A Critical Role of Zinc Importer AdcABC in Group A Streptococcus-Host Interactions During Infection and Its Implications for Vaccine Development

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1. Introduction

Nutrient acquisition by pathogens during infection is imperative for bacterial proliferation and disease development (Porcheron et al., 2016). The host exploits the nutritional requirements of the pathogen to limit growth by altering the availability of micronutrients at the colonization surface in a process referred to as nutritional immunity. The delicate balance between zinc sufficiency and toxicity is maintained by bacterial metal homeostasis systems (Klaus, 2005; Moore and Helmann, 2005). Although insights into the role of nutritional immunity in host defenses against pathogenic microorganisms are rapidly emerging (Corbin et al., 2008; Damo et al., 2013; Kehl-Fie and Skaar, 2010; Ong et al., 2014). The primary GAS zinc uptake system, AdcABC, is composed of a cell surface-exposed zinc-binding protein (AdcA), an inner membrane permease (AdcB), and a cytosolic ATPase (AdcC) that provides the energy for zinc import by ATP hydrolysis. GAS also employs additional factors such as AdcAII/Lmb, PhtD, and PhtY to overcome zinc limitation (Sanson et al., 2015). During zinc sufficiency, the zinc-bound AdcR represses the expression of target genes, whereas the apo-AdcR relieves the repression during zinc deficiency. When cells encounter zinc limitation, GAS upregulates genes encoding zinc acquisition systems (adcA, adcAll/lmb, adcbC, phdT, and phtY), and zinc sparing responses (rpsN2 and adh) (Sanson et al., 2015). The primary GAS zinc uptake system, AdcABC, is composed of a cell surface-exposed zinc-binding protein (AdcA), an inner membrane permease (AdcB), and a cytosolic ATPase (AdcC) that provides the energy for zinc import by ATP hydrolysis. GAS also employs additional factors such as AdcAll/Lmb, PhtD, and PhtY to overcome zinc limitation (Sanson et al., 2015). Both AdcA and AdcAll share homologous N-terminal ZnuA-like extracellular zinc-binding domains. However, AdcA has an additional C-terminal ZntT-like...
domain and the role of the ZnT-like domain of AdcA in GAS homeosta-
sis is yet to be elucidated. Although AdcAII/Lmb and Pht proteins con-
tribute to streptococcal adaptive responses to zinc limitation (Bayle et
al., 2011; Moulin et al., 2016; Tedde et al., 2016), the exact mechanisms
by which they facilitate zinc acquisition remain unknown. Nevertheless,
the significance of the AdcR signaling pathway to GAS pathogenesis is
underscored by the observation that inactivation of AdcR caused dysreg-
ulation of zinc homeostasis and significantly attenuated GAS virulence
(Sanson et al., 2015).

The host recruits calprotectin (CP), an S100A8/A9 heterodimer, at
microbial colonization surfaces and inhibits bacterial proliferation by
sequestration of zinc, and manganese (Corbin et al., 2008; Damo et al.,
2013; Diaz-Ochoa et al., 2016; Liu et al., 2012; Lusistani et al., 2003).
The antibacterial activity of CP against bacterial and fungal pathogens
has been demonstrated (Corbin et al., 2008; Damo et al., 2013;
Diaz-Ochoa et al., 2016; Liu et al., 2012; Sohnle, et al., 1991; Urban, et
al., 2009), but its role against most of the streptococcal pathogens is un-
known. Emerging evidence indicates that GAS encounters host-mediat-
ed zinc immune mechanisms (Brenot et al., 2007; Ong et al., 2014).
However, molecular details underlying host defense mechanisms, bac-
terial countermeasures, and their role in GAS pathogenesis are lacking.
Using a multidisciplinary approach, we discovered that CP is a major
host defense factor against GAS infection in different niches and medi-
ates growth inhibition primarily by zinc sequestration. Conversely,
GAS employs a high-affinity zinc uptake system and a refined sensory
system to overcome CP-mediated growth inhibition. To realize the
translational potential of our findings, we assessed and validated the ex-
tracellular component of the zinc importer, AdcA, as a potential GAS
vaccine candidate.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Supple-
mentary Table S1. Strain MGAS10870 is representative of serotype M3
strains that cause invasive infections whose genome has been fully se-
quenced and has wild-type sequences for all major regulatory genes
(Beres et al., 2010). Details of isogenic mutants construction using the
parental serotype MGAS10870 is described in the Supplemental Exper-
imental Procedures. Escherichia coli DH5α strain was used as the host
for plasmid constructions and BL21(DE3) strain was used for recombi-
nant protein overexpression. GAS was grown routinely on Trypticase
SoY agar containing 5% sheep blood (BSA; Becton Dickinson) or in
Todd–Hewitt broth containing 0.2% (w/v) yeast extract (THY; DIFCO).
The Escherichia coli was grown in Luria-Bertani broth (LB broth; Fisher
Scientific).

