T300A variant impaired...
Figure 5. Loss of Atg16l1 or Atg7 but not Atg14 or Epg5 leads to urothelial architecture alterations. (a-d, 1 and 2) IF imaging analysis of urothelium from Atg16l1<sub>fl/fl</sub> Upk3a Cre<sup>−</sup> and Atg16l1<sub>fl/fl</sub> Upk3a Cre<sup>+</sup> (A1-A2), Atg7<sub>fl/fl</sub> Upk3a Cre<sup>−</sup> and Atg7<sub>fl/fl</sub> Upk3a Cre<sup>+</sup> (B1-B2), Atg14<sub>fl/fl</sub> Upk3a Cre<sup>−</sup> and Atg14<sub>fl/fl</sub> Upk3a Cre<sup>+</sup> (C1-C2) and WT and epg5<sup>−/−</sup> mice (D1-D2). Green, vesicles; magenta, CDH1; blue, bisbenzimide DNA stain; red arrowhead, vesicular congestion. Bar: 10 µm. (A-D, 3 and 4) TEM of superficial cell ultrastructure in Atg16l1<sub>fl/fl</sub> Upk3a Cre<sup>−</sup> and Atg16l1<sub>fl/fl</sub> Upk3a Cre<sup>+</sup> (A3-A4), Atg7<sub>fl/fl</sub> Upk3a Cre<sup>−</sup> and Atg7<sub>fl/fl</sub> Upk3a Cre<sup>+</sup> (B3-B4), Atg14<sub>fl/fl</sub> Upk3a Cre<sup>−</sup> and Atg14<sub>fl/fl</sub> Upk3a Cre<sup>+</sup> (C3-C4) and WT and epg5<sup>−/−</sup> mice (D3-D4). Red arrowhead, multivesicular bodies; blue arrow, lysosomes. n = 3 mice. Bar: 2 µm. (a-d, 5) Quantification of MVBs of Atg16l1<sub>fl/fl</sub> Upk3a Cre<sup>−</sup> and Atg16l1<sub>fl/fl</sub> Upk3a Cre<sup>+</sup> mice (A5), Atg7<sub>fl/fl</sub> Upk3a Cre<sup>−</sup> and Atg7<sub>fl/fl</sub> Upk3a Cre<sup>+</sup> mice (B5), Atg14<sub>fl/fl</sub> Upk3a Cre<sup>−</sup> and Atg14<sub>fl/fl</sub> Upk3a Cre<sup>+</sup> mice (C5) and WT and epg5<sup>−/−</sup> mice (D5) urothelium. Bars represent mean ± SEM. (A-D, 6) Quantification of lysosomes of Atg16l1<sub>fl/fl</sub> Upk3a Cre<sup>−</sup> and Atg16l1<sub>fl/fl</sub> Upk3a Cre<sup>+</sup> (A6), Atg7<sub>fl/fl</sub> Upk3a Cre<sup>−</sup> and Atg7<sub>fl/fl</sub> Upk3a Cre<sup>+</sup> (B6), Atg14<sub>fl/fl</sub> Upk3a Cre<sup>−</sup> and Atg14<sub>fl/fl</sub> Upk3a Cre<sup>+</sup> (C6) and WT and epg5<sup>−/−</sup> mice urothelium (D6). Bars represent mean ± SEM.
autophagy during UTI, we measured LC3-II at 3 hpi and found equivalent levels of LC3-II between bladders from WT and T300A mice (Figure 7(d)). Thus, the T300A variant does not alter bulk autophagy activity in the bladder.

Presence of the T300A variant alters expression of RAB GTPases that are important for UPEC expulsion

Collectively, our findings suggest that the intersection of UPEC with the autophagy machinery occurs at the
phagophore elongation phase wherein ATG16L1 plays a critical role as a central adaptor [38,39]. Given the important role of ATG16L1 in urothelial intracellular membrane trafficking and UPEC persistence, we sought to investigate how the T300A variant might alter the RAB GTPase network in the urothelium. Network mapping of autophagy pathways has identified proteins, notably, a Golgi-resident small GTPase, RAB33B, which interacts directly with ATG16L1 and modulates autophagosome formation [32,40,41]. Thus, we hypothesized that RAB33B could be implicated in the altered UPEC pathogenesis in T300A mice. To test this, we examined expression levels of Rab33b in WT and T300A bladders before and after UPEC infection. Before infection, we found no significant change in expression levels of Rab33b. However, upon induction of a UTI, we noted a significant increase in Rab33b expression in T300A bladders (Figure 8(a)), in contrast to its downregulation in WT controls.

Recent studies have demonstrated that specific small secretory RAB GTPases, RAB27B and RAB11A, are highly enriched in superficial urothelial cells and are important for vesicle trafficking, UPK recycling, and expulsion of intracellular UPEC [23–31]. Examination of Rab27b and Rab11a mRNA levels in WT and T300A mice revealed that the T300A variant was again associated with increases in expression levels of both Rab27b and Rab11a upon induction of a UTI in contrast to decreases in expression in WT bladders (Figure 8(a)). Thus, secretory RABs associated with ATG16L1 and autophagosome formation and/or UPK recycling appear to be altered in T300A mice, a result consistent with a role for this variant in modulating urothelial architecture. Given the established role of Rab27b and Rab11a in urothelial stretch-induced trafficking [25–27] and in UPEC exocytosis from human BECs [29,30], we mechanistically determined whether inhibition of RAB activity would lead to increased bacterial load in BECs. We treated human BECs with 3-pyridinyl ethylidene hydroxyl phosphonocarboxylate (3-PEHPC), an inhibitor of RABGGT (Rab geranylgeranyl transferase) that inhibits all secretory RAB GTPase activity [42–44]. Pretreatment of human BECs with 3-PEHPC prior to infection with UPEC revealed a significant increase in intracellular bacteria load upon infection (Figure 8(b)). The highest load was noted at 6 hpi, which corresponds to the time point shown to be the peak of exocytosis of UPEC via Rab27b [30]. Further, we depleted Rab33b, Rab27b and Rab11a via siRNA-mediated gene-silencing in human BECs. We found that silencing of Rab33b, Rab27b and Rab11a led to a significant increase in the intracellular bacterial load in human BECs at 3 and 6 hpi (Figure 8(c)), which is consistent with previous findings that RAB small GTPases mediate bacterial clearance from the urothelium [29]. Taken together, our in vivo and in vitro data suggest that changes in Rab33b,
The presence of the T300A variant differentially regulates genes encoding RAB GTPases upon UPEC infection. (a) RT-qPCR analysis of mRNA levels of Rab27b, Rab11a, and Rab33b in bladders from WT and T300A mice before infection and 24 hpi. (b) Quantification of intracellular UPEC CFU in human BECs treated with vehicle and 3-PEHPC at the indicated time post infection. *P < 0.05; **P < 0.01 by Mann-Whitney test. (c) Quantification of intracellular UPEC CFU in human BECs treated with control siRNA and siRNAs directed against RAB33B, RAB27B, and RAB11A. *P < 0.05; **P < 0.01 by two-way ANOVA with Bonferroni post-test.
RAB27B and RAB11A levels due to the presence of the T300A variant likely contribute to reduced UPEC persistence in urothelium.

**Humans carrying the ATG16L1 T300A variant display aberrant urothelial cell architecture in bladder tissue biopsies**

Given that UPEC persistence correlates with urothelial-specific aberrant vesicular trafficking (and not with canonical autophagy pathways or non-cell-autonomous immune cell effects) in mice carrying the human T300A variant, we next determined whether the incidence of the T300A variant in human populations would be correlated with similar urothelial architectural alterations. We obtained bladder tissues from patients undergoing transurethral resections for bladder cancer diagnosis and examined them in a blinded fashion for the presence/absence of vesicles. Remarkably, our analysis revealed that 33% of individuals with the GG (homozygous carriage of the T300A allele) or AG (heterozygous) genotypes displayed urothelial vesicular congestion, whereas only 14% of those with the AA (homozygous for the WT ATG16L1 allele) genotype had this phenotype (Figure 9(a-c)). Thus, the strong association between the presence of the T300A variant and increased urothelial vesicular abnormalities in mouse and human bladder urothelium indicates a key role for the T300A variant in the bladder mucosa with significant implications for UPEC pathogenesis.

