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Daptomycin resistant *Staphylococcus aureus* clinical isolates are poorly sensed by dendritic cells.

Running Title: Innate sensing of antibiotic-resistant bacteria

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Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) presents an increasing threat to public health, with antimicrobial resistance on the rise and infections endemic in the hospital setting. Despite a global research effort to understand and combat antimicrobial resistance, less work has focused on understanding the nuances in the immunopathogenesis of clinical strains. In particular, there is a surprising gap of knowledge in the literature pertaining to how clinical strains are recognised by dendritic cells (DC). Here, we show that the activation of DC is compromised in response to MRSA strains resistant to the last line antibiotic

daptomycin. We found a significant reduction in the secretion of proinflammatory cytokines including TNF- α , IL-6, RANTES and MIP-1 β , as well as decreased expression of CD80 by dendritic cells responding to daptomycin-resistant MRSA. We further demonstrate that this phenotype is coincident with the acquisition of specific point mutations in the cardiolipin synthase gene *cls2*, and, partly, in the bifunctional lysylphosphatidylglycerol flippase/synthetase *mprF*, which are genes that are often mutated in clinical daptomycin-resistant strains. Therefore, throughout infection and antibiotic therapy, MRSA has the capacity to not only develop further antibiotic resistance, but also develop resistance to immunological recognition by DC, due to single amino acid point mutations occurring under the selective pressures of both host immunity and antibiotic therapy. Understanding the diversity of clinical MRSA isolates and the nuances in their immune recognition will have important implications for future therapeutics and the treatment of these infections.

Introduction

Staphylococcus aureus is a Gram-positive bacterium associated with serious infections in the community and hospital setting. In hospitals, *S. aureus* is the causative pathogen in one in every five bloodstream infections¹. Resistance to antibiotics is on the rise, with rates of methicillin-resistant *S. aureus* (MRSA) now accounting for over 25% of healthcare-associated staphylococcal bloodstream infections². Increased mortality has been associated with antibiotic resistance, with MRSA infections having a mortality rate almost double that of methicillin-sensitive *S. aureus* (MSSA)³. Infections caused by antibiotic-resistant *S. aureus*, including to last-line agents such as vancomycin and daptomycin, have been associated with complex and more persistent infections⁴⁻⁶, suggesting that *S. aureus* likely adapts in vivo to both antibiotic and host immune selection pressures.

Daptomycin is a lipopeptide antibiotic with activity against Gram-positive organisms that targets the bacterial cell membrane to induce bactericidal effects⁷. Resistance to daptomycin has been associated with mutations in genes related to membrane phospholipid biosynthesis⁸. Point mutations in *mprF*, which encodes a protein responsible for the lysinylation of membrane phosphatidylglycerol and the translocation of lysyl-

phosphatidylglycerol to the outer leaflet of the membrane, have most commonly been reported⁸⁻¹². These mutations were initially proposed to alter the composition and charge of the cell membrane, facilitating the repulsion of positively charged daptomycin complexes⁸. Mutations in *mprF* have also been demonstrated to reduce susceptibility to host cationic antimicrobial immune peptides with a similar mechanism of action to that of daptomycin^{9,13}. In addition to *mprF*, the gene responsible for cardiolipin synthase, *cls2*, has also been associated with daptomycin resistance^{8,14}. Cardiolipin is normally converted from phosphatidylglycerol during progression to stationary phase or upon phagocytosis by neutrophils¹⁵, with *cls2* thought to play the predominant role in these processes. Our recent study confirmed that clinically relevant *cls2* point mutations lead to enhanced cardiolipin biosynthesis, resulting in membrane phospholipid profile changes that deter daptomycin penetration and membrane disruption¹⁴. Furthermore, the membrane lipid changes appeared to compromise neutrophil recruitment to a localised site of infection, providing more support for the immune evasion mechanisms that evolve in the context of antibiotic resistant *S. aureus* infections¹⁴. While the mechanisms by which wall alterations induce antibiotic resistance are an active area of research^{8,9,14,16}, studies investigating how such alterations affect immune recognition are lacking. Innate detection of bacteria relies on recognition by pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs), of which more than half recognise components of the bacterial cell membrane and wall¹⁷.

Dendritic cells (DC) are a heterogeneous population of innate sentinel cells that phagocytose pathogens and present antigens on MHC, therefore linking innate and adaptive immunity. The two main classes of DC, conventional (c)DC and plasmacytoid (p)DC, have distinct phenotypes and functional characteristics. Functionally, cDC are distinct in their potent presentation of antigen and their ability to prime naïve T-cells¹⁸. The cDC can be further subdivided into 2 subsets: cDC1, that are specialized in cross-presentation of antigens and are superior in the activation of cytotoxic T cells; and cDC2, that are superior in the activation of CD4+ T cells. These distinct specialisations of cDC are critical for the clearance of bacterial infections, and have been well reviewed¹⁹. In contrast to cDC, the pDC have limited capacity to present antigen to T-cells, but rather possess a unique ability to rapidly produce abundant Type-I Interferons (IFN)¹⁸. While IFN has classically been associated with the induction of anti-viral responses, its role in regulating immunity to

bacterial pathogens has been well reviewed^{20,21}. The DC subsets express distinct and overlapping sets of PRRs, ensuring both exclusive and overlapping responses to different pathogenic stimuli (reviewed in ¹⁸).

