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Methicillin-resistant *Staphylococcus aureus* causes sustained collecting lymphatic vessel dysfunction

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of morbidity and mortality worldwide and is a frequent cause of skin and soft tissue infections (SSTIs). Lymphedema—fluid accumulation in tissue caused by impaired lymphatic vessel function—is a strong risk factor for SSTIs. SSTIs also frequently recur in patients and sometimes lead to acquired lymphedema. However, the mechanism of how SSTIs can be both the consequence and cause of lymphatic vessel dysfunction is not known. Intravital imaging in mice revealed both an acute reduction in lymphatic vessel contractility and lymph flow after localized MRSA infection. Moreover, chronic lymphatic impairment is observed long after MRSA is cleared and inflammation is resolved. Associated with decreased collecting lymphatic vessel function was the loss and disorganization of lymphatic muscle cells (LMCs), which are critical for lymphatic contraction. *In vitro*, incubation of MRSA-conditioned supernatant led to LMC death. Proteomic analysis identified several accessory gene regulator (*agr*)-controlled MRSA exotoxins that contribute to LMC death. Infection with *agr* mutant MRSA resulted in sustained lymphatic function compared to animals infected with wild type MRSA. Our findings suggest that *agr* is a promising target to preserve lymphatic vessel function and promote immunity during SSTIs.

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Introduction

Skin and soft tissue infections (SSTIs) are caused by microbial invasion of the epidermis, for which there are 14 million out-patient visits annually in the United States (1). Patients are generally treated with oral and topical antibiotics. However, complicated infections may additionally require intravenous antibiotics, leading to nearly 500,000 hospital admissions each year in the US (2, 3). The most common pathogens associated with bacterial infections of the skin and underlying tissues are beta-hemolytic *Streptococci* and *Staphylococcus aureus*, including strains of methicillin-resistant *Staphylococcus aureus* (MRSA) (4). The resistance of MRSA to antibiotics, along with an increasing incidence of infections, has become a growing public health concern for individuals in the community and hospital settings (5), and underscores the need for treatments that target both MRSA colonization and its pathogenesis (6). These infections can often recur and are frequent in patients with impaired lymphatic function, including those with lymphedema (7, 8). Further SSTIs can also lead to secondary lymphedema (7), suggesting that SSTIs can be both the consequence and cause of impaired lymphatic function. However, little is known about how bacteria interact with lymphatic vessels. Understanding this relationship will be critical to developing future therapies and vaccination strategies that maintain lymph flow to improve clearance of MRSA and toxins as well as promote antigen delivery to the lymph node for robust immune responses.

The lymphatic vasculature consists of a network of vessels critical for maintenance of tissue homeostasis, fluid balance, immune function, absorption of dietary fat and lipid transport (9). Disruption or malfunction of lymphatic vessels can result in lymphedema, presented as local fluid retention and tissue swelling (10). Primary lymphedema has a genetic etiology that leads to lymphatic insufficiency (11). In contrast, secondary lymphedema is more common and is triggered by obstruction or damage to lymphatic vessels caused by iatrogenic damage, inflammation or infection—either bacterial or parasitic (12). Individuals with lymphedema are prone to develop SSTIs, both erysipelas and underlying tissue cellulitis (13).

Lymph flow is controlled by a combination of factors that propel lymph to the lymph nodes for immune surveillance and filtration (14). Primarily, the organized lymphatic muscle cells (LMCs) that cover collecting lymphatic vessels (CLVs) periodically contract to drive lymph forward (15). Calcium (Ca^{2+}) signaling in LMCs drives contraction in response to chemical and mechanical stimuli such as endothelium-derived factors and lymph flow, respectively (16, 17). Nitric oxide, a vasodilator produced by the endothelium in normal and inflammatory conditions, has been shown to have a context-dependent effect on lymphatic contraction. In normal physiology, loss of nitric oxide has been shown to strengthen, blunt, or have no effect on lymphatic contraction (18–20). Perturbations in either Ca^{2+} or nitric oxide signaling, such as blocking Ca^{2+} channels, increasing nitric oxide production or causing sterile inflammation, have been shown to significantly compromise lymphatic contraction (18, 19, 21, 22). Here, we present a murine infection model to determine whether MRSA infections inhibit lymphatic vessel function and to define the molecular mechanisms related to this response.

Results

Acute inhibition of lymphatic function in a localized MRSA infection model

To examine a potential relationship between MRSA infection and lymphatic function, we established a MRSA infection model of the mouse hindlimb, a location analogous to the commonly affected lower extremities in human SSTIs (23). Neutrophils infiltrated the hindlimb and draining lymph node (fig. S1, A to D) within 24 hours post-infection. Lymphangiography revealed dilated afferent lymphatic vessels to the popliteal lymph node, which we designate here as PLVs, compared to PLVs of uninfected mice (fig. S1, A and B, fig. S1, A and E).

Since dilated lymphatic vessels are associated with lymphatic dysfunction (24), we measured the ability of PLVs to contract using intravital fluorescence microscopy (Fig. 1A) (25). MRSA infection impaired lymphatic contraction compared to mock-infected animals (Fig. 1, A and B, and Videos S1 and S2). The strength of PLV contraction, as measured by ejection fraction—the theoretical fractional volume of lymph expelled with each vessel contraction—declined significantly after infection (Fig. 1C) ($p < 0.05$). PLVs from infected animals also showed a significant decrease in phasic contraction frequency compared to uninfected controls (Fig. 1D) ($p < 0.05$).

To determine if lymph flow inside PLVs was also decreased, we employed a recently developed label-free method to identify PLVs (Fig. 1E) and directly measure instantaneous total lymph velocity (basal and pulsatile flow) *in vivo* by Doppler optical coherence tomography (DOCT) (26). In uninfected mice, 90% of the lymph velocity measurements showed distinct peaks in flow in the PLV (Fig. 1E). Four days after infection, only 33% of the lymph velocity measurements were pulsatile, with others showing constant, yet reduced lymph velocity compared to uninfected animals (Fig. 1, E and F). Taken together, mice infected with MRSA demonstrated significant inhibition of both lymphatic vessel contraction and lymph velocity during active infection ($p < 0.05$).