**Discussion**

In this report, we demonstrated that the common ATG16L1 T300A variant confers protection from UPEC persistence primarily due to an alteration in urothelial cell architecture and consequent depletion of UPEC-protective niches. We found that mice carrying the human ATG16L1 T300A variant phenocopied urothelial abnormalities noted in mice carrying a hypomorphic allele of Atg16l1 with an 80% knockdown of the protein. Both Atg16l1<sup>HMS</sup> and T300A mice harbored fewer persistent UPEC reservoirs than WT mice, underscoring a pro-pathogenic role for ATG16L1 in the bladder mucosa.

Interestingly, we observed substantial differences at the early stages of UPEC infection between Atg16l1<sup>HMS</sup> and T300A mice. In T300A mice, as opposed to Atg16l1<sup>HMS</sup> mice, we did not observe faster clearance of bacteria from the urine, increased neutrophil infiltration, or increased IL1B/IL-1β production by macrophages. These differences are likely due to the means of generation of these mice. Atg16l1<sup>HMS</sup> mice were generated using gene-trap mutations to introduce a false splice acceptor in the intronic regions adjacent to exon 6 or 10, resulting in truncated core protein. Conversely, the T300A mutation occurs in the WD repeat domain of ATG16L1 which is not associated with canonical autophagy [10,13,14]. Our study demonstrated that T300A-associated urothelial alterations are independent of bulk autophagy activity, which is consistent with previous findings that the ATG16L1 T300A variant does not reduce basal autophagy [15–17]. Boada-Romero et al., recently showed that the ATG16L1 T300A variant also alters the ability of the WD repeat domain to interact with a transmembrane protein, TMEM59, and thereby affects TMEM59-mediated endosome trafficking. This impairment does not affect canonical autophagy and does not require caspase processing of ATG16L1 [12]. Our findings are consistent with these observations because the presence of the T300A variant does not sensitize caspase-dependent cleavage of ATG16L1 upon UPEC infection. In other words, the level of ATG16L1 in the bladder of T300A mice is not affected by the mutation.

T300A mice exhibited alterations in the recycling of intracellular membranes in superficial urothelial cells, increased expression of UPKs, and accumulation of MVBs, which is

![Figure 9.](image)

**Figure 9.** The ATG16L1 T300A variant leads to superficial cell architectural alterations in human bladders. (a and b) Hematoxylin and eosin staining of human bladder tissue biopsies from participants carrying ATG16L1 rs2241880 AA (a) and GG (b) alleles. Red arrowhead, vesicles. (c) Percentage of human bladders of the indicated genotype that showed a vesicular congestion phenotype.
consistent with an important role of ATG16L1 in governing the secretory pathway in epithelial cells [13,22,45]. Because MVBS are required for degrading and recycling of UPKs [46], we suggest that accumulation of MVBS in the urothelium may impair intracellular trafficking of UPKs and UPEC such that the bacteria are rerouted to exosomes for exocytosis [30] rather than to phagophores where they can persist within the cell (i.e., inside autophagosomes). RAB27B is important for stretch-induced exocytosis in bladder umbrella cells [26]. RAB11A, a protein that labels recycling endosomes, recruits WIP12 for ATG16L1-mediated LC3 conjugation and autophagosome formation [47] and thus may be involved in ATG16L1T300A-induced vesicle congestion in the urothelium. Recent work by Miao, et al., has demonstrated that concerted actions of the secretory RAB GTPases, RAB11A and RAB27B are involved in expulsion of UPEC from bladder epithelial cells [29]. Indeed, our findings that the T300A variant was associated with increased expression of Rab33b, Rab27b, and Rab11a, may explain the reduced UPEC persistence in T300A bladders. This notion is further underscored by significantly higher intracellular UPEC loads in bladder epithelial cells treated with a pan-secretory RAB inhibitor or depleted of RAB33b, RAB27b, or RAB11A by siRNA. Further mechanistic studies are needed to understand how ATG16L1 deficiency or T300A polymorphism alters urothelial trafficking and how this may be mediated via RAB33b, RAB27b, or RAB11A. Nevertheless, it is clear that this variant is associated with decreased UPEC persistence, likely via disruption of UPEC trafficking to phagophores, allowing the bacteria to persist within autophagosomes.

Our findings are similar to a recent study demonstrating that the T300A variant is associated with structural defects in Paneth cells caused by defects in secretory autophagy due to the presence of this variant. Interestingly, the consequences of T300A variant-induced structural defects in Paneth cells and superficial bladder cells are quite the opposite. Bel et al., showed that disrupted secretory autophagy in Paneth cells in T300A mice is associated with increased Salmonella enterica serovar Typhimurium infection [48]. However, in the bladder, T300A variant-induced structural changes are beneficial to the host as these alterations impair the ability of UPEC to persist in the urothelium. Thus, our data highlight a positive role for secretory autophagy in response to uropathogenic infection and point towards the tissue- and cell-type-specific roles of the T300A variant.

We demonstrate that UPEC adopt a unique strategy by selectively exploiting a specific set of autophagy proteins to establish latency, confirming that bacterial pathogens can utilize different host cell compartments and pathways during their intracellular cycle. Autophagy gene-dependent cellular functions in controlling intracellular pathogens have been reported in a variety of disease models. For example, Starr et al., showed selective subversion of autophagy initiation proteins by Brucella abortus [49]. Huang et al., showed that IFNG/IFN-γ inhibits murine norovirus growth in macrophages in a manner dependent on Atg5, Atg7, and Atg16l1, but not on Atg14 [50]. More recently, Park et al., showed that loss of autophagy genes including Atg5, Atg16l1, Atg7, and Atg14 but not Atg6b in myeloid cells results in inhibition of MHV68 reactivation from latently infected macrophages [51]. Additionally, Kimmy et al., demonstrated a unique role for Atg5 but not for other autophagy genes in modulating Mycobacterium tuberculosis infection [52].

The exact mechanism by which UPEC hijacks ATG16L1 and ATG7 and the bacterial proteins that are involved in mediating this process remain poorly described. We suggest that the absence of ATG16L1 or ATG7 leads to alteration of intracellular membrane trafficking, which in turn reroutes UPEC to compartments where they cannot persist. Urothelium-specific loss of these autophagy genes appears sufficient to induce urothelial vesicular congestion and confer resistance to UPEC persistence, further supporting our conclusion that regulation of QIR formation is not governed by loss of autophagy gene-mediated inflammatory responses. Loss of autophagy genes in myeloid cells or in the adaptive immune compartment does not affect intracellular membrane trafficking or bacterial persistence within the urothelium [22]. Furthermore, we previously reported that Atg16l1−/− macrophages had increased CASP1-dependent secretion of IL1B upon UPEC infection [37]. Likewise, recent studies showed that the T300A polymorphism was associated with increased IL1B production in macrophages due to increased CASP3-mediated degradation of ATG16L1 in the context of metabolic stress or infection [13,14]. However, we did not find any differences in production of IL1B or other proinflammatory mediators between macrophages from ATG16L1T300A or WT mice before or in response to UPEC infection. Together, our findings suggest that the T300A variant specifically affects urothelial cells and indicate an epithelial-intrinsic role of autophagy genes in intracellular trafficking of UPEC inside the urothelium.

UTIs have been described as early as 1550 B.C., are the second most common infection in humans, and are the most common infection in women worldwide. It is remarkable that presence of the T300A variant in humans with no known bladder disease is sufficient to confer structural alterations in urothelial cells. This association is reminiscent of the observation that the harmful sickle cell trait is maintained in humans because it confers resistance to another common infection, malaria, by structurally altering red blood cells, thus limiting the ability of the parasite to invade [53]. Together, our findings underscore a key role for ATG16L1 and its associated human polymorphism, T300A, in maintaining bladder epithelial architecture and altering the trafficking patterns of intracellular UPEC, thus having significant clinical implications for understanding the etiology of recurrent UTIs.