It is well established that DC can induce strong Th1/Th17 responses¹⁸ and that these responses are indispensable for the clearance of *S. aureus* infection^{22,23}. However, whilst the essential PRRs required for the recognition of *S. aureus* by the main DC classes have been characterised²⁴, most available research utilises the IL-4 and GM-CSF inducible DC culture system²⁵⁻²⁸ and/or laboratory strains of MRSA^{25,29,30}. A problem with the former approach being that these DC culture systems do not recapitulate primary DC subsets, and as such the use of these systems to model DC function is contentious³¹⁻³³. Jin and colleagues³⁴ were the first group to extensively phenotype human blood DC in response to stimulation with the *S. aureus* model strain SH100. Their study indicated that conventional BDCA1+ DC (cDC2) are the sole blood DC subset able to efficiently phagocytose *S. aureus*, consequently upregulating surface activation markers and secreting IL-12p70³⁴. However, conflicting studies have demonstrated human pDC are able to phagocytose *S. aureus*, consequently upregulating CD86 and secreting IL-6, TNF- α and IFN- α ^{29,30,35}. The discrepancies between cDC2 and pDC activation in these studies highlight the intricacies of DC function, but may also be indicative of a broader issue in using laboratory bacterial strains for functional studies of primary infection.

In this study we utilise the murine FMS-like tyrosine kinase 3 (flt3-L) inducible DC model, which recapitulates *bona fide* murine spleen-like DC subsets, pDC, cDC1 and cDC2 in appreciable numbers, overcoming the limitations of directly isolating DC direct from primary tissue. Here, for the first time to our knowledge, we have phenotyped the response of *bona fide* murine DC to clinically derived, antibiotic-resistant *S. aureus*. This work demonstrates that the magnitude of DC responses is highly variable between distinct clinical isolates of MRSA and MSSA. Further, DC responses can be downregulated in response to MRSA isolates that have acquired antibiotic resistance mutations during daptomycin therapy. These results have important implications for the understanding of MRSA pathogenesis and innate immune evasion strategies, which is critical for the development of novel non-antibiotic immunotherapies and vaccines.

RESULTS

Phenotype of flt3-L induced DC (FLDC) in response to bacterial stimuli

Although DC responses to mimics of bacterial products such as CpG-DNA are well described¹⁸, a comprehensive evaluation of FLDC responses to bacteria has not been carried out. Therefore, we first sought to establish a panel of cytokines and surface markers that were useful measures of activation of FLDC in response to bacteria. As a point of reference, we examined the response of FLDC to a panel of pathogenic and non-pathogenic strains of Gram-negative and Gram-positive heat-killed bacteria equivalent to a multiplicity of infection (MOI) of 10. Stimulation of total FLDC with these bacterial stimuli induced secretion of a range of pro-inflammatory cytokines and chemokines including: RANTES (CCL5), MDC (CCL22), MIP-1 α (CCL3), MIP-1 β (CCL4), IL-6 and TNF- α (Supplementary Figure 1a). Bacterial stimulation of FLDC also triggered secretion of IFN- β (Supplementary Figure 1a), but not IFN- α (Supplementary Figure 1a). Further, we observed dose dependent production of IFN- λ in response to stimulation with higher concentrations of *S. aureus*, *Escherichia coli* and *Listeria monocytogenes*, particularly at a MOI greater than 25 (Supplementary Figure 1b), but IFN- α was still not detected (data not shown). A caveat to these data was that the survival of pDC, the likely source of IFN- α in response to bacterial stimuli, was poor.

In response to bacterial stimuli both cDC1 and cDC2 subsets were found to upregulate canonical surface activation markers required for T-cell activation; CD40, CD86, MHC-II (data not shown) and CD80 (Supplementary Figure 1c and d). We further examined checkpoint inhibitory ligands (PD-L1 and PD-L2), which are negative regulators of activated and mature DC and PD-1 expressing lymphocytes^{36,37}. In response to most bacterial stimuli, the cDC1 exhibited PD-L1 and PD-L2 expression similar to the positive control (CpG1668, a type B-oligonucleotide (ODN) with CpG motifs, used to mimic purified bacterial DNA, Supplementary Figure 1c). Conversely, bacterial stimuli were found to induce lower expression of PD-L1 and PD-L2 on cDC2, than did the CpG control in these cultures (Supplementary Figure 1d).

