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.

RESULTS

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

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

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

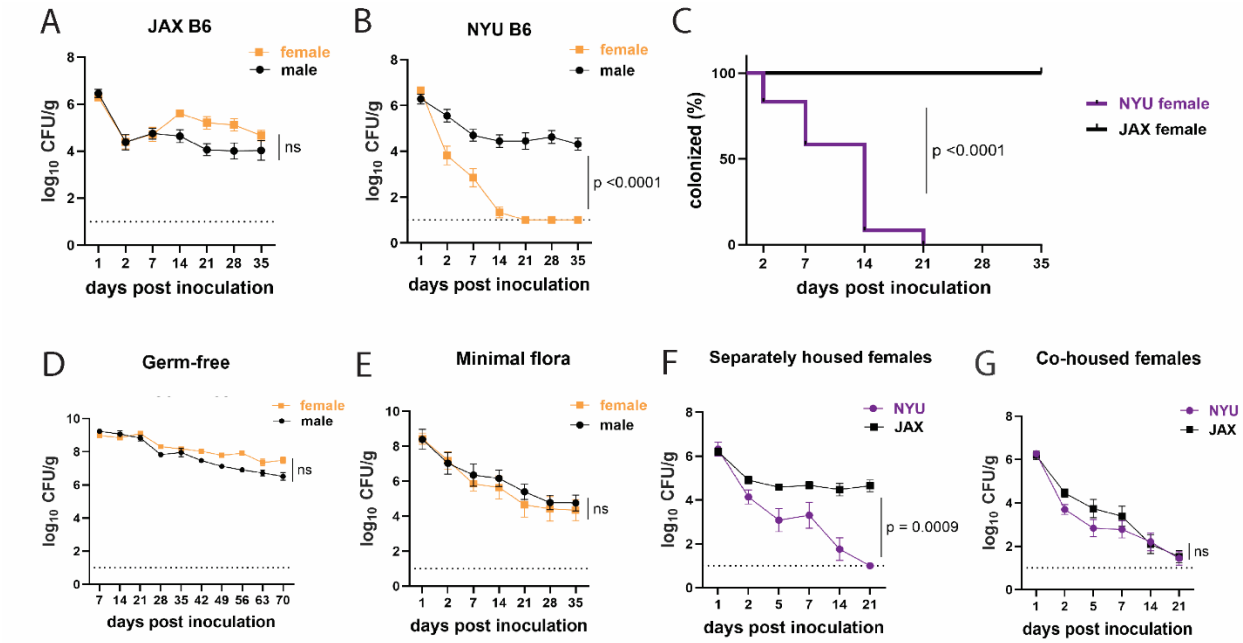

Fig.1 Female mice are protected from MRSA gastrointestinal colonization in a microbiota dependent manner.

(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

two independent experiments. Statistical analysis: area under the curve followed by a two-tailed t-test for (A), (B), (D)-(G) and Log-rank Mantel Cox test for (C). ns: not significant.

Microbiota is not sufficient to explain sex bias in MRSA GI colonization.

To compare the microbiome composition of male and female NYU and JAX mice, we performed 16S ribosomal DNA sequencing of stool collected prior to inoculation with MRSA. Principal Coordinates Analysis (PCoA) of operational taxonomic units (OTUs) showed that samples were distinguished based on the source of mice (NYU versus JAX) rather than sex **(Fig2 A)**. Overall alpha diversity as measured by Shannon index was increased in JAX mice compared to NYU mice prior to MRSA inoculation, as well as in JAX females compared to NYU females **(Fig2 B)**. Alpha diversity by Shannon index between NYU males and females shows a similar spread when comparing sexes **(Fig2 B)**. JAX females had higher abundance of *Clostridiaceae* and *Lachnospiraceae* family members, while NYU female microbiomes were dominated by the *Muribaculaceae* family **(Fig2 C)**. The NYU male and female microbiomes clustered together **(Fig2 A)** and had similar relative taxonomic abundances **(Fig2 D).** These findings suggest that the observed sex bias in colonization resistance may not be due to microbiome composition alone.