Chronic inhibition of lymphatic function after clearance of MRSA and resolution of inflammation

Subcutaneous infection of mice with MRSA led to a transient infection, with the peak of bacterial burden in the infected skin and underlying muscle, as well as in the lymph node, at 4 days post-infection (Fig. 2A). Consistent with previous results for subcutaneous MRSA infections in mice, MRSA in skin (27) and ipsilateral popliteal lymph node (PLN) was not detected by colony formation assays 30 days post-infection (Fig. 2A). Similar results were obtained using qPCR to detect *mecA* and *thermonuclease*—both *Staphylococcal*-specific genes (fig. S2, A and B). Concomitant with the presence of inflammatory cells during early infection (fig. S2, A to D), a gene array measured an increase in several key genes that mediate the host inflammatory response (Fig. 2B). Notably, the transcript for IL-1 β —a protein essential for host defense against *S. aureus* (28)—was the most highly upregulated gene four days after infection. *Il-1 β* upregulation was separately confirmed by qPCR (fig. S2C). In contrast, 60 days after infection, the inflammatory gene profile (Fig. 2B), including *il-1 β* (fig. S2C) was comparable to the expression profile of uninfected mice. These data

show MRSA clearance and resolution of inflammation by 30 and 60 days post-infection, respectively.

By 120 days post-infection, lymphatic vessel diameter had returned to the normal range found in most uninfected animals (fig. S2D). Surprisingly, the strength (Fig. 2C) and frequency (Fig. 2D) of PLV contraction remained impaired after clearance of infection at day 30 and this impairment was sustained 120 days post-infection ($p<0.05$). Using DOCT to measure lymph flow (Fig. 2E), we found pulsatile lymph flow in only 50% of the mice measured after MRSA clearance (35 days post-MRSA infection) compared to 90% of mice in the control group (Fig. 2E). Additionally, measurements made 35 days post-MRSA infection showed limited net flow velocity (Fig. 2F), comparable to flow velocity measurements 4 days post-infection. Moreover, we found that only 2 out of 7 mice at 260 days post-MRSA infection showed pulsatile lymph flow. Consistent with this finding, the net lymph velocity remained decreased in MRSA-infected mice relative to age-matched controls 260 days post-infection (fig. S2E) ($p<0.05$).

Effect of nitric oxide inhibition on lymphatic function during infection

We hypothesized that nitric oxide produced by infiltrating cells during MRSA infection could disrupt the temporal and spatial nitric oxide gradients established by lymphatic endothelial cells (19, 29), similar to sterile inflammation (19). Using immunofluorescence, inducible nitric oxide synthase (iNOS) was identified in inflammatory cells in the skin and within the abscess (Fig. 3A). iNOS-positive cells were often found in proximity to lymphatic vessels (Fig. 3A), suggesting a potential role for iNOS in inhibiting lymphatic contraction. Unexpectedly, the majority of iNOS-positive cells did not stain positively for Gr-1, a marker of neutrophils and myeloid-derived suppressor cells (Fig. 3B). Depletion of Gr-1⁺ cells led to greater reduction of lymphatic contraction (fig. S3A), likely due to the critical role of Gr-1⁺ cells in host defense during acute infection (30). These findings are in contrast to the role of Gr-1⁺ cells in a model of sterile inflammation (19). Next, we tested whether MRSA-derived lipoteichoic acid (LTA), through production of iNOS (31), inhibited lymphatic contraction during MRSA infection. Purified LTA injected into the mouse hindlimb decreased lymphatic contraction in a dose-dependent manner (Fig. 3C), but did not impair lymphatic contraction in iNOS deficient mice (iNOS KO) (Fig. 3C). This suggests that LTA acts through iNOS to reduce the strength of lymphatic function, similar to sterile inflammation (19). Although the concentration of LTA at day 4 of MRSA infection was comparable to purified LTA at the same time point (fig. S3B), iNOS KO mice infected with MRSA maintained a significant reduction in lymphatic contraction (Fig. 3D) ($p<0.05$). Notably, 60 days after MRSA infection, iNOS was undetectable in hindlimb tissue compared to 4 days after infection (fig. S3C), and animals injected with LTA showed normal lymphatic contraction 30 and 60 days after LTA injection (fig. S3D).

Another source of nitric oxide is MRSA itself, which use nitric oxide synthase oxygenase (saNOS) to catalyze nitric oxide production from L-arginine (32). Infection of wild-type mice with MRSA lacking saNOS (ΔsaNOS) resulted in reduced lymphatic vessel contraction (Fig. 3D). Similarly, infection of iNOS KO mice with ΔsaNOS MRSA did not improve PLV contraction (Fig. 3D). These results suggest that nitric oxide—produced by

iNOS or bacterial saNOS—is not the sole inhibitor of lymphatic contraction during and after MRSA infection.

Effect of inhibiting inflammation on lymphatic function during infection

The inflammatory cytokines IL-1 β , TNF- α (both elevated in Fig. 2B) and IL-6 have been previously shown to decrease lymphatic contraction and lymph flow (33). Animals null for MyD88 (MyD88 KO), an intracellular adaptor protein for inflammatory signaling pathways downstream of Toll-like receptors, show attenuated production of these cytokines after intravenous *S. aureus* challenge (34, 35). However, lymphatic contraction in MRSA-infected MyD88 KO mice revealed no improvement in lymphatic function (measured by intravital microscopy) 4 days after infection, compared to infected WT mice (fig. S3E). Additionally, prophylactic and therapeutic neutralization of TNF- α yielded no benefit in lymphatic function during MRSA infection (fig. S3F). Finally, mice treated prophylactically and during active infection with non-steroidal anti-inflammatory drugs (NSAIDs)—the cyclooxygenase inhibitors acetylsalicylic acid (aspirin) or etodolac—did not exhibit enhanced lymphatic contraction (fig. S3G) relative to vehicle control.