Antibiotic resistance acquired by MRSA affects DC activation

To determine the response of DC to clinically-derived *S. aureus* isolates, we analysed the established panel of cytokines, IFNs and cell surface markers to test the response of DC to two MSSA clinical isolates and one MRSA clinical isolate (Figure 1a). The two MSSA strains induced IL-6, and IFN- β secretion by FLDC (Figure 1a and data not shown), similar to heat killed bacterial strains analysed in Supplementary Figure 1. The clinical MRSA isolate activated DC at less efficient levels compared with the two MSSA isolates (Fig 1a).

This MRSA strain, A8819, was isolated from a patient with bloodstream infection before treatment with daptomycin. While on daptomycin treatment, resistance emerged leading to the daptomycin-resistant (DapR) daughter strain, A8817^{8,38}. We have recently shown that DapR strains are associated with persistent infections and impaired neutrophil chemotactic responses compared to their DapS parental strains¹⁴. To further examine if persistent infections caused by DapR *S. aureus* are partly due to compromised DC responses, we tested the ability of A8819 (DapS) and A8817 (DapR) cells to stimulate IFN- λ production from FLDC in bulk culture. Comparing the titrated DapS and DapR MRSA strains, it was clear that maximal DC activation occurred at an MOI of 10. We found that the sensitive strain (DapS) triggered far greater IFN- λ production by FLDC than the resistant strain (DapR) (Figure 1b). This difference was not due to lower DC viability in the DapR cultures, since the DapR and DapS cultures yielded similar DC survival (Figure 1c). Hence, based on the maximal secretion of IFN- λ (Figure 1b) and viability of FLDC (Figure 1c), MOI of 10 was selected for all subsequent analyses.

Given differential expression of IFN- λ by FLDC following stimuli with the daptomycin exposed clinical pair, we next examined a broader cytokine panel in response to these MRSA isolates. These analyses revealed that FLDC exposed to DapR MRSA produced significantly less RANTES, MIP-1 α , MIP-1 β , TNF- α and IL-6 than FLDC exposed to DapS MRSA (Figure 2a). On the other hand, MDC was produced to significantly higher levels in response to the DapR strain (Figure 2a). IL-10 and IFN- β were similarly produced in response to both DapS and DapR strains whilst we were unable to detect IFN- α production from the FLDC in response to MRSA (data not shown). Heat inactivation of these strains resulted in rescue of some cytokines in response to DapR MRSA, most notably RANTES (Figure 2a). However, significant differences in IFN- β and MIP-1 α were observed following heat inactivation (Figure 2a). Importantly, with the exception of RANTES, the cytokine profile was dampened overall

following stimuli with heat inactivated bacteria, highlighting the need to use live isolates for such immunogenicity studies.

CD80 is differentially expressed by DC in response to daptomycin sensitive and resistant clinical isolates of MRSA

To define the response of individual DC subsets to MRSA strains we sorted cDC1, cDC2 and pDC from FLDC cultures. Despite the differences in cytokine production observed in Figures 1 and 2a, the surface phenotype of DC following stimulus with DapR MRSA was surprisingly similar to stimulation with DapS MRSA (Figure 2b). The upregulation of CD40, CD86, MHC-II, PD-L1 and PD-L2 by cDC1, cDC2 and pDC subsets were equivalent following stimulus with DapS and DapR MRSA (Figure 2b and data not shown). However, we observed a small differential expression of CD80 by cDC1, cDC2 and notably very few pDC responded with CD80 upregulation in the DapR cultures (Figure 2b). Collectively, these data demonstrate a differential stimulation of all FLDC subsets to the MRSA clinical pair, with the DapR strain able to impede both cytokine secretion in bulk culture and surface activation marker upregulation by FACS sorted pDC.

cDC1 dominate the inflammatory cytokine and chemokine response to MRSA

All sorted DC subsets produced some inflammatory cytokines and chemokines in response to the DapS and DapR isolates and the same trend of higher response to DapS than to DapR was observed, particularly with cDC1. Indeed, the cDC1 stood out as the dominant responders and the only subset able to produce bioactive IL-12p70 in response to MRSA (Figure 2c). Further, cDC1 were the major producers of IFN in response to MRSA, producing substantially more IFN- β and IFN- λ than cDC2 or pDC (Figures 2c and d). We were unable to detect IFN- α production from any DC subset in response to MRSA (data not shown). Of note, the sorted pDC produced substantial levels of chemokines MIP-1 α and MIP-1 β as well as TNF- α , illustrating sufficient viability to respond, despite producing minimal IFNs (Figures 2c and d). These analyses of the sorted DC subsets (Figures 2c and d) revealed that the differential secretion of inflammatory mediators in response to DapS compared to DapR strains, was mediated largely by the cDC1 subset, but did not reach significance with the

sorted cells. This suggested that cross talk between the subsets was required to maximise the differential response to DapS compared to DapR strains.