Materials and methods

**Mice**

Atg16l1−/− and Atg14−/− mice were kindly provided by Dr. Herbert Virgin (Washington University School of Medicine). Atg7−/− mice were a kind gift from Dr. Masaaki Komatsu (Tokyo Metropolitan Institute of Medical Science). Upk3a-GFP-CreERT2 mice were purchased from Jackson laboratory. Atg16l1−/−, Atg7−/− or Atg14−/− mice were bred to Upk3a-GFP-CreERT2 mice to generate Atg16l1−/−Upk3a CreERT2, Atg7−/− or Atg14−/− mice.
Mice were sacrificed at 14 dpi and the bladders were processed as hpi for acute stage of infection and 14 dpi for persistent stage) collected before infection and at indicated time points (3 collected at 24 hpi to measure bacterial titers. Bladders were (PBS; Sigma-Aldrich, D8537). Urine and bladders were collecting units (CFUs) of UTI89 in phosphate-buffered saline and their times over the course of 1 wk. The mice were then infected 1 wk after the last tamoxifen treatment. egpS−/− mice were kindly provided by Dr. Hong Zhang (Chinese Academy of Sciences) [54]. T300A mice were generated as described previously [13]. Experiments were performed according to approved protocols. In all experiments, adult female mice, 8–10 wk of age were used. Mice were maintained under specified pathogen-free conditions in a barrier facility and under a strict 12-h light/dark cycle. All experimental procedures were approved by the animal study committee of the Washington University School of Medicine (Animal Welfare Assurance #A-3381–01).

**Bacterial strains, mouse inoculations, and urinalysis**

UTI89, a clinical UPEC isolate from a patient with cystitis [55] was grown statically for 17 h in Luria-Bertani broth (10 g/L tryptone, 5 g/L yeast extract, and 10g/L NaCl) at 37°C prior to infection in all studies [22]. Mice were anesthetized and inoculated via transurethral catheterization with 107 colony forming units (CFUs) of UTI89 in phosphate-buffered saline (PBS; Sigma-Aldrich, D8537). Urine and bladders were collected at 24 hpi to measure bacterial titers. Bladders were collected before infection and at indicated time points (3–24 hpi for acute stage of infection and 14 dpi for persistent stage) following bacterial inoculation.

**Quantification of QIRs**

Mice were sacrificed at 14 dpi and the bladders were processed as described previously [22]. Eight separate 5 μm step sections over a thickness of 300 μm were immunostained with antibodies against E. coli (United States Biological, E3500-26), LAMP1 (Developmental Studies Hybridoma Bank, 1D4B), and CDH1/E-cadherin (BD Transduction Laboratories, 610182). The total number of LAMP1-positive UPEC reservoirs were counted using a 63x oil objective on a Zeiss Apotome microscope and reported as number of QIRs/bladder.

**Immunofluorescence and immunoblotting analysis**

Mouse bladders were processed as described previously [22]. The following primary antibodies were used: rabbit polyclonal antibodies to E. coli, mouse monoclonal antibody to CDH1/E-cadherin, rat monoclonal antibody to LAMP1 (Developmental Studies Hybridoma Bank, 1D4B), and mouse anti-UPK3/uroplakin III (Fitzgerald, 10R-U103A). Antibigen-antibody complexes were detected with Alexa Fluor 488-, 594-, and 647-conjugated secondary antibodies (ThermoFisher, A32731, A-21209 and A32728). Images were obtained on a Zeiss Apotome microscope using 10-20x and 40-63x (oil) objectives.

Blots were probed with the following primary antibodies: rabbit polyclonal to ATG16L1 (Abgent, AP1817B), ATG7 (Sigma-Aldrich, A2856), LC3B (Novus Biologicals, NB600-1384), and CASP3/caspase3 (Cell Signaling Technology, 9664), and rabbit monoclonal to GAPDH (Cell Signaling Technology, 2118). Secondary antibodies used were goat anti-rabbit IgG-HRP (sc-2004) and donkey anti-goat IgG-HRP (sc-2020) from Santa Cruz Biotechnology. The protein bands on the developed film were quantified with ImageJ (NIH: http://rsb.info.nih.gov/ij/).

**Transmission electron microscopy (TEM) and measurement of organelle density**

Bladders were fixed with fixative containing 2% glutaraldehyde and 3% paraformaldehyde in 0.1 M sodium cacodylate. Samples were rinsed 3 times in sodium cacodylate buffer and post-fixed in 1% osmium tetroxide for 1 h, stained in 1% uranyl acetate for a further hour, then rinsed, dehydrated, and dried using critical point drying. Samples were then gold-coated and viewed in an ULTRA 55 FEG (Zeiss). TEM images were taken from the superficial cells area at 2500X. The number of lysosomes and MVBs and the surface area of the superficial cells was counted using ImageJ. The densities of MVBs and lysosomes were reported as the number of MVBs and lysosomes per surface area examined (n = 200–300 TEM sections from 3 mice).

**Quantitative PCR (qPCR)**

Bladders from WT and T300A mice were collected before infection and 24 hpi. RNA was isolated from bladder tissue by using TRIzol (Invitrogen, 15596026) and treated with DNase I (Invitrogen, AM1907) to remove contaminating DNA. cDNAs were synthesized from 1 μg of total RNA using Superscript II reverse transcriptase (Invitrogen, 18064014). Gene expression was detected by real-time PCR using a CFX 96 Real-Time System (Bio-Rad, Hercules, CA, USA) and SYBR green PCR master mix (Bio-Rad, 172574). Each sample was measured in triplicate. Relative quantification was determined by using the comparative threshold cycle (CT) method with Rn18s/18S expression as a control.

**Neutrophil and monocyte counts in urine**

Urine samples were collected before infection and 24 hpi. HEMAVET® 950, Veterinary 5 part WBC Hematology System (Americas Drew Scientific Inc, Waterbury, CT, USA; Department of Comparative Medicine, WUSM) was used to determine neutrophil and monocyte counts.

**Isolation, differentiation and challenge of bone marrow-derived macrophages (BMDMs)**

Differmatiation of BMDMs from WT and T300A mice was performed as described previously [37]. Briefly, bone marrow was aseptically isolated and differentiated on non-tissue-culture-treated petri dishes for 7 d at 37°C with 5% ambient CO2 in DMEM (Gibco, 11330–032) with 15% FBS, 30% L929-conditioned medium (prepared as described previously [56]), 1% Glutamax [Gibco, 35050–061], 1% Na-pyruvate, and 1% penicillin and streptomycin. On day 7 post-isolation, non-attached cells were aspirated, and the medium was changed to DMEM with 10% FBS, 1% Glutamax, and 1%
Na-pyruvate. On day 8, cells were incubated with ice-cold DPBS (Gibco, 14190–136), removed with a cell scraper, counted, and plated at 5 × 10^5 cells/ml. On day 9, BMDMs were challenged with a multiplicity of infection of 0.1 of UTI89. BMDMs were incubated with UTI89 for 2 h, treated with medium containing 100 µg/ml gentamicin for 1 h, and then incubated with medium containing 10 µg/ml gentamicin for 21 h. Then, the supernatant was collected for ELISAs. DuoSet ELISA kits for IL1B (DY401), IL6 (DY406), and TNF/TNFα (R&D systems, DY 410) were used according to the manufacturer’s instructions.