As male and female mice are housed separately post weaning, and weaning is associated with microbiome changes[41], we tested if the colonization resistance displayed by female NYU mice could be transferred to male NYU mice. Instead of co-housing male and female mice, which could introduce variables through social stress or changes in hormone levels due to mating,[42] we performed fecal microbiota transplantations (FMT) by repetitively gavaging recipient mice with donor stool. JAX female mice receiving a FMT from NYU female, but not male donor mice, displayed colonization resistance to MRSA. However, an FMT using NYU female donor stool was unable to confer colonization resistance to JAX male recipients **(Fig2 E)**. Therefore, the microbiota contributes to the difference between female JAX and NYU mice, but other factors mediate the divergence of female and male NYU mice. From here on, we use NYU bred mice to investigate host factors that mediate this sex specific colonization resistance.

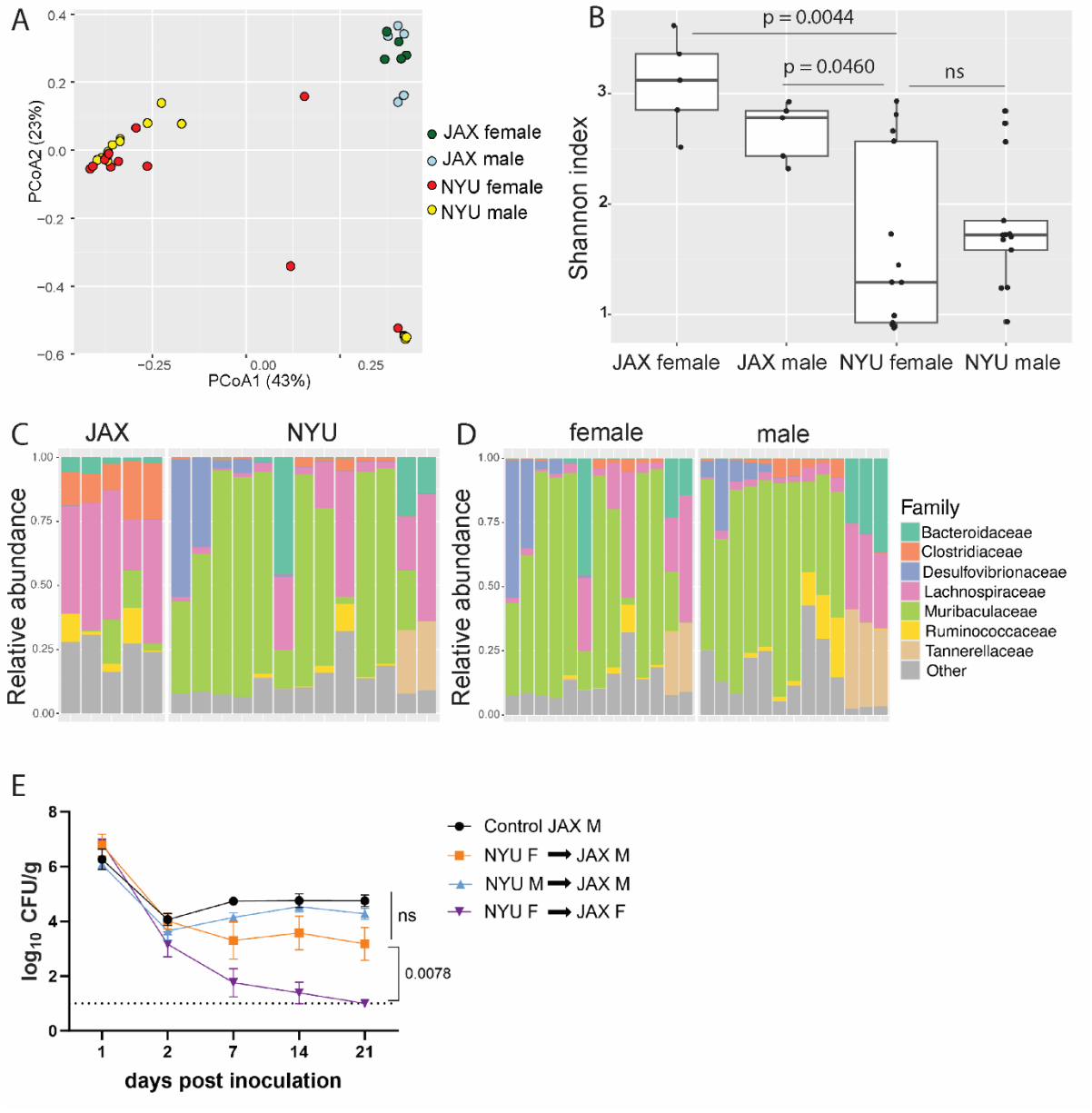

Fig. 2 Microbiome is not sufficient to explain sex bias in MRSA GI colonization. **(A)** Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances of 16S sequences obtained from stool of JAX and NYU male and female mice prior to MRSA inoculation. Proportion of variance explained by each axes 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