Loss of lymphatic muscle cells after MRSA infection

Since reducing host inflammation and inflammatory cytokines did not improve lymphatic function (Fig 3, fig. S3, A, E and G), we analyzed the cellular integrity of PLVs after MRSA infection. To this end, we performed immunohistochemical staining on hindlimb tissue. Using CD31 to identify blood and lymphatic vessels (fig. S4A), we found decreased α -SMA staining of the PLV and adjacent posterior tibial artery 36 and 96 hours after infection (Fig. 4A, fig. S4C). However, no apoptotic α -SMA⁺ cells were detected at these times using TUNEL staining (Fig. 4A, fig. S4C). Since this finding suggested decreased lymphatic muscle and smooth muscle cell coverage, we next used mice that express DsRed under the control of the α -smooth muscle actin gene promoter (α SMA-DsRed) (19) to identify the LMCs on lymphatic vessels highlighted by FITC-dextran lymphangiography (Fig. 4B). LMC coverage of the PLV was decreased and DsRed localization was altered 4 days post-infection (Fig. 4, B and C) ($p < 0.05$). Additionally, loss of SMC coverage was measured in the posterior tibial artery (Fig. 4, A and B, fig. S4C). Interestingly, LMC coverage remained abnormal 30 days post-infection (Fig. 4, C and D) and LMCs showed an elongated pattern of α SMA distribution after infection (Fig. 4C). To confirm LMC loss, we analyzed the expression of α SMA by immunofluorescence using optically cleared hindlimb tissue. In agreement with findings from α SMA-DsRed reporter mice, infection of wild type mice led to reduced LMC coverage 4 days post infection ($p < 0.05$) with partial recovery 30 days after infection (Fig. 4, E and F). Surprisingly, 260 days post-infection, decreased LMC coverage persisted ($p < 0.05$) and LMCs displayed abnormal morphology and distribution of α SMA compared to the corresponding uninfected contralateral leg of the same animal (Fig. 4, G and H).

MRSA-induced lymphatic muscle cell death

MRSA can damage biological membranes in several cell types, leading to cell death (36). We investigated whether MRSA could cause death of LMCs. To this end, we isolated LMCs from murine PLVs and cultured them *in vitro* (fig. S5A). After only 6 hours of incubation

with MRSA-conditioned supernatant, LMC viability *in vitro* was significantly diminished compared to control media (tryptic soy broth, TSB) (Fig. 5A) ($p < 0.05$). Since LMCs are critical for lymphatic contraction, we sought to further assess the mechanism of LMC death. To test the relevance to human disease mechanisms, we also added MRSA-conditioned supernatant, to human smooth muscle cells hSMCs (Fig. 5B). To determine if the relevant secreted factor(s) in the MRSA-conditioned supernatant were proteins, we pretreated MRSA-conditioned supernatant with trypsin. Trypsin abolished the lethality of MRSA-conditioned supernatant on hSMCs (Fig. 5B), suggesting that protein(s) mediate the observed cell death. Within abscesses, we found Gr-1⁺ cells that were also TUNEL-positive (fig. S6, A and B). Since we did not identify TUNEL-positive LMCs (Fig. 4A), we next asked if cellular lysis represented a mechanism of MRSA-induced LMC death by measuring the release of lactate dehydrogenase (LDH) into the media. The LDH release assay indicated MRSA cytolysis of hSMCs as early as 6 hours after exposure to MRSA-conditioned media (Fig. 5C).

Death of lymphatic muscle cells from MRSA toxins

Since MRSA-conditioned supernatant was able to kill LMCs (Fig. 5A), we next used mass spectrometry to analyze the cell-free conditioned media from the MRSA strain USA300 JE2 and found 233 proteins that were highly enriched in the conditioned media compared to control media (Fig. 6A). Toxin expression in the MRSA-conditioned supernatant was abundant (see full list of proteins identified in table S1), accounting for 9% of identified proteins with known function (fig. S7A). Expression of many *S. aureus* toxins is primarily dependent on the accessory gene regulator (*agr*) operon (36) (Fig. 6B). Based on their high expression in MRSA supernatant, we chose to investigate the effect of delta hemolysin, alpha-hemolysin and phenol soluble modulins (PSMs) on LMCs.

To this end, we added recombinant delta hemolysin, alpha hemolysin or PSM α 1 to LMCs *in vitro*. Incubation of each toxin with LMCs led to cell lysis, with PSM α 1 causing the greatest degree of cytolysis (Fig. 6C). However, genetic deletion of delta hemolysin (*hld*) or alpha hemolysin (*hla*) did not render LMCs viable after incubation with conditioned media from these respective mutants (Fig. 6D). Incubation of conditioned media from a genetic deletion of *psma* (including *psma* 1 through *psma* 4) resulted in minimal improvement in LMC viability, also seen in a mutant with combined disruption of the *psma*, *psm* β , and *hld* (combined deletion) loci (Fig. 6D). *In vivo*, the strength (Fig. 6E) and frequency (Fig. 6F) of lymphatic contraction in mice infected with *hld*, *psma*, and combined deletion mutants remained significantly reduced after 30 days relative to PBS sham infected mice ($p < 0.05$). LMC coverage after infection with mutants was comparable to that of WT MRSA (Fig. 6G). Taken together, these data suggest a role for additional *agr*-dependent factors towards LMC lethality. To this point, an alpha hemolysin neutralizing antibody was sufficient to prevent hSMC viability loss from recombinant alpha hemolysin (fig. S7B) ($p < 0.05$), but had no effect on the lethality from MRSA-conditioned supernatant (fig. S7C) ($p > 0.05$).