### Cell culture, bacterial infection, and gentamycin protection assays

Human bladder epithelial cell line 5637 (BECs; ATCC, HTB-9) were used for all experiments. BECs were seeded into 24-well plates and grown to confluency in RPMI 1640 medium (GIBCO, 11875-085) supplemented with 10% FBS, infected with UTI89 at a multiplicity of infection of 100 and incubated for 1 h at 37°C. Extracellular bacteria were then removed from the BECs by washing twice with PBS and incubating with medium containing 0.1 mg/ml gentamicin. At the indicated times, BECs were washed 3 times in PBS and lysed in 1 ml of PBS containing 0.1% Triton X-100 (Sigma, 9002-93-1). Lysates were plated on LB agar plates to determine numbers of surviving intracellular bacteria.

### siRNA transfection

Human BECs were transfected using Trans-X2 (Mirus, MIR 6003) reagent with siRNA targeting ATG16L1 (IDs30071), ATG7 (IDs20651), ATG14 (ID22135), EPgp5 (ID148797), RAB33B (ID120671), RAB27B (ID120374), RAB11A (ID120400) or non-targeting control siRNA (Life Technologies, 4390844).

### RAB inhibitor studies

3-Pyridinyl ethyldiene hydroxyl phosphonocarboxylate (3-PEHPC), an analog of the bisphosphonate drug risedronate was generated as previously described [43,44]. The 5637 cells were treated with 5 mM 3-PEHPC for 24 h before being infected with UPEC. The cells were collected at 0.5, 3 and 6 hpi for quantification of intracellular bacterial load using the gentamicin protection assay.

### Tissue analysis of human bladder biopsy samples

Paraffin-embedded bladder biopsy samples from patients who underwent transurethral tissue resections were retrieved from the Pathology service of Barnes-Jewish Hospital, St. Louis, MO, USA. All samples were de-identified, and tissues with pathological evidence of malignancy were excluded as intact superficial urothelial cells were not evident. Tissues were sectioned and stained with hematoxylin & eosin and screened in a blinded manner for vesicular congestion by bright field differential interference contrast microscopy at 40X and 100X magnification. The presence/absence of vesicles was scored. Images were acquired with Olympus DP71 software.

### rs2241880 genotyping

Genomic DNA was extracted from paraffin-embedded bladder sections using the QIAamp DNA FFPE Tissue Kit (Qiagen, 56404). Genotyping to detect the rs2241880 polymorphism in ATG16L1 was performed using the TaqMan 5'-exonuclease allelic discrimination assay, which contains sequence-specific forward and reverse primers to amplify the polymorphic sequences and 2 probes labeled with VIC and FAM dyes to detect both alleles of each polymorphism (Applied Biosystems, 4351379). PCR reactions were carried out with TaqMan universal PCR Master Mix (Applied Biosystems, 4304437) following the manufacturer’s instructions in a Step-One Plus Real-time PCR system.

### Statistical analyses

To assess the significance of differences between mouse groups, unpaired t-test or non-parametric Mann-Whitney U test or two-way ANOVA with Bonferroni post-test was performed with Graph Prism software. P < 0.05 was considered significant.

### Acknowledgments

We thank Drs. Herbert Virgin (Washington University in St Louis School of Medicine) for Atg16l1<sup>fl/fl</sup> and Atg14<sup>fl/fl</sup> mice; Hong Zhang (Chinese Academy of Science) for epg5<sup>+/−</sup> mice; Masaaki Komatsu (Tokyo Metropolitan Institute of Medical Science) for Atg<sup>−/−</sup> mice; Frank Ebetino (University of Rochester) for providing 3-PEHPC; Wendy Beatty for TEM assistance; Jason Mills, Deborah Frank, Brooke Liang for comments.

### Disclosure statement

No potential conflict of interest was reported by the authors.

### Funding

This work was funded by NIH grants DK097485 and U19AI109725 (to RJX) and R01 DK100644 (to IUM) and a pilot and feasibility grant (NIDDK P30 DK092574) (to IUM). MML was supported by NIH training grants T32 AI007172 and T32 GM007200. KAB was supported by a training grant in Reproductive Sciences (T32-HD049305). Image analyses were performed in part through the Washington University Center for Cellular Imaging (WUCCI) supported by Washington University School of Medicine, TheChildren’s Discovery Institute of Washington University and St. Louis Children’s Hospital (CDI-CORE-2015-505) and the National Institute for Neurological Disorders and Stroke (NS086741); HHS | NIH | National Institute for Diabetes and Digestive and Kidney Diseases [R01 DK100644]; DH | National Institutes for Health [K12AI109725]; HHS | NIH | National Institute of Child Health and Human Development (NICHD) [HD049305]; HHS | NIH | National Institute of Allergy and Infectious Diseases [NIAID] [AI007172]; HHS | NIH | National Institute of General Medical Sciences (NIGMS) [GM007200]; Childrens Discovery Institute [CDI-CORE-2015-505].

### References


Appendix 3: The impact of methenamine hippurate treatment on bladder barrier function and inflammation in aged mice and women with urinary tract infections.

This appendix is accepted for publication in Female Pelvic Medicine and Reconstructive Surgery. MML contributed to the experimental design, mouse experiments, and manuscript revisions.

ABSTRACT

Importance: Antibiotics are commonly used to treat and prevent urinary tract infection (UTI), but resistance is growing. Non-antibiotic prophylaxis such as methenamine hippurate (MH) show clinical promise, but its impact on bladder factors influencing recurrent UTIs (rUTI) is not well-described.

Objectives: To examine the effect of MH on bladder inflammation and barrier function in aged mice and women with rUTI.

Study Design: This study included urine samples from an experimental study involving aged female mice with and without methenamine treatment as well as women with rUTI who received either no prophylaxis; MH alone; vaginal estrogen therapy (VET) and/or d-mannose (DM) alone; or MH in addition to VET and/or DM. We performed comprehensive cytopathological analysis, which included enzyme-linked immunosorbent assay for immunoglobulin A (IgA), interleukin 6 (IL-6, in human samples), and FITC-Dextran permeability assay (in mice) to assess for urothelial permeability.

Results: In the aged mice model, there was a decreased urothelial permeability (as seen by retention of FITC-Dextran fluorescence in superficial cells) and increased urinary IgA in mice treated with MH compared to controls. There was no significant difference in urothelial shedding (p>0.05). In human samples, there was significantly increased urinary IgA in those taking MH alone compared to no prophylaxis (704.1±248.4 vs 469.2±259.6 ng/mL, p=0.04), but no significant difference in IL-6.

Conclusions: MH appears to enhance barrier function as evidenced by decreased urothelial permeability and increased urinary IgA levels, without worsening inflammation. This may reflect another beneficial mechanism by which MH helps to prevent rUTI.
INTRODUCTION

Urinary tract infections (UTIs) are one of the most commonly treated infections that disproportionately affect women.\textsuperscript{1-3} Furthermore, recurrence occurs in over 25\% of women,\textsuperscript{4,5} with up to 50\% of recurrence occurring in postmenopausal women.\textsuperscript{6} Recurrent UTIs (rUTI) can result in severe morbidity, mortality, and economic burden.\textsuperscript{2,7} Since women, particularly older women, experience the highest burden of disease, they are also more vulnerable to complications from UTI and antibiotic use. Thus, rUTI prevention strategies are of utmost importance.

Although antibiotics are frequently used to prevent rUTIs, antibiotics expose patients to risk of adverse effects; damage commensal bacteria; promote infections like \textit{C. difficile}; and induce resistance.\textsuperscript{8,9} Antibiotic resistance contributes to 3 million illnesses and 23,000 deaths per year.\textsuperscript{10} Non-antibiotic prophylaxis can avoid such complications. We have previously found that vaginal estrogen decreases risk of rUTI by decreasing bladder inflammation, as evidenced by decreased interleukin-6 (IL-6), leukocyte influx, and urothelial shedding.\textsuperscript{11} Other promising non-antibiotic prophylaxis include D-mannose (DM) and methenamine hippurate (MH), a promising urinary antiseptic.\textsuperscript{12} However, inadequate knowledge of their impact on the bladder environment limits our ability to optimize non-antibiotic UTI prophylaxis.