Resistance to antibiotics in MRSA correlates with reduced cytokine activation by cDC1 without differences in surface activation

To extend our observed findings of reduced innate responses by cDC1 to a clinical DapR (A8817) strain compared to its DapS (A8819) parent strain, we tested four other clinical pairs that we have characterised previously (Table 1)⁸. Similar to A8819/A8817 (Figure 3a), all of the isogenic DapR and DapS pairs activated DC, as shown by upregulation of CD40, CD80, CD86 and MHC-II (Figure 3a and data not shown). Similar to A8819/A8817, there was also a decrease in the induction of CD80 after stimulation with the DapR isolate A9764 compared with A9763 (DapS) (Figure 3a), again most obvious in the pDC response. The A9764 DapR, like A8817 DapR, has mutations in *cls2* and *mprF* genes in comparison to its isogenic DapS parent strain⁸. This same isogenic pair (A9763/A9764) also induced a cytokine response that was similar to the A8819/A8817 pair ~~with a trend of reduced cDC1 cytokine responses when comparing the response to the A9763 (DapS) strain with the isogenic A9764 DapR (Figure 3b).~~

Importantly, the other clinical isolates of MRSA were extremely poor at inducing cytokine responses from DC (A9719 / A9744; A9796 / A9799 and A9754 / A9757). Given that all MRSA strains tested induced surface phenotype activation of DC, it remains that simply analysing surface phenotype of DC after exposure to MRSA is not indicative of effective DC activation.

Single point mutation in *cls2* impacts recognition by DC

We have previously identified that the DapR daughter strain (A8817) acquired non-synonymous point mutations in *cls2* and *mprF* during a clinical infection treated with daptomycin⁸. The independent role of these mutations in causing daptomycin resistance was confirmed by recreating these mutations into the DapS parental strain A8819, creating A8819_{Cls2-T33N} (Cls2_{mut}) as previously published¹⁴, and A8819_{MprF-T345I} (MprF_{mut}). Here, we

studied the impact of these point mutations in mediating altered immune recognition by DCs.

FLDC were stimulated with each of the four strains: A8819 (DapS), A8819 (DapR), *ClS2_{mut}* and *MprF_{mut}*. A contribution of mutations in both the *cls2* and *mprF* genes was seen in the reduced ability of FLDC to respond to the *MprF_{mut}* and *ClS2_{mut}* strains, compared to the DapS strain (Fig 4a). However, stimulation of sorted DC subsets showed a predominant effect of *ClS2*, since the *ClS2_{mut}* induced responses that mirrored the response of cDC1 to DapR and also decreased the chemokine response of cDC2 compared with DapS, without having a negative effect on cell viability (Supplementary Figure 2b).

To confirm responses that were directly due to the *cls2* mutation, we further stimulated FLDC with a reversion strain of *ClS2_{mut}* (*ClS2_{res}*), restored with the wild-type *cls2* gene¹⁴. Repair of the *cls2* mutation back to wild-type led to a restoration of DC activation with TNF- α , RANTES, MIP-1 α , and IFN- β levels equivalent to that of stimulation with DapS (Figure 4b). Furthermore, the rescue of *cls2* was able to induce CD80 expression equivalent to or greater than that of the DapS strain on all sorted DC subsets (supplementary Figure 2c).

Overall, these data demonstrate that the reduced cytokine production following stimulation of DC with MRSA is not an artefact of reduced viability, but genuinely representative of reduced activation due at least in part to mutations in the *cls2* gene. In contrast to the cytokine profile, the lower expression of CD80 in response to DapR was only partially recreated following stimulus with *ClS2_{mut}*. Thus, the compromised response of DC to the DapR strain is largely due to a mutation in *cls2*, but also other factors that are not replicated by mutation of either *cls2* or *mprF* alone.

Discussion

Our analyses of mouse FLDC responses to clinical isolates of MSSA and MRSA indicated that small changes to antibiotic-resistance genes can have major effects on the recognition by and activation of DC. At the cell surface, the DC appeared activated by all isolates of

MSSA and MRSA that we analysed. However, the analyses of cytokine production gave us an insight into fundamental differences amongst recognition of each of the isolates.

Importantly, viability of DC following stimulation with the isolates did not correlate with differences in cytokine secretion. Indeed DC survival tended to be higher following stimuli with DapR and *cls2* mutant strains compared to DapS strains (Fig 1 & Supplementary Fig 2b). These results show that the differences in cytokine secretion are not an artefact of cell viability, but rather are underestimated when considering cytokine secretion on a per cell basis.

Surprisingly, we were unable to detect the production of IFN- α to any of the isolates analysed or the heat killed bacteria (Supplementary Fig 1a). This is in contrast to earlier studies where we demonstrated high IFN- α produced by pDC in response to Pansorbin (heat killed and lyophilised *S. aureus*)³⁹. It is likely that the relative MOI using Pansorbin was much higher than we achieved with heat killed bacteria, likely explaining these discrepancies.