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

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

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

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

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

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

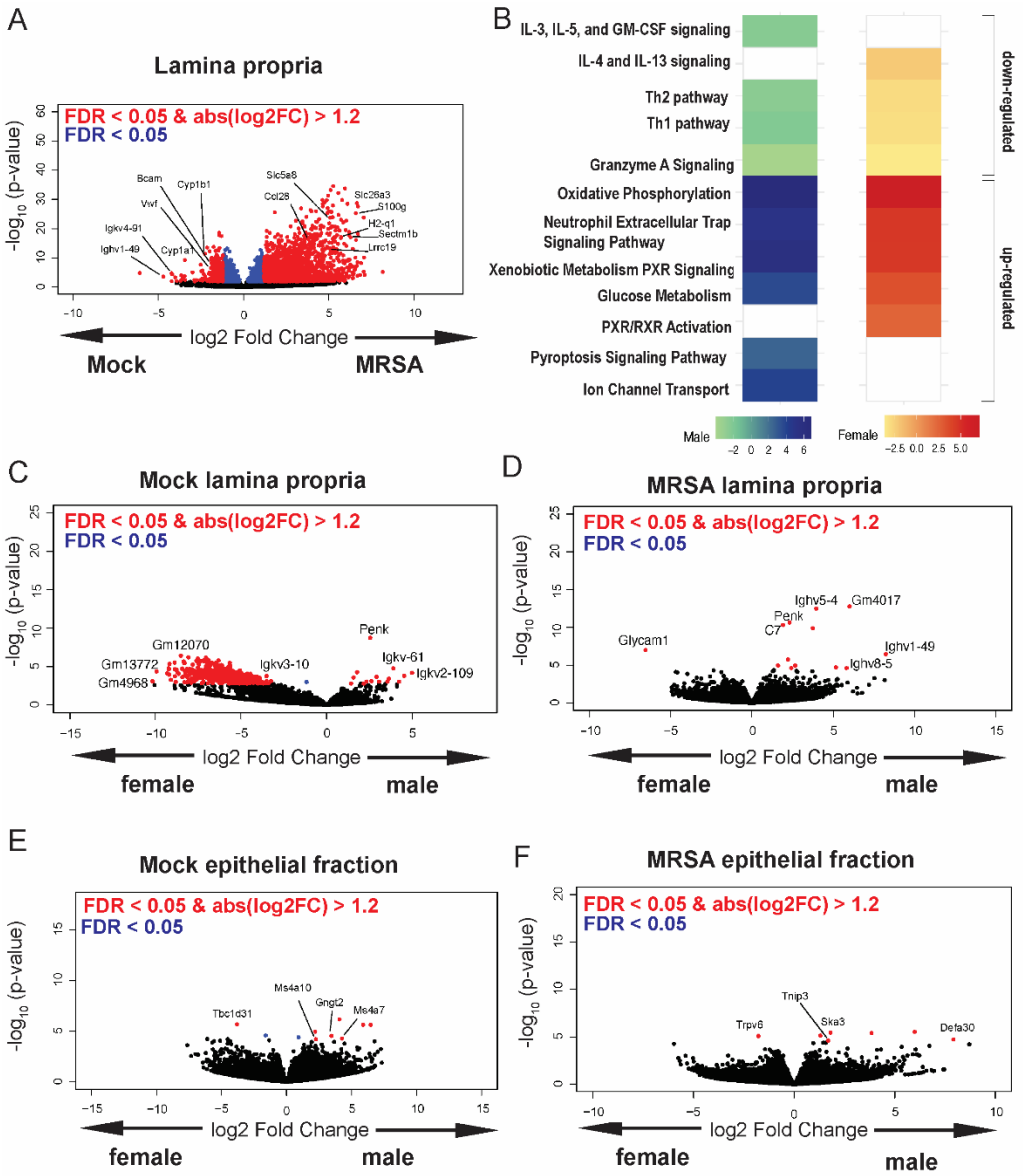

Fig 3. MRSA GI colonization elicits sex-dependent gene expression changes in the gut. **(A)** Volcano plot of differentially expressed genes identified by RNA-seq analyses of the intestinal lamina propria of NYU mice 2 dpi with MRSA compared with PBS mock inoculated NYU mice. **(B)** Ingenuity Pathway 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)**

Volcano plot of differentially expressed genes in the intestinal epithelial fraction of males and females 2 dpi with MRSA. Four mice were used for each sequencing experimental condition. Genes shown in red have a false discovery rate (FDR) of <0.05 and an absolute log2 fold change (abslog2FC) of >1.2. Genes shown in blue have a FDR of <0.05. Genes linked to X and Y chromosomes were removed from volcano plots.