Effect of MRSA on additional cells associated with blood and lymphatic vasculature

The loss of smooth muscle cells on blood vessels during acute MRSA-infections (Fig. 4, A and B) suggested that additional cell types may be affected by MRSA toxins. We tested the

ability of MRSA conditioned media to kill other vascular cell types, including both murine and human vascular SMCs as well as human lymphatic endothelial cells (LECs). MRSA-conditioned supernatant killed human and murine SMCs (fig. S7, D and E), and cultured primary human lymphatic endothelial cells (LECs) (fig. S7F). Taken together, these data suggest that MRSA cytotoxicity is not limited to LMCs and explain the loss of smooth muscle cells of the posterior tibial artery. Interestingly, the requirement of alpha hemolysin, delta hemolysin and PSMs for cell toxicity was cell-specific, as loss of delta hemolysin and PSM α led to less killing of human (fig. S7, D and G) and murine (fig. S7E) SMCs relative to LMCs (Fig. 6D) *in vitro*. Further, deletion of *hla* and *psm β* in MRSA resulted in improved viability of lymphatic endothelial cells (fig. S7F).

***agr*-dependent inhibition of collecting lymphatic vessel function**

Next, we employed an *agr* mutant of the USA300 strain of MRSA, which has diminished production of several virulence-associated genes including *hld* and *hla* (37). Further, the *agr* mutant has no detectable PSM production (38). Unlike WT MRSA-conditioned supernatant, *agr* mutant-conditioned supernatant was not cytotoxic and did not cause death of LMCs, SMCs or LECs (Fig. 7, A and B, fig. S7, B to D). *In vivo*, mice infected with *agr* mutant MRSA had LMC coverage comparable to uninfected mice (Fig. 7C). Further *agr* mutant-infected mice showed stronger lymphatic function relative to mice infected with the parental strain at day 30, which is after clearance of MRSA (Fig. 7D and Videos S3 and S4). The strength of PLV contraction (ejection fraction) in *agr* mutant-infected mice was 58% stronger relative to the contraction of animals infected with WT MRSA ($p<0.05$). Several *agr* mutant-infected animals showed contraction strength similar to that of uninfected mice (Fig. 7E). Moreover, the frequency of lymphatic vessel contraction was significantly faster in *agr* mutant-infected mice compared to WT MRSA (Fig. 7F) ($p<0.05$). Of note, the contraction frequency of *agr* mutant-infected mice was increased even relative to uninfected mice, potentially compensating for the moderately lower ejection fraction. Taken together, these data suggest that *agr*-dependent MRSA toxins are primarily responsible for long-term inhibition of CLV function.

Discussion

MRSA compromises and evades host immune responses by several mechanisms, including, but not limited to, suppressing chemotaxis of leukocytes, toxin-mediated killing of leukocytes and the production of superantigens to impair the immune response (39). Here, we used the endemic USA300 strain of CA-MRSA, a common cause of serious bacterial infections in the United States, to investigate its effect on lymphatic vessels in a murine model of infection. Our data show that a single MRSA infection inhibits lymphatic vessel contraction and lymph flow long after the infection clears, providing another mechanism of how MRSA compromises immune responses.

Several mechanisms have been shown to alter the strength of lymphatic contraction. These include physical, neural and humoral influences (15). In particular, nitric oxide and inflammatory cytokine concentrations are able to dictate lymphatic contraction frequency and amplitude (19, 40, 41). Although we measured a marked increase in inflammatory

cytokine production and nitric oxide-producing infiltrating cells at the peak of MRSA infection, several lines of evidence support the conclusion that nitric oxide and other inflammatory signaling mediators are not the cause of long-term lymphatic dysfunction after MRSA infections. The absence of inflammatory molecules, even though CLV function was still impaired after MRSA clearance, suggested underlying damage to CLVs established during the acute stage of infection.

From this study, we report a new mechanism of lymphatic impairment. *In vitro* and *in vivo*, we show that MRSA causes LMC cell death, suggesting a causal connection between LMC loss and CLV dysfunction. We found that *agr*-dependent proteins, including alpha hemolysin, delta hemolysin and PSM α (38) are able to kill LMCs. Consistent with protection of LMCs from cytotoxicity caused by *agr* mutant conditioned media *in vitro*, animals infected with an *agr* null mutant exhibited normal CLV function.

In agreement with other studies (42), the β -type PSM peptides were less cytolytic towards LMCs and SMCs in our study than the α -type PSM peptides. Several PSMs are able to lyse a variety of eukaryotic cell types (42). It has been proposed that this is due to membrane perturbation of cells in a receptor independent manner (43). Alpha-hemolysin also showed the ability to kill LMCs *in vitro*, but deletion of alpha-hemolysin was not sufficient to rescue LMCs from MRSA-induced death *in vitro* and *in vivo*. Alpha-hemolysin is known to cause indiscriminate cell death at high concentrations (44), and this may explain the discrepancy in our results with recombinant alpha hemolysin and mutants deficient in alpha hemolysin. Taken together, our results suggest that *agr*-dependent production of alpha hemolysin, delta hemolysin and PSM α cause LMC death *in vitro*. However, we cannot rule out synergism or redundancy with other *agr*-controlled toxins in contributing to LMC death *in vivo*, particularly as inhibiting the production of many toxins by mutating *agr* led to the maintenance of lymphatic function.

The death of LMCs and the apparent slow regeneration of LMCs explain how CLV function can be impaired long after MRSA have been cleared and the toxins are no longer present in the system. Further, the coupling of slow LMC regeneration with MRSA recurrence may explain the cyclical pattern of lymphatic deterioration and reinfection in some SSTI patients. Investigations into therapeutic approaches to accelerate LMC regeneration may provide an alternative treatment to restore lymphatic function in patients with recurrent SSTIs.