We detected low IL-10 production from the DC in response to the clinical isolates (Figure 2a). High IL-10 responses have previously been associated with monocyte-derived DC responses to lab strains of MRSA⁴⁰. However, the cytokine responses that we were able to detect from MSSA, and those with the biggest differential between DapS and DapR strains of MRSA, were the classic mouse cDC1 response of IFN- λ and IL-12 p70, together with strong IL-6, TNF- α and chemokine production (Figure 1, Figure 2 and data not shown). The cDC1 were major contributors to all cytokines and chemokines tested. It should be noted that the cell surface activation of cDC2 and pDC was also strong, and that it is possible that other factors are produced from these cells that we have not analysed in our cytokine array.

Specific mutations in the *cls2* and *mprF* genes were associated with a decrease in the ability of FLDC to produce cytokines in response to clinical isolates (Figure 4). When the DC were sorted, only mutations in the *cls2* gene seemed to have an effect on the cDC1 responses (Supplementary Fig 2). It is possible that other factors produced by cDC2 or pDC and not tested in this study, were differentially produced in response to the DapR strains and these factors affected the overall FLDC response. An unbiased analysis of transcriptomic responses of DC in response to MRSA DapS and DapR strains will help to resolve this issue.

The readout of an involvement of *cls2* and/or *mprF* genes was only obvious in clinical isolates of MRSA that induced relatively high production of cytokines from DC *before* acquiring daptomycin resistance (Figure 3 and Table 1). Recently published data demonstrate that *cls2* mutation leads to reduced neutrophil recruitment to the site of infection during MRSA infection¹⁴. This current study further demonstrates that the same mutation also impedes early innate immune recognition and activation by DC. Collectively, these data demonstrate that a single mutation, acquired under host immune and antimicrobial selective pressures, is able to alter the early innate immune recognition by DC and subsequent recruitment of immune effector cells.

We are currently continuing work to understand the mechanisms of differential DC functions in response to the different clinical isolates that we have analysed, including the dependence on specific PRRs and how this correlates to human DC. We contend that future work should focus on elucidating DC responses to primary clinical isolates of *S. aureus*, as opposed to the commercial lab strains, with our current view that each clinical MRSA isolate should be treated individually with respect to how it may induce innate and influence adaptive immune responses through the activation of DC.

These findings show that cDC1 are the major inflammatory responders to MRSA, and that mutations within *cls2* specifically, acquired by MRSA during daptomycin therapy, are able to reduce the inflammatory response of this DC subset.

Methods

Mice and bone marrow isolation

C57BL/6J mice were obtained from the Monash Animal Research Platform (Melbourne, VIC, Australia), and handled in accordance with the Monash University Animal Ethics Committee (ethics number MARP/2016/027). Fresh bone marrow harvested from male mice between 6 and 12 weeks of age following sacrifice by CO₂ asphyxiation and cervical dislocation. Bone marrow was flushed from the femur and tibia in cold media using a 21-gauge needle.

Culture of FLDC

Murine bone marrow cells were cultured in a humidified atmosphere at 37°C in 5% CO₂ in complete media (CM: Gibco RPMI-1640 Glutamax (Thermo Fisher Scientific; Waltham, USA) medium supplemented with 10% FCS (In Vitro Technologies; Noble Park, Australia), 100µM 2-Mercaptoethanol (Thermo Fisher Scientific) and 0.01% (w/v) Penicillin/Streptomycin (Thermo Fisher Scientific) adjusted to mouse tonicity 308 mOsm/L for 8 days in the presence of 100 ng mL⁻¹ Flt3-ligand (FL, Bio X Cell; Lebanon, USA) as previously described⁴¹. The differentiation of FLDC, *bona fide* counterparts to primary splenic CD8+, CD8- and pDC, was verified at day 8 via flow cytometry.

In vitro stimulations with bacterial strains

Paired clinical isolates of MRSA are listed in Table 1. Bacterial strains were grown in Brain Heart Infusion (BHI) broth overnight and the bacterial density was adjusted to 4 x 10⁹ CFU mL⁻¹, which was estimated by optical density of 600nm (OD₆₀₀) = 8. The bacterial cell suspension was serially diluted and plated on BHI agar to confirm the desired bacterial cell density was achieved. Where applicable, heat inactivation of clinical isolates was performed at 95°C for 30 minutes. Control strains of *L. monocytogenes*, *L. rhamonusus*, *H. pylori*, *S. aureus*, *S. epidermis*, *S. pneumoniae* and *E. coli* were purchased as heat inactivated lyophilised bacteria (InvivoGen; San Diego, USA). Bulk cultures of FLDC were maintained in CM at 1 X 10⁶ cells mL⁻¹, and sorted FLDC maintained in CM supplemented with 10 ng mL⁻¹ GM-CSF at 0.5 X 10⁶ cells mL⁻¹. DC were stimulated for 18 hours in a humidified atmosphere at 37°C in 5% CO₂ in CM at an equivalent MOI of 10 unless otherwise indicated. Tissue culture supernatants were collected to assess inflammatory cytokine secretion at endpoint, and cells analysed by flow cytometry.