2.2. Animal Infection Studies

Mouse experiments were performed according to protocols ap-
proved by the Houston Methodist Hospital Research Institute Institu-
tional Animal Care and Use Committee. These studies were carried out
in strict accordance with the recommendations in the Guide for the
Care and Use of Laboratory Animals, 8th edition. Mouse infections stud-
ies, analysis of colony-forming units (CFU), and histopathology of the
infected tissues were carried out as detailed in the Supplemental Experi-
mental Procedures.

2.3. Preparation of Total Protein Extracts From the Infected Tissues

To ensure that the CP levels measured are predominantly the secret-
ed fraction, not the neutrophil cytosolic fraction, we employed methods
to minimize contamination with intact neutrophils, neutrophil lysis,
and acquisition of cytosolic CP. Typically, the abscess fluid is mostly
acellular, and contains predominantly necrotized neutrophils. Intact
neutrophils are likely to be around the edges of abscesses bordering
with the healthy tissues and were not included in the sample prepara-
tion. The abscess fluid from the GAS infected tissues were carefully aspi-
rated by puncturing the lesion and the purulent fluid was resuspended
(30 mg of lesion/ml) in sterile PBS supplemented with a protease inhib-
itor cocktail pellet. Subsequently, the lesions were homogenized gently
on ice and cell debris was removed by centrifugation at 20,000 × g for
30 min. The clarified supernatant was collected, filtered with 0.22 μm
filter, and the total protein concentration was assessed by Bradford
assay. The supernatant containing equal total protein concentration
was assayed for CP by immunoblotting and ELISA assays.

2.4. Identification of Calprotectin in Infected Tissues by Immunoblotting

Equal concentrations of the total protein extracts from the tissue
samples were resolved on a 15% SDS-PAGE gel, transferred to a nitrocel-
lulose membrane, and probed with rat monoclonal anti-mouse S100A8,
S100A9 (R&D Systems), and polyclonal anti-mouse GAPDH (Invitrogen)
antibodies. Detection was accomplished by goat anti-rat secondary
antibody conjugated with horseradish peroxidase (R&D Systems), and vi-
ualized by chemiluminescence.

2.5. Quantification of Calprotectin in Infected Tissues by ELISA

ELISA assays to measure calprotectin levels in the murine tissues
were performed according to the manufacturer’s instructions (Hycult
Biotech). The calibration curve was generated using the calprotectin
standard in the kit, and this curve was used to derive the concentration
of calprotectin in the tissue samples. Samples were analyzed in triplicates
and data were graphed as calprotectin concentration in micro-
grams per ml of total protein extract.

2.6. Bacterial Growth Studies in the Presence of CP

Recombinant human calprotectin was overexpressed and purified as
detailed in Supplemental Experimental Procedures (Fig. S1). A 1:100 di-
lution of overnight GAS growth was inoculated into THY broth supple-
mented with calprotectin buffer (20 mM Tris pH 7.5, 0.1 M NaCl,
10 mM (γ-mercaptopoethanol, 3 mM CaCl₂). The indicated concentrations
of CP were added to the starter culture and growth was monitored by
measuring A600 with a microplate reader. Samples were analyzed in
triplicate and at least two different CP preparations were used.