CD4+ T cells mediate colonization resistance in female mice.

Female mice have been shown to be protected from dermonecrosis in a model of invasive MRSA skin infection due to reduced expression of genes associated with the NLRP3 inflammasome (*Nlrp3* and *Il-1β*) compared to males[14]. However, we did not observe sex-specific differences in NLRP3-associated transcripts with RNA-seq **(Supplemental Fig3 A,B)** and female *Nlrp3*$^{-/-}$ mice displayed similar resistance to MRSA GI colonization as female *Nlrp3*$^{+/-}$ controls **(Supplemental Fig3 C)**. Thus, the mechanism of sex specific resistance to MRSA colonization of the GI tract may be different from that observed during a skin infection where barrier breach triggers an exaggerated innate immune response that may cause more harm than benefit.

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

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

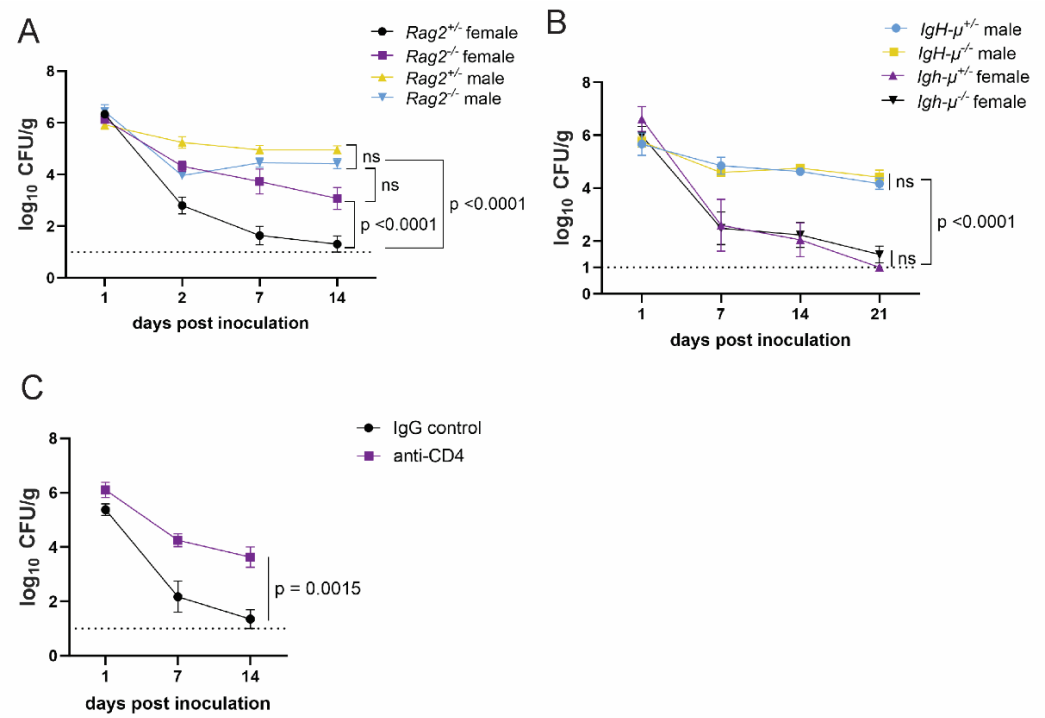

Fig 4. CD4+ T cells mediate colonization resistance in female mice.
(A) MRSA CFU in stool following oral inoculation of *Rag2*$^{-/-}$ and *Rag2*$^{+/-}$ mice bred at NYU. Male *Rag2*$^{-/-}$ n=12, male *Rag2*$^{+/-}$ n=12, female *Rag2*$^{-/-}$ n=12, female *Rag2*$^{+/-}$ n=11. **(B)** MRSA CFU in stool 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