Cytokine assays

IFN-β, IL-6, IL-12p70, MIP-1α, MIP-1β, RANTES and TNF-α levels were quantified from either fresh or thawed tissue culture supernatants using a custom LEGENDplex (BioLegend) flow cytometry based bead array system per manufacturer's instructions (BioLegend). IFN-α and IFN-λ levels were quantified from either fresh or frozen tissue culture supernatants by ELISA as per manufacturer's instructions, for IFN-α (Invivogen) and IFN-λ (R&D Systems; Minneapolis, USA).

FACS sorting and flow cytometry

Day 8 FLDC were stained for cell sorting (Supplementary Figure 2) or analyses with the following antibodies: Brilliant Ultra Violet (BUV) 395- anti-CD172 (clone P84; Becton Dickinson), Brilliant Violet (BV) 421- anti-CD11c (clone N418; BioLegend, San Diego, USA), BV711- anti-CD24 (clone M1/69; Becton Dickinson), Biotin anti-CD317 (clone 120G.8), PE-Cyanine7- (PE-Cy7) streptavidin (Becton Dickinson) and allophycocyanin-cyanine7 (APC-Cy7) anti-CD45R (clone RA3-6B2; BioLegend). Following bacterial stimulation DC were stained with the following antibodies: BV605- anti-PD-L1 (clone 10F.9G2; Becton Dickinson), PE-anti-CD80 (clone 16-10A1; Becton Dickinson), PE-Cy7- anti-CD86 (clone GL1; BioLegend), APC- anti-PD-L2 (clone TY25; Becton Dickinson), APC- anti-CD40 (clone 3/23; BD) and APC-Cy7 anti-MHC-II (clone M5/114.15.2; BioLegend). Viability was quantified via differential staining in propidium iodide (Sigma, Rowville, Australia). Cell sorting and analyses were performed on a BD Influx and BD Fortessa (Becton Dickinson) respectively.

Genetic manipulation of *S. aureus*

The vector pIMAY-Z and the *E. coli* strain DC10B were used to genetically manipulate *S. aureus* isolate A8819 following the published protocol⁴². DNA fragments were amplified from A8819 genomic DNA using forward primer 5' CCTCACTAAAGGGAACAAAAGCTGGGTACC AGCAATCACATTGTATCGGGAGT 3', and reverse primer 5' CGACTCACTATAGGGCGAATTGGAGCTCAGTCGCTCTAAATCCACGGC 3' followed by PCR purification. The purified DNA fragments were then joined with pIMAY-Z using Seamless Ligation Cloning Extract (SLICE) cloning⁴³ and transformed into *E. coli* strain DC10B. Colony PCR with the forward primer 5' TACATGTCAAGAATAAACTGCCAAAGC 3' and reverse primer 5' AATACCTGTGACGGAAGATCACTTCG 3' were used to detect pIMAY-Z in *E. coli* and *S. aureus* transformants⁴⁴. Point mutation of *mprF* was confirmed by DNA sequencing using primer 5' GCGTTTCAACCTACGTGCTC 3'. Daptomycin E-tests (BioMerieux) were conducted according to manufacturer's recommendations to confirm the resistance to daptomycin of the genetically engineered strain A8819_{MprF-T345I}.

Statistical analysis

Statistical significance was determined using paired parametric two-tailed t-tests or one-way ANOVA using Tukey's test for to correct for multiple comparisons, calculated with Prism (GraphPad Software, La Jolla, USA).

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Conflict of Interest

The authors have no conflicts of interest to declare

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Table 1 Clinical and genomic characteristics of daptomycin exposed *Staphylococcus aureus* isolates, as previously reported by Peleg *et. al.*, (2012).

Strain	Clinical diagnosis	Dp MIC ($\mu\text{g mL}^{-1}$) [†]	Genomic mutations [‡]	Mutated genes affecting cell wall or membrane	Ref
A8796	Bacteraemia	0.5	2	<i>mprF</i>	Peleg <i>et. al.</i> , (2012) ⁸
A8799	Vertebral osteomyelitis	2		<i>citZ</i>	
A9719	Bacteraemia	0.25	6	<i>mprF</i>	Peleg <i>et. al.</i> , (2012) ⁸
A9744	Endocarditis	2		<i>cls2</i> <i>atl</i>	
A9754	Bacteraemia	0.5	9	<i>mprF</i>	Peleg <i>et. al.</i> , (2012) ⁸
A9757	Endocarditis	4			
A9763	Bacteraemia	0.5	3	<i>mprF</i>	Peleg <i>et. al.</i> , (2012) ⁸
A9764	Osteomyelitis, Prosthetic joint infection	4		<i>cls2</i>	
A8819	Bacteraemia	0.25	5	<i>mprF</i> <i>cls2</i>	Peleg <i>et. al.</i> , (2012) ⁸
A8817	Osteomyelitis, septic arthritis	2			
A8819 _{Cls2-T33N} (Cls2 _{mut})	N/A	2	N/A	<i>cls2</i>	Jiang <i>et. al.</i> , (2019) ¹⁴
A8819 _{Cls2-T33NN33T} (Cls2 _{res})	N/A	0.5	N/A	<i>none</i>	Jiang <i>et. al.</i> , (2019) ¹⁴
A8819 _{MprF-T345I} (MprF _{mut})	N/A	2	N/A	<i>mprF</i>	This study