The identification of a bacterial-derived mechanism for LMC death represents a critical first step in a mechanistic analysis of how MRSA impairs CLV function. It is unknown whether the altered morphology of LMCs that regenerate after infection is indicative of functional differences compared to LMCs of uninfected mice. We also cannot rule out effects of MRSA on lymphatic endothelial cells *in vivo*, which also contribute to the maintenance of lymphatic vessel contraction. However, prophylactic treatment of recurrent cellulitis infections (45) by targeting *agr* signaling may overcome the effects exerted by MRSA toxins on multiple different cell types and lead to the preservation of CLV function and immunity. Several studies have focused on identifying chemical inhibitors of *agr* to combat the virulence of bacterial infections and to limit antibiotic use (46–50). While these inhibitors

broadly limit pathological tissue damage, it is unknown whether they also help maintain lymphatic function.

Although the ability to evaluate CLV function provides insight into the effect of MRSA post-infection, our model is limited in that it is not a recurrent model of infection. Further, animals are maintained in a controlled sterile environment and mice do not develop secondary chronic lymphedema. Another limitation of this study is that it does not address a role, if any, for *Streptococci* and methicillin susceptible *S. aureus* (MSSA) on lymphatic impairment, as Group A *Streptococci* and MSSA are also common pathogens associated with SSTIs (4). It is likely that MSSA can blunt CLV function through *agr*-mediated toxin production, albeit MSSA toxin production is lower than that of MRSA (37).

In lymphedema patients, recurring episodes of SSTIs lead to further damage of the lymphatic system, resulting in lymph flow deterioration (51–53). Emerging evidence suggests both T and B lymphocyte-mediated adaptive immune responses are generated after *S. aureus* infection (54). In patients with pre-existing lymphedema or progressive lymphedema as a result of recurring infections, we expect local immune deficiency due to the ineffective transport of antigen to the local draining lymph nodes, representing another mechanism by which *S. aureus* inhibits the adaptive immune response. When lymphatic function is impaired, the protein-rich and stagnant lymphatic fluid may facilitate bacterial growth. Further, impaired lymphatic function also inhibits bacterial clearance through the lymphatic system, resulting in bacterial and toxin accumulation that will impair both innate and adaptive immunity and increase the risk of recurrent infections. Our data provide a mechanistic framework for the chronic reduction of lymph flow in patients after MRSA infection. Neutralization of MRSA pathogenicity as part of the treatment of MRSA-induced cellulitis may help intervene in the vicious cycle of SSTI, lymph flow deterioration, lymphedema and recurrent infection in these patients.

Materials and Methods

Study design

The goal of this study was to determine the effect of MRSA infection on CLV function. Using a model of community-associated MRSA infection in the hindlimb of mice, we assessed CLV function by performing lymphatic contraction experiments previously established (19,25). We deemed a 25% difference in lymphatic contraction or lymph flow with an anticipated standard deviation of 20% as biologically significant. At an $\alpha=0.05$ and a beta of 0.80, we determined sample size based on prior and gained experience regarding the success rate of lymphatic contraction and lymph flow, respectively. No animals were excluded from lymphatic contraction studies pending successful surgical exposure of lymphatic vessel. The criteria for exclusion of animals for lymph flow experiments with DOCT are described in the materials and methods and involved the ability to obtain a clear image of the vessel and calculate a Doppler angle of less than 85 degrees. α SMA-DsRed transgenic mice were visualized and immunofluorescence staining of α SMA⁺ lymphatic muscle cells of WT mice was performed to analyze LMC coverage of lymphatic vessels on multiple days post-infection. Each analysis represents a terminal experiment, not a longitudinal study of the same animal. Researchers were blinded to the details of the

infection during image analysis. Both male and female mice were used and were age-matched between all comparator groups. Cell viability and cell lysis assays were used to measure killing of LMCs, hSMCs, mSMCs and LECs by MRSA-conditioned supernatant from strain(s) simultaneously grown overnight in similar conditions. Cell culture experiments to investigate the effects of MRSA-conditioned supernatant or recombinant *S. aureus* toxins on cells were performed in duplicate or triplicate in three to five independent experiments. All data are presented as means $\pm$ SEM, unless stated otherwise in the figure legend. The methods used to support these findings are described in the Materials and Methods/Supplementary Materials and Methods. Statistical tests used to establish significance and associated *p* values are described in the figure legends. Primary data are located in table S2.

Animals

In vivo studies were initiated in 5–8 week old male and female C57BL/6, iNOS^{-/-} C57BL/6, MyD88^{-/-} C57BL/6 (generous gift from Dr. Alessio Fassano), LysM^{gfp/gfp} C57BL/6 mice (generous gift from Dr. Thomas Graf) and α SMA^P-DsRed/C57BL/6 mice. All mice were bred and maintained in our Cox-7 gnotobiotic animal colony or at the Center for Comparative Medicine and Animal Laboratory Resources at Massachusetts General Hospital. All procedures were performed following the guidelines of the Institutional Animal Care and Use Committee of the Massachusetts General Hospital.

S. aureus strains

The clinical MRSA isolate, CA-MRSA USA300 LAC, had 3 non-essential plasmids removed to create the CA-MRSA USA300 JE2 strain (55). Additionally, the NE27 mutant (lacking functional *saNOS*) was created by mutagenesis from *mariner*-based transposon *bursa aurealis* resulting in an erythromycin-resistant deletion strain of JE2. Both isolates were obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA; Chantilly, VA). Wild type and isogenic deletion mutants of the *agr* regulatory system, alpha hemolysin, delta hemolysin, psm α , psm β , and psm α β hld (TKO) in the LAC (USA300) strain are described previously (37, 38, 56–58).

Infection

MRSA strains were subcultured until they reached their exponential growth phase, washed with sterile PBS once and resuspended in sterile PBS. Mice were anesthetized with intraperitoneal (i.p.) administration of ketamine/xylazine (10 mg ketamine and 100 mg xylazine/kg). Fur was shaved from the hindlimb. $2\text{--}4 \times 10^6$ c.f.u. of MRSA were injected subcutaneously (using a 29 gauge 1/2" insulin syringe) into the hindlimb, proximal to the ankle, in a 50 μ L volume of sterile PBS. A similar procedure was performed with lipoteichoic acid (Invivogen, Cat # tlrl-pslta) after resuspension in sterile PBS.