2.7. Metal Content Analysis by Inductively Coupled Plasma Mass Spectrom-
etry (ICP-MS)

Metal concentration in CP-treated growth medium, intracellular
metal content of different GAS strains and variants of recombinant
AdcR were analyzed by ICP-MS as described in Supplemental Experi-
mental Procedures.

2.8. Transcript Level Analysis

GAS strains were grown to the indicated OD₆₀₀ and incubated with
two volumes of RNAprotect (Qiagen) for 10 min at room temperature.
RNA isolation and purification were performed using an RNAeasy kit
(Qiagen). cDNA was synthesized from the purified RNA using Sup-
script III (Invitrogen) and Taqman quantitative RT-PCR was performed
with an ABI 7500 Fast System (Applied Biosystems). Comparison of
transcript levels was performed by the ΔCt method of analysis using
tufA as the endogenous control gene. The Taqman primers and probes
used in this study are listed in Supplementary Table S2.
2.9. Overexpression and Purification of AdcR

The adcR gene of strain MGAS10870 was cloned into plasmid pET-21b and pET-15b. Protein overexpression and purification was performed as described previously and detailed in Supplemental Experimental Procedures.

2.10. Crystallization and Structure Determination of AdcR

Crystallization and structure determination of AdcR was performed by standard methods and detailed in the Supplemental Experimental Procedures.

2.11. Site-directed Mutagenesis of Recombinant AdcR

Plasmid pET21b-adcR or pDC-adrC containing the wild-type adcR-coding region was used as template for the site-directed mutagenesis. Quick change site-directed mutagenesis kit (Stratagene) was used to introduce single amino acid substitutions within the adcR coding region and substitutions were confirmed by DNA sequencing. Mutant proteins were overexpressed and purified as described above.

2.12. Overexpression and Purification of AdcA-NTD

The coding sequence of the extracellular domain of adcA without the secretion signal sequence (amino acids 32–320) from strain MGAS10870 was amplified and cloned into NdeI and BamHI restriction sites of E. coli overexpression vector pTYB21 (New England BioLabs). The details of AdcA-NTD purification is described in the Supplemental Experimental Procedures.

2.13. Mouse Immunization Studies

Female 3–4 week-old CD1 mice (Harlan Laboratories) were immunized with 10 μg of purified AdcA-NTD with either 2% aluminum hydroxide gel (Accurate Chemical and Scientific Corporation) or Sigma adjuvant system (Sigma-Aldrich) administered intramuscularly at 14, 28, and 42 days. The control group mice were immunized with either 2% aluminum hydroxide gel or Sigma adjuvant system mixed with PBS. Blood samples were collected after each round of immunization and the sera were separated.

2.14. Subcellular Fractionation of GAS Proteins

To maximize the expression of AdcA, chemically defined medium devoid of zinc was used for bacterial growth. GAS strains were grown to the late exponential growth phase (A600 ~ 1.0) and washed twice with PBS. Individual subcellular fractions of GAS growth were prepared as described in Supplemental Experimental Procedures. Equal concentration of the samples was probed for AdcA with anti-mouse AdcA serum by standard immunoblotting.

2.15. Western Immunoblotting Analysis

Purified AdcA-NTD was resolved by SDS-PAGE and transferred to nitrocellulose membranes. After blocking the membrane overnight with 4% nonfat milk, the membrane was incubated with mouse serum in TBS-T for 1 h at room temperature and rinsed with TBS-T for 15 min 3 times. The membrane was incubated for 1 h with goat anti–mouse horseradish peroxidase (HRP)–conjugated secondary antibodies diluted at 1:5000. Antibody-antigen interaction was visualized by chemiluminescence.

2.16. ELISA

To measure antibody titers of immune sera from mice vaccinated with recombinant AdcA-NTD, microtiter plates (Corning) were coated with purified recombinant AdcA-NTD at a concentration of 0.5 μg/ml and antibody levels in serum samples were determined using standard protocols. Plates were washed 3 times with TBS-T, and the uncoated surface was blocked with 2% skim milk (v/v) for 3 h at