[†]Daptomycin resistance is defined as MIC > 1 $\mu\text{g mL}^{-1}$

[‡] Total number of point mutations observed in the clinical daptomycin resistant daughter isolate with reference to the susceptible parent strain

Figure Legends

Figure 1 Differential response of FLDC to live clinical isolates of MSSA and MRSA. **(a)** IL-6 and IFN- β production (pg mL^{-1}) by FLDCs after 18 hour stimulus with two unrelated MSSA clinical isolates and daptomycin sensitive (DapS) MRSA, at an MOI of 10. Data show mean and standard error of the mean (SEM) of FLDC preparations from 8 individual mice pooled from three independent experiments ($n = 8$). Significance shown as * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ from a one-way ANOVA using Tukey's test for to correct for multiple comparisons **(b)** IFN- λ production (pg mL^{-1}) by FLDCs after 18 hour stimulus with 2-fold serial dilutions of DapS and DapR clinical isolates of MRSA from an MOI of 20. Data shown from one experiment, representative of at least three independent experiments. **(c)** FLDC viability after 18 hour stimulus with 2-fold serial dilutions of paired DapS and DapR clinical isolates of MRSA from an MOI of 160. Data show the mean and range of technical replicates from one experiment, representative of two independent experiments.

Figure 2 Activation phenotype of FLDCs exposed to clinical paired DapS and DapR MRSA. **(a)** Cytokine production ($\text{pg}^{-1} \text{ mL}$) by total FLDCs after 18 hour stimulus with MRSA, equivalent to MOI = 10. Bars show the mean and SEM for each bacterial stimuli (MOI = 10), CpG ODN 1668 ($0.5 \mu\text{M}$) or media alone. Heat inactivated bacterial stimuli, $n = 5$, and all other stimuli, FLDC preparations from 11 individual mice ($n=11$), pooled from at least three independent experiments. Significance shown as * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ in paired two-tailed t -tests. **(b)** Histogram overlays showing CD40, CD80 and MHC-II and PD-L1 expression by FACS sorted cDC1, cDC2 and pDC after 18 hour stimulus with DapS (grey line) and DapR (black filled) MRSA. Fluorescence minus one background control (FMO) is shown by dotted black line. Data shown are representative of three independent experiments on sorted FLDC ($n = 3$). **(c)** Cytokine production (pg mL^{-1}) by FACS sorted DCs after 18 hour stimulus with DapS (grey bars) and DapR (black bars) paired clinical isolates of MRSA. Bars show the mean and SEM with replicates pooled from four independent experiments. **(d)** IFN- λ production (pg mL^{-1}) by sorted FLDC subsets following 18 hours stimulus with DapS and DapR MRSA (MOI = 10) or CM. Bars show the mean and SEM with replicates from four independent experiments.

Figure 3 Reduced activation of DCs correlates with resistance to antibiotics. **(a)** Histogram overlays showing CD80 expression by FACS sorted FLDC subsets after 18 hour stimulus with DapS (grey line) or DapR (black filled) MRSA clinical pairs. Data shown are representative of two independent experiments with sorted FLDC. **(b)** Cytokine production (pg mL^{-1}) by FACS sorted FLDC after 18 hours stimulus with paired MRSA clinical isolates

(grey bars: DapS; black bars: DapR). Bars show the mean (circles) and range of replicates pooled from the same 2 independent experiments as in (a).

Figure 4 Point mutations in *cls2* and *mprF* in MRSA affect the activation of DC. **(a)** Cytokine secretion (pg mL^{-1}) by FLDC following 18 hour stimulus with DapS and DapR MRSA clinical isolates, *Cls2*_{mut} and *MprF*_{mut} strains (MOI=10). Data show the mean and SEM of replicates from 8 individual mouse FLDC preparations ($n = 8$), pooled from three independent experiments. Significance shown as * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ from a one-way ANOVA using Tukey's test for to correct for multiple comparisons. **(b)** Cytokine secretion (pg mL^{-1}) by FLDC following 18 hour stimulus with MRSA clinical isolates, *Cls2*_{mut} as in 'a', and the rescue strain, *Cls2*_{res} (MOI=10). Data show the mean and SEM of replicates from 8 individual mouse FLDC preparations ($n = 8$), pooled from three independent experiments (data shown in a and b from same set of experiments). Significance shown as * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ from a one-way ANOVA using Tukey's test for to correct for multiple comparisons. **(c)** Histogram overlays showing CD80 expression by FACS sorted cDC1, cDC2 and pDC after 18 hour stimulus with DapS and DapR clinical isolates, and *Cls2*_{mut} and *MprF*_{mut} strains. Data shown from one experiment representative of three independent experiments **(d)** Geometric mean fluorescence intensity (gMFI) of CD80 expression by DC subsets as in 'c', showing the mean and SEM of expression pooled from three independent experiments. Significance shown as * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ from a one-way ANOVA using Tukey's test for to correct for multiple comparisons.