Isolation of murine lymphatic muscle cells and blood smooth muscle cells

While in solution of Hanks Balanced Saline Solution, fat and surrounding connective tissue were removed from the posterior tibial artery and afferent lymphatic vessels adjacent to the popliteal lymph node. To make 5 mL of enzyme solution, Collagenase Type II and Soybean

Trypsin Inhibitor (Worthington) were added to Hanks Balanced Saline Solution at 1 mg/mL. 35 μ L of elastase (Worthington) and 50 μ L Penicillin/Streptomycin were added. Next, the enzyme solution was added to minced fragments of popliteal artery or lymphatic vessels and allowed to incubate at 37°C, 5% CO₂ for 4 hours with occasional agitation. Cell outgrowth was allowed to proceed for 5 days.

Preparation of MRSA-conditioned supernatant

Planktonic culture-conditioned medium was prepared by growing an overnight culture of indicated MRSA strain in Trypticase Soy Broth (TSB; BD Biosciences) under shaking conditions (200 rpm) at 37°C for 12–18 hrs. Afterwards, the bacteria were centrifuged at 6,000 rpm for 10 min and subsequently the supernatant was filtered through a 0.22- μ m pore-size polyethersulfone (PES) filter (Olympus Plastics) to produce cell-free conditioned medium.

The cell-free conditioned MRSA medium was mixed at a 1:1 dilution with muscle cell culture medium. A 1:1 dilution of cell culture medium with TSB medium was used as a control in parallel. Where indicated, MRSA-conditioned medium was treated with 0.0625% trypsin (final percentage in solution) for 18–24 h at 37°C to degrade proteins. Trypsin was heat-inactivated at 100°C for one hour and allowed to cool to room temperature before supernatant was incubated with cells.

Lymphatic contraction model

Mice were anesthetized with ketamine/xylazine and 3 μ L of 2% FITC-Dextran (2 million molecular weight; ThermoFisher Scientific Cat. #D7137) was interstitially injected in the footpad. Surgical preparation and intravital imaging were performed as described (19, 25). Briefly, the leg skin and underlying connective tissue near the afferent lymphatic vessel to the popliteal lymph node (PLV) were carefully excised. Time-lapse images (360 images separated by 210 ms) of the exposed afferent lymphatic vessels to the popliteal lymph node were captured while imaging with an inverted fluorescence microscope. For each mouse, we imaged two to three lymphatic segments and took images every 15 minutes for four time points. Using in-house MATLAB codes, time-lapse images were analyzed to track the position of the vessel wall and to measure frequency using the peak-and-valley method as described previously (19, 25). For most analyses, we collected 6–10 measurements of ejection fraction values from a single mouse. The mean of the measurements was used to represent lymphatic contraction for that animal.

Measuring lymphatic flow

To measure lymph flow inside murine CLVs, we employed a recently developed label-free method for directly measuring lymph flow velocity *in vivo* by Doppler optical coherence tomography (DOCT) (26). This technique measures the velocity of lymph fluid, both basal and pulsatile, and relies only upon the absolute movement of lymph relative to the laser beam. Mice were anesthetized with Ketamine/Xylazine. The surgical procedure to expose the PLV was performed as described previously (19, 25). Anesthetized mice were transferred to a heating pad to maintain body temperature at 37°C.

Hydration of the PLV and surrounding tissue was maintained with physiological saline while measuring lymph flow. Repeated five minute DOCT measurements were acquired from a fixed location within a lymphangion, in between lymphatic valves. Next, the measurements were processed to produce a structural image and the scattering signals within the lymphatic vessel were analyzed using an algorithm designed specifically to measure the lymph flow signal. Three-dimensional imaging was used to measure the lymphatic vessel orientation and thereby the Doppler angle of the flow, the latter being mandatory to calculate flow speed (from Doppler shift). For these measurements, a structural image showing a clear vessel-tissue boundary and absence of signal artifact within the vessel lumen was of paramount importance. In addition, a Doppler angle less than 85 degrees and a lymphatic vessel within 100 microns from the tissue surface was needed to guarantee trustworthy results. Data not adhering to these criteria were excluded for analysis.

Twenty-four 8–12 week old C57BL/6 female mice were infected with $2-4 \times 10^6$ c.f.u. of MRSA-USA 300 JE2 and lymphatic flow was measured at either four days (n=8), 35 days (n=16) or 260 days after infection (n=7). Eight age-matched mice served as uninfected controls for each time point. Lymphatic flow was measured using M-Mode acquisition until two measurements per mouse were obtained without consequential x, y or z drift, as judged by the investigators. Mouse limbs were positioned lower than the abdomen on a 15 degree tilted stage and measurements were acquired and subsequently analyzed as described previously (26). In the control group 19/25 measurements were successful in 6/8 mice. Out of eight mice four days after CA-MRSA infection, four could not be imaged due to excessive postoperative bleeding resulting from severe inflammation during active bacterial infection. In the remaining four mice, 3/13 measurements were successful, all from the same mouse. After 35 days, 17/46 measurements were successful in 6/12 mice. In this group, four additional mice did not show intact collecting vessels within 100 microns from the tissue surface and therefore no measurements could be acquired.

Cell viability assays

10,000–20,000 hSMCs or 1,000–5000 mLMCs, delivered in 200 μ l of respective media were allowed to adhere overnight to the bottom of a 48-well plate. After 24 to 48 hours of exposure to MRSA-conditioned supernatant or TSB, the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was performed according to instructions. Briefly, 100 μ l of medium was removed from the wells of the plates containing medium, and 100 μ l of room temperature CellTiter-Glo® Reagent was added. Plates were rocked for two minutes in the dark and incubated for an additional eight minutes or longer (while protected from light) before reading luminescent output on an FLUOstar® Omega series luminescence microplate reader.