Our objective was to determine the impact of MH on bacteriuria, bladder inflammation, and bladder barrier function: all important factors that impact rUTI. Our primary aim was to examine the impact of MH treatment on bladder inflammation and barrier function markers in an aged female mouse model infected with a uropathogenic E coli isolate. Our parallel primary aim was to examine the impact of MH alone or in combination with other non-antibiotic UTI prophylaxis on bladder inflammation and barrier function correlates in women with rUTI. Our secondary aim was to measure bacterial load, pH, and directly measure barrier function via
urothelial permeability in aged, infected mice after MH treatment. We hypothesized that in both models, MH works by dampening inflammation and improving barrier function (decreased urothelial permeability, increased IgA).

**MATERIALS AND METHODS**

This study involved two models: a prospective cohort study involving aged female mice (18 months old, which represent humans at around 65 years of age) and urine samples from a biorepository of women recruited from Female Pelvic Medicine and Reconstructive Surgery (FPMRS) and general Obstetrics & Gynecology (OBGYN) clinics at Washington University School of Medicine since 2018 (IRB #201712113).

**Mouse Model**

Our aged mouse model has successfully modeled UTIs seen in older women, with findings significant for delayed bacterial clearance, increased inflammatory response, and impaired urinary barrier function.\(^{13,14}\) Our protocol was approved by Washington University School of Medicine Institutional Animal Care and Use Committee (#20-0056).

**Methenamine hippurate (MH) supplementation**

Human level dosage of MH (2 grams daily) was converted to murine dosage by the Division of Comparative Medicine. We treated one group of aged female mice with murine dose-adjusted MH and another group with water as control for 4 weeks.

**Inoculation with UT189**

At the end of 4 weeks, mice were inoculated with UT189, a uropathogenic E.coli clinical cystitis isolate from a frozen glycerol stock grown statically at 37°C for 24hr in 20 mL of Luria-Bertani broth.\(^{15}\) Mice were anesthetized and transurethrally catheterized with 50 uL of \(10^7\) CFU of
the bacterial suspension diluted in PBS.\textsuperscript{15} Urine was collected at days 1, 3, 7, 10, and 14 post-inoculation. Mice were sacrificed on day 14 post-inoculation.

**Urine Analysis**

During treatment, daily urinary pH was measured, as urinary pH of <5.85 produces the optimal bacteriostatic concentration of formaldehyde from MH.\textsuperscript{16}

Urine cytology was obtained by centrifuging 10uL of urine and 40uL sterile PBS onto slides to count exfoliated urothelial cells per high powered field using Papanicolaou staining and light microscopy.

Urine specimens were cultured by serially diluting urine in sterile PBS. Then, 5uL of each dilution was seeded on agar plates for a total of six replicates. Bacterial colonies were counted and bacterial load calculated as colony forming units (CFU)/mL. Urine was considered to be non-infected when log-transformed bacterial colony count was \(<10^3./mL\).

Urinary levels of secretory IgA were quantified using Invitrogen Mouse IgA Uncoated ELISA Kit (Thermo Scientific, Vienna, Austria) according to manufacturer’s instructions, which has a detection range of 0.39-25 ng/mL.

**Assessment of Barrier function**

After sacrifice, bladders were fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid), embedded in paraffin, and 5 µm thick sections cut for further analysis.

Barrier function as reflected by urothelial permeability was measured by injecting 50 uL of 10 mg/mL 10 kD Fluorescein isothiocyanate-conjugated dextran (FITC-Dextran) (D1821, Invitrogen) transurethrally into mouse bladders.\textsuperscript{17} After sacrifice, frozen bladder tissue sections were made and dipped in 1:1 methanol-acetone and PBS, then cover-slipped with Prolong Diamond Antifade with DAPI (P36971, Invitrogen). Images were acquired using a fixed exposure.
set to detect fluorescence. Location and intensity of fluorescence permeating through the urothelium was quantified by averaging the mean gray value for FITC channel in 3 random squares from 2 separate images for each mouse using ImageJ software. Higher mean gray values indicated concentration of fluorescence in the superficial cell layers of the urothelium (decreased permeability). Values were combined for each mouse for statistical analysis.

**Human urinary samples**

**Sample Collection**

Human urine samples were obtained through an IRB approved biorepository prospectively collected from clinic patients who consented to have samples stored for further study. Informed consent was obtained from all participants. Enrolled women provided baseline medical, surgical, and demographic information; history of rUTI; and any use of UTI prophylaxis with vaginal estrogen (VET), d-mannose (DM), and/or methenamine hippurate (MH) at the time of sample collection. Urine samples were collected via voided midstream clean catch or straight catheterization based on clinical indications. Urine samples were treated with protease inhibitor (EDTA-free, Halt; Thermo Scientific, Waltham, Massachusetts) and stored at -80°C.

**Sample Selection**

Samples from subjects were included if subjects had a history of rUTI and documentation of any non-antibiotic UTI prophylaxis use. Subjects were excluded if they had no history of rUTI; were on antibiotic prophylaxis or antibiotic treatment; or declined use of their banked samples for other studies. Samples from these women were categorized into four groups:

1) women who were not on MH, VET, or DM prophylaxis at time of sample collection (NP);

2) women on MH alone;
3) women on VET and/or DM alone (VET±DM); and

4) women on MH in addition to VET and/or DM (MH + VET±DM).

To assess the impact of any MH treatment, we compared samples from group 1 to group 2 or group 4. We also compared samples from group 3 to group 4 to assess the impact of MH to effects already produced by VET and/or d-mannose. Using Gower’s method, we matched samples from comparison groups based on baseline characteristics, medical comorbidities, and medications that could impact local bladder and systemic inflammation (Supplementary Method and Table 1).

Sample Processing

Thawed urine (100 µL) was cyto-centrifuged onto positively charged glass slides. Urothelial sloughing and urinary secretory IgA were measured with the Invitrogen Mouse IgA Uncoated ELISA Kit (Thermo Scientific, Vienna, Austria), which has a detection range of 1.6-100 ng/mL. Because we were able to collect a greater amount of urine from human women than from mice, we were also able to analyze urinary IL-6 to examine urinary inflammation using the R&D Systems DuoSet Human IL-6 ELISA according to manufacturer’s instructions (R&D, Minneapolis, Minnesota, sensitivity 0.6 pg/mL).

Sample Size Determination – Of Mice and Women

As there were no prior studies examining MH and IgA (reflecting barrier function) in mice, we based our sample size on our prior work on the impact of VET on bladder inflammation in mice.\textsuperscript{13} Based on a standard deviation of the log-transformed distribution of IL-6 of 0.75 ng/ml, 10 mice per group would give us 80% power to detect a minimum 2.7-fold difference between MH treated and untreated mice.

We also based our sample size for human urinary samples on IL-6 data from a prior study.\textsuperscript{11} Assuming that the standard deviation of the log-transformed distribution of change in IL-6 is no
greater than 1.7 ng/ml, 10 women per baseline group would give us 91% power to detect a difference between groups with and without MH treatment.

**Statistical Analysis**

Comparison between groups for both mouse and human samples were analyzed using ANOVA with Tukey’s multiple-comparison posttest for continuous variables, while categorical variables were compared using $\chi^2$ test and Fisher exact test. P values of $<0.05$ were considered statistically significant.

**RESULTS**

**Aged mouse model**

**pH**

During the treatment period, mean daily urinary pH ranged from pH 4.1-4.9 for MH-treated aged mice (N=8) and pH 4.3-5.1 for controls (N=8). There was no significant difference between groups (Overall pH 4.6±0.3 vs 4.8±0.2, mean difference -0.17, p=0.21).

**Bacteriuria**

There was no difference in bacteriuria between aged mice treated with MH and controls during 14 days of observation post-inoculation (Figure 1A). There was a non-significant trend towards decreased infectivity in aged mice treated with MH compared to control over the course of 14 days (Figure 1B, p=0.06).
**Inflammation: Urothelial shedding**

MH treatment did not affect urothelial shedding (56.2±88.4 vs 4.4±8.2 mean urothelial cells/50 uL for control and MH groups respectively, p=0.71, day 1 post-inoculation; 117.5±249.7 vs 25.8±42.1, p=0.9, day 3 post-inoculation).

