Tissue-resident memory T cells mediate mucosal immunity to recurrent urinary tract infection

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Abstract

Urinary tract infection (UTI) is one of the most prevalent human bacterial infections. Due to the rapid rise of multidrug-resistant uropathogens in global circulation, new therapeutic approaches, including vaccination and immunotherapy, are urgently needed. However, the development of these therapies is impeded by an incomplete understanding of how memory develops during UTI. Here, we found that reducing bacteria load early in infection, by reducing the quantity of bacteria used for infection or with antibiotics, completely abrogated the protective memory response. As we observed that a mixed T helper cell polarization bias, comprised of Th1, Th2, and Th17 T cells, develops during a primary infection, we hypothesized that reducing antigen load altered T helper cell polarization. Surprisingly, T helper cell polarization was unchanged in these scenarios. However, we uncovered a population of tissue resident memory (T_{RM}) T cells that was significantly reduced in the absence of sufficient antigen, demonstrating that this population is necessary for protection. Supporting that T_{RM} T cells are sufficient to protect against recurrent infection, depletion of systemic T cells, or treatment with FTY720 to block migration of memory lymphocytes from lymph nodes to infected tissue, did not compromise the adaptive immune response to a second infection. Thus, our findings point to an unappreciated mechanism of adaptive immunity to UTI and key role for T_{RM} cells in the memory response to bacterial infection in this mucosa, providing a target for non-antibiotic-based immunotherapy and/or new vaccine strategies to prevent recurrent UTI.

Introduction

Development of immune memory, naturally after infection or by immunomodulatory approaches such as vaccination, protect an organism from experiencing the same infection more than once. Failure to develop adequate immune memory results in repeated or recurrent infection. Mucosal surfaces, which are contiguous with the environment, are particularly at risk for infection. Sterilizing immune memory, however, often fails to develop at these sites, for reasons that are incompletely understood.

The bladder is one such mucosal surface that, in a large proportion of the population, will be infected repeatedly, often with the same pathogen (Flores-Mireles et al., 2015). Indeed, urinary tract infection (UTI), caused predominantly by uropathogenic *E. coli* (UPEC), is one of the most prevalent human bacterial infections (Litwin et al., 2007). Women experience UTI at a significantly higher rate than men; and children, the elderly, pregnant women, and immuno-compromised individuals are at even greater risk than the general population for complications such as chronic infection, pyelonephritis, and kidney damage (Chromek and Brauner, 2007; De Nisco et al., 2019; Dielubanza and Schaeffer, 2011; Hoepelman et al., 1992; Mazor-Dray et al., 2009). Approximately one in two women will have a UTI in her lifetime, with the highest risk between the ages of 16 and 35, when women are 35 times more likely than men to be infected (Foxman, 2002). Additionally, nearly half of all infected women will have a recurrent infection, defined by similar or identical symptoms, within 6 months of their first UTI, suggesting that immune memory fails to develop following infection of the bladder (Ikaheimo et al., 1996). Although the incidence of UTI is much lower in adult men (~1%), in this population, it is always considered a complicated infection, with considerable risk of becoming a recurrent or chronic UTI (Lipsky, 1989). Finally, the prevalence of UTI increases substantially in older individuals, bringing with it increased morbidity and mortality in an aging population (Ruben et al., 1995).

Given the diversity of at-risk populations, the frequency of recurrence, and the considerable increase in multi-drug resistant uropathogen isolates in global circulation, new approaches, such as immune modulation to augment mucosal immunity, are needed to defend against chronic or recurrent UTI (Klein and Hultgren, 2020; Lacerda Mariano and Ingersoll, 2020). First line antibiotic therapy only reduces acute bacterial burden, and no treatment or therapy that protects against initial or recurrent UTI exists (Lacerda Mariano and Ingersoll, 2020). Experimental vaccination in humans and mice was reported to induce antibody production correlating with lower bacterial colonization more than 30 years ago, however, these attempts have not lead to a successful clinically available vaccine (Alteri et al., 2009; Billips et al., 2009; Klein and Hultgren, 2020; Langermann et al., 1997; Uehling et al., 1997; Uehling et al., 2001; Uehling et al., 1994; Uehling et al., 1991). Recurrent UTI likely arises due to several factors, including inadequate development of memory responses after a primary infection, but also due to development of reservoirs in the bladder and gut, which potentially escape elimination by antibiotics (Mora-Bau et al., 2015; Mysorekar and Hultgren, 2006; Spaulding et al., 2017). As the associated costs

of UTI, including treatment and lost productivity, are more than $3 billion in the US (Sivick and Mobley, 2009), with similar expenditures in Europe, a better understanding of how immune memory arises and how it can be targeted to improve the adaptive response to UTI would have a profoundly positive societal and economic impact, as well as improve the quality of life of those suffering from recurrent infection.

Notably, how adaptive immunity develops in response to bladder infection is still incompletely understood (Ingersoll and Albert, 2013; Lacerda Mariano and Ingersoll, 2020). An early key observation, that mice infected with an ovalbumin-expressing UPEC strain have ovalbumin-specific antibodies in the serum and reduced bacterial burden following a second or challenge infection, demonstrates that protective memory can develop during UTI (Thumbikat et al., 2006). We reported that acute bacterial burden in the bladder following a challenge infection is lower than that observed during a first or primary UTI (Mora-Bau et al., 2015). By contrast, bacterial burden is not reduced after challenge infection in RAG2$^{-/-}$ mice, dendritic cell-depleted mice, and CD4^{+} and CD8^{+} T cell-depleted mice, supporting that the lower bacterial burden observed after a challenge infection in wildtype mice is mediated by an adaptive immune response (Mora-Bau et al., 2015). Together, these data support that immune memory to UTI does arise, however, similar to the clinical picture in humans, immunity is not sterilizing in the bladder.

Whether T cells or antibodies are the key players in bacterial clearance in the bladder is unclear. A recent study suggested that a Th2-biased T cell response develops during UTI to promote bladder epithelial repair, at the expense of bacterial clearance (Wu et al., 2020). While this is seemingly a potential explanation for why the memory response to UTI is nonsterilizing, this work fails to consider that although a type 2 biased response develops in female mice, Th2 polarization is not observed in male mice (Zychlinsky Scharff et al., 2019). Importantly, despite the absence of a Th2-bias in male mice, female and male mice exhibit the same degree of nonsterilizing protection against a challenge UTI (Zychlinsky Scharff et al., 2019). Additionally, the early cytokine response to UTI, which is robust in female mice and largely absent in male mice, suggests that a Th2 T cell bias is not the determinant factor in memory development after bladder infection (Mora-Bau et al., 2015; Zychlinsky Scharff et al., 2019).

Thus, to understand how a memory response arises following UTI, we investigated the T cell response, finding that a mixed Th1, Th2, and Th17 bias arose during a first and second infection. Antigen persistence was necessary for immunity, as memory was abrogated after early antibiotic treatment or when mice were infected with a smaller inoculum. Surprisingly, limiting antigen persistence did not change T cell polarization, but did lead to a significant reduction in a population of T cells, identified as tissue resident memory (T_{RM}) T cells. Depleting T cells from circulation or blocking lymphocyte egress from lymph nodes did not impair memory, supporting that local mucosal memory responses, and not systemic immunity, determine protection against UTI, revealing a specific target for new therapeutics.

110

111 **Results**

112 *CD4 or CD8 T cells are sufficient for immune memory to UTI*

113 C57BL/6 mice have significantly lower bacterial burdens in their bladders after a second or challenge
114 infection compared to a primary infection with UPEC (Lacerda Mariano et al., 2020; Mora-Bau et al.,
115 2015; Zychlinsky Scharff et al., 2019). Supporting that this is due to immune memory, bacterial burdens
116 are not significantly reduced after a challenge infection in the absence of B or T cells, such as in RAG2^{-}
117 $^{/-}$ mice or in CD4^{+} and CD8^{+} T cell-depleted C57BL/6 mice (Mora-Bau et al., 2015). Given that urine
118 from women with a history of UTI inhibits UPEC binding to human urothelial cells and that antibody
119 depletion of this urine abrogates this inhibitory capacity (Svanborg-Eden and Svennerholm, 1978), we
120 hypothesized that immune memory to UTI is mediated by a specific B cell response.

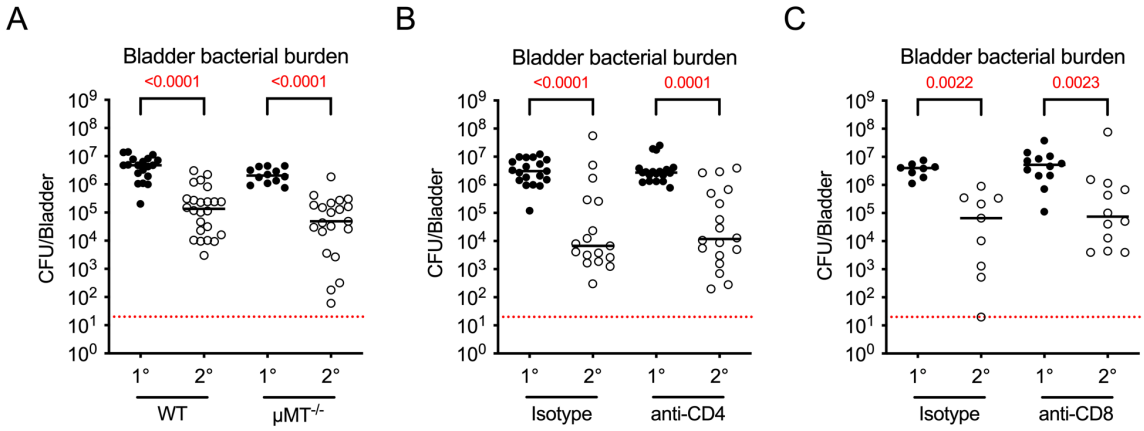
121

122 To test this hypothesis, we intravesically instilled 6 to 8-week-old wildtype female C57BL/6 mice or
123 μMT$^{-/-}$ mice, which lack mature B cells (Kitamura et al., 1991), with 10^7 colony forming units (CFU) of
124 1 of 2 isogenic UPEC strains with resistance to ampicillin (UTI89-GFP-amp^{R}) or kanamycin (UTI89-
125 RFP-kana^{R}). Half of the mice from each group were sacrificed 24 hours post-infection (PI) to assess the
126 bacterial burden in the bladder after the primary infection. Resolution of infection was monitored in the
127 remaining mice by urine sampling twice per week for 4 weeks. The sustained absence of bacteria in the
128 urine was considered to be a resolved infection. At 4 weeks PI, all resolved mice were challenged with
129 10^7 CFU to model a recurrent UTI, using the isogenic UPEC strain not used for the primary infection.
130 The distinct antibiotic resistance markers between the first and second infection allow us to distinguish
131 UPEC that establish tissue reservoirs following primary infection from those causing the second
132 infection (Mora-Bau et al., 2015). We sacrificed mice 24 hours post-challenge infection to assess
133 bacterial CFU in the bladder. As expected, bacterial burden was significantly reduced 24 hours post
134 challenge (2°) compared to the burden following primary infection (1°) in wildtype mice. Surprisingly,
135 a comparable, statistically significant reduction in CFU was also observed in μMT$^{-/-}$ mice after challenge
136 infection, suggesting that B cells are dispensable for memory to UTI (**Figure 1A**).

137

138 Having ruled out a role for B cells, we tested which T cells (CD4^{+} or CD8^{+}) were necessary for immune
139 memory to UTI. Six- to eight-week-old female C57BL/6 mice were treated with anti-CD4 or anti-CD8
140 depleting antibodies, or relevant isotype controls, and then infected three days later with 10^7 CFU UPEC.
141 As above, bacterial burden was assessed in half of the animals at 24 hours PI and the remaining mice
142 were challenged with 10^7 CFU of an isogenic UPEC strain following resolution of infection at 4 weeks.
143 In the isotype control groups, bacterial burdens 24 hours after challenge infection were statistically
144 significantly reduced compared to primary UTI, as expected. Unexpectedly, bacterial burdens were also
145 significantly reduced in mice treated with anti-CD4 or anti-CD8 depleting antibodies (**Figure 1B, C**).

146 Together, our findings support that B cells are dispensable and either $CD4^+$ or $CD8^+$ T cells are sufficient
147 for immune memory to UTI.



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149 **Figure 1. CD4 or CD8 T cells mediate protection against a second UTI**. Six- to eight-week-
150 old female C57BL/6 or $\mu MT^{-/-}$ mice were instilled intravesically with 10^7 CFU of UPEC strain
151 UTI89 and bladder bacterial burden was assessed 24 hours post-primary infection (1°, filled
152 circles). Urine was monitored twice per week for 4 weeks and all resolved mice with sterile urine
153 were challenged with an isogenic UTI89 strain and bacterial burden was assessed 24 hours after
154 challenge infection (2°, open circles). Graphs depict 1° and 2° CFU/bladder in (**A**) C57BL/6 or
155 $\mu MT^{-/-}$ mice, (**B**) isotype or anti-CD4 antibody-treated mice, or (**C**) isotype or anti-CD8 antibody-
156 treated mice. Data are pooled from 2-5 experiments, n=2 to 7 mice/group in each experiment.
157 Each circle is a mouse, lines are medians. Dotted red lines depict the limit of detection of the
158 assay, 20 CFU/bladder. Significance (adjusted *p*-value) was determined using a Kruskal-Wallis
159 test comparing bacterial burden 24 hours post 1° to 2° within a treatment group, with a Dunn's
160 post hoc test to correct for multiple comparisons. *p*-values <0.05 are in red.

161

162 *T cell polarization is mixed during primary and challenge UTI*

163 Having determined that either $CD4^+$ or $CD8^+$ T cells were sufficient for immune protection, we assessed
164 the T cell bias arising during infection. It was proposed that a Th2-dominated, type 2 immune response
165 mediates nonsterilizing memory to UTI in female mice (Wu et al., 2020). However, female mice express
166 a wide variety of cytokines, including those associated with type 2 immunity, but also type 1 and 3
167 polarization (Mora-Bau et al., 2015; Zychlinsky Scharff et al., 2019). Thus, we hypothesized that
168 polarization was potentially more complicated than previously appreciated.

169

170 To test this idea, we determined bladder-infiltrating $CD4^+$ T cell polarization over time after primary
171 and challenge UTI. We focused on $CD4^+$ T cells because $CD4^+$ T cell infiltration was more than 100
172 times higher than $CD8^+$ T cell infiltration following infection of naïve mice (**Supplementary Figure**
173 **1**). Following infection with 10^7 CFU of UPEC, six-week-old female C57BL/6 mice were sacrificed at

174 day 1, 3, and 7 post primary and challenge infection. Using intracellular staining and flow cytometry,
175 we determined T cell polarization by transcription factor and cytokine expression (T-bet$^+$, IFNγ$^+$: Th1;
176 Gata-3$^+$, IL-4$^+$: Th2; RORγT$^+$, IL-17$^+$: Th17). Compared to naïve animals (day 0), total CD4$^+$ T cells
177 increased approximately 10-fold in the bladder over time after primary infection (**Figure 2A**). CD4$^+$ T
178 cell numbers also increased between mice 7 days post-challenge UTI compared to resolved (R) mice
179 before a challenge infection, although the baseline in the resolved animals was significantly higher than
180 in naïve mice (**Figure 2A**). Instead of a distinct polarization, a mixed bias was clearly apparent among
181 infiltrating CD4$^+$ T cells following primary and challenge UTI (**Figure 2A**). Of the global CD4$^+$ T cell
182 population, T-bet$^+$ Th1 T cells made up ~10-20% of cells, Gata-3$^+$ Th2-polarized T cells represented
183 ~5-15% of cells, and RORγt$^+$ Th17 T cells made up ~3-30% of cells over time in the primary and
184 challenge infections (**Figure 2B**). In addition to Th1, Th2, and Th17 T cells, a large number of FoxP3$^+$
185 regulatory T cells (Tregs) infiltrated the bladder after primary UTI, remained elevated compared to naïve
186 levels after challenge infection, and made up 10-30% of total CD4$^+$ T cells over time (**Figure 2C**). We
187 observed a similar mixed bias among T cells in the draining lymph nodes after primary and challenge
188 UTI with increased numbers of Th2, Th17, IFNγ$^+$ Th1, and IL-17$^+$ Th17 T cells in resolved mice
189 compared to naïve animals (**Supplementary Figure 2A, B,** and **C**). Strikingly, in the bladder no T
190 helper cell subset dominated the response, and indeed, only one mouse in all experiments performed
191 had more than 50% Th2-biased T cells in a single timepoint. Furthermore, while the numbers of IFNγ$^+$
192 Th1 T cells, IL-17$^+$ Th17 T cells, and Tregs were significantly increased after primary infection, we
193 detected no IL-4-expressing Th2-biased T cells (**Figure 2D**), in line with our previous finding that IL-
194 4 levels are 10-100 times lower than any other cytokine measured (Zychlinsky Scharff et al., 2019).
195
196 We were struck by the large number of infiltrating Tregs, and given that a balanced Treg/Th T cell ratio
197 is important for immune tolerance, and also an efficient immune response against pathogens (Falivene
198 et al., 2015; Yordanova et al., 2019), we calculated the ratio of Tregs to all CD4 Th cells. In the bladder,
199 the ratio of Tregs to all Th cells was less than 1 for all timepoints measured (**Figure 2E**). The ratio of
200 Tregs to Th1, Th2, or Th17 T cells individually was ≤1 in naïve mice (Day 0) and mice infected for 24
201 hours. However, the ratios increased over 1 at day 3 and 7 post-primary infection, and remained around
202 1 during the second infection (**Figure 2F**). In the lymph nodes, the ratio was close to 2 during primary
203 UTI and ≥ 1 during the second infection (**Supplementary Figure 2D**). The ratio of Treg to each Th
204 subset was greatly increased over 1 (medians from 3.5 to 32) over time, however this was more
205 pronounced during the primary infection compared to the second UTI (**Supplementary Figure 2E**).
206 Thus, Treg T cells are highly prevalent during UTI, and given their abundance, likely influence the
207 development of memory T cells. Together, these data support that a dominant T helper bias does not
208 develop during UTI. Interestingly, all CD4$^+$ Th cell subsets that increased during the first UTI remained

209 significantly elevated over naïve levels in resolved mice in the bladder, suggesting that these T cells
210 may have undergone tissue-specific imprinting after a primary UTI.
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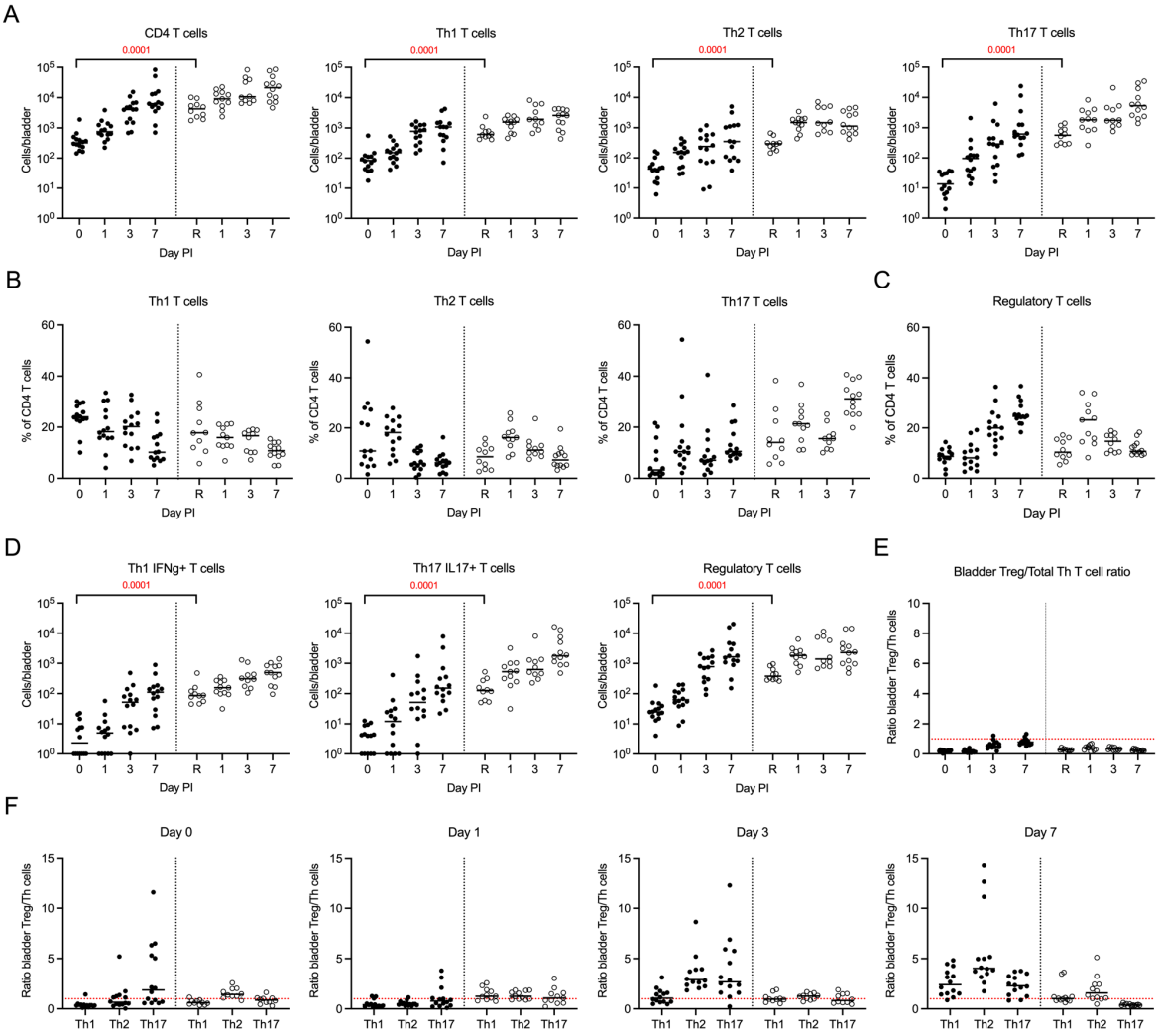

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213

214 **Figure 2. No distinct CD4$^+$ Th cell bias is apparent during primary and recurrent UTI.** Six-
215 week-old infected female C57BL/6 mice were sacrificed at the indicated timepoints post-primary
216 (filled circles), or post-challenge infection (open circles). Day 0 are naïve mice and 'R' denotes
217 animals that resolved their primary UTI but were not challenged with a second infection. The
218 graphs depict (**A**) total CD4$^+$, Th1, Th2, and Th17 T cells, (**B**) percentage of the global CD4 T
219 cell population for the indicated Th cell subset, (**C**) percentage of the global CD4 T cell population
220 for Treg, (**D**) total IFNγ$^+$ Th1, IL-17$^+$ Th17 T, and Treg cells in the bladder at the indicated day
221 post primary or challenge infection. Treg/Th cell ratios for the indicated days post-primary (filled
222 circles), or post-challenge infection (open circles) were calculated using the cell numbers
223 presented in **A, D**. (**E**) Ratio of Treg/total CD4$^+$ Th cells and (**F**) ratio of Treg/indicated CD4$^+$ Th
224 cell subset in the bladder. Data are pooled from 2 experiments, n=5 to 7 mice/group in each

experiment. Each circle is a mouse and lines are medians. Nonparametric Mann-Whitney tests comparing cell numbers between '0' and 'R' were performed. All *p*-values were corrected for multiple comparisons across all populations shown using the false discovery rate (FDR) method and *p*-values <0.05 are in red.

Antigen persistence determines the development of adaptive immunity during UTI

We observed that Tregs outnumbered Th cell subsets, potentially inducing a suppressive or tolerant environment in response to UTI. As tolerance can be induced following suboptimal (too much or too little) antigen stimulation (Xiao and Link, 1997; Yoshida et al., 1997), we hypothesized that the abundant Treg infiltration reflected antigen quantity (*i.e.*, bacterial inoculum). We reported that increasing the inoculum from 10^7 CFU/mouse to 10^8 or 10^9 CFU/mouse does not promote a stronger memory response to a second UTI, supporting that 10^7 CFU/mouse is sufficient antigen to induce an adaptive immune response, but may be too high leading to some degree of tolerance (Mora-Bau et al., 2015). Thus, we tested whether decreasing the inoculum, to reduce antigen load, would induce a stronger memory response. We intravesically infected female C57BL/6 mice with 10^7, 10^5, or 10^3 CFU of UPEC. One cohort of mice from each group was sacrificed 24 hours post-primary infection to assess bladder bacterial burden and a second cohort was monitored for resolution over 4 weeks. Resolved animals were challenged 28 days later with 10^7 CFU of the isogenic UPEC strain and we assessed CFU in the bladder 24 hours post-challenge infection. Mice infected with 10^5 or 10^3 CFU resolved their primary infection faster than those infected with 10^7 CFU (**Figure 3A**). Notably, compared to the control infection with an inoculum of 10^7 CFU, mice infected with 10^5 or 10^3 CFU for the primary infection were no longer protected against a challenge infection and had higher bladder-associated bacterial burdens after their second UTI compared to the control group (**Figure 3B**). Indeed, mice infected with 10^3 CFU during their primary infection had markedly reduced primary bacterial burdens near or at the limit of detection of the assay and, after a challenge infection, had bacterial burdens closely resembling the level observed after infection of naïve mice. Together, this supports that immune memory requires persistent and robust bacterial colonization in the bladder, and low-level exposure to UPEC cannot induce immune memory.

To directly test whether antigen persistence was necessary for immune memory to UTI, we infected mice with the same inoculum and treated them with antibiotics at specific days PI. In this scenario, the initial antigen exposure of 10^7 CFU is the same, but antigen persistence is disrupted at different timepoints post-infection. The advantage of this approach is that antibiotics are first line therapy to treat UTI, and thus, we modeled the impact of a clinical scenario on immune memory. UPEC-infected female C57BL/6 mice were given trimethoprim/sulfamethoxazole, an antibiotic combination commonly prescribed for UTI, in the drinking water for 5 days starting at 1, 2, 3, or 5 days PI. All resolved animals were challenged with an isogenic UPEC strain 28 days PI and bacterial burdens at 24 hours post-challenge infection were compared to CFU at 24 hours post-primary infection. When antibiotics were

262 given at day 1 post-primary infection, immune memory was abrogated, whereas when antibiotics were
263 delayed until day 2, 3, or 5 post-primary infection, bacterial burdens were significantly reduced in
264 challenge infection, similar to that observed in the control untreated group (**Figure 3C**). These findings
265 support that antigen persistence is necessary for the development of memory in UTI. Additionally, they
266 raise the important question as to whether a previous UTI is actually a risk factor for recurrent infection
267 (Hooton et al., 1996), or rather, antibiotic treatment contributes to an individual's risk for recurrent UTI
268 due to poor immune memory.
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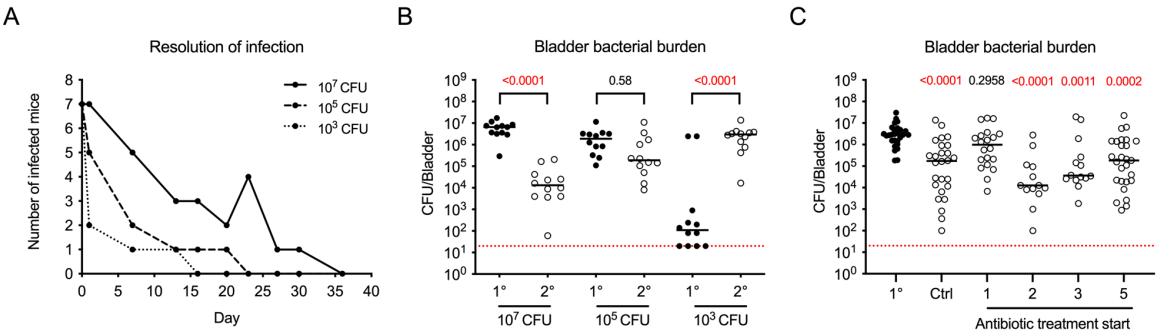

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271 **Figure 3. Antigen persistence is necessary for immune memory to UTI.** 6 to 8-week-old
272 female C57BL/6 mice were infected with (**A-B**) 10^7 (solid line), 10^5 (dashed line), or 10^3 CFU
273 (dotted line) of UPEC isolate UTI89-GFP-amp^R or UTI89-RFP-kan^R and challenged with 10^7
274 CFU of an isogenic strain after resolution, or (**C**) infected with 10^7 CFU UPEC isolate UTI89-
275 GFP-amp^R or UTI89-RFP-kan^R, treated with trimethoprim/sulfamethoxazole 1, 2, 3, or 5 days PI,
276 and challenged with 10^7 CFU of an isogenic strain after resolution. (**A**) Resolution after primary
277 infection was monitored twice per week by assessing the presence of UPEC in the urine. The
278 graph depicts the number of infected mice over time and an increase in the number of infected
279 mice in the course of the experiment is due to a spontaneous recurrent UTI. (**B-C**) Mice were
280 sacrificed 24 hours post-primary (1°, filled circles) or post-challenge infection (2°, open circles)
281 for CFU assessment. The graphs show bladder bacterial burdens following (**B**) primary infection
282 with the indicated inocula and challenge infection with 10^7 CFU UPEC and (**C**) primary infection
283 and challenge infection in mice treated with trimethoprim/sulfamethoxazole at the indicated days
284 post-primary UTI. The control (Ctrl) group did not receive antibiotics. **A** depicts representative
285 data from 1 experiment of 2 (n=7 mice/group). Data in **B** are pooled from 2 experiments, n=5 to
286 7 mice/group in each experiment. Data in **C** are pooled from 2-4 experiments, n=6 to 7 mice/group
287 in each experiment. Each circle is a mouse, lines are medians, dotted red lines depict the limit of
288 detection of the assay, 20 CFU/bladder. In **B,** significance was determined by Kruskal-Wallis test
289 comparing bacterial burden 24 hours post 1° to 2° within each inoculum group and in **C**, each 2°
290 group was compared to the 1° CFU, with Dunn's post hoc test to correct for multiple comparisons
291 and adjusted *p*-values for each comparison are presented, *p*-values <0.05 are in red.

292

293 *Limiting antigen persistence does not impact Th cell polarization*

294 When antigen load was reduced, by decreasing the inoculum or giving antibiotics on day 1 PI, protection
295 against a second infection was lost. Previously, we reported that depletion of tissue resident
296 macrophages, prior to challenge infection, results in increased infiltration of Th1 T cells and improved
297 bacterial clearance in the bladder (Lacerda Mariano et al., 2020). To test whether the loss of immune
298 memory observed in the two infection scenarios was due to a change in the proportions of polarized Th
299 cell populations, we either infected mice with 10^7 or 10^3 CFU of UTI89, or we infected a cohort of mice
300 with 10^7 CFU of UTI89 and treated half of these mice with antibiotics at day 1 PI. 28 days later, we
301 challenged all resolved mice with 10^7 CFU of UTI89 and assessed $CD4^+$ T cell polarization 24 hours
302 post-challenge. In mice infected with 10^3 CFU for their first UTI, we observed a significant reduction
303 in the total number of Th1, Th2, and Th17 T cells, as well as decreases in $IFN\gamma^+$ Th1 T cells, $IL\text{-}17^+$
304 Th17 T cells, and Tregs compared to the control group (**Figure 4A**). Disproving our hypothesis,
305 however, all Th cell subsets and Tregs decreased similarly, so proportion of each subset and therefore,
306 the global Th cell polarization was the same between mice infected with 10^7 CFU or 10^3 CFU (**Figure**
307 **4B**). Further supporting our conclusion that the loss of immune memory was not due to a change in T
308 cell polarization, we observed no differences in the numbers of Th1, Th2, or Th17 T cell subsets; $IFN\gamma^+$
309 Th1 T cells or $IL\text{-}17^+$ Th17 T cells; or Tregs between groups of mice that were antibiotic-treated or not
310 (**Figure 4C**). As the number of T cells in each subset did not change in antibiotic-treated mice, the
311 proportions of each Th cell subset were unchanged compared to the control condition (**Figure 4D)**.
312 Supporting that memory was not lost due changes in the systemic T cell compartment, the draining
313 lymph nodes also had no differences among any of the parameters measured in the experimental groups
314 compared to the control conditions (**Supplementary Figure 3**). Notably, although both experimental
315 groups had abrogated immune memory (**Figure 3**), in one scenario, T cell infiltration was globally
316 reduced (**Figure 4A**), whereas in the second, T cell infiltration was comparable to the control group
317 (**Figure 4C**). That there was no change in proportions of polarized Th cells or, in the case of antibiotic
318 treatment, no change in the number of Th cell subsets present, supports that the polarization and quantity
319 of infiltrating Th cells in response to UTI are not major determinants of memory development in UTI.

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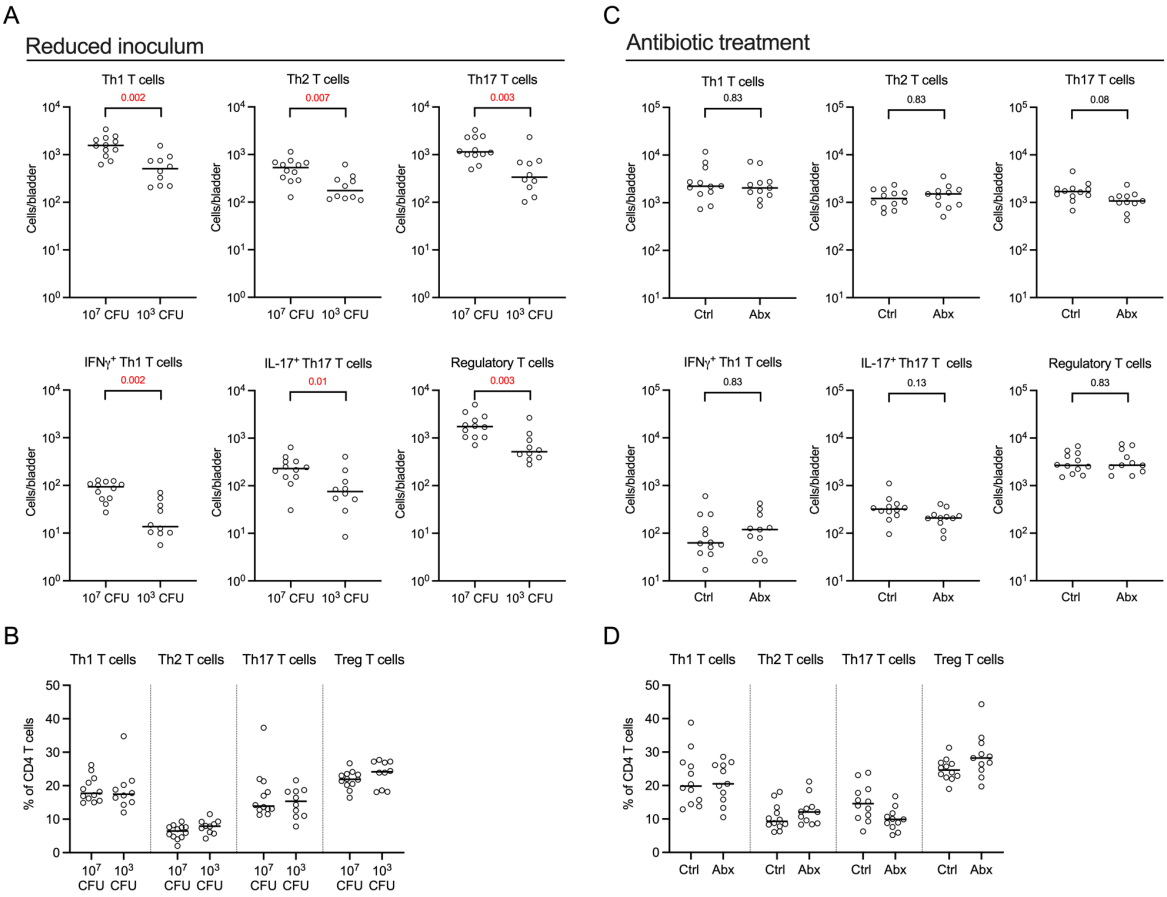
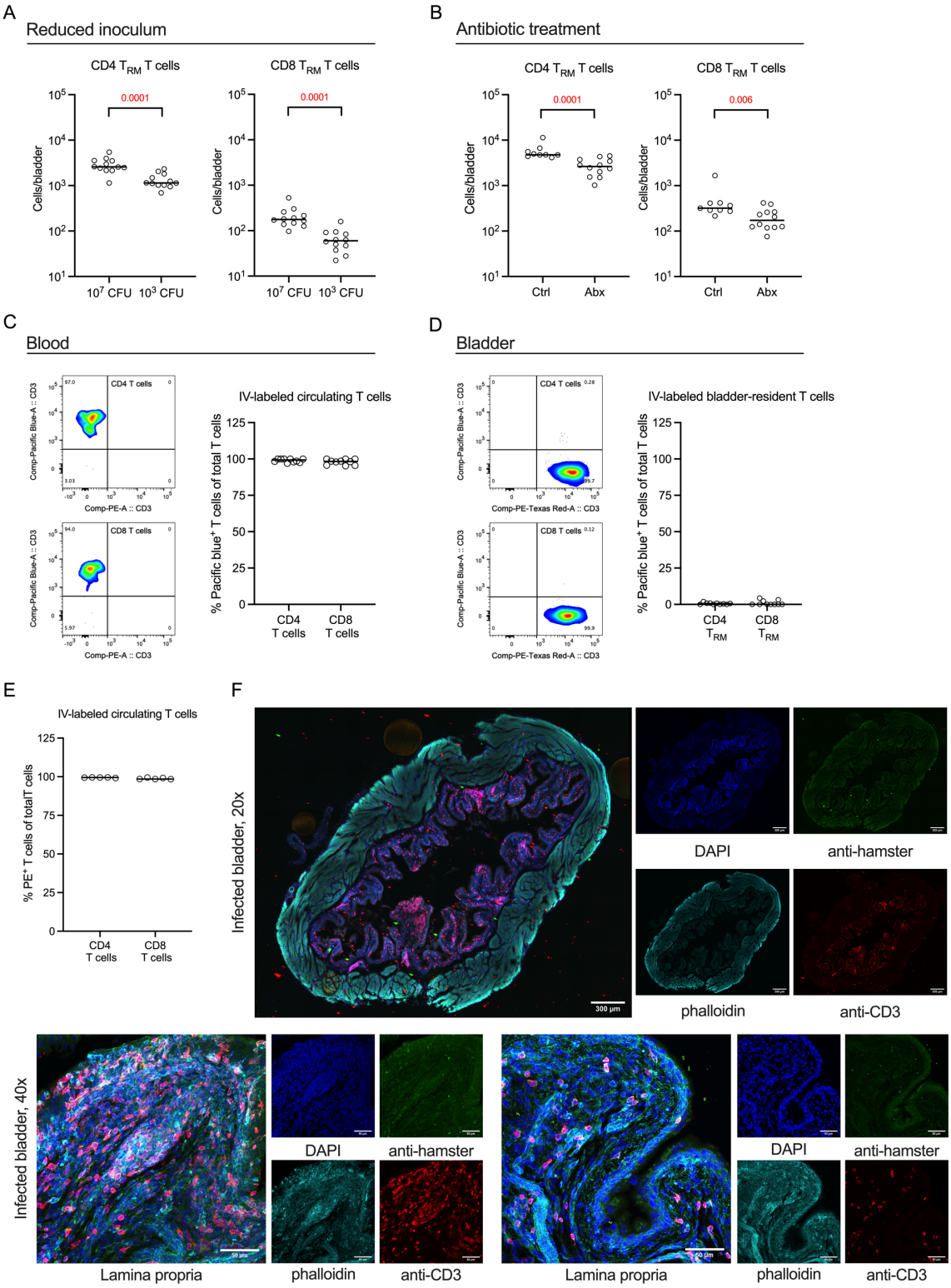


Figure 4. Th T cell polarization is unchanged when antigen persistence is limited. (**A-B**) Six-week-old female C57BL/6 mice were intravesically infected with either 10^7 or 10^3 CFU of UTI89, and at day 28, resolved mice were challenged with 10^7 CFU of UTI89 and bladders analyzed by flow cytometry 24 hours post-challenge infection. Graphs depict (**A**) the total number of the specified Th cell populations per bladder and (**B**) the frequency among CD4 T cells of the Th cell subsets in **A**. (**C-D**) Six-week-old female C57BL/6 mice were infected with 10^7 CFU of UTI89. One cohort of mice was treated 24 hours PI with antibiotics and all resolved mice were challenged with 10^7 CFU of UTI89 28 days later. Bladders were analyzed by flow cytometry 24 hours post-challenge infection. Graphs show (**C**) the total number of the specified Th cell populations per bladder and (**D**) the frequency of each Th cell subset among $CD4^+$ T cells. Data are pooled from 2 experiments, n=4 to 6 mice/group in each experiment. Each circle represents a mouse and lines are medians. In **A** and **C,** nonparametric Mann-Whitney tests comparing cell numbers between each condition were performed and all *p*-values were corrected for multiple comparisons among the same experiment using the false discovery rate (FDR) method. In **B** and **D,** significance was determined by Kruskal-Wallis tests comparing the frequency of indicated T cell subsets for each condition with Dunn's post hoc test to correct for multiple comparisons were performed. *p*-values <0.05 are in red.

340 *Tissue resident memory T cells are necessary to mediate immune memory to recurrent UTI*
341 We were surprised that Th cell polarization was not changed when immune memory to a recurrent UTI
342 was abrogated following approaches that limited antigen persistence. To understand this unexpected
343 result, we considered that immunological memory can be mediated through B cells, circulating central
344 (T_{CM}) and (T_{EM}) effector memory T cells, or a more recently described subset of long-lived stationary T
345 cells called tissue resident memory T cells (T_{RM}) (Akkaya et al., 2020; Mueller et al., 2013). Our prior
346 work, as well as our findings in **Figure 1**, ruled out a role for B cells, and demonstrated T cells are
347 necessary for an adaptive response (Mora-Bau et al., 2015). This T cell-mediated memory response to
348 UTI is rapid as a 2-log reduction in bacterial burden is observed in the bladder at 24 hours post challenge
349 infection (Lacerda Mariano et al., 2020; Mora-Bau et al., 2015; Zychlinsky Scharff et al., 2019). Given
350 the speed of this response, we hypothesized that T_{RM} cells mediate memory to recurrent UTI.
351
352 To test this hypothesis, we infected mice with 10^7 or 10^3 CFU of UTI89, or we treated mice infected
353 with 10^7 CFU of UTI89 with antibiotics 1 day PI. 28 days post-primary infection, we challenged all
354 resolved mice with 10^7 CFU of UTI89 and analyzed bladder resident T cell populations by flow
355 cytometry. Defining these cells as $CD3^+$, $CD4^+$, $CD8^+$, $CD44^{high}$, $CD62L^-$, and expressing CD69 and/or
356 CD103, we observed a significant reduction in the number of $CD4^+$ and $CD8^+$ T_{RM} cells in mice infected
357 with 10^3 CFU compared to mice infected with 10^7 CFU (**Figure 5A**) or in mice treated with antibiotics
358 at 1 day PI compared to control-treated mice (**Figure 5B**). Supporting that it is specifically bladder-
359 associated T_{RM} cells that mediate memory, there were no differences in the number of T cells with a T_{RM}
360 phenotype in the draining lymph nodes in any of the tested conditions (**Supplementary Figure 4**). Thus,
361 reducing antigen load or persistence negatively impacted only local bladder T_{RM} cell populations,
362 correlating with abrogation of protection following a recurrent UTI.
363
364 One defining feature of T_{RM} cells is that their tissue resident status renders them inaccessible to
365 intravascular staining strategies. Therefore, to provide supporting evidence that the
366 $CD3^+CD4^+CD44^{high}CD62L^-$, or $CD3^+CD8^+CD44^{high}CD62L^-$ cells expressing CD69 and/or CD103 were
367 tissue resident, we injected fluorescently-labelled anti-CD3 antibody intravenously (IV) after resolution
368 of a primary infection and collected blood and bladders 3 minutes after injection. Circulating $CD4^+$ and
369 $CD8^+$ T cells were nearly universally labeled by the injected antibody (**Figure 5C**). By contrast, bladder-
370 associated $CD4^+$ and $CD8^+$ T cells, including $CD4^+$ and $CD8^+$ T_{RM} cells, were almost entirely negative
371 for the injected antibody IV, but were identified by using an anti-CD3 antibody with a different
372 fluorophore after bladder digestion for flow cytometry (**Figure 5D**). As a complementary approach to
373 test whether these cells were tissue resident while avoiding the caveats of tissue disruption, we used
374 confocal microscopy following IV-labeling of T cells. Again, blood $CD4^+$ and $CD8^+$ T were uniformly
375 positive for an IV-injected fluorophore labeled anti-CD3 antibody (**Figure 5E**). However, using an anti-
376 hamster secondary antibody to amplify the IV-injected primary anti-CD3 antibody signal in the bladder,

377 we did not detect any T cells labeled by IV antibody administration (**Figure 5F**). The absence of signal
378 with anti-hamster labeling was not due to a lack of T cells as when we used an anti-CD3 antibody to
379 immunostain bladder tissue sections, we observed an abundance of T cells in the urothelium and lamina
380 propria of post-infected tissue (**Figure 5F**). Altogether, these data support that $CD3^{+}CD4^{+}$, $CD8^{+}$,
381 $CD44^{high}$, $CD62L^{-}$ cells expressing CD69 and/or CD103 are T_{RM} cells.
382



383

384

385 **Figure 5: Tissue resident memory T cell accumulation requires antigen persistence.** Graphs
386 depict the total number of the specified T_{RM} cell populations per bladder, identified by flow
387 cytometry, after (**A**) six-week-old female C57BL/6 mice were instilled intravesically with 10^7 or
388 10^3 CFU of UTI89, and following resolution, challenged with 10^7 CFU of UTI89 and sacrificed
389 24 hours post challenge infection or (**B**) mice were infected with10^7 CFU of UTI89 and half of
390 the animals were treated with antibiotics 24 hours PI. All resolved mice were challenged with 10^7
391 CFU of UTI89 28 days later and sacrificed 24 hours post-challenge infection. (**C-F**) Six-week-
392 old female C57BL/6 mice were instilled intravesically with 10^7 CFU of UTI89 and following
393 resolution 28 days later, received 3μg of anti-CD3 antibody intravenously. Blood and bladders
394 were collected and analyzed by flow cytometry or confocal microscopy. (**C**) Representative dot
395 plots and graph show the proportion of IV-injected Pacific blue-labeled anti-CD3 antibody
396 staining in circulating T cells. (**D**) Representative dot plots and graph show the proportion of IV-
397 injected Pacific blue-labeled anti-CD3 antibody staining and post-tissue digestion PE-CF594 anti-
398 CD3 antibody staining in bladder-associated T cells. (**E**) Graph shows the proportion of IV-
399 injected PE-labeled hamster anti-CD3 antibody staining in circulating T cells. (**F**) Representative
400 confocal images of 3 different bladders from C57BL6/J mice at 20x and 40x magnification.
401 Merged images and single channels with the target of interest are shown. DAPI, 4',6'-diamidino-
402 2-phenymlindole. Data in **A** and **B** are pooled from 2 experiments, n=3 to 6 mice/group in each
403 experiment. Data in (**C-D**) are pooled from 2 experiments n=5 mice/group in each experiment.
404 (**E** and **F**) are from 1 experiment with 5 mice. Each circle is a mouse, lines are medians.
405 Significance was determined in **A** and **B** by nonparametric Mann-Whitney tests comparing cell
406 numbers between each condition and calculated *p*-values were corrected for multiple comparisons
407 using the false discovery rate (FDR) method. *p*-values <0.05 are in red.

408

409 *Tissue resident memory T cells are sufficient to mediate immune memory to recurrent UTI*

410 To formally demonstrate that T_{RM} cells mediate protection against a recurrent UTI, we hypothesized
411 that their elimination should abrogate immune memory. However, there are currently no specific tools
412 that target T_{RM} cells without also impacting circulating T cell populations. As an alternative approach,
413 we chose to eliminate all T cells subsets except T_{RM} cells. Similar to their inaccessibility to intravascular
414 labeling, T_{RM} cells are protected from depleting antibodies when administered using certain doses or
415 schedules (Davies et al., 2017; Galvez-Cancino et al., 2018; Gebhardt et al., 2011; Turner et al., 2018).
416 Thus, we created a scenario in which the only memory T cells present during a challenge infection would
417 be T_{RM} cells in the bladder. Mice were infected with UPEC and a cohort of animals was sacrificed 24
418 hours PI infection. After resolution of infection, at day 28, mice received anti-CD4 and CD8 depleting
419 antibodies or their respective isotype controls. Mice were challenged 24 hours later with 10^7 CFU of
420 UPEC (**Figure 6A**). In T cell-depleted but uninfected mice, we measured T cell depletion in the blood,

421 draining lymph nodes, and bladder. $CD4^+$ and $CD8^+$ T cells were significantly reduced to nearly zero in
422 the circulation of antibody-treated mice (depicted in red) compared to control isotype-treated animals
423 (**Figure 6B**). In bladder-draining lymph nodes of antibody-depleted mice, $CD4^+$ and $CD8^+$ T cells with
424 a T_{RM} cell phenotype (**Figure 6C**), as well as $CD4^+$ and $CD8^+$ T_{CM} and T_{EM} cells (**Figure 6D**) were
425 significantly reduced to nearly zero, indicating depleting antibodies accessed the lymph nodes and any
426 memory T cells resident in the lymph nodes were not available to infiltrate the bladder upon challenge
427 infection to mediate protection. Importantly, analysis of the T_{RM} populations in the bladder revealed that
428 T_{RM} cells were protected from antibody depletion and there was no reduction of $CD4^+$ or $CD8^+$ T_{RM} cells
429 in the bladders of mice that received CD4 and CD8 depleting antibodies compared to mice that were
430 treated with isotype controls (**Figure 6E**). Thus, only bladder T_{RM} cells were present in the mice when
431 we challenged them with a second UPEC infection.
432
433 Following challenge UTI, isotype-treated mice had bacterial burdens that were significantly reduced
434 compared to bacterial CFU measured after a primary infection, as expected. Remarkably, anti-CD4 and
435 CD8 antibody-treated mice were also protected to the same extent from challenge infection (depicted
436 by red open circles), with significantly reduced bacterial CFU in the bladder despite a near global
437 circulating T cell depletion, demonstrating that immune memory was intact in these animals (**Figure**
438 **6F**). As an alternative approach, we used FTY720, an inhibitor of the sphingosine-1-phosphate receptor
439 pathway (Chiba et al., 1998), which prevents lymphocyte migration from the lymph nodes. Mice were
440 infected with UPEC, and after resolution of infection at day 28, mice received 3 doses of either control
441 diluent or FTY720 prior to challenge infection (**Figure 6A**). Control or FTY720-treated mice (depicted
442 by blue open circles) were equally protected 24 hours post-challenge with no significant difference in
443 their bacterial burdens after a second UTI (**Figure 6G**). Together, these results support that T_{RM} cells,
444 specifically in the bladder, and not peripheral memory T cells, mediate protection against recurrent UTI.
445

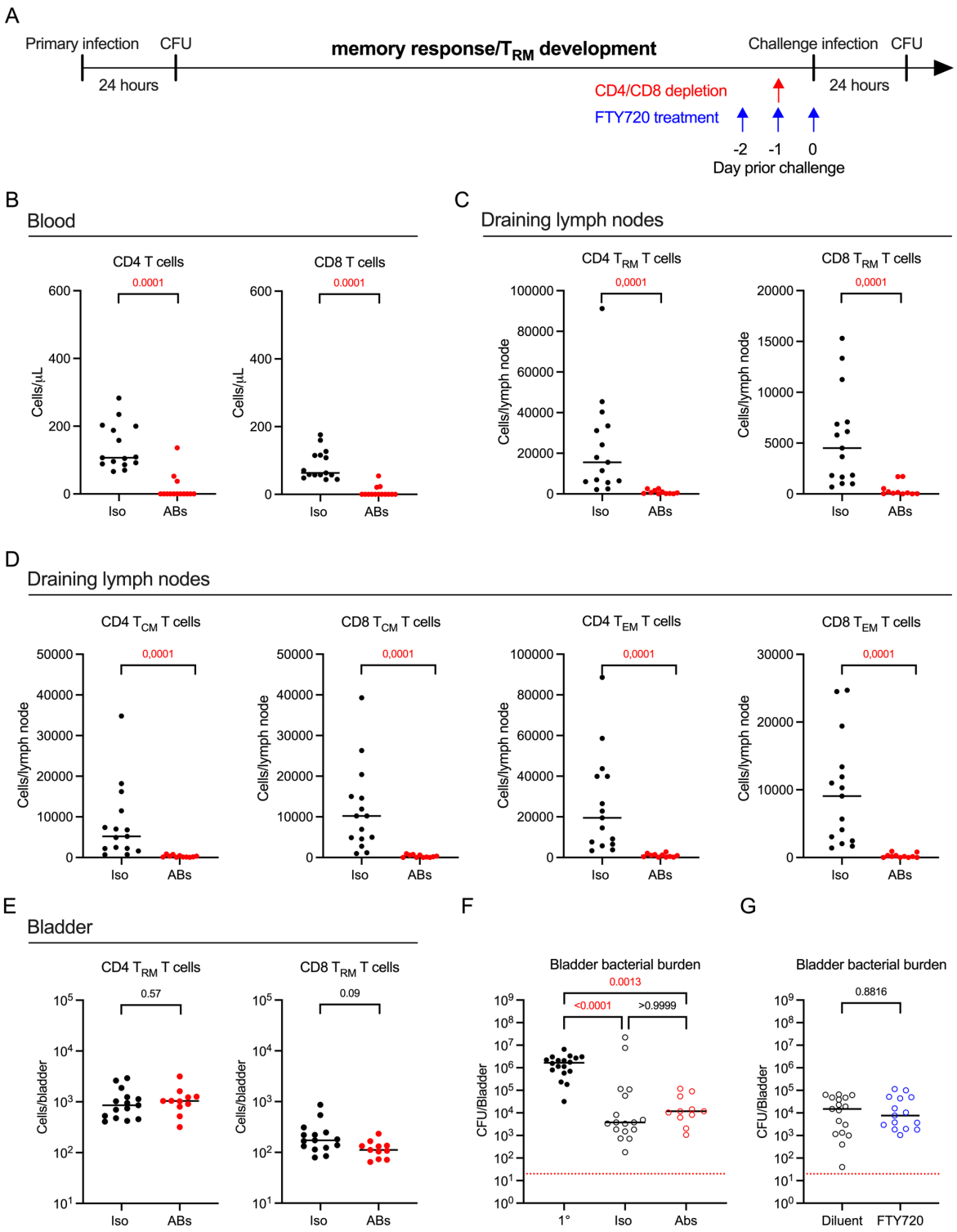


Figure 6: Tissue resident memory T cells mediate protection against recurrent UTI (A) Schematic of experiments in which six-week-old female C57BL/6 mice were infected with 10^7 CFU of UTI89 and at 28 days post-infection treated with either (**B-F**) anti-CD4 and anti-CD8 antibodies or their respective isotype controls or (**G**) FTY720 or a control diluent. Resolved mice were sacrificed (**B-E**) to assess depletion or (**F-G**) challenged with 10^7 CFU of UTI89 and

analyzed 24 hours post-challenge infection. Graphs depict the total number of specified cell populations in (**B**) blood, (**C** and **D**) bladder-draining lymph nodes, and (**E**) bladder in resolved but unchallenged mice. Graphs show bacterial burden in (**F**) control and antibody-depleted mice and (**G**) control and FTY720-treated mice. Data in (**B-F**) are pooled from 3 experiments, n=3 to 6 mice/group in each experiment. Data in **G** are pooled from 4 experiments, n=3 to 5 mice/group in each experiment. Each circle is a mouse, lines are medians. In **F** and **G**, dotted red lines depict the limit of detection of the assay. Significance in **B-E** was determined by nonparametric Mann-Whitney tests comparing cell numbers between each condition and all calculated *p*-values were corrected for multiple comparisons using the false discovery rate (FDR) method, in **F** by Kruskal-Wallis test comparing bladder bacterial burden 24 hours post primary infection (filled circles) to bacterial CFU 24 hours post challenge infection (open circles) in isotype (black open circles) or depleting antibody-treated mice (red open circles), with Dunn's post hoc test to correct for multiple comparisons, and in **G** by nonparametric Mann-Whitney test comparing bacterial burden 24 hours post challenge between diluent (black open circles) and FTY720-treated mice (blue open circles). *p*-values <0.05 are in red.

Discussion

Overwhelmingly, studies of immunity to UTI focus on innate immunity, and despite how common recurrent UTI is, we have a poor understanding of how memory develops after an acute infection (Lacerda Mariano and Ingersoll, 2020). Supporting that humoral responses are important, UPEC-specific antibody production is observed in children with pyelonephritis (Jodal et al., 1974) and, many years ago, antibodies were proposed as a biomarker for human pyelonephritis (Percival et al., 1964). Thus, it was surprising to rule out a role for B cells in our model of recurrent UTI. Notably, however, in a similar mouse UTI model, antibodies to UPEC arise only when bacteria colonized both the bladder and kidneys (Chan et al., 2013). We used C57BL/6 mice in our studies, and given that this background has a very low incidence of vesicoureteral reflux and kidney colonization (Murawski et al., 2010), and that antibodies are present specifically in cases of human pyelonephritis, our findings support that B cells are likely dispensable for memory to cystitis. This has consequences for future vaccines or other immunomodulatory strategies for recurrent UTI. Indeed, it may be that targeting the bladder to promote antibody production will not provide efficacious protection against cystitis, and may explain why, despite more than 25 years of research, vaccination strategies for UTI have so far not been successful.

Immune memory to UTI develops, but it is not sterilizing (Mora-Bau et al., 2015). Inadequate or inefficient memory responses likely play a role in the frequency of recurrent UTI. Recently, a study proposed that a type 2 immune biased response during UTI in mice is the reason for why memory is not sterilizing (Wu et al., 2020). While a Th2 biased response would be expected to favor tissue repair over

490 strong cellular immunity, our data show that immune memory can be ablated without changing the
491 proportion, or even the number, of Th1, Th2, or Th17 subsets and that there is no prevailing T helper
492 cell bias in the first 7 days of a primary or recurrent infection. In addition, female and male mice develop
493 comparable protective memory responses after an initial UTI, although male mice do not express type
494 2 cytokines (Zychlinsky Scharff et al., 2019). In trying to understand this paradox, we found through
495 immunophenotyping that the immune response is not specifically biased towards any single Th subset,
496 but rather that a mixed Th1, Th2, and Th17 response develops, with a robust infiltration of regulatory T
497 cells. While this mixed response was not previously appreciated, it is not entirely surprising, as in the
498 first 24 hours of infection, UPEC induces extensive tissue damage, including robust inflammation and
499 urothelial cell exfoliation, and will be both intracellular and extracellular (Anderson et al., 2003;
500 Ingersoll et al., 2008; Justice et al., 2004; Mora-Bau et al., 2015; Mulvey et al., 1998; Zychlinsky Scharff
501 et al., 2019), necessitating a Th2 response to mediate repair, regulatory T cells to control effector cell
502 activation, Th1-polarized T cells to eliminate intracellular pathogens and a Th17 response to target
503 mucosal and extracellular pathogens concomitantly (Gieseck et al., 2018; Kaiko et al., 2008; Shevyrev
504 and Tereshchenko, 2019; Vignali et al., 2008; Zhu et al., 2010). Indeed, this mixed response reflects the
505 complexity of the host-pathogen interaction and immune response to UTI, as well as the fine equilibrium
506 that must be established between resolution of infection and maintenance of bladder integrity.
507
508 Adding to our understanding of memory to UTI, the quantity and persistence of bacteria is a key
509 parameter in the development of an adaptive immune response, and a minimum exposure of greater than
510 24 hours is needed for the development of adaptive immunity after a primary UTI. While additional
511 studies are necessary to determine the mechanisms underlying this result, alarmingly, it may be that the
512 rapid availability of antibiotics to individuals with a UTI actually places them at risk for recurrent
513 infections by impeding the development of immune memory. Determining whether delaying antibiotic
514 treatment improves the memory response is feasible and may ultimately decrease the incidence of
515 recurrence. Treatments that ameliorate symptoms but do not eliminate bacteria themselves, followed by
516 delayed antibiotic therapy in those not at risk for pyelonephritis, may be a better therapeutic approach
517 for lasting memory. Supporting this idea, while nonsteroidal anti-inflammatory drugs (NSAIDs)
518 administered in place of antibiotics are not superior to antibiotic treatment (Gagyor et al., 2015;
519 Kronenberg et al., 2017; Vik et al., 2018), a meta-analysis of 962 patients comparing antibiotic treatment
520 to placebo for uncomplicated cystitis did not find a difference in the development of pyelonephritis
521 (Falagas et al., 2009). This suggests that delaying antibiotics may not put individuals at greater risk for
522 progression to pyelonephritis, and may support the development of a better memory response. Further
523 supporting this alternative treatment approach, NSAID-treated individuals had a significant reduction
524 in the number of recurrent UTI in their first month post-treatment compared to those treated with
525 antibiotics (Bleidorn et al., 2016). Thus, it would be of great value to test whether briefly delaying

526 antibiotic treatment promotes a better memory response and a reduction in recurrent UTI in humans,
527 which would, in turn, reduce reliance on antibiotics for this infection.
528
529 We previously reported that macrophage depletion before a primary or a challenge infection improves
530 bacterial clearance during the challenge infection (Lacerda Mariano et al., 2020; Mora-Bau et al., 2015).
531 This phenotype is T cell-dependent, and macrophage depletion specifically before a second UTI leads
532 to an increase in type 1-biased effector immune cell infiltration (Lacerda Mariano et al., 2020; Mora-
533 Bau et al., 2015). Thus, when we observed that memory was negatively impacted by decreasing the
534 inoculum or administering antibiotics, we expected a change in T cell polarization, potentially towards
535 a Th2 bias. We were surprised to observe changes only in the numbers of T_{RM} cell populations, and not
536 in the proportions of Th subsets, which suggests to us that resident macrophages directly influence
537 bladder resident T cell development or maintenance, potentially as a mechanism to maintain bladder
538 integrity. Bladder resident macrophages likely modulate the microenvironment, which shapes the local
539 T_{RM} cell response. In other mucosal tissues, lung resident macrophages limit the development of
540 influenza-specific $CD8^+$ T_{RM} cells (Goplen et al., 2019). In *Yersinia pseudotuberculosis* infection, type
541 I IFN and IL-12 in the intestinal microenvironment is key for differentiation and persistence of T_{RM}
542 cells, and depletion of $CCR2^+$ IL-12-producing cells, including macrophages or monocytes, impedes
543 T_{RM} cell differentiation (Bergsbaken et al., 2017). Deciphering the mechanisms by which macrophages
544 control local T cell responses in the bladder will be key to design immunomodulatory approaches.
545
546 The discovery of T_{RM} cells localized in non-lymphoid tissues, which are anatomically well positioned
547 to detect and respond to a second infection faster than T_{CM} or T_{EM} cells, has driven development of
548 vaccine strategies to promote their differentiation. UTI needs new therapeutic approaches to combat the
549 rapid global spread of multidrug resistant bacteria. Vaccination strategies promoting the differentiation
550 of bladder T_{RM} cells would likely be of great benefit for patients suffering from recurrent UTI and would
551 have a positive economic impact by reducing the cost of medical care. The failure of previous vaccine
552 strategies for UTI may be because the correct vaccination strategy to promote the differentiation of T_{RM}
553 cells has not been identified. For example, a BCG mucosal vaccination better protects mice against
554 tuberculosis compared to a subcutaneous vaccination, and adoptive transfer of T_{RM} cells from vaccinated
555 mice protects naïve animals from disease (Perdomo et al., 2016). Similarly, a mucosal vaccine approach
556 provides superior protection against coronavirus infections compared to a subcutaneous route (Zhao et
557 al., 2016). In addition to the route of vaccination, the type of vaccine used is also a key parameter.
558 Indeed, an attenuated influenza vaccine promotes T_{RM} cell generation in the lung, inducing long term
559 protection, whereas an inactivated vaccine does not, despite intranasal delivery (Zens et al., 2016).
560 Intranasal immunization with *Chlamydia trachomatis* promotes uterine mucosa protection and oral or
561 gastrointestinal vaccination with *Chlamydia muridarum* protects the genital tract from infection (Stary

562 et al., 2015; Wang et al., 2018). These observations will likely inform vaccine strategies for UTI and
563 suggest that the bladder mucosa does not need to be directly targeted.
564
565 In sum, we demonstrated that bladder T_{RM} cells mediate memory to recurrent UTI, and do so in the
566 absence of circulating or lymph node-resident T cells. Thus, we uncovered a specific, targetable
567 population for development of new therapeutic approaches for this common infection plagued by
568 antibiotic resistance. These findings greatly improve our understanding of adaptive immunity to UTI
569 and provide valuable information for the design and development of much needed new non-antibiotic-
570 based therapies.
571
572
573 **Materials and methods**
574 *Study design*
575 This study was conducted using a preclinical mouse model in controlled laboratory experiments to
576 investigate adaptive immunity UTI. Our objective was to determine how an adaptive immune response
577 develops after a UTI. Mice were assigned to groups by random partition into cages. In all experiments,
578 a minimum of 2 and a maximum of 7 mice made up an experimental group and all experiments were
579 repeated 2 to 5 times, with the exception of confocal imaging, in which five mice were assessed in total.
580 Data from all repetitions were pooled before any statistical analysis to reach a predetermined *n* of at
581 least 10 mice per group. As determined *a priori*, all animals with abnormal kidneys (atrophied, enlarged,
582 and/or white in color) at the time of sacrifice were excluded from all analyses, as we have observed that
583 abnormal kidneys correlate with the inability to resolve infection. Animals with a recurrent or
584 unresolved UTI, defined as having persistent bacteria in the urine up to 1 day prior to challenge infection,
585 were also excluded. End points were determined before the start of experiments and researchers were
586 not blinded to experimental groups.
587
588 *Ethics Statement*
589 Animal experiments were conducted in accordance with approval of protocol number 2012-0024 and
590 2016-0010 by the *Comité d'éthique en expérimentation animale Paris Centre et Sud* and the *Comités*
591 *d'Ethique pour l'Expérimentation Animale* Institut Pasteur (the ethics committee for animal
592 experimentation), and APAFIS #34290 by SC3 - CEEA34 – Université de Paris Cité, at Institut Cochin,
593 in application of the European Directive 2010/63 EU. In all experiments, mice were anesthetized by
594 intraperitoneal injection of 100 mg/kg ketamine and 5 mg/kg xylazine and sacrificed by carbon dioxide
595 inhalation or cervical dislocation after isoflurane inhalation.
596
597 *Mice*
598 Female mice between the ages of 6 and 8 weeks were used in this study. Female C57BL/6J mice were

599 obtained from Charles River Laboratories France. μMT$^{-/-}$ mice were bred in house at Institut Pasteur,
600 Paris, and were a kind gift from Claude Leclerc.
601
602 *UTI and determination of bacterial burden*
603 Female mice were anesthetized as described above, catheterized transurethrally, and infected with
604 1×10^7, 1×10^5, or 1×10^3 colony forming units (CFU) of UTI89-GFP-amp^R or UTI89-RFP-kan^R in 50 μL
605 PBS as previously described (Mora-Bau et al., 2015). UTI89-GFP-amp^R and UTI89-RFP-kan^R infect
606 with equal efficiency and were used interchangeably (Mora-Bau et al., 2015). For flow cytometry, only
607 the nonfluorescent parental UTI89 strain was used. Infection was monitored by detection of bacterial
608 growth from urine samples collected twice per week. 2 μL of urine were diluted directly into 8 μL PBS
609 spotted on agar plates containing antibiotics as appropriate (kanamycin (50 μg/ml) or ampicillin (100
610 μg/ml)). The presence of any bacterial growth was counted as positive for infection. The limit of
611 detection (LOD) for this assay is 500 bacteria per mL of urine. Mice were sacrificed at indicated time
612 points, bladders homogenized in sterile PBS, serially diluted, and plated on LB agar plates with
613 antibiotics, as appropriate, to determine CFU. The LOD for CFU in the bladder is 20 CFU per bladder
614 and is indicated by a dotted line in graphs. All sterile organs are reported at the LOD.
615
616 *Flow cytometry of bladder tissue, draining lymph nodes and blood*
617 Samples were acquired on a BD LSRFortessa using DIVA software (v8.0.1), and data were analyzed
618 by FlowJo (Treestar) software. Bladder and blood analyses were performed as described previously
619 (Lacerda Mariano et al., 2020; Mora-Bau et al., 2015; Zychlinsky Scharff et al., 2017). Briefly, bladders
620 were dissected, cut into small pieces, and digested using Liberase (0.34 U/ml) in phosphate-buffered
621 saline (PBS) at 37°C for 1 hour with robust manual agitation every 15 min. Digestion was stopped by
622 adding FACS buffer (PBS supplemented with 2% fetal bovine serum and 0.2 μM EDTA). Single cell
623 suspensions were washed and resuspended in brilliant stain buffer (BD) with anti-mouse CD16/CD32
624 to block Fc receptors. Antibody mixes (**Table 1**) in brilliant stain buffer were added directly to the
625 samples after 10 minutes. Total cell counts were determined by addition of AccuCheck counting beads
626 to a known volume of sample after staining, just before cytometer acquisition. To determine cell
627 populations in the circulation, whole blood was incubated with BD PharmLyse and stained with
628 antibodies (**Table 1**). Total cell counts per μL of blood were determined by the addition of AccuCheck
629 counting beads to 10 μl of whole blood in 1-step BD Fix/Lyse Solution. Draining lymph nodes were
630 disrupted with 27G needles and passed through 70 μm filters (Miltenyi). Single cell suspensions were
631 washed and resuspended in brilliant stain buffer with anti-mouse CD16/CD32 to block Fc receptors and
632 subsequently stained as above for bladders. Total cell counts were determined as they were for the
633 bladder. Memory T cell staining was performed at 37°C for 30 minutes to enhance CCR7 staining
634 (Berhanu et al., 2003).
635

636 For intracellular staining, single cell suspensions were resuspended in 1 mL of Golgi Stop protein
637 transport inhibitor diluted 1:1500 in RPMI with 10% FBS, 1% sodium pyruvate, 1X HEPES, 1X
638 nonessential amino acid, 1% penicillin-streptomycin, phorbol 12-myristate 13-acetate (50 ng/ml), and
639 ionomycin (1 μg/ml), and incubated for 4 hours at 37°C. Samples were washed once with FACS buffer
640 and Fc receptors blocked with anti-mouse CD16/CD32. Samples were stained with antibodies listed in
641 **Table 1** against surface markers and fixed and permeabilized with 1X fixation and permeabilization
642 buffer and incubated at 4°C for 40 to 50 min protected from light. After incubation, samples were
643 washed two times with 1X permeabilization and wash buffer from the transcription factor buffer kit (BD
644 Biosciences) and stained with antibodies against IFN-γ, IL-17, IL-4, and the transcription factors
645 RORγT, Gata-3, T-bet, and FoxP3 (**Table 1**), diluted in 1X permeabilization and wash buffer at 4°C for
646 40 to 50 min protected from light. Finally, samples were washed two times with 1X permeabilization
647 and wash buffer and resuspended in FACS buffer. Total cell counts were determined by addition of
648 counting beads to a known volume of sample after staining, just before cytometer acquisition.
649
650 **Table 1: Antibodies used for flow cytometry in this study**

Antibodies	Clone	Source	Identifier
Anti-mouse NK1.1 APC	PK136	BD Biosciences	cat# 550627
Anti-mouse B220 AF700	RA3-6B2	BD Biosciences	cat# 557957
Anti-mouse I-A/I-E (MHCII) APC-efluor780	MS/114.15.2	Invitrogen	cat# 47-5321-82
Anti-mouse CD8 FITC	53-6.7	BD Biosciences	cat# 553031
Anti-mouse GR1 PerCP-Cy5.5	RB6-8C5	BD Biosciences	cat# 552093
Anti-mouse CD3 Pacific Blue	500A2	BD Biosciences	cat# 558214
Anti-mouse CD45 BV605	30-F11	BD Biosciences	cat# 563053
Anti-mouse CD115 PE	AFS98	Invitrogen	cat# 12-1152-82
Anti-mouse SiglecF PE-CF594	E50-2440	BD Biosciences	cat# 562757
Anti-mouse CD4 PE-Cy7	GK1.5	ebioscience	cat# 25-0041-82
Anti-mouse NK1.1 AF700	PK136	BD Biosciences	cat# 560515
Anti-mouse CD127 APC-eFluor780	A7R34	Invitrogen	cat# 47-1271-82
Anti-mouse CD69 FITC	H1.2F3	BD Biosciences	cat# 553236
Anti-mouse CD8a PerCP-Cy5.5	53-6.7	BD Biosciences	cat# 551162
Anti-mouse Ly6C BV421	HK1.4	Biolegend	cat# 128032

Anti-mouse CD103 BV510	M290	BD Biosciences	cat# 563087
Anti-mouse CD4 BV605	RM4-5	Biolegend	cat# 100548
Anti-mouse CCR7 BV650	4B12	BD Biosciences	cat# 564356
Anti-mouse KLRG1 BV711	2F1	BD Biosciences	cat# 564014
Anti-mouse CD45 BUV395	30-F11	BD Biosciences	cat# 564279
Anti-mouse CD44 BUV737	IM7	BD Biosciences	cat# 612799
Anti-mouse **γδ** TCR PE	eBioGL3 (GL3)	Invitrogen	cat# 12-5711-82
Anti-mouse CD3 PE-CF594	145-2C11	Biolegend	cat# 100348
Anti-mouse CD62L PE-Cy7	MEL-14	Invitrogen	cat# 25-0621-82
Anti-mouse CD25 APC	PC61	BD Bioscience	cat# 557192
Anti-mouse CD11b AF700	M1/70	BD Biosciences	cat# 557960
Anti-mouse FoxP3 AF488	MF-14	Biolegend	cat# 126406
Anti-mouse ROR**γ**T BV421	Q31-378	BD Bioscience	cat# 562894
Anti-mouse **γδ** TCR BV510	GL3	BD Biosciences	cat# 563218
Anti-mouse IL-17A BV650	TC11-18H10	BD Biosciences	cat# 564170
Anti-mouse NK1.1 BV711	PK136	BD Biosciences	cat# 740663
Anti-mouse IFN**γ** BV785	XMG1.2	Biolegend	cat# 505837
Anti-human/mouse T-bet PE	eBio4B10 (4B10)	Invitrogen	cat# 12-5825-80
Anti-mouse CD3 PE-CF594	145-2C11	Biolegend	cat# 100348
Anti-mouse Gata-3 PE-Cy7	L50-823	BD Biosciences	cat# 560405
Anti-mouse IL-4 BV711	11B11	BD Biosciences	cat# 564005

Immune cell depletion

To deplete T cells, 100 µg of CD4 and/or 100 µg of CD8 (clone GK1.5, clone YTS 169.4, respectively, Bio X Cell) depleting antibodies were injected intraperitoneally in 100 µl sterile PBS per mouse 3 days before primary infection, or 24 hours before challenge infection. 100 µg or 200 µg of the isotype control (clone LTF-2, Bio X Cell) were injected intraperitoneally in 100 µl of sterile PBS per mouse at the same time in control groups. Antibody injections given before the primary infection were repeated 7 days after the first injection, *i.e.*, day 4 PI.

Intravascular staining

Female mice were anesthetized as describe above, and 3µg of Pacific Blue anti-CD3 (clone 500A2, BD bioscience), or 3µg of PE anti-CD3 (clone 145-2C11, BD bioscience) were injected intravenously in the retroorbital sinus. 3 minutes later mice are sacrificed as described above, blood and bladder are collected and analyzed by flow cytometry or by confocal microscopy.

Histological and immunostaining for confocal microscopy

Whole bladders were fixed with 4% paraformaldehyde (PFA) in PBS for 1 hour and subsequently washed with PBS. Samples were then dehydrated in 30% sucrose in PBS for 24 hours. Samples were cut transversally and embedded in optimal cutting temperature compound, frozen, and sectioned at 30 µm. Sections were blocked for 1 hour with blocking buffer [3% bovine serum albumin (BSA) + 0.1% Triton X-100 + goat serum (1:20) in PBS] and washed three times. Immunostaining was performed using rat anti-mouse CD3 (1:100) in staining buffer (0.5% BSA + 0.1% Triton X-100 in PBS) overnight. Sections were washed and stained with phalloidin (1:350), goat anti-hamster (1:350) and goat anti-rat (1:1000) antibodies in staining buffer for 4 hours. Last, sections were washed and stained with 4′,6-diamidino-2-phenylindole. Confocal images were acquired on a Leica SP8 confocal microscope. Final image processing was done using Fiji (version 2.0.0-rc-69/1.52p).

FTY720 treatment

Female mice received by an intraperitoneal injection 50µl of aqueous solution, or FTY720 at 0,4mg/ml diluted in aqueous solution (0,02mg/mouse ≈ 1mg/kg) at 3 consecutive days (day -2, -1, and at the time of challenge infection).

Statistical analysis

Statistical analysis was performed in GraphPad Prism 9 (GraphPad, USA) for Mac OS X applying the nonparametric Mann-Whitney test for unpaired data in the case of two group comparisons. To correct for comparisons made within an entire analysis or experiment, calculated *p*-values were corrected for multiple testing with the false discovery rate (FDR) method to determine the FDR-adjusted *p*-value. In the case that more than two groups were being compared, Kruskal-Wallis tests were performed, with Dunn's post hoc test to correct for multiple comparisons. All adjusted *p*-values are shown in the figures.

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Author contributions

Conceptualization: MR and MAI; Methodology: MR, LLM, TC, MAI; Investigation and data analysis: MR, LLM, TC, MAI; Writing - Original Draft: MR and MAI; Writing - Review & Editing: MR, LLM, TC, MAI; Funding Acquisition: MAI; Supervision: MAI.

Competing interests

The authors declare no competing interests.

Data and materials availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by Matthieu Rousseau (matthieu.rousseau@inserm.fr) and Molly A. Ingersoll (molly.ingersoll@pasteur.fr).

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Supplementary Information for

Tissue-resident memory T cells mediate mucosal immunity to recurrent urinary tract infection

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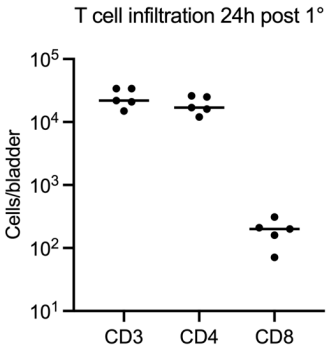

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911 **Supplementary Figure 1: CD8 T cells poorly infiltrate the bladder.** Graph shows the total number
912 of the specified T cell populations per bladder 24 hours post primary infection. A representative
913 experiment from 3 experiments is presented. Each circle is a mouse and lines are medians.

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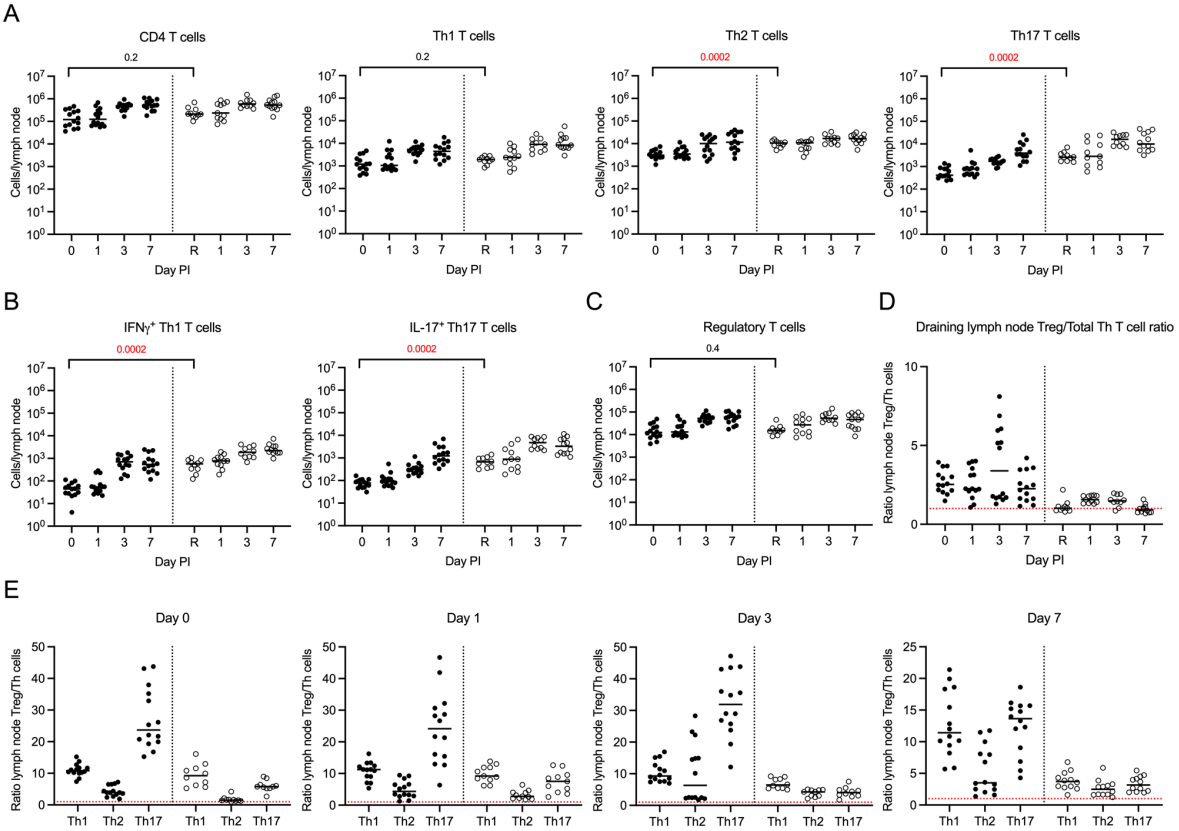
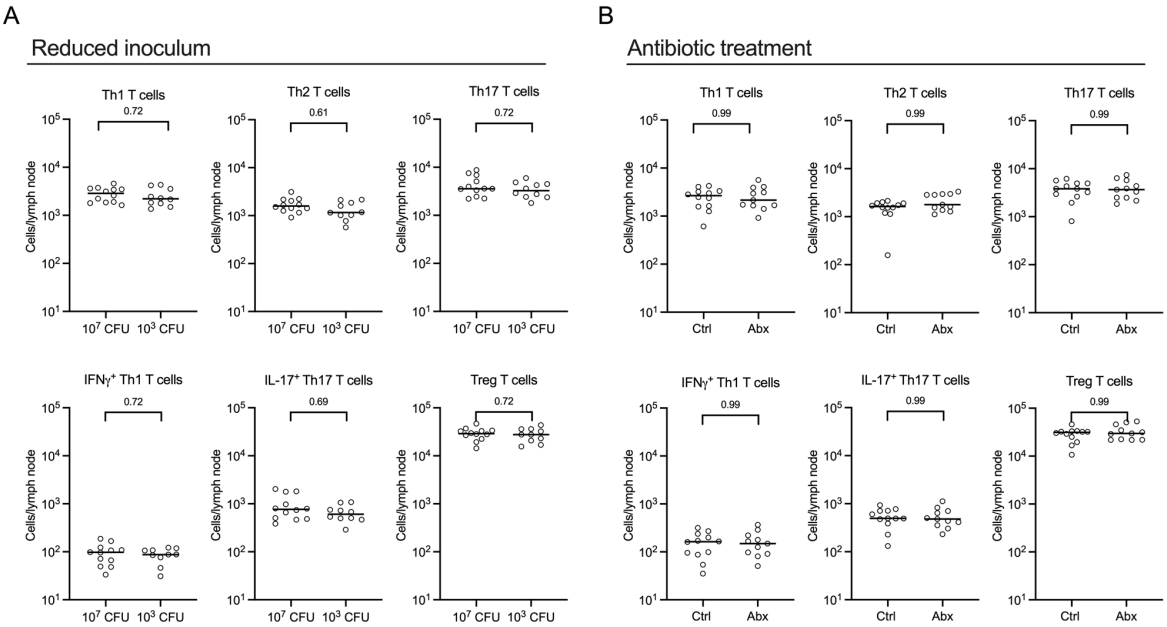
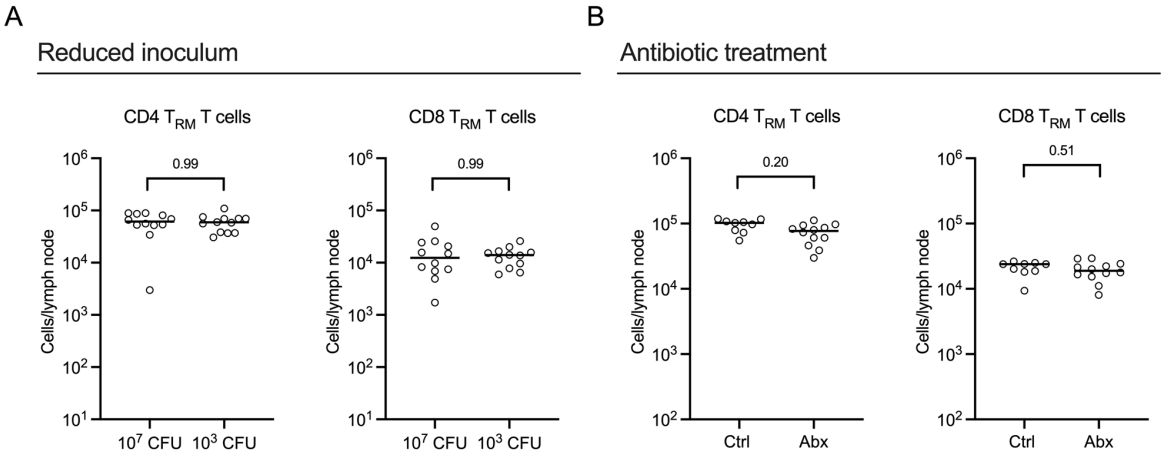


Supplementary Figure 2: No distinct CD4$^+$ Th cell bias arises during primary and recurrent UTI in the lymph nodes. Six-week-old infected female C57BL/6 mice were sacrificed at the indicated timepoints post-primary (filled circles), or post-challenge infection (open circles). Day 0 are naïve mice and 'R' denotes animals that resolved their primary UTI but were not challenged with a second infection. The graphs depict total (**A**) CD4$^+$, Th1, Th2, or Th17 T cells, (**B**) IFNγ$^+$ Th1 or IL-17$^+$ Th17 T cells, and (**C**) Tregs, in the draining lymph nodes at the indicated day post primary or challenge infection. Treg/Th cell ratios for the indicated days post-primary (filled circles), or post-challenge infection (open circles) were calculated using the cell numbers in **Supplementary Figure 2A and C**. (**D**) Ratio of Treg/total CD4$^+$ Th cells and (**E**) ratio of Treg/indicated CD4$^+$ Th cell subset in the draining lymph nodes. Data are pooled from 2 experiments, n=5 to 7 mice/group in each experiment. Results from the bladders of these mice are shown in **Figure 2**. Each circle is a mouse and lines are medians. Nonparametric Mann-Whitney tests comparing cell numbers between '0' and 'R' were performed. All *p*-values were corrected for multiple comparisons across all populations shown using the false discovery rate (FDR) method and *p*-values <0.05 are in red.



Supplementary Figure 3. Limiting antigen persistence does not change Th cell polarization in bladder-draining lymph nodes. Graphs show the total number of specified cell populations per bladder-draining lymph nodes from mice presented in **Figure 4A-D** in which (**A**) shows draining lymph nodes of mice presented in **Figure 4A** and **B, (B**) shows draining lymph nodes of mice presented in **Figure 4C** and **D,** analyzed by flow cytometry. Data are pooled from 2 experiments, n=4 to 6 mice/group in each experiment (**A**), or n=5 to 6 mice/group in each experiment (**B**). Each circle is a mouse and lines are medians. Nonparametric Mann-Whitney tests comparing cell numbers between each condition were performed. *p*-values were corrected for multiple comparisons using the false discovery rate (FDR) method. Corrected *p*-values are shown.



Supplementary Figure 4. Limiting antigen persistence does not impact bladder-draining lymph node T cells with a tissue resident memory phenotype. (**A** and **B**) Graphs show the total number of specified cell populations per bladder draining lymph nodes from mice (**A**) presented in **Figure 5A** and (**B**) presented in **Figure 5B**. Data are pooled from 2 experiments, n=3 to 6 mice/group in each experiment. Nonparametric Mann-Whitney tests comparing cell numbers between each condition were performed. *p*-values were corrected for multiple comparisons using the false discovery rate (FDR) method. Corrected *p*-values for each comparison are presented.