Sex-dependent gastrointestinal colonization resistance to MRSA is microbiota and Th17 dependent

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ABSTRACT

Gastrointestinal (GI) colonization by methicillin-resistant *Staphylococcus aureus* (MRSA) is associated with a high risk of transmission and invasive disease in vulnerable populations. The immune and microbial factors that permit GI colonization remain unknown. Male sex is correlated with enhanced *Staphylococcus aureus* nasal carriage, skin and soft tissue infections, and bacterial sepsis. Here, we established a mouse model of sexual dimorphism during GI colonization by MRSA. Our results show that in contrast to male mice that were susceptible to persistent colonization, female mice rapidly cleared MRSA from the GI tract following oral inoculation in a manner dependent on the gut microbiota. This colonization resistance displayed by female mice was mediated by an increase in IL-17A+ CD4+ T cells (Th17) and dependent on neutrophils. Ovariectomy of female mice increased MRSA burden, but gonadal female mice that have the Y chromosome retained enhanced Th17 responses and colonization resistance. Our study reveals a novel intersection between sex and gut microbiota underlying colonization resistance against a major widespread pathogen.

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is a major global health concern due to its multidrug resistance, wide range of infections, and high morbidity and mortality rates[1–4]. Gastrointestinal *S. aureus* colonization is present in an estimated 20% of the healthy population[5,6], and an estimated 1-6% are persistently colonized with MRSA[5,6]. MRSA carriage increases the likelihood of invasive infections, especially while in the hospital or post discharge[7,8]. Intestinal carriage is associated with higher rates of infections and bacteremia than nasal carriage alone[4,9]. The risk of transmission to other individuals in the hospital and community settings through fomite spread is increased by intestinal carriage[10,11].

A better understanding of why a subset of individuals are susceptible to colonization may inform decolonization strategies[5–7]. Although little is known mechanistically, population level studies have identified correlates of colonization. For example, male sex is correlated with *S. aureus* nasal carriage, skin and soft tissue infections and bacterial sepsis in adulthood[12–15]. High free testosterone levels are a risk factor for *S. aureus* throat carriage in women[12]. However, experimental systems are necessary to clarify to what extent male sex as a risk factor for carriage is biological or behaviorally based.

Sex steroid hormones and sex chromosomes can modulate the scale and type of immune response[16–18]. Sex hormones such as estrogen and testosterone can directly influence immune cell activation, proliferation and cytokine response through receptor signaling[19–23]. In general, males are more susceptible to gastrointestinal and respiratory infections, whereas females are more affected by autoimmune diseases in part due to the pro-inflammatory effect of estradiol, but these responses are tissue and cell specific[17,19]. In the few studies examining GI MRSA colonization in mice, the focus has been on the adaptation of *S. aureus* to the host[24–27]. These studies generally rely on depletion of the mouse microbiota with antibiotics to establish long-term colonization[24,25,28]. To investigate mechanisms of colonization resistance in a setting with an intact microbiota, we established a GI colonization model that does not rely on antibiotic treatment, better recapitulating MRSA colonization in a healthy host. We report that following MRSA oral inoculation, female mice with a non-permissive microbiota were resistant to sustained colonization compared with male mice that remain persistently colonized. Colonization resistance displayed by female mice was mediated by an increase in T helper 17 (Th17) cells and associated with sex hormones rather than sex chromosomes. Thus, our study demonstrates a role for the Th17 response and microbiota in GI colonization resistance against MRSA, while also highlighting the sex-dependent susceptibility to this major pathogen.

68 **RESULTS**

69 **Female mice are protected from MRSA gastrointestinal colonization in a microbiota**
70 **dependent manner.**

71 We and others have shown that inbred laboratory mice from different sources, or even
72 the same institution, can display substantial differences in mucosal immune responses and
73 susceptibility to GI colonization by microorganisms[29–34]. Therefore, when we set out to establish
74 a model of GI MRSA colonization, we examined two sources of C57BL/6J (B6) mice - those
75 bred in Jackson Laboratory (JAX) and genetically identical mice bred within our institutional
76 animal facility (referred to as NYU mice). Adult B6 mice received a single oral gavage with 10^8
77 colony forming units (CFU) of MRSA USA300 LAC, the predominant MRSA clone responsible
78 for community associated MRSA infections and a growing number of hospital-acquired
79 infections in the United States[2]. Prior to inoculation, we confirmed the absence of pre-existing *S.*
80 *aureus* colonization by plating stool from individual mice on ChromAgar plates which select for
81 *S. aureus* growth. We confirmed that mice inoculated with MRSA do not display signs of
82 disease such as weight loss **(Supplemental Fig1 A)**. Consistent with previous findings[35], we did
83 not observe extraintestinal dissemination of bacteria or histological signs of intestinal
84 inflammation **(Supplemental Fig1 B-D).**

85 In JAX mice, we detected 10^4 to 10^5 CFU per gram of stool of MRSA on day 2 that
86 remained stable for at least 35 days post-inoculation (dpi) **(Fig1 A)**. However, we noticed
87 bimodal distribution of the data for NYU mice where some mice remained stably colonized while
88 detectable MRSA burden diminished rapidly in others. Segregation of the groups by sex
89 revealed that NYU mice that displayed resistance to MRSA colonization were females. Despite
90 originating from the same litters, male NYU mice displayed sustained colonization at levels
91 similar to male and female JAX mice, while MRSA levels were already lower by day 2 and
92 undetectable by 2-3 weeks post inoculation in female NYU mice **(Fig1 B-C)**.
93 Commensal microbes that are constituents of the microbiota can inhibit *S. aureus* nasal, skin,
94 and intestinal colonization[36–38]. In support of a role for commensal microbes in promoting
95 colonization resistance in NYU female mice, we observed stable MRSA GI colonization in germ-
96 free (GF) mice of both sexes **(Fig1 D).** We next examined germ-free mice that were colonized
97 with a defined consortium of 15 bacterial strains representative of a mouse gut microbiota
98 (Oligo-MM_{12} + FA3), previously shown to be sufficient to confer colonization resistance against
99 the enteric pathogen *Salmonella enterica* serovar Typhimurium[39]. We observed sustained
100 MRSA GI colonization and no sex difference in these minimal flora mice, indicating that
101 additional intestinal commensals contribute to sex-dependent colonization resistance **(Fig1 E).**

Given these results, we examined whether we could transfer the MRSA resistance displayed by NYU female mice to permissive JAX female mice through co-housing mice in the same cage, which allows for the transfer of microbiota between mice[40]. JAX and NYU female mice were co-housed together for a week prior to MRSA inoculation and kept together for the duration of the experiment. When housed together, JAX female mice cleared MRSA colonization similarly to their NYU cage mates **(Fig1 F,G)** indicating that the microbiota of NYU female mice promotes resistance to colonization and that this protective property can be transferred.

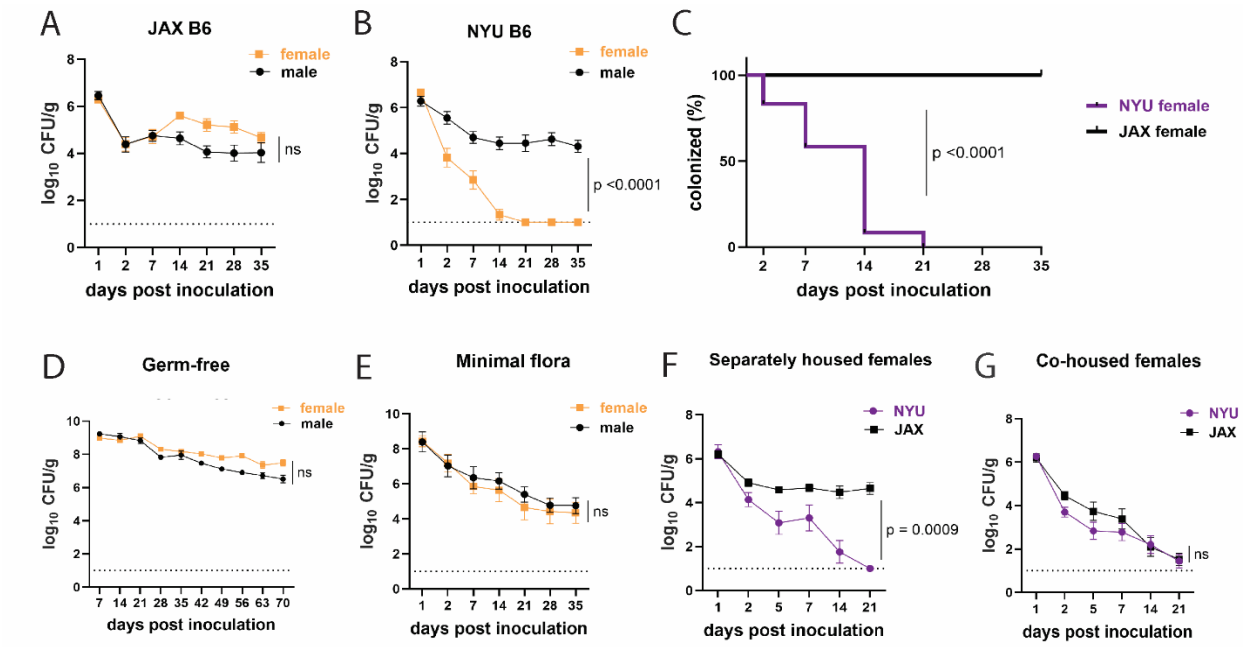

Fig.1 Female mice are protected from MRSA gastrointestinal colonization in a microbiota dependent manner.