**Barrier function**

We noted significantly deeper FITC-Dextran permeability through the urothelium in aged mice, indicating worse barrier function (Figure 2A). When comparing aged mice with and without MH treatment, there was decreased permeability in MH-treated aged mice compared to control (Figure 2A), as seen by the FITC-Dextran retention in superficial cells versus deeper permeation in controls. Quantification of FITC-Dextran fluorescence permeability revealed greater mean gray values in MH-treated mice, confirming that there was significantly decreased permeability of FITC-Dextran into the deeper urothelial layers (mean value 19.5±3 vs 45.6±12.1 for control and MH, respectively, Figure 2B, p=0.0007).

We further evaluated barrier function by measuring urinary IgA post-inoculation. When compared to aged, untreated mice, aged mice treated with MH had greater urinary IgA levels (Figure 3).

**Human urinary samples**

We identified 8 samples from group 2 (MH) and 29 samples from group 4 (MH + VET±DM). These were matched as described to a total of 78 samples from women categorized as group 1 (NP) and 62 samples from group 3 (VET±DM). Baseline demographics and medical characteristics can be found in Supplementary Table 2.
**pH**

There was no significant difference in mean urinary pH between NP and MH groups, nor between VET±DM and MH + VET±DM groups. However, mean urinary pH was significantly lower in the NP group compared to the MH + VET±DM group (pH 5.8±0.9 vs 6.3 ±0.9, p=0.04).

**Inflammation: IL-6 levels**

When comparing urine IL-6 levels between matched groups, there was no significant difference between NP and MH; VET±DM and MH + VET±DM; or NP and MH + VET±DM groups (Figure 4).

**Barrier function**

There was no difference in IgA levels between VET±DM and MH + VET±DM groups or NP and MH + VET±DM groups (Figure 5B and C). However, there was a statistically significant increase in urinary IgA in the MH group compared to the NP group (704.1±248.4 vs 469.2±259.6 ng/mL, p=0.04, Figure 5A).

**DISCUSSION**

In our study, MH appears to increase IgA levels without increasing bladder inflammation in aged mice and humans. In aged mice, while MH did not affect pH or bacteriuria, it appeared to decrease urothelial permeability, as evidenced by decreased infiltration of FITC-Dextran in MH-treated mice versus controls. While we were limited by our sample size and heterogeneity of human samples, we also observed increased urinary IgA in the MH group compared to NP. The agreement between these two models of UTI suggests that MH may work by improving urothelial barrier function.
Methenamine hippurate is bacteriostatic in human urine. It is hydrolyzed to formaldehyde in acidic urine, but does not generate significant levels of formaldehyde in the gut or other tissues. MH is overall well tolerated and does not induce bacterial resistance. Thus, MH is a promising bladder antiseptic for rUTI prophylaxis. In metaanalysis, MH was associated with a reduced risk of UTI in patients with normal urinary tracts (RR 0.24, 95% CI 0.07-0.89). An upcoming RCT may shed further light on its clinical efficacy for rUTI prevention.

While MH appears to be effective clinically, few studies have examined its impact on other factors associated with rUTI. The observation of potential barrier function improvement in our study may be due to the effect of formaldehyde, which “fixes” cells by denaturing proteins and cross-linking amino groups. However, the mechanism by which MH improves urothelial barrier function requires further investigation. Urothelial barrier function is important to rUTIs development, as UPEC can establish quiescent intracellular reservoirs within the urinary bladder epithelium through invasion of the superficial facet cells, and thus evade eradication and enable reemergence and recurrent infection. If MH does reinforce the bladder mucosal barrier, it may also decrease rUTIs by limiting UPEC’s ability to invade the urothelium.

Furthermore, our study indicated that urinary IgA levels may be elevated with MH treatment. This may reflect improved barrier function, as secretory IgA is normally found in the healthy bladder. On the other hand, promoting IgA may be another way by which MH improves barrier function. In the gut, secretory IgA recognizes and cross-links antigenic epitopes on surfaces, preventing epithelial penetration. In a study involving probiotics bound to IgA, secretory IgA was associated with improved probiotic adhesion and increased phosphorylation of tight junction proteins; thus, IgA may improve barrier function by promoting tight junction function. Secretory IgA increases with acute UTI and is higher in aged mice, potentially in response to
chronic, age-related inflammation. Lastly, in other mucosal surfaces, secretory IgA also helps to control commensal bacteria. While we did not study MH’s impact on the bladder microbiome, if methenamine does increase secretory IgA, this may help to promote normalization of the urinary microbiota.

In older clinical practice, concentrated formaldehyde was used to superficially necrose and coagulate the bladder to stop severe hemorrhage, which resulted in significant fibrosis on pathological analysis. Animal studies have also noted initial urothelial necrosis, edema, and leukocytic infiltration after concentrated formaldehyde exposure. Thus, our concern was that MH, though resulting in significantly lower levels of formaldehyde, could adversely affect bladder inflammation. However, we found no significant difference in IL-6 between women receiving MH treatment versus other groups. Likewise, urothelial sloughing, a correlate of bladder inflammation, was not different between aged mice with and without MH treatment. While our findings are limited by our small sample size, this is encouraging preliminary evidence that MH does not adversely impact inflammation in the bladder environment.

MH’s potential mechanism of action is contrasted with that of vaginal estrogen. Studies suggest that VET may prevent rUTI in older women by dampening their enhanced urinary inflammatory response, as excessive inflammation may impair barrier function and promote rUTI. This suggests that MH could be used synergistically with other non-antibiotic prophylaxis to increase efficacy of rUTI prophylaxis as different therapies would act through multiple mechanisms. Our results did not suggest further benefit to inflammation and barrier function with the addition of MH to VET and/or DM. However, this may reflect our small sample size. Furthermore, effect size may have been dampened by the inclusion of women with either VET,
DM, or both into the same group. Larger clinical studies would be more useful to study the efficacy of a multimodal, non-antibiotic regimen compared to monotherapy.

This is one of the first studies to explore the impact of MH on the bladder factors that may lead to rUTI. Our translational approach allowed us to compare our findings in both mice and human models. We further matched patients for factors that could influence systemic inflammation to decrease bias.

Our small sample size may have limited our ability to detect statistically significant differences between groups, particularly in the MH only group. Our human samples were also limited by collection from women with and without UTI at the time of collection, as well as unknown UTI symptom status at time of collection. This limits our ability to compare these results with the results from mice, which were all infected prior to sample collection. Our human samples were also impacted by lack of therapeutic randomization and clinical prescription practices, as most women were offered VET and/or DM prior to MH. Thus, women who opted for prophylaxis with MH alone may represent a different population of women or have characteristics that we were unable to control through matching.