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

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

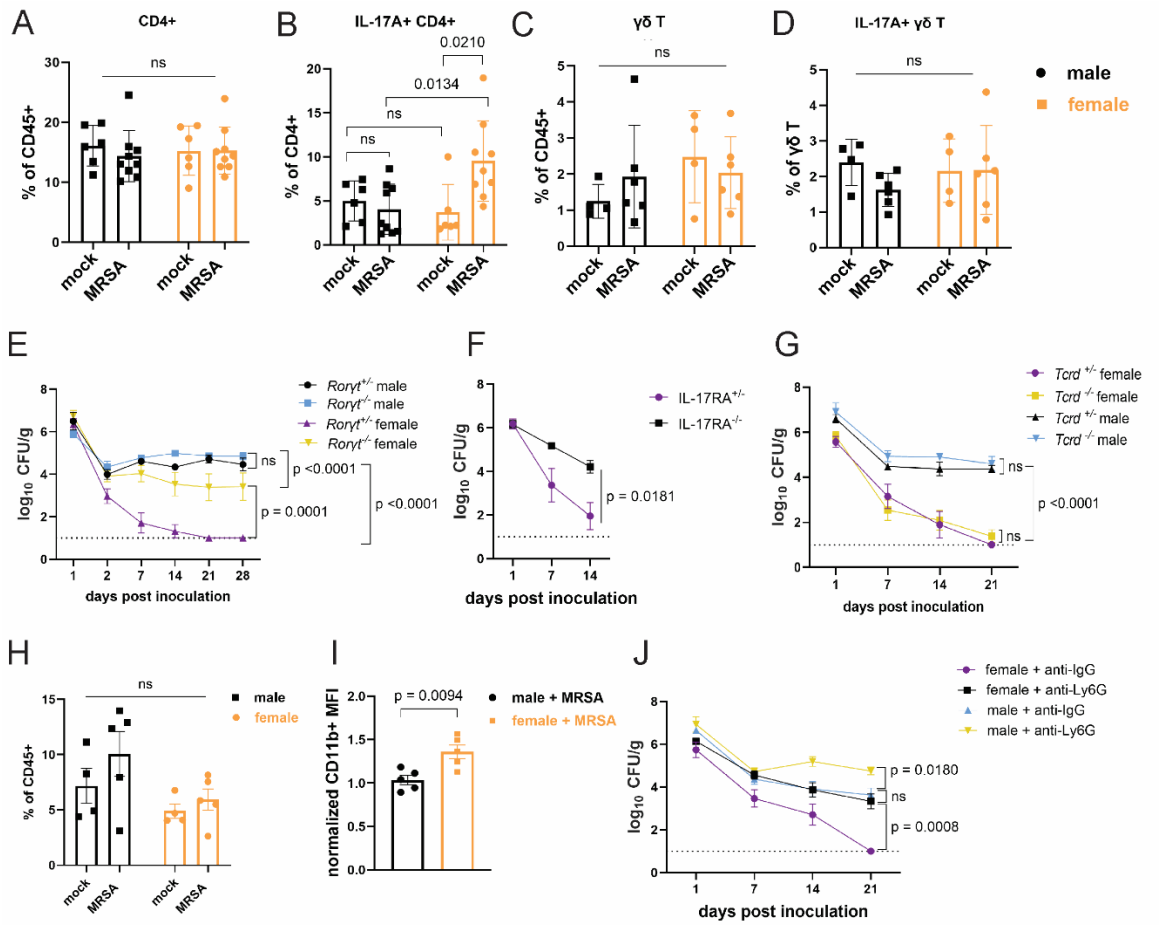

Fig 5. Female mice have increased IL-17A+ CD4+ T cells and require neutrophils for MRSA clearance.