Statistical Methods

Statistical analyses were completed with Prism 7 (Graph Pad). Statistical significance was determined with either a one-way analysis of variance (ANOVA) with the Fisher's Least Significance Difference post hoc test, or two-tailed unpaired Student's *t* test, as appropriate. Statistical significance was set at $p < 0.05$. All statistical tests were two-sided.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Collecting popliteal lymphatic vessels exhibit diminished contraction and lymph velocity following MRSA infection

C57BL/6 mice were infected subcutaneously in the hindlimb with $2-4 \times 10^6$ c.f.u. of MRSA for four days or left uninfected. (A) Representative sequential images from intravital microscopy of PLVs perfused with FITC-Dextran 2 million MW; s=seconds. **Scale bar**=50 μ m (See Supplementary Videos 1 and 2). (B) Representative traces from PLV wall measurements show lymphatic diameter and contraction over time, s=seconds; (n=8–11 each group). (C) Ejection fraction, indicating strength of lymphatic vessel contraction, in uninfected mice and mice infected with WT MRSA after 4 days (n=8–11 each group). (D) Frequency indicates the counts per minute (cpm) of PLV contractions in uninfected mice and mice infected with WT MRSA after 4 days (n=7–10 each group). (E) Top Panel: Representative depth vs. time image of the optical coherence tomography intensity signal (grayscale) from a fixed transverse location that is used to identify the upper and lower boundaries of PLVs in uninfected animals and during active infection (Day 4). LV, lymphatic vessel; s=seconds. Instantaneous lymph velocity (color) in the PLV in uninfected animals and during active infection (Day 4) is overlaid on structural OCT depth scan. Bottom Panel: Representative trace of instantaneous velocity of lymph flow averaged over the PLV cross-sectional area in uninfected animals and during active infection (Day 4). (F) Time-averaged mean velocity of lymph flow in uninfected animals and during active infection (n=3–11 measurements each group). For C, D and F, statistical analysis was performed using Student's unpaired two-sided *t* test. Error bars show SEM. * *p* < 0.05

Fig. 2. Collecting lymphatic vessel dysfunction persists after clearance of MRSA

(A) Infectious burden measured by colony formation assay in the draining popliteal lymph node (left) at indicated time points (n=6–14 all groups). Infectious burden in the skin and underlying muscle tissue (right) from the site of infection at indicated time points (n=4–5 all groups). (B) Gene expression array of mouse inflammatory cytokines and receptors comparing tissue from the site of infection collected at day 4 (left) and 60 (right) post-MRSA infection. CT was calculated with normalization of raw data to housekeeping genes. The graph plots normalized gene expression levels (log₁₀) from control (uninfected) skin and muscle tissue (n=2) on the x-axis versus normalized gene expression levels (log₁₀) of day 4 (n=2) or day 60 (n=2) post-infection skin and muscle tissue on the y-axis. Circles above the upper gray line identify significantly up-regulated genes (p<.05); the blue line and lower gray line represent the boundary for genes unchanged and significantly downregulated, respectively. Individual samples were loaded into each qPCR array plate and normalized to skin from uninfected mice. (C) Ejection fraction (n=3–8) and (D) frequency (n=3–10) show the strength and number (counts per minute, cpm), respectively, of PLV contractions over time. Each time point represents a different cohort of mice; days 1, 2, 4, 30, 60 and 120 were measured). (E) Representative depth vs. time image of the depth-resolved optical coherence tomography intensity from a fixed transverse location that is used to identify the upper and lower boundaries of PLVs in uninfected animals and after clearance of infection (D35). Instantaneous lymph velocity (color) in the lymphatic vessel in uninfected animals and after clearance of infection (D35) is overlaid on structural OCT depth scan. (F) Time-averaged mean velocity of lymph in uninfected animals and after clearance of infection (D35; n=11–17 measurements in each group); s=seconds. Statistical analysis was performed using Student's unpaired two-sided *t* test (F). Error bars show SEM. *p < 0.05

Fig. 3. Inhibition of iNOS is not sufficient to restore collecting lymphatic vessel contraction during MRSA infection

(A) Representative sections of control skin (from uninfected mice) and skin 4 days post-MRSA infection. Lymphatic vessels were identified with anti-LYVE-1 antibody (red) and nuclei with DAPI (blue). Anti-iNOS antibody (green) identified iNOS-positive cells; $n=4$. Scale bar=300 µm. (B) Serial sections to the images in (A) was stained with anti-Gr-1 antibody (blue), anti-iNOS antibody (green) and anti-Ly6G antibody (red); $n=4$. Scale bar=50 µm. (C) 50 µL of sterile PBS or 50 µL of lipoteichoic acid (LTA) suspended in sterile PBS was injected into the hindlimb of C57BL/6 mice and C57BL/6-iNOS^{-/-} mice. On day 4 post-injection, the ejection fraction shows the strength of PLV contraction from indicated concentrations of LTA ($n=3-4$ each group). (D) C57BL/6 mice were injected subcutaneously in the hindlimb with PBS (uninfected) or infected subcutaneously in the hindlimb with $2-4 \times 10^6$ c.f.u. of WT MRSA or MRSA deficient in nitric oxide production (ΔsaNOS). C57BL/6-iNOS^{-/-} mice were infected subcutaneously in the hindlimb with $2-4 \times 10^6$ c.f.u. of WT MRSA or ΔsaNOS MRSA. Ejection fraction four days after infection shows the strength of PLV contraction among respective groups ($n=8-11$ each group). For C, and D, One-Way ANOVA comparison with Fisher's least significant difference (LSD) post-hoc analysis was used to determine significance. Error bars show SEM. * $p < 0.05$

Fig. 4. Infection-induced collecting lymphatic vessel dysfunction is associated with decreased lymphatic muscle cell coverage