Overall, this study provides new insight into mechanisms by which MH may prevent UTIs. MH’s impact on urothelial barrier function and urine IgA suggests that it may improve barrier function and possibly play a role in regulating urinary microbiota, important factors in rUTI prevention. Our findings also suggest that MH may work best in synergy with other non-antibiotic prophylaxis that work through other distinct mechanisms, such as VET. Though our evidence is preliminary, methenamine hippurate appears to have therapeutic advantages for UTI prophylaxis beyond bacteriostasis.
Figure 1. Bacteriuria between aged mice with and without methenamine hippurate treatment. Filled dots: aged mice without MH treatment (NP). Empty squares: aged mice treated with MH (MH). A. There was no significant difference between bacterial colony forming units per milliliter of urine between aged mice with and without MH treatment. Over time, there was a non-significant trend towards decreased proportion of mice considered infected in aged mice treated MH (p=0.06).
Figure 2. FITC-Dextran permeability assay between aged and young mice with and without methenamine hippurate treatment. The white dashed line indicates the margin of the bladder urothelium. The nuclei stained blue compromise the bladder urothelium that faces the lumen. ***p ≤ 0.001. Filled dots: aged mice without MH treatment (NP). Empty squares: aged mice treated with MH (MH) A. Aged mice without MH treatment showed greater permeability of fluorescence into deeper layers of the urothelium (as delineated by black arrow), while young mice and aged mice treated with MH showed decreased permeability, with more fluorescence retained in the superficial level of the urothelium (as shown by the white arrow). B. Greater mean gray values were seen in aged mice treated with MH compared to controls (p ≤ 0.001).
Figure 3. Urinary secretory IgA levels in aged mice with and without methenamine hippurate treatment. NS, Non-significant, p >0.05. *p ≤0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001. Filled dots: aged mice without MH treatment (NP). Empty squares: aged mice treated with MH (MH). There was a significant difference between urinary IgA levels between aged mice treated with MH compared to controls at most time points after infection.
Figure 4. Urinary IL-6 levels in women with and without methenamine hippurate treatment. Filled dots: women without MH, VET, or DM prophylaxis (NP). Empty squares: women receiving MH alone (MH). Filled triangles: women receiving VET and/or DM (VET±DM). Empty triangles: women receiving MH in addition to VET and/or DM (MH + VET±DM). There was no significant difference in urinary IL-6 levels between A. women receiving no prophylaxis with MH, DM, or VET (NP) versus those receiving MH alone (MH); B. women receiving VET and/or DM (VET±DM) versus those receiving MH in addition to VET and/or DM (MH + VET±DM); and C. women receiving no prophylaxis with MH, DM, or VET (NP) versus those MH in addition to VET and/or DM (MH + VET±DM).
Figure 5. Urinary secretory IgA levels in women with and without methenamine hippurate treatment. NS, Non-significant, p >0.05. *p ≤0.05. Filled dots: women without MH, VET, or DM prophylaxis (NP). Empty squares: women receiving MH alone (MH). Filled triangles: women receiving VET and/or DM (VET±DM). Empty triangles: women receiving MH in addition to VET and/or DM (MH + VET±DM) A. Women receiving MH alone (MH) had significantly higher levels of urinary IgA compared to women not receiving MH, VET, or DM prophylaxis (NP). However, there was no significant difference in urinary IgA levels between B. women receiving no prophylaxis with MH, DM, or VET (NP) versus those receiving MH alone (MH) or C. women receiving VET and/or DM (VET±DM) versus those receiving MH in addition to VET and/or DM (MH + VET±DM).
Supplementary Method 1:
Gower’s distance is a nearest neighbor matching method suitable for matching mixed numeric/categorical data. In matching, subjects are matched based on the smallest “distance” in characteristics between subjects – however, since in this case, both numeric and categorical variables are involved, “hierarchical clustering” methods such as Gower’s distance must be used. The Gower distance between two items is computed by calculating the absolute value of the difference or Euclidian distance between two items divided by the range if the element is numeric.¹ If the element is non-numeric, the term is 1 if the elements are different or is 0 if the elements are the same. The Gower distance is the average of the terms. Since each term is divided by its range, the results for each individual term will be between 0.0 and 1.0, where a distance of 0.00 means that the two items are the same and a distance of 1.00 means that they are quite different. In this study, every subject was coded for characteristics stored each item in a dataset is compared to all the data in the second dataset and the distances between them are computed. Using previously described methods,¹,² we compared patient characteristics between comparison groups by calculating Gower’s coefficient for each variable, and then combined those distances to a single value per record-pair using Excel. Subjects with the smallest Gower’s coefficient were selected as the most matched pair for analysis, and were matched on a 1:2 case:control basis.

Supplementary Table 1. Baseline characteristics and medical factors for matching between groups

<table>
<thead>
<tr>
<th><strong>Baseline Characteristics</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Race</td>
</tr>
<tr>
<td>Body mass index</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Medical Factors</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary incontinence (presence or absence)</td>
</tr>
<tr>
<td>Menopausal status (Pre or post-menopausal)</td>
</tr>
<tr>
<td>Tobacco use (presence or absence)</td>
</tr>
<tr>
<td>Alcohol use (presence or absence)</td>
</tr>
<tr>
<td>Illicit drug use (presence or absence)</td>
</tr>
<tr>
<td>Presence or absence of any medical factors associated with elevated IL-6</td>
</tr>
<tr>
<td>• Diabetes</td>
</tr>
<tr>
<td>• Cardiovascular disease</td>
</tr>
<tr>
<td>• Stroke</td>
</tr>
<tr>
<td>• Venous thromboembolism</td>
</tr>
<tr>
<td>• Chronic kidney disease</td>
</tr>
<tr>
<td>• Inflammatory bowel disease</td>
</tr>
<tr>
<td>• Irritable bowel syndrome</td>
</tr>
<tr>
<td>• Rheumatologic diseases</td>
</tr>
<tr>
<td>• History of radiation</td>
</tr>
<tr>
<td>• History of cancer</td>
</tr>
<tr>
<td>• Chronic pelvic pain</td>
</tr>
<tr>
<td>• Fibromyalgia</td>
</tr>
<tr>
<td>• Endometriosis</td>
</tr>
<tr>
<td>Positive or negative use of any medications with possible impact on the bladder OR medications associated with local and/or systemic inflammation</td>
</tr>
<tr>
<td>• Antidepressants</td>
</tr>
<tr>
<td>• Diuretics</td>
</tr>
<tr>
<td>• Anticholinergics</td>
</tr>
<tr>
<td>• Antihistamines</td>
</tr>
<tr>
<td>• Hormone therapy (other than VET)</td>
</tr>
<tr>
<td>• Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>• Immunosuppressants</td>
</tr>
<tr>
<td>• Systemic or inhaled corticosteroids</td>
</tr>
<tr>
<td>• Antiplatelet therapy</td>
</tr>
</tbody>
</table>

*For medical factors, subjects were dichotomized into the presence or absence of the variables listed in the table. For example, subjects were dichotomized into those with 1 or more medical factor that could impact IL-6 versus those without any medical factors that could impact IL-6. In a similar fashion, subjects were dichotomized into those with 1 or more medication that could impact the bladder or inflammation versus those without medications that could impact the bladder or inflammation.
Supplementary Table 2: Baseline characteristics and medical factors for matching between groups for human samples