(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

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

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

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

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

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

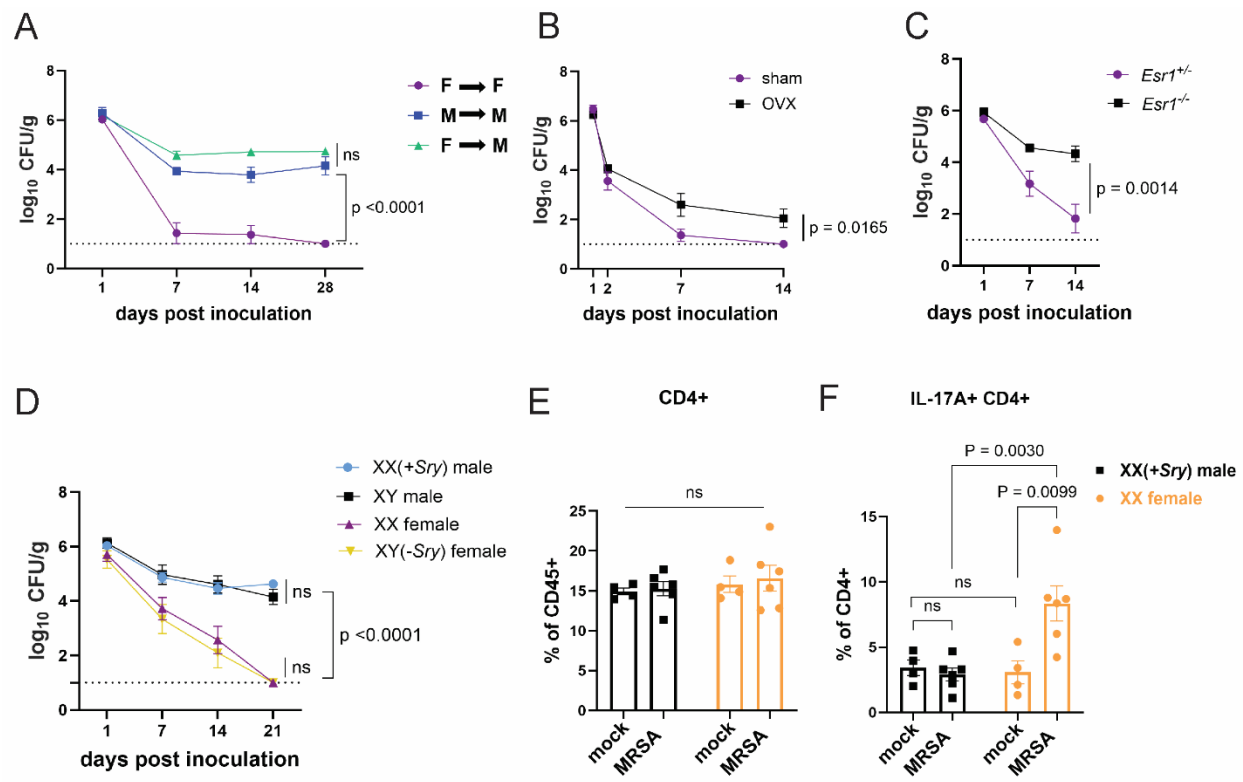

Fig 6. Sex hormones, not sex chromosomes, mediate MRSA colonization resistance in female mice.

(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.

DISCUSSION

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Mice

Mice designated as JAX refer to male and female 6- to 8-week-old C57BL/6J mice purchased from Jackson Laboratory and used directly for experiments. NYU C57BL/6J breeders were originally purchased from Jackson Laboratory and bred onsite at New York University Grossman School of Medicine to generate littermate male and female mice for comparison. Every six months breeders were replaced by using a new male from Jackson Laboratory to pair with a NYU female to reduce genetic drift. *Rag2*$^{-/-}$, *Tcrd*$^{-/-}$, *Igh-μ*$^{-/-}$, *Ptprc*^{a} *(B6 CD45.1),* and *Esr1*$^{tm1Ksk+/-}$ mice were purchased from Jackson Laboratory. *Rorc(γt)–enhanced GFP* (*Roryt*$^{-/-}$) mice were previously described[85]. *Il17ra*$^{-/-}$ mice were generated by Amgen Inc. (Seattle, WA) and provided by Dr. Jeffrey Weiser. Littermate heterozygous controls for *Rag2*$^{-/-}$, *Igh-μ*$^{-/-}$, and *Roryt*$^{-/-}$ mice were generated by initially crossing homozygous knockout mice with wild-type C57BL/6J mice to establish heterozygotes that were then used to generate homozygous and heterozygous breeder pairs.

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

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

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

MRSA intestinal colonization in mice

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

Intestinal lamina propria and epithelial cell isolation

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

Flow cytometry

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

Antibody mediated depletion experiments

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

Bone marrow chimera experiments

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

Ovariectomy surgery

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

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

RNA-sequencing

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

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

DNA extraction from stool

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

16S library preparation and sequencing analysis

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

Statistical analysis

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

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

DATA AVAILABILITY

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

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COMPETING INTERESTS

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

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