Supplementary Figure 1 Activation of FLDCs exposed to a panel of heat inactivated bacteria. **(a)** Cytokine production (pg mL^{-1}) by FLDCs after 18 hour stimulus with heat inactivated bacteria, equivalent to MOI = 10. Responses to media (CM and CpG control are shown in right panel). Data shown are from two independent experiments. Mean and range are shown for each stimuli ($n = 2$). **(b)** IFN- λ production (pg mL^{-1}) by FLDC following 18 hours stimulus with heat inactivated bacteria from an MOI = 100, CpG 2216 (0.5 μM), polyIC (100 $\mu\text{g mL}^{-1}$) or CM. Bars show the mean and range of biological duplicates from one experiment ($n = 1$). **(c, d)** CD40, CD80, CD86 and MHC-II expression by cDC1 **(c)** and cDC2 **(d)** subsets in FLDC bulk cultures after 18 hour stimulus with heat inactivated bacteria, equivalent to MOI = 10 (grey filled) or media (black line). FMO is shown as dotted line. Data shown are from 1 experiment, representative of two independent experiments ($n = 2$).

Supplementary Figure 2 Repair of the *Cls2*_{mut} strain restores DC activation. **(a)** Cytokine production (pg mL^{-1}) by FACS sorted FLDC subsets; cDC1, cDC2 and pDC, 18 hours after stimulus DapS and DapR clinical isolates, *Cls2*_{mut} and *MprF*_{mut} strains. Bars show the mean and SEM of replicates from individual mouse FLDC preparations from three independent experiments ($n = 3$). **(b)** Percentage viability of FACS sorted FLDC subsets stimulated as in (b). Viability quantified by flow cytometry via differential staining with propidium iodide at 18 hours post stimulus. Bars show the mean and standard deviation of replicates from 3 independent experiments ($n = 3$). **(c)** Histogram

overlays showing CD80 expression by FLDC following 6 hour stimulus with DapS (light grey shaded) clinical isolates, the rescue strain Cls2_{res} (light grey trace), and media alone (black trace). Data shown from 1 experiment, representative at least three independent experiments on FLDC and splenic DC (n = 3).

Supplementary Figure 3 Sequential gating strategy for FACS sort of day 8 FLDCs. **(a)** Doublet exclusion based of forwards scatter height and area (FSC-H and FSC-A). **(b)** Exclusion of debris based on FSC-A and side scatter area (SSC-A). **(c)** Live cell gate isolating live cell sized events negative for propidium iodide viability dye. **(d)** Gating of most mature conventional and plasmacytoid DC subsets based on expression of highest levels of CD11c and CD45R, differentiating cDCs (CD11c⁺, CD45R⁻) and pDCs (CD11c^{int}, CD45R⁺). Note in inset histogram that approximately 90% of cells in the culture express CD11c. The black line is the unstained control. **(e)** Gating of cDC1 and cDC2 from the most mature conventional subset based on CD24 and CD172 α expression, differentiating cDC1 (CD24^{hi}, CD172 α ⁻) and cDC2 (CD24^{int}, CD172 α ⁺). **(f)** Those 'cDC' that express lower levels of CD11c (yellow box) are predominantly CD172 α ⁺ cDC2. With another 1-2 days of culture these cells mature and upregulate CD11c (not shown). **(g)** Apart from the cDC1 (black circle) and mature cDC2 (red box) gated in (e), there is also a population of CD11c⁺ cells within the mature cDC gate of the FLDC Day 8 cultures that have the phenotype CD11c⁺CD24^{int}CD172 α ^{-/+} (blue box). These cells, many are also present with lower levels of CD11c in (f), are immediate precursors of the mature DC populations, as we have previously shown (Naik SH et al. Nat Immunol. 2007 Nov;8(11):1217-26.). As shown in the histogram to the left, this CD11c⁺CD24^{int}CD172 α ^{-/+} population has the phenotype MHCII⁺CD86^{lo} (blue line in histogram below dot plot), quite distinct from the mature cDC1 shown in the black line. Grey line is unstained control. As shown in the histogram to the right these cells are small, low side scatter, express low levels of CD11b and CD317 and do not express XCR1. The cDC1 (black) and cDC2 (red) phenotypes are shown for comparison.

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