<table>
<thead>
<tr>
<th>Matched Comparison Groups</th>
<th>MH N=8</th>
<th>NP N=18</th>
<th>MH+ VET±DM N=29</th>
<th>NP N=59</th>
<th>MH+ VET±DM N=29</th>
<th>VET±DM N=61</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UTI at the time of collection</td>
<td>0 (0%)</td>
<td>4 (22.2%)</td>
<td>8 (27.6%)</td>
<td>14 (23.7%)</td>
<td>8 (27.5%)</td>
<td>15 (24.6%)</td>
</tr>
<tr>
<td>UTI status unknown at time of collection</td>
<td>5 (62.5%)</td>
<td>3 (16.7%)</td>
<td>9 (31%)</td>
<td>9 (15.2%)</td>
<td>9 (31%)</td>
<td>21 (34.4%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52.9 (28.7)</td>
<td>52.7 (18.4)</td>
<td>67.2 (14.4)</td>
<td>65.6 (13.6)</td>
<td>67.2 (14.4)</td>
<td>66.4 (13.5)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>5 (62.5%)</td>
<td>15 (83.3%)</td>
<td>25 (86.2%)</td>
<td>55 (93.2%)</td>
<td>25 (86%)</td>
<td>54 (88.5%)</td>
</tr>
<tr>
<td>Black</td>
<td>2 (25%)</td>
<td>3 (16.7%)</td>
<td>1 (3.4%)</td>
<td>4 (6.8%)</td>
<td>1 (3.4%)</td>
<td>2 (3.3%)</td>
</tr>
<tr>
<td>Other</td>
<td>1 (12.5%)</td>
<td>0</td>
<td>3 (10.3%)</td>
<td>0 (0%)</td>
<td>3 (10.3%)</td>
<td>5 (8.2%)</td>
</tr>
<tr>
<td>Body mass index</td>
<td>28.1 (11.6)</td>
<td>27.9 (8.2)</td>
<td>29 (8.5)</td>
<td>30.4 (7.1)</td>
<td>29 (8.5)</td>
<td>30.1 (7.2)</td>
</tr>
<tr>
<td><strong>Medical Factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any urinary incontinence</td>
<td>0 (0%)</td>
<td>1 (6%)</td>
<td>9 (31%)</td>
<td>13 (22%)</td>
<td>9 (31%)</td>
<td>16 (26.2%)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>3 (42.9%)</td>
<td>11 (61.1%)</td>
<td>26</td>
<td>52</td>
<td>26 (89.7%)</td>
<td>55 (90.2%)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>1 (12.5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>3 (5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Any alcohol use</td>
<td>6 (75%)</td>
<td>12 (66.7%)</td>
<td>13 (44.9%)</td>
<td>22</td>
<td>13 (44.8%)</td>
<td>24 (39.3%)</td>
</tr>
<tr>
<td>Illicit drug use</td>
<td>0 (0%)</td>
<td>1 (5.6%)</td>
<td>0 (0%)</td>
<td>1 (1.7%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Presence of any medical factors associated with elevated IL-6</td>
<td>3 (37.5%)</td>
<td>8 (44.4%)</td>
<td>17 (58.6%)</td>
<td>43 (72.9%)</td>
<td>17 (58.6%)</td>
<td>42 (68.9%)</td>
</tr>
<tr>
<td>Any use of medications with impact on bladder or inflammation</td>
<td>7 (87.5%)</td>
<td>17 (94.4%)</td>
<td>22 (75.9%)</td>
<td>52 (88.1%)</td>
<td>22 (75.9%)</td>
<td>49 (80.3%)</td>
</tr>
</tbody>
</table>

Mean (SD) or N (%) unless otherwise specified
REFERENCES


Marianne Morris Ligon

EDUCATION & TRAINING

06/14/22 - 06/30/26  Residency, Anesthesiology, University of Pittsburgh Medical Center, Pittsburgh, PA

06/16/14 - 05/20/22  M.D., Ph.D., Molecular Microbiology & Microbial Pathogenesis, Washington University in St. Louis School of Medicine, St. Louis, MO

08/11/10 - 05/09/14  B.S. Cellular Biology, B.S. Microbiology, University of Georgia, Athens, GA  
First Honor Graduate (4.0 GPA), summa cum laude, with Highest Honors (Honors Program)

AWARDS & SCHOLARSHIPS

2019  Society for Mucosal Immunology Travel Award to International Congress on Mucosal Immunology

2019  Center for Reproductive Health Sciences Travel Award

2019  American Association of Immunologists Travel Award to AAP/ASCI/APS Annual Joint Meeting

2017  Infectious Disease Training Grant Award

2017  MMMP Qualifying Exam Passed with Distinction

2016  IDWeek Mentorship Program Travel Award

2014  MSTP Distinguished Young Scholar Award

Nationally Recognized - Undergraduate

2014  Phi Beta Kappa Honor Society

2013  Blue Key Honor Society

2012  Barry M. Goldwater Scholarship

University of Georgia

2014  Alpha Epsilon Delta Pre-Medical Honor Society Cohen Award

2014  Department of Cellular Biology Grace Thomas Award

2012  Foundation Fellowship

2012  Honors International Scholarship

2011  Center for Undergraduate Research Opportunities (CURO) Summer Fellowship

2010  Bernard Ramsey Honors Scholarship

2010  CURO Honors Scholarship

RESEARCH EXPERIENCE

06/2014 – 05/2022  
**Washington University in St. Louis**, Dept of OB/GYN, St. Louis, MO  
PhD Thesis Mentor: Indira Mysorekar, PhD  
**PhD Thesis:** Tertiary lymphoid tissue in the urinary bladder of aging mice and women
09/2010 – 05/2014
University of Georgia, Dept of Biochemistry and Molecular Biology, Athens, GA
Research Mentor: Michael Terns, PhD
Honors Thesis: Biochemical architecture and function of thermophilic CRISPR-Cas effector complexes

01/2013 – 06/2013
University of Oxford, Sir William Dunn School of Pathology, Oxford, United Kingdom
Research Mentor: Kevin Maloy, PhD
Mechanisms of the unfolded protein response in acute and chronic colitis

06/2012 – 08/2012
New York University, Skirball Institute of Biomolecular Medicine, New York, NY
Research Mentor: Dan R. Littman, MD, PhD
Tools for investigating Segmented Filamentous Bacteria (SFB) genetics and antigens stimulating Th17 cells

07/2009 – 07/2010
Clemson University, Dept of Biological Sciences, Clemson, SC
Research Mentor: Kimberly Paul, PhD
Genetics and inhibition of Trypanosoma brucei fatty acid acquisition and synthesis

PUBLICATIONS


**TEACHING EXPERIENCE**

2021-2022  **Clinical mentoring**, for 1st year medical students, Washington U. in St. Louis
2019  **Supervisor/organizer**, Benign Urology Center Summer Undergraduate Research, Washington U. in St. Louis
2018  **Tutor**, Pathology, Washington University in St. Louis
2017-2019  **Instructor**, MCAT Biology, MCAT Organic Chemistry, Washington University in St. Louis
2016-2017  **Teaching Assistant**, Cell and Organs System Biology – Histology, Washington U. in St. Louis
2016  **Course Liaison**, Infectious Diseases, Washington University in St. Louis
2014-2018  **Mentor**, Young Scientist Program
2012  **Teaching Assistant**, Honors Program, University of Georgia

**ORAL PRESENTATIONS**

2019  International Congress on Mucosal Immunology. Brisbane, Australia.
2019  Medical Scientists Training Program Work in Progress. Washington University in St. Louis.
2019  Center for Reproductive Health Sciences Seminar Series. Washington University in St. Louis.
2018  Infectious Disease/Basic Microbiological Mechanisms Seminar. Washington University in St. Louis.
2018  Pelvic Floor Disorders Week 2018. American Urogynecology Society. Chicago, IL.
2018  Center for Reproductive Health Sciences Seminar Series. Washington University in St. Louis.
2012  Leadership Alliance National Symposium. Hartford, CT
2012  CURO Symposium. University of Georgia. Athens, GA.
2011  CURO Summer Fellowship Forum. University of Georgia. Athens, GA.
2010  SC Junior Science & Humanities Symposium. 5th Place. Columbia, SC

POSTER PRESENTATIONS
2019  Collaborating for the Advancement of Interdisciplinary Research in Benign Urology. Kansas City, MO.
2019  International Congress on Mucosal Immunology. Brisbane, Australia.
2019  AAP/ASCI/APSA Annual Joint Meeting. Chicago, IL
2018  MMMP Annual Retreat. Washington University in St. Louis.
2018  Medical Scientist Training Program Annual Retreat. Washington University in St. Louis.
2018  6th Annual Global Health & Infectious Disease Conference. Washington U. in St. Louis
2017  MMMP Annual Retreat. Washington University in St. Louis
2017  NIH Aging and Immunity Symposium. Rockville, MD
2017  Medical Scientist Training Program Annual Retreat. Washington University in St. Louis
2017  5th Annual Global Health & Infectious Disease Conference. Washington U. in St. Louis
2012  Southeastern Medical Scientists Symposium/American Physician Scientists Association Southeast Regional Meeting. Vanderbilt University. Nashville, TN
2012  Summer Undergraduate Research Program Symposium. New York University
2010  CTEGD 20th Annual Molecular Parasitology and Vector Biology Symposium. University of Georgia. Athens, GA.

PROFESSIONAL MEMBERSHIPS
Society for Mucosal Immunology
American Medical Association
American Society of Anesthesiologists
American Physician Scientists Association