(A) MRSA colony forming units (CFU) per gram of stool following oral inoculation of B6 mice purchased from Jackson Laboratory (JAX). Males n=10, females n=13. **(B)** MRSA CFU in stool following oral inoculation of B6 mice generated from breeders in the NYU animal facility. Males n=13, females n=13. **(C)** Proportion of JAX and NYU female mice with detectable MRSA GI colonization over time. **(D)** MRSA CFU stool burden following oral inoculation of germ-free mice. Males n=8, females n=7. **(E)** MRSA CFU in stool following oral inoculation of mice with a defined minimal microbiota. Males n=12, females n=11. **(F)** MRSA CFU stool burden of female NYU or JAX mice housed separately or **(G)** co-housed with each other. NYU n=9, JAX n=11, cohoused NYU n=14, cohoused JAX n=14. Data points represent mean ± SEM from at least

122 two independent experiments. Statistical analysis: area under the curve followed by a two-tailed
123 t-test for (A), (B), (D)-(G) and Log-rank Mantel Cox test for (C). ns: not significant.
124
125 **Microbiota is not sufficient to explain sex bias in MRSA GI colonization.**
126 To compare the microbiome composition of male and female NYU and JAX mice, we
127 performed 16S ribosomal DNA sequencing of stool collected prior to inoculation with MRSA.
128 Principal Coordinates Analysis (PCoA) of operational taxonomic units (OTUs) showed that
129 samples were distinguished based on the source of mice (NYU versus JAX) rather than sex
130 **(Fig2 A)**. Overall alpha diversity as measured by Shannon index was increased in JAX mice
131 compared to NYU mice prior to MRSA inoculation, as well as in JAX females compared to NYU
132 females **(Fig2 B)**. Alpha diversity by Shannon index between NYU males and females shows a
133 similar spread when comparing sexes **(Fig2 B)**. JAX females had higher abundance of
134 *Clostridiaceae* and *Lachnospiraceae* family members, while NYU female microbiomes were
135 dominated by the *Muribaculaceae* family **(Fig2 C)**. The NYU male and female microbiomes
136 clustered together **(Fig2 A)** and had similar relative taxonomic abundances **(Fig2 D).** These
137 findings suggest that the observed sex bias in colonization resistance may not be due to
138 microbiome composition alone.
139 As male and female mice are housed separately post weaning, and weaning is
140 associated with microbiome changes[41], we tested if the colonization resistance displayed by
141 female NYU mice could be transferred to male NYU mice. Instead of co-housing male and
142 female mice, which could introduce variables through social stress or changes in hormone
143 levels due to mating,[42] we performed fecal microbiota transplantations (FMT) by repetitively
144 gavaging recipient mice with donor stool. JAX female mice receiving a FMT from NYU female,
145 but not male donor mice, displayed colonization resistance to MRSA. However, an FMT using
146 NYU female donor stool was unable to confer colonization resistance to JAX male recipients
147 **(Fig2 E)**. Therefore, the microbiota contributes to the difference between female JAX and NYU
148 mice, but other factors mediate the divergence of female and male NYU mice. From here on, we
149 use NYU bred mice to investigate host factors that mediate this sex specific colonization
150 resistance.

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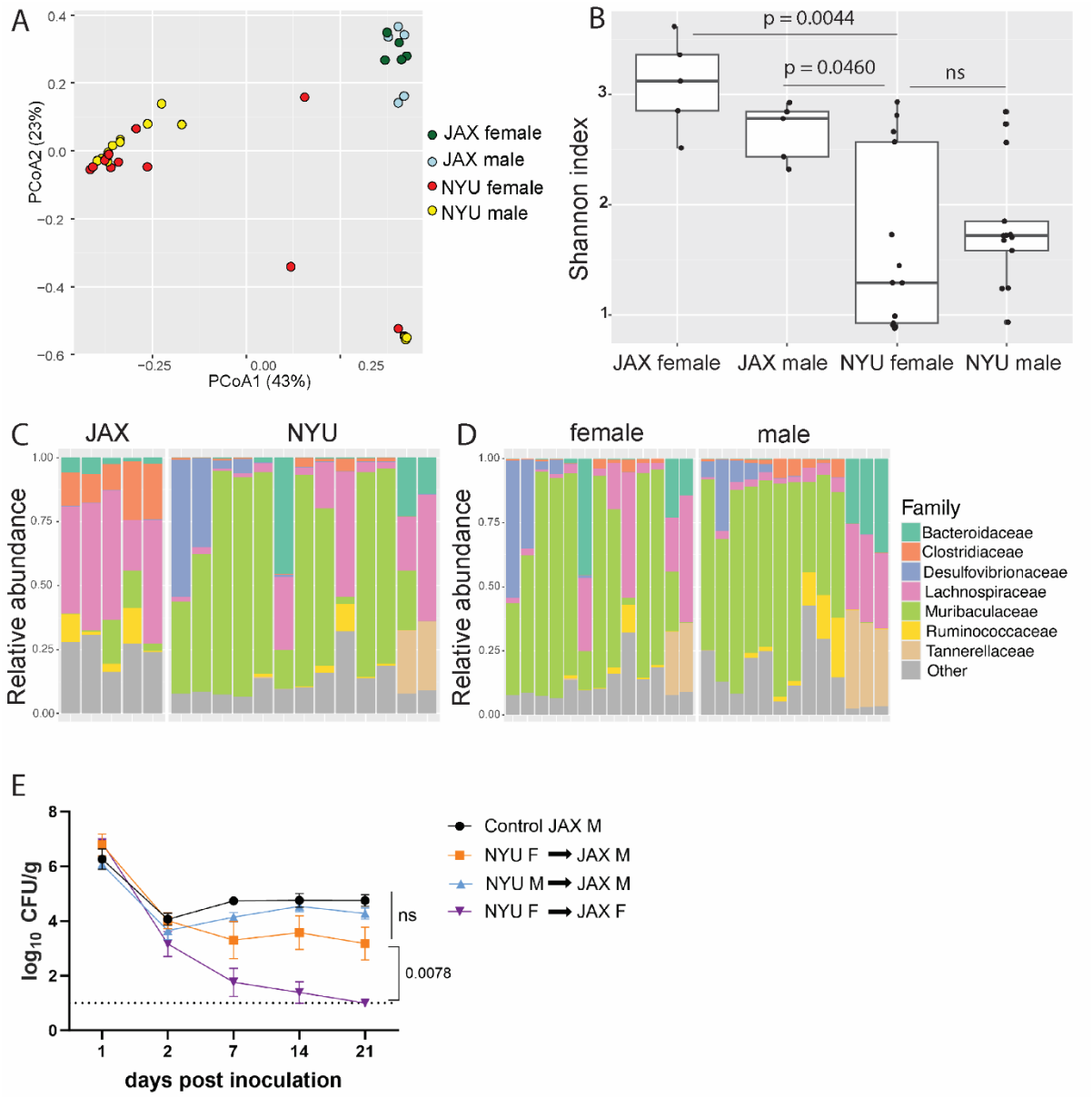

Fig. 2 Microbiome is not sufficient to explain sex bias in MRSA GI colonization. **(A)** Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances of 16S sequences obtained from stool of JAX and NYU male and female mice prior to MRSA inoculation. Proportion of variance explained by each axes shown in parentheses. JAX M n=5, JAX F n=5, NYU M n=11, NYU F n=13. **(B)** Alpha diversity of the microbiomes of male and female JAX and NYU mice. Wilcoxon rank sum test used for pairwise statistical comparisons to NYU females; ns: not significant. **(C)** Relative abundance of bacterial families in female NYU and JAX mice prior to MRSA inoculation. **(D)** Relative abundance of bacterial families in male and female NYU mice prior to MRSA inoculation. **(E)** MRSA colony forming units (CFU) in stool following oral

162 inoculation of JAX female (JAX F) and male (JAX M) recipients of fecal microbiome transplants
163 (FMT) from female (NYU F) and male (NYU M) donor mice compared with JAX male controls
164 (control JAX M) that did not receive an FMT. Mean MRSA burden ± SEM. Area under the curve
165 analysis + 1 way ANOVA Sidak's multiple comparisons test for (E). Control M n=6, M +NYU F
166 stool n=7, M +NYU M stool n=6, F +NYU F stool n=6. ns: not significant.