(A) Representative immunohistochemical images of uninfected mouse hindlimb and mouse hindlimb 36 hours after infection stained with α SMA (crimson, arrowhead) and TUNEL (brown); $n=4$ mice per group. **Scale bar**=40 μ m. (B) Representative intravital image from segment of PLV of an α SMA^{DsRed}/C57BL/6 mouse interstitially injected with FITC-Dextran; $n=4$ mice per group. Arrowhead indicates lymphatic vessel. Arrow points to blood vessel. **Scale bar**=100 μ m. (C) Representative intravital image from PLV segments of α SMA^{DsRed}/C57BL/6 mice on indicated days. **Scale bar**=100 μ m. (D) Quantification of α SMA-positive cells per 100 μ m of PLV; $n=4-8$ mice per group. (E) Representative image from PLV segments of C57BL/6 mice, stained with anti- α SMA, on indicated days. **Scale bar**=100 μ m. (F) Computer-automated quantification of % α SMA-positive area of PLV; $n=3-4$ mice for each group. (G) Representative image from a segment of PLV stained with anti- α SMA (red) 260 days post-infection and corresponding control (contralateral) PLV. **Scale bar**=25 μ m. Arrowheads indicate adjacent blood vessel (posterior tibial artery). (H) Computer-automated quantification of % α SMA coverage of PLV 260 days post-infection or corresponding age matched control (contralateral) popliteal lymphatic vessel ($n=5$ both groups). For **D** and **F**, statistical analysis was performed using One-Way ANOVA comparison with Fisher's least significant difference (LSD) post-hoc analysis. For **H**,

statistical analysis was performed using Student's unpaired two-sided *t* test. Error bars show SEM. * $p < 0.05$

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Fig. 5. MRSA protein causes muscle cell death

(A) Cell viability (RLU, relative luciferase units) analysis of human SMCs after exposure for 24 hours to control media (tryptic soy broth), MRSA-conditioned media, or MRSA conditioned media incubated for 18–24 hours at 37°C with trypsin; n=3. (B) Graph depicting lactate dehydrogenase (LDH) release from hSMCs (left Y-axis) and cell viability (RLU, right Y-axis) after exposure for 6 hours to control media (tryptic soy broth), MRSA-conditioned media, or doxycycline; n=3. (C) Mouse lymphatic muscle cell (LMC) viability analysis (RLU) after exposure for 6 hours to control media (tryptic soy broth), or (WT) MRSA-conditioned media; n=3. For A, One-Way ANOVA comparison with Fisher's least significant difference (LSD) post-hoc analysis was performed. For B, statistical analysis was performed using multiple t tests for comparators indicated. For C, statistical analysis was performed using Student's unpaired two-sided *t* test compared to the control group. Error bars show SEM. **p* < 0.05. N.D. = not detected

Fig. 6. Effect of MRSA toxins on lymphatic muscle cells and collecting lymphatic vessel function (A) Identification by mass spectrometry of the relative abundance of proteins (area) from <100 kDa fraction of MRSA-conditioned media. (B) Heat map of the most highly expressed toxins, as identified by mass spectrometry; also depicted is the corresponding toxin expression level in the absence of the accessory gene regulator (*agr*). (C) Graph depicting lactate dehydrogenase (LDH) release from LMCs (left Y-axis) and cell viability (RLU, relative luciferase units (right Y-axis)) after incubation for 24 hours with normal growth media only (–), α-hemolysin (1.0 μg/mL), δ-hemolysin (100 μg/mL) or PSMα1 (100 μg/mL). Statistical analysis was performed using multiple t tests relative to untreated control for cytotoxicity and cell viability. * $p < 0.05$; $n = 3$. (D) Cell viability analysis (RLU, relative luciferase units) of murine lymphatic muscle cells after exposure for 24 hours to control media (tryptic soy broth), MRSA-conditioned media or conditioned media of isogenic MRSA mutants, as indicated; $n = 3$. * $p < 0.05$ relative to control media. hla=alpha hemolysin, hld=delta hemolysin. (E) Lymphatic ejection fraction ($n = 4–5$ mice for each group) and (F) frequency ($n = 5–6$ mice for each group) of lymphatic vessel contraction was measured in mice infected with wild type or indicated mutant MRSA strains. (G) Computer-automated quantification of % αSMA-positive area of PLV in animals 30 days post infection with wild type or indicated mutant MRSA strain; $n = 3–4$ mice for each group. For D–G, statistical

analysis was performed by One-Way ANOVA comparison with Fisher's Least Significance Difference post-hoc analysis. Error bars show SEM. * $p < 0.05$. N.D. = not detected

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Fig. 7. MRSA virulence proteins cause lymphatic muscle cell death and diminished collecting lymphatic vessel function

(A) Mouse lymphatic muscle cell (LMC) viability analysis (RLU, relative luciferase units) after exposure for 24 hours to control media (tryptic soy broth), *agr* mutant-conditioned media or MRSA-conditioned media; n=3. (B) Lactate dehydrogenase (LDH) release from LMCs incubated with conditioned medium from WT or *agr* mutant MRSA; n=3. (C) Computer-automated quantification of % αSMA-positive area of PLV in uninfected animals and animals 30 days post-*agr* mutant MRSA infection; n=3–4 mice for each group. (D) Representative traces from lymphatic vessel wall measurements show lymphatic diameter and contraction over time (s, seconds) in uninfected mice and mice infected with WT or *agr* mutant MRSA for 30 days. (E) Ejection fraction shows the strength of lymphatic vessel contraction in uninfected mice and mice infected with WT or *agr* mutant MRSA for 30 days; n=8–17. (F) Frequency indicates the cpm (counts per minute) of lymphatic vessel contractions in uninfected mice and mice infected with WT or *agr* mutant MRSA for 30 days; n=8–15. For E and F, statistical analysis was performed by One-Way ANOVA comparison with Fisher's Least Significance Difference post-hoc analysis. Error bars show SEM. *p < 0.05. N.D. = not detected