167

168 **MRSA GI colonization elicits sex-dependent gene expression changes in the gut**

169 To gain insight into the host response, we performed bulk RNA-seq analysis of cecal-
170 colonic tissue obtained two days post inoculation (2 dpi) of NYU mice (both sexes) with either
171 MRSA or PBS (mock), as 2 dpi is the timepoint at which MRSA burden begins to diverge
172 between males and females. The lamina propria compartment, enriched with immune cells, and
173 the epithelial cell fraction were isolated and sequenced separately. In the lamina propria, 795
174 genes were upregulated in both male and female mice following MRSA inoculation, with an
175 additional 352 genes upregulated in females only and 648 genes upregulated in males only, as
176 determined by a log2 fold change cutoff of 1.2 and a false discovery rate (FDR) of 0.05
177 (**Supplemental Fig2 A**). Following MRSA inoculation in both sexes, genes with the largest
178 downregulation included those involved with immunoglobulins (*Ighv1-19*, *Igkv4-91, Bcam*),
179 cytochrome p450 genes (*Cyp1a1, Cyp1b1*), and von Willebrand factor (*Vwf*), a secreted protein
180 that MRSA binds to disrupt platelet recruitment and coagulation[43] **(Fig3 A)**. Interestingly,
181 constitutive cytochrome P4501A1 (*Cyp1a1*) expression impairs the intestinal immune response
182 against enteric pathogens[44], and its downregulation in mice inoculated with MRSA may be
183 supportive of a mucosal immune response. Genes that were upregulated in the lamina propria
184 following MRSA inoculation included those involved in T cell and neutrophil activity such as
185 *Ccl28*[45], *Sectm1b*[46], *S100g*[47], *Lrrc19*[48], and *H2-q1*[49] **(Fig3 A)**.

186 Pathway analysis of the lamina propria fraction indicated that both males and females
187 have downregulation of immune related Th1, Th2, and granzyme A signaling pathways, and
188 upregulation of oxidative phosphorylation, neutrophil extracellular trap formation, glucose
189 metabolism, and xenobiotic metabolism pathways **(Fig3 B)**. Downregulation of interleukin (IL)-4
190 and IL-13 pathway and upregulation of retinoid X receptor (RXR) and the pregnane X receptor
191 (PXR) pathways were specific to female mice. Unique to male mice was the downregulation of
192 IL-5, IL-3, and GM-CSF signaling, and upregulation of the pyroptosis and ion channel transport
193 pathways.

194 Known X and Y chromosome linked genes *Xist*, *Ddx3y*, *Eif2s3y*, *Kdm5d*, and *Uty* were
195 differentially present in females and males as expected but were removed from male vs female
196 analysis plots to highlight other differentially expressed genes. Other than known sex
197 chromosome linked genes, when comparing male and female mice prior to MRSA inoculation,
198 we observed several genes without a descriptive name or suggested pseudogenes such as
199 *Gm12070, Gm13772,* and *Gm4968* **(Fig3 C)**. In the MRSA condition, there were 13 genes
200 differentially expressed in the intestinal lamina propria between male and female mice.
201 *Glycosylation-dependent cell adhesion molecule-1* (*Glycam1*), which mediates lymphocyte
202 trafficking to lymphoid tissues[50], was upregulated in female mice **(Fig3 D)**. Genes selectively
203 upregulated in male mice 2 dpi included *Complement protein C7* and several immunoglobulin
204 heavy chain variants (*Ighv5-4, Ighv1-49, Ighv8-5*).

205 In the intestinal epithelial fraction, we observed only 15 genes upregulated in males 2 dpi
206 with MRSA, while 2,037 were upregulated in females with no overlap between sexes
207 (**Supplemental Fig2 B**). Among the most differentially downregulated genes following MRSA
208 inoculation when combining both sexes were *Irf5*, a pivotal transcription factor in the type I IFN
209 pathway, *Csf2rb*, part of the receptor for IL-3, IL-5, and CSF signaling, and *Ciita*, a mediator of
210 major histocompatibility complex activation. Among those upregulated were genes involved in
211 limiting inflammation like *Cav1*, involved in epithelial barrier maintenance, *Cfh*, a regulator of the
212 complement pathway, and *Slpi* which functions to protect tissue from the detrimental
213 consequences of inflammation **(Supplemental Fig2 C)**. Pathway analysis indicated that both
214 sexes had upregulation of cell cycle and rRNA processing pathways and downregulation of
215 neutrophil degranulation. Unique to females was upregulation of PTEN signaling and
216 SUMOlyation of DNA damage response pathways **(Supplemental Fig2 D)**. There were few
217 differentially expressed genes between males and females in the mock epithelial condition **(Fig**
218 **3E)** but following MRSA inoculation males had increased expression of the antimicrobial peptide
219 *Defa30* and *Tnip3*, a negative regulator of NFκβ and Toll-like receptors **(Fig 3F)**. Thus, MRSA
220 inoculation elicits distinct transcriptional responses in males and females in both the lamina
221 propria and epithelial fractions, and many of the differentially regulated genes have known
222 functions in neutrophils and lymphocyte migration and function.
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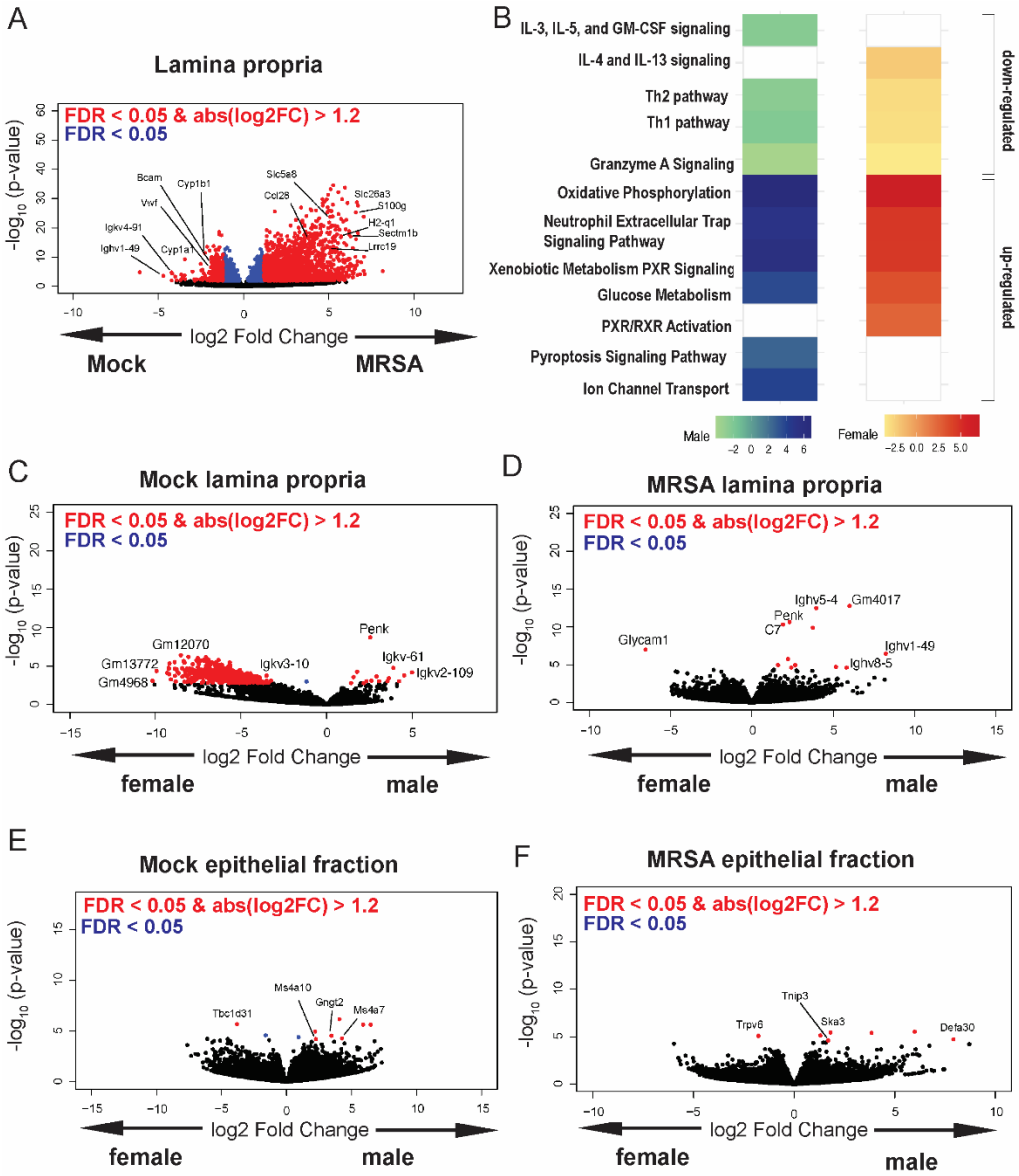

Fig 3. MRSA GI colonization elicits sex-dependent gene expression changes in the gut. **(A)** Volcano plot of differentially expressed genes identified by RNA-seq analyses of the intestinal lamina propria of NYU mice 2 dpi with MRSA compared with PBS mock inoculated NYU mice. **(B)** Ingenuity Pathway Analysis (IPA) of downregulated and upregulated genes in the intestinal lamina propria upon MRSA inoculation of male and female mice. **(C)** Volcano plot of differentially expressed genes in the intestinal lamina propria comparing mock treated males and females. **(D)** Volcano plot of differentially expressed genes in the intestinal lamina propria comparing males and females 2 dpi with MRSA. **(E)** Volcano plot of differentially expressed genes in the intestinal epithelial fraction of males and females prior to MRSA inoculation. **(F)**

235 Volcano plot of differentially expressed genes in the intestinal epithelial fraction of males and
236 females 2 dpi with MRSA. Four mice were used for each sequencing experimental condition.
237 Genes shown in red have a false discovery rate (FDR) of <0.05 and an absolute log2 fold
238 change (abslog2FC) of >1.2. Genes shown in blue have a FDR of <0.05. Genes linked to X and
239 Y chromosomes were removed from volcano plots.
240
241 **CD4+ T cells mediate colonization resistance in female mice.**
242 Female mice have been shown to be protected from dermonecrosis in a model of
243 invasive MRSA skin infection due to reduced expression of genes associated with the NLRP3
244 inflammasome (*Nlrp3* and *Il-1β*) compared to males[14]. However, we did not observe sex-specific
245 differences in NLRP3-associated transcripts with RNA-seq **(Supplemental Fig3 A,B)** and
246 female *Nlrp3*$^{-/-}$ mice displayed similar resistance to MRSA GI colonization as female *Nlrp3*$^{+/-}$
247 controls **(Supplemental Fig3 C)**. Thus, the mechanism of sex specific resistance to MRSA
248 colonization of the GI tract may be different from that observed during a skin infection where
249 barrier breach triggers an exaggerated innate immune response that may cause more harm
250 than benefit.

251 The T cell signature in the RNA-seq analyses of the intestinal lamina propria of MRSA
252 colonized mice was unexpected given that the day 2 time point is earlier than what is typically
253 required for an adaptive immune response. To test the role of lymphocyte responses in GI
254 colonization resistance, we measured bacteria in stool following oral inoculation with MRSA of
255 *recombination activating gene 2* (*Rag2*) deficient mice, which lack mature B and T cells. We
256 observed no difference in burden between male *Rag2*$^{-/-}$ and *Rag2*$^{+/-}$ controls **(Fig4 A).**
257 However, female *Rag2*$^{-/-}$ mice had significantly increased MRSA burden compared to *Rag2*$^{+/-}$
258 littermates **(Fig4 A).** These data suggest that a B or T cell mediated response is required for the
259 sex bias we observe in colonization resistance.

260 B cell associated *Ighv* genes were differentially regulated between males and females.
261 However, *Igh-μ*$^{-/-}$ (*μ*MT) mice, which lack mature B lymphocytes, were similar to *Igh-μ*$^{+/-}$
262 controls and retained sex differences; males displayed prolonged colonization and females were
263 resistant **(Fig4 B)**. In contrast, antibody-mediated depletion of CD4+ T cells **(Supplemental**
264 **Fig3 D)** substantially increased MRSA GI colonization of female mice, similar to *Rag2*$^{-/-}$ mice
265 **(Fig4 C).** Therefore, CD4+ T cells are required for MRSA colonization resistance associated
266 with female sex.
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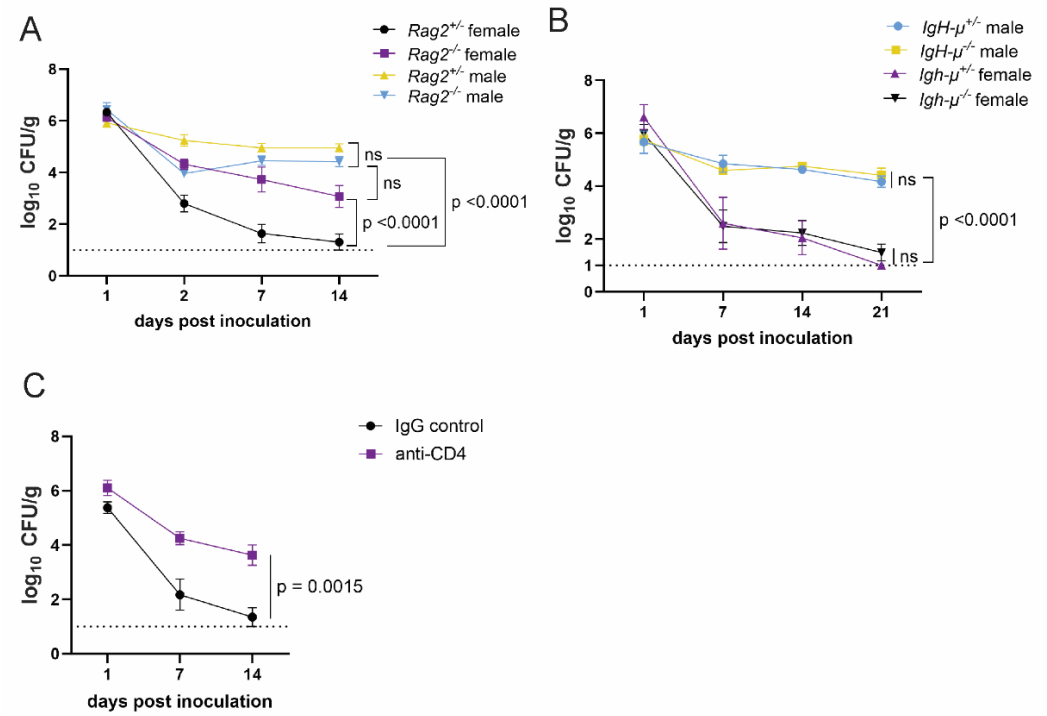

Fig 4. CD4+ T cells mediate colonization resistance in female mice.
(A) MRSA CFU in stool following oral inoculation of *Rag2*$^{-/-}$ and *Rag2*$^{+/-}$ mice bred at NYU. Male *Rag2*$^{-/-}$ n=12, male *Rag2*$^{+/-}$ n=12, female *Rag2*$^{-/-}$ n=12, female *Rag2*$^{+/-}$ n=11. **(B)** MRSA CFU in stool following oral inoculation of *Igh-μ*$^{-/-}$ and *Igh-μ*$^{+/-}$ mice bred at NYU. Male *Igh-μ*$^{-/-}$ n=6, male *Igh-μ*$^{+/-}$ n=6, female *Igh-μ*$^{-/-}$ n=8, female *Igh-μ*$^{+/-}$ n=5. **(C)** MRSA CFU in stool following oral inoculation of female NYU B6 mice injected I.P. with 250 μg of anti-CD4 depleting antibody or anti-IgG control. Anti-IgG n=8, anti-CD4 n=10. Data points represent mean ± SEM from at least two independent experiments. Statistical analysis: area under the curve analyzed by a 1 way ANOVA with Sidak's multiple comparison test for (A) and (B) and a two-tailed t test for (C). ns: not significant.

MRSA colonization resistance in female mice is dependent on Th17 cells and neutrophils.

In addition to T cells, our transcriptome analysis showed upregulation of genes associated with neutrophil function **(Fig3 B)**. In mouse models of nasal colonization, clearance is mediated by neutrophil influx downstream of the type 17 response, which includes interleukin-17A (IL-17A) producing lymphoid cells such as Th17 cells[51,52]. As an extracellular Gram-positive bacterium at the mucosal surface in the gut, MRSA may be subject to regulation by pre-existing Th17 cells[53–55]. Although the total numbers of CD4+ T cells were similar across conditions, we

288 observed an increase in IL-17A+ CD4+ T cells in the small intestine and colon of female mice
289 2dpi with MRSA that was not observed in males **(Fig5 A,B, Supplemental Fig4 A,B and 5**
290 **A,B)**. There were no differences in γδ T cells and innate lymphoid cells (ILCs) or the proportion
291 of these lymphoid subsets that were IL-17A+ **(Fig5 C,D** and **Supplemental Fig5 C,D)**. To
292 determine the importance of the type 3 immune response that encompasses Th17 cells, we
293 inoculated *RAR-related orphan receptor gamma* (*Rorγt*) deficient mice that lack the
294 transcriptional regulator required for the differentiation of these cell types. Although there was
295 no difference in MRSA burden between male *Rorγt*$^{+/-}$ and *Rorγt*$^{-/-}$ mice that remained
296 colonized, female *Rorγt*$^{-/-}$ mice had higher MRSA burden compared to *Rorγt*$^{+/-}$ controls **(Fig5**
297 **E).** Responses to IL-17 itself was required, because female mice deficient in the IL-17 receptor
298 (*IL-17ra*) had increased MRSA burden compared to heterozygous littermates **(Fig5 F)**. There
299 was no difference in MRSA burden between *Tcrd*$^{-/-}$ and *Tcrd*$^{+/-}$ male or female mice **(Fig5 G)**,
300 confirming that γδ T cells were not required for colonization resistance. Taken together, these
301 data implicate Th17 cells and IL-17 in colonization resistance against MRSA observed in female
302 mice.

303 Th17 cells and IL-17 can recruit and promote antimicrobial functions of neutrophils[56].
304 Neutrophils from female mice and humans display signs of increased maturity and activation[57–
305 60]. Although we did not observe significant differences in the number of neutrophils between
306 conditions **(Fig5 H)**, neutrophils from female mice 2 dpi with MRSA had increased levels of
307 CD11b, a sign of activation[61], compared to males **(Fig5 I)**. Antibody- mediated depletion of
308 neutrophils **(Supplemental Fig5 E)** led to increased MRSA burden compared with isotype
309 control treated mice **(Fig5 J)**. Although colonization was increased in males, neutrophil
310 depletion in female mice led to an earlier and more pronounced increase in MRSA colonization
311 that was prolonged. These results are consistent with the RNA-seq analysis suggesting that a
312 neutrophil response occurs in both sexes. It is likely that neutrophils control bacterial burden to
313 some degree but fail to promote clearance in males. In contrast, neutrophils are required for
314 clearance of MRSA in the gut of female mice.

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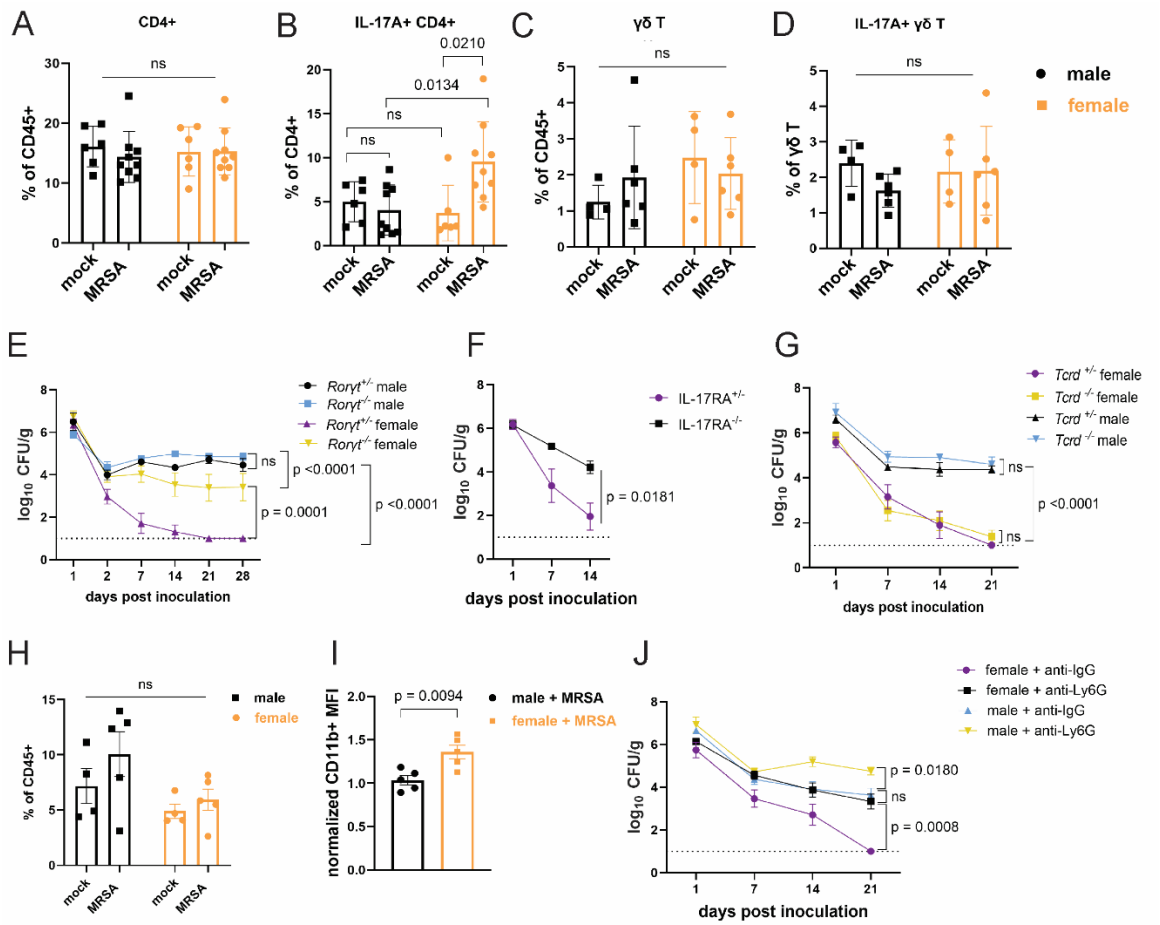

Fig 5. Female mice have increased IL-17A+ CD4+ T cells and require neutrophils for MRSA clearance.

(A) Flow cytometry of cecal-colonic lamina propria CD4+ T cells as a percentage of CD45+ cells in male and female NYU mice treated with PBS or MRSA 2 dpi. **(B)** Flow cytometry of cecal-colonic lamina propria IL17A+ CD4+ T cells as a percentageof total CD4+ T cells in male and female NYU mice treated with PBS or MRSA 2 dpi. **(C)** Flow cytometry of cecal-colonic lamina propria γδ T cells as a percentage of CD45+ cells in male and female NYU mice treated with PBS or MRSA 2 dpi. **(D)** Flow cytometry of cecal-colonic lamina propria IL17A+ γδ T cells as a percentage of total CD4+ T cells in male and female NYU mice treated with PBS or MRSA 2 dpi. **(E)** MRSA CFU in stool following oral inoculation of *Roryt*$^{-/-}$ and *Roryt*$^{+/-}$ mice bred at NYU. Male *Roryt*$^{+/-}$ n=5, male *Roryt*$^{-/-}$ n=7, female *Roryt*$^{+/-}$ n=9, female *Roryt*$^{-/-}$ n=9. **(F)** MRSA CFU in stool following oral inoculation of female *IL-17ra*$^{+/-}$ and *IL-17ra*$^{-/-}$ mice bred at NYU. *IL-17ra*$^{+/-}$ n=6, *IL-17ra*$^{-/-}$ n=6. **(G)** MRSA CFU in stool following oral inoculation of *Tcrd*$^{+/-}$ and *Tcrd*$^{-/-}$ mice bred at NYU. Male *Tcrd*$^{+/-}$ n=6, male *Tcrd*$^{-/-}$ n=6, female *Tcrd*$^{+/-}$ n=8, and female *Tcrd*$^{-/-}$ n=12. **(H)** Flow cytometry of cecal-colonic lamina propria Ly6G+CD11b+ neutrophils as a percentage

334 of CD45+ cells in male and female NYU mice treated with PBS or MRSA 2 dpi. **(I)** Quantification
335 of mean fluorescence intensity (MFI) of surface CD11b on neutrophils by flow cytometry
336 normalized to mock treated controls. **(J)** MRSA CFU in stool following oral inoculation of NYU
337 B6 mice treated with anti-Ly6G neutrophil depleting antibody or anti-IgG control. Male anti-IgG
338 n=6, male anti-Ly6G n=8, female anti-IgG n=12, and female anti-CD4+ n=15. Data points
339 represent mean ± SEM from at least two independent experiments. Statistical analysis: 2 way
340 ANOVA + Sidak's multiple comparisons test for (A)-(D) and (H), area under the curve followed
341 by a 1 way ANOVA + Sidak's multiple comparisons test for (E), (G), (J) or a two-tailed t test for
342 (F) and a two-tailed t test for (I). ns: not significant.
343

344 **Sex hormones, not sex chromosomes, mediate MRSA colonization resistance in female**
345 **mice**

346 Sex bias in immunity could be mediated by genes that are encoded on sex
347 chromosomes. Many X linked genes are involved in immunity, such as pattern recognition
348 receptors, and incomplete X inactivation can lead to higher expression of such genes[16]. To test
349 whether colonization resistance was due to sex chromosomes within a hematopoietic cell type,
350 we generated chimeras in which wildtype male mice were reconstituted with T-lymphocyte
351 depleted bone marrow (BM) from female donors and compared with male and female mice that
352 received BM from same sex donors. The reciprocal chimera in which female mice are
353 reconstituted with male BM was not feasible due to transplant rejection. Although female mice
354 that received female donor BM were resistant to MRSA, male mice that received male or female
355 donor BM remained colonized **(Fig6 A)**. Thus, it is unlikely that a gene expressed on a sex
356 chromosome in immune cells such as T cells mediates colonization resistance in females,
357 raising the possibility of a role for sex hormones in our model. The hormones estrogen,
358 progesterone, and testosterone are known to modulate trafficking and function of immune cells.
359 For example, estrogen can enhance responses to extracellular pathogens[21,62,63]. To test
360 whether sex hormones are involved in GI colonization resistance to MRSA, female mice at six
361 weeks of age were ovariectomized (OVX) or given a sham operation (SO) and allowed to
362 recover for two weeks prior to oral inoculation with MRSA. OVX mice had increased MRSA
363 burden and duration of carriage compared to SO controls **(Fig6 B)**. Estrogen receptor-α (ERα,
364 encoded by *Esr1*) signaling increases Th17 cell proliferation and production of IL-17[21,64]. We
365 confirmed *Esr1* was expressed in cecal-colonic immune cells, and that it displayed a non-
366 significant trend suggesting an increase upon MRSA inoculation **(Supplemental Fig6 A)**.

367 Female *Esr1*$^{-/-}$ mice had increased MRSA burden compared to heterozygous littermates **(Fig6**
368 **C)** indicating that estrogen mediates the enhanced colonization resistance in female mice.

369 To formally decouple the role of sex hormones and sex chromosome encoded gene
370 products, we used the mouse four core genotype (FCG) model in which the gene sex
371 determining region Y (*Sry*) that defines male sex has been moved from the Y chromosome to an
372 autosome[65]. Thus, the sex chromosome complement (XX or XY) does not relate to gonadal sex
373 in the FCG model. We found that XY*(-Sry)* gonadal female mice lose MRSA carriage analogous
374 to XX females, consistent with prior studies showing that they display similar estradiol levels[66,67],
375 and both XY and XX*(+Sry)* gonadal male mice remain persistently colonized at similar levels
376 **(Fig6 D)**. Like their wildtype counterparts, we do not observe a change in the proportions of
377 CD4+ T cells in the lamina propria between XX males and XX females with and without MRSA
378 **(Fig6 E)**. Instead, XX*(-Sry*) females displayed an increase in IL17A+ CD4+ T cells in the lamina
379 propria compared with XX(+*Sry*) males 2 dpi MRSA inoculation **(Fig6 F).** These findings further
380 support a hormone mediated effect on the Th17 cell response in female mice rather than a
381 chromosomal one.

382

383

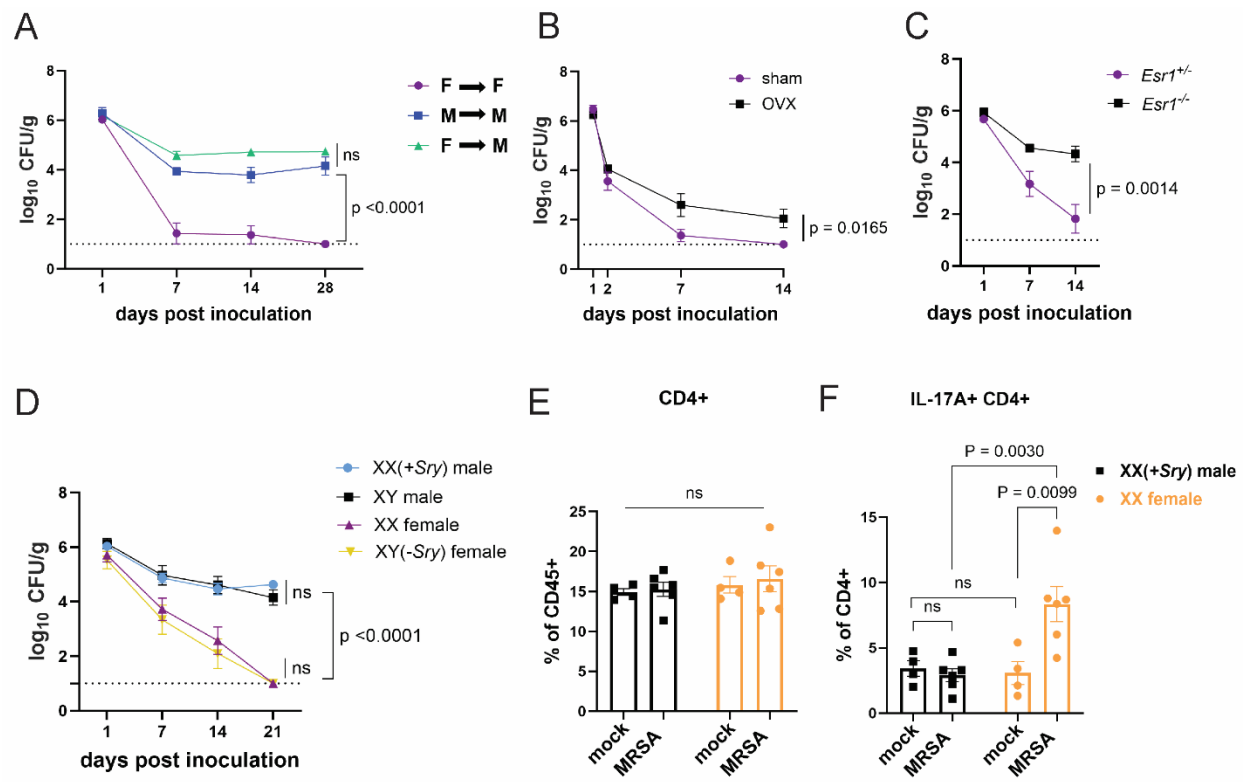

Fig 6. Sex hormones, not sex chromosomes, mediate MRSA colonization resistance in female mice.

(A) MRSA CFU in stool following oral inoculation of male or female mice that were irradiated and reconstituted with bone marrow (BM) from donor male or female mice. Female BM into female recipients (F --> F) n=8, male BM into male recipients (M--> M) n=7, female BM into male recipients (F--> M) n=8. **(B)** MRSA CFU in stool following oral inoculation of ovariectomized female mice or sham operated littermate controls. OVX n=10, sham n=10. **(C)** MRSA CFU in stool following oral inoculation of *Esr1*$^{+/-}$ and *Esr1*$^{-/-}$ female mice bred at NYU. *Esr1*$^{+/-}$ n=6, *Esr1*$^{-/-}$ n=6. **(D)** MRSA CFU in stool following oral inoculation of four core genotype mice. XX n=5, XY(-*Sry*) n=5. XY n=5, XX(+*Sry)* n=4. **(E)** CD4+ T cells from cecal-colonic lamina propria of XX females and XX(+*Sry*) males 2 dpi inoculation with MRSA or mock control. **(F)** Percentage of IL-17A+ CD4+ T cells in cecal-colonic lamina propria of XX females and XX(+*Sry*) males 2dpi inoculation with MRSA or mock control. Data points represent mean ± SEM from at least two independent experiments. Statistical analysis: area under the curve followed by a 1 way ANOVA with Sidak's multiple comparisons for (A),(C) and two-tailed t-test for (B),(F) and 2 way ANOVA + Sidak's multiple comparisons test for (D)-(E). ns: not significant.

402 **DISCUSSION**

403 *S. aureus* intestinal carriage is common and associated with infection, but how variables
404 such as sex contribute to colonization susceptibility have been obscure. We established a
405 mouse model to investigate MRSA intestinal colonization, which revealed a sex-specific effect in
406 which female mice rapidly cleared MRSA, while their male counterparts remained colonized.
407 This protection was microbiota dependent because mice lacking a microbiota or those with a
408 less diverse microbiota were susceptible to persistent colonization. Microbiota composition
409 alone, however, was insufficient to explain the sex dependent colonization resistance observed.
410 Females displayed an enhanced immune response to MRSA colonization characterized by
411 increases in Th17 cells and neutrophil activation. Increase in MRSA burden in ovariectomized or
412 ERα-deficient females and XX (+*Sry*) gonadal male mice indicate that this effect of the female
413 sex is hormonally mediated rather than dependent on genes present on sex chromosomes.
414 Collectively, our results support a model in which GI colonization resistance against MRSA in
415 female mice is dependent on the microbiota and an enhanced Th17 response downstream of
416 sex hormones.

417 Sex steroid hormones have well documented effects on the immune response, including
418 CD4+ T cells[21–23,68–70]. Our results are consistent with studies showing that ERα signaling
419 increases differentiation and cytokine production of Th1 and Th17 cells[21,71,72], although higher
420 levels of estrogen characteristic of pregnancy can stimulate immunosuppressive regulatory
421 CD4+ T cell conversion[73]. Recent work identified a parallel role for the male sex hormone
422 androgen in suppressing lymphoid and neutrophil responses during intradermal infection of
423 mice with *S. aureus*[22,23]. In this context, introduction of a complex microbiota into germ-free mice
424 amplified the skin type 17 sex bias in females[22]. Sex biases in neutrophil function, including
425 increased phagocytosis and extracellular trap formation in female mice have been
426 observed[14,62,74,75]. In line with our findings of increased CD11b on intestinal neutrophils isolated
427 from female mice exposed to MRSA, studies examining MRSA skin and soft tissue infections
428 found that bone marrow neutrophils from female mice have an enhanced ability to kill MRSA *ex*
429 *vivo* compared to those from male mice, which was linked to increases in surface CD11b and
430 antimicrobial production[14,75].

431 Although sex difference was not reported, colonization resistance against *S. aureus* in
432 the nares is also associated with Th17 cells and neutrophils[51,52], supporting the general
433 importance of the CD4+ T cell response over antibodies and B cells in determining susceptibility
434 to colonization. The absence of a sterilizing B cell response may reflect immune evasion

435 strategies such as the bacterially produced Protein A that binds to the Fcγ portion of
436 immunoglobulins, protecting *S. aureus* from opsonophagocytic killing[76,77]. Our finding that
437 female mice lacking mature B cells were still able to resist MRSA GI colonization is mirrored by
438 a human volunteer study demonstrating that antibodies prior to intranasal inoculation did not
439 block persistent colonization by their cognate *S. aureus* strains and that antibody levels did not
440 distinguish intermittent and non-carriers[78]. In contrast, low CD4+ T cell counts in HIV+
441 individuals are a risk factor for *S. aureus* nasal colonization[79], and rates of colonization were
442 higher in HIV+ males compared to HIV+ females[80]. Understanding the cellular response to
443 MRSA colonization and the impact of host sex may inform vaccination strategies[81].

444 We observed an increase in IL-17A+ CD4+ T cells in colonization resistant females at
445 two days post inoculation with MRSA, a duration that is typically insufficient for a *de novo*
446 antigen-specific adaptive immune response. It is likely that the gut harbors pre-existing T cells
447 that can rapidly respond. The microbiota-dependence of colonization resistance provides a
448 potential explanation for this observation. Superantigens from *S. aureus* bind directly to Vβ
449 regions of T cell receptors (TCRs) and major histocompatibility complex (MHC) class II on
450 antigen-presenting cells, resulting in hyperactivation of T lymphocytes and
451 monocytes/macrophages. *S. aureus* superantigens induce a robust IL-17 response from
452 memory Th17 cells from adult humans but not from naive T cells[82], raising the possibility that
453 such a mechanism can act on Th17 cells selected by the microbiota. Also, CD4+ T cells in the
454 gut, including Th17 cells, can recognize antigens that are shared by taxonomically diverse
455 bacteria in the gut[53]. In the colon of healthy humans and mice, MHC-II restricted CD4+ T cells
456 with Th17 functionality have been identified that are responsive to commensal microbial
457 antigens in an innate-like manner[83]. Additionally, intestinal colonization by the fungus *Candida*
458 *albicans* favors Th17 polarization associated with increased IL-17 and neutrophils that protect
459 mice from intravenous infection with MRSA[84,85]. Thus, it is possible that we are observing either
460 a cross reactive Th17 response or a bystander effect initiated by a commensal.

461 Investigating the antigen specific response to MRSA is challenging due to bacterially-
462 encoded superantigens and other secreted virulence factors, such as α-hemolysin and LukED,
463 that induce cell death of lymphocytes[86]. Therefore, the relationship between the microbiota and
464 sex-dependent colonization resistance remains unanswered in our model. Other possibilities
465 include microbiota-mediated regulation of estrogen and its receptor or signaling. However, given
466 the strong connection between microbiota and Th17[33,87], we propose a speculative model in
467 which initial microbiota differences prime the gut for a Th17 and neutrophil response to MRSA

468 that is enhanced by estrogen. Identifying the microbe(s) that are necessary for colonization
469 resistance in females and careful examination of the dynamic regulation of sex hormones in this
470 model will be insightful.

471 Animal models that incorporate sex as a variable are critical to conduct rigorous,
472 translational science and build towards personalized medicine. Our study reveals an interplay
473 between host microbiota, immune response, and sex steroid hormones in response to intestinal
474 exposure to MRSA, a common commensal and major source of life-threatening invasive
475 infections. Given the importance of the gut as a site of colonization and transmission for many
476 medically important infectious agents, we suggest careful documentation of sex specific effects
477 in future studies examining this host-microbe interface.

478

479 **MATERIALS AND METHODS**

480 **Mice**

481 Mice designated as JAX refer to male and female 6- to 8-week-old C57BL/6J mice
482 purchased from Jackson Laboratory and used directly for experiments. NYU C57BL/6J breeders
483 were originally purchased from Jackson Laboratory and bred onsite at New York University
484 Grossman School of Medicine to generate littermate male and female mice for comparison.
485 Every six months breeders were replaced by using a new male from Jackson Laboratory to pair
486 with a NYU female to reduce genetic drift. *Rag2*$^{-/-}$, *Tcrd*$^{-/-}$, *Igh-μ*$^{-/-}$, *Ptprc*^{a} *(B6 CD45.1),* and
487 *Esr1*$^{-/-}$ mice were purchased from Jackson Laboratory. *Rorc(γt)–enhanced GFP* (*Roryt*$^{-/-}$) mice
488 were previously described[85]. *Il17ra*$^{-/-}$ mice were generated by Amgen Inc. (Seattle, WA) and
489 provided by Dr. Jeffrey Weiser. Littermate heterozygous controls for *Rag2*$^{-/-}$, *Igh-μ*$^{-/-}$, and
490 *Roryt*$^{-/-}$ mice were generated by initially crossing homozygous knockout mice with wild-type
491 C57BL/6J mice to establish heterozygotes that were then used to generate homozygous and
492 heterozygous breeder pairs.

493 Germfree (GF) C57BL/6J were bred and maintained in flexible-film isolators at the New
494 York University Grossman School of Medicine Gnotobiotics Animal Facility[88–90]. Absence of
495 fecal bacteria was confirmed monthly by evaluating the presence of 16S rDNA in stool samples
496 by qPCR. Minimal flora mice harboring the consortium of 15 bacteria (Oligo-MM_{12} + FA3)[39] were
497 maintained in a separate isolator as previously described[88,89]. For inoculation with bacteria, GF

498 mice and minimal flora mice were housed in Bioexclusion cages (Tecniplast) with access to
499 sterile food and water.

500 The sample size for animal experiments was chosen based on previous data generated
501 in the laboratory. All animal studies were performed according to protocols approved by the
502 NYU Grossman School of Medicine Institutional Animal Care and Use Committee

503 **MRSA intestinal colonization in mice**

504 Prior to inoculation, stool from C57BL/6J mice was homogenized and plated on
505 CHROMagar *Staph aureus* (CHROMagar, Paris, France) selective plates to ensure mice were
506 free of *S. aureus* carriage. C57BL/6J mice were orally gavaged with ~1×10^8 colony-forming
507 units (CFU) of CA-MRSA strain USA300 (LAC). Stool samples were collected from each mouse
508 on indicated days post inoculation. Screw-cap tubes (2 mL) filled with 1.0 mm beads were
509 weighed before and after the addition of stool to determine weight. Sterile phosphate-buffered
510 saline (PBS) (1 mL) was added to each tube, which were vigorously shaken in a bead-beater
511 (MP Biomedicals, Santa Ana, CA) for 60 s. Stool aliquots were diluted and plated to enumerate
512 viable bacteria. Samples were plated on CHROMagar MRSA II (BBL) /Mannitol salt agar plates,
513 incubated at 37ºC for 24 hours, and colonies were counted to determine MRSA burden per
514 gram of stool.

515 **Intestinal lamina propria and epithelial cell isolation**

516 Colonic and cecal tissues were flushed with HBSS (Gibco), fat and Peyer's patches
517 were removed, and the tissue was cut into 6-8 pieces. Tissue bits were incubated first with 20
518 mL of HBSS with 2% HEPES (Corning), 1% sodium pyruvate (Corning), 5mM EDTA, and 1 mM
519 dithiothreitol (DTT) (Sigma-Aldrich) for 15 min at 37°C with shaking, and then with new 10 mL of
520 HBSS with 2% HEPES, 1% sodium pyruvate, 5mM EDTA for 10 min at 37°C with shaking. The
521 samples were filtered by 40 µm cell strainer (BD) and the supernatant containing intestinal
522 epithelial cells was collected and subjected to gradient centrifugation using 40% and 80%
523 Percoll (Sigma-Aldrich). The upper layer containing epithelial cells between the 40% and 80%
524 gradients was collected. Tissue bits were washed in HBSS + 5% FCS, minced, and then
525 enzymatically digested with collagenase D (0.5 mg/mL, Roche) and DNase I (0.01 mg/mL,
526 Sigma-Aldrich) for 30 min at 37°C with shaking. Digested solutions were passed through a 40
527 mm cell strainer and cells were subjected to gradient centrifugation using 40% and 80% Percoll
528 (Sigma-Aldrich). The lower layer containing immune cells between the 40% and 80% gradients
529 was collected.

530 **Flow cytometry**

531 Lamina propria cells from either cecal and colonic or small intestinal tissue were
532 harvested as described. For intracellular cytokine staining, cells were stimulated using the
533 eBioscience cell stimulation cocktail for 4 h at 37 °C. The cells were fixed and permeabilized
534 using the Biolegend fixation and permeabilization buffer. The following antibodies (clones) were
535 used for staining: CD45 (30-F11), Ly6G (1A8), TCR-β (H57-597), CD4 (GK1.5), CD8 (53–6.7),
536 IL-17A (TC11-18H10.1), TCR γ/δ (GL3), CD127(SB/199), and CD11b (M1/70). All samples were
537 blocked with Fc Block (TruStain FcX). Zombie UV Fixable Viability Kit (Biolegend) was used to
538 exclude dead cells prior to gating for other markers. Samples were run on BD FACS Symphony
539 A5 and FACsFlowJo v.10 was used to analyze the flow cytometry data.

540 **Antibody mediated depletion experiments**

541 C57BL/6J mice bred at NYU were injected intraperitoneally (I.P.) with either 200 µg rat
542 anti-mouse Ly6G or rat IgG2a isotype control antibody to deplete neutrophils and 250 µg rat
543 anti-mouse CD4 or rat IgG2a isotype control antibody to deplete CD4+ T cells (Bio-X-Cell, West
544 Lebanon, NH). Anti-Ly6G injections occurred 1 day prior to MRSA inoculation and then every 3
545 days until mice were sacrificed 14 days post inoculation. Anti-CD4 injections occured 3 days
546 prior to inoculation and every 7 days after initial injection until mice were sacrificed 14 days post
547 inoculation.

548 **Bone marrow chimera experiments**

549 Eight-week-old recipient CD45.1 congenic C57BL/6J mice bred at NYU received 550
550 rads in 2 doses over 2 sequential days and were injected retro-orbitally with 2×10^6 T
551 lymphocyte-depleted bone marrow cells from either female or male CD45.2 congenic C57BL/6J
552 donors bred at NYU. Mature T lymphocytes were depleted from bone marrow cell suspension
553 using the CD3ε MicroBead Kit (Miltenyi Biotech). Mice were allowed 8 weeks for reconstitution
554 before oral inoculation with 1×10^8 CFU MRSA. Bone marrow reconstitution of the CD45+
555 compartment in CD45.1 mice was confirmed by flow cytometry analysis of CD45.2+ cells in the
556 bone marrow at the time of sacrifice.

557 **Ovariectomy surgery**

558 6-week-old female mice bred at NYU were anesthetized with isoflurane and placed in
559 ventral recumbency with tail towards surgeon. Ophthalmic ointment was applied bilaterally and
560 heat support was provided throughout the procedure. The dorsal mid-lumbar area was shaved

561 in an area 150% greater than anticipated incision length and swabbed three times with
562 alternating scrubs of betadine and alcohol. A 1-1.5 cm dorsal midline skin incision was made
563 halfway between the caudal edge of the ribcage and the base of the tail. A single incision of 5-
564 7mm long was made into the muscle wall on both the right and left sides approximately 1/3 of
565 the distance between the spinal cord and the ventral midline. The ovary and the oviduct were
566 exteriorized through the muscle wall. A hemostat was clamped around the uterine vasculature
567 between the oviduct and uterus. Each ovary and part of the oviduct was removed with single
568 cuts through the oviducts near the ovary. The hemostat was removed and the remaining oviduct
569 was assessed for hemorrhage. Hemostasis was confirmed prior to placing the remaining tissue
570 into the peritoneal cavity. The ovary on the other side was removed in a similar manner. The
571 muscle incision was closed with monofilament absorbable suture in a cruciate pattern.
572 Bupivacaine was applied on the closed muscle layer. The skin incision was closed in a cruciate
573 pattern with sterile skin sutures (monofilament nonabsorbable) and a small amount of tissue
574 glue. The skin sutures were removed 10 days after surgery. Sham surgery mice underwent the
575 same surgery but did not have their ovaries removed. Mice were administered Carprofen SQ
576 every 24 hours for 3 days following the procedure. Mice were allowed two weeks to fully recover
577 before oral inoculation with $1x10^8$ CFU of MRSA.

578 **RNA-sequencing**

579 RNA was extracted from lamina propria cells and epithelial fraction isolated from the
580 cecum-colon using RNeasy Plus Mini Kit (Qiagen). Four male and four female B6 mice bred at
581 NYU were used for each experimental condition. An RNA library was prepared using the
582 Illumina TruSeq RNA sample preparation kit and sequenced with the Illumina HiSeq 2000 using
583 the TruSeq RNA v.2 protocol. Illumina CASAVA v.1.8.2 was used to generate FASTQ files
584 containing 29.5–53.5 million qualified reads per sample. Alignment and gene expression count
585 were computed using default settings, which aligns reads to the union of all RefSeq-annotated
586 exons for each gene.

587 RNA-seq results were processed using the v.4 R package DESeq2 v.3 to obtain
588 variance stabilized count reads, fold changes relative to specific condition, and statistical P
589 value. Analysis of the transcriptome focused on differentially expressed genes (DEGs), defined
590 as the genes with an absolute log2 fold change relative to specific condition >1.2 and an
591 adjusted P value < 0.05. Enriched pathways for the DEGs were analyzed by Ingenuity Pathway
592 Analysis (Qiagen). The analyses were visualized using R package ggplot2[91].

593 **DNA extraction from stool**

594 DNA were extracted from stool using the MagMAX™ Microbiome Ultra Nucleic Acid
595 Isolation Kit (Thermo Fischer) following the manufacturer's instructions with modifications. An
596 initial bead-beating step using differentially sized beads (glass beads, 0.5–0.75 mm; zirconia
597 beads, <100 μm) was included and lysozyme (20 mg mL–1) was added to the lysis buffer.

598 **16S library preparation and sequencing analysis**

599 Bacterial 16S rRNA genes were amplified at the V4 region using 16S universal primer
600 pairs and amplicon sequencing was performed on the Illumina MiSeq system, yielding 150-bp
601 paired reads.
602 16S Amplicon PCR Forward Primer = 5'
603 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG,
604 16S Amplicon PCR Reverse Primer = 5'
605 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC
606 The sequencing reads were processed using the DADA2 v.1 pipeline in the QIIME2 v.2022.2
607 software package. The cloud based platform Nephele v.2 was used to run QIIME2 analysis[92].
608 The minimum Phred quality score of 20 was applied to ensure high-quality sequence data. An
609 open-reference clustering algorithm was used to identify operational taxonomic units (OTUs)
610 based on a 97% similarity threshold to a reference database. Chimera removal was conducted
611 to eliminate artifacts in the data. Taxonomic classification was performed using a search-based
612 method to assign taxonomy to the OTUs. Barplots were generated with a minimum frequency
613 filter of 1000 to visualize the abundance of microbial taxa. A percentage identity threshold of
614 97% was used to assign taxonomy at the genus level using the SILVA rRNA database. Principal
615 coordinates analysis (PCoA) and calculation of Shannon index were performed using the
616 phyloseq R package[93] version 1.46 after rarefaction to a depth of 1000.

617 **Statistical analysis**

618 The number of animals per group is annotated in corresponding figure legends.
619 GraphPad Prism v.10 was used to generate graphs and assess significance for bacterial
620 burden, weight loss, and flow cytometry data were analyzed using FlowJo v.10. The MRSA
621 burden curves were analyzed using area under the curve followed by a 1 way ANOVA with
622 Sidak's multiple comparisons test when comparing more than 2 experimental conditions and a
623 two-tailed t-test when comparing 2 experimental conditions. An unpaired two-tailed Student's t-
624 test was used to evaluate the differences between two groups. Welch's correction was used

625 when variances were significantly different between groups. A 2-way ANOVA with Sidak's
626 multiple comparisons test was used to evaluate experiments involving multiple groups. All P
627 values are shown in the figures.

628 **DATA AVAILABILITY**

629 The sequencing accession number for the bulk RNA sequencing is PRJNA1134782 and
630 the accession number for the 16S rRNA sequencing is PRJNA1135964.

631

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645

646 **COMPETING INTERESTS**

647 KC has received research support from Pfizer, Takeda, Pacific Biosciences, Genentech, and
648 Abbvie. KC has consulted for or received an honoraria from Puretech Health, Genentech, and
649 Abbvie. KC is an inventor on U.S. patent 10,722,600 and provisional patent 62/935,035 and
650 63/157,225. V.J.T. has received honoraria from Pfizer and MedImmune and is an inventor on
651 patents and patent applications (US8431,687B2; US2019135900-A1; EP4313303A1) filed by
652 New York University, which are currently under commercial license to Janssen Biotech Inc.
653 Janssen Biotech Inc. provides research funding and other payments associated with a licensing
654 agreement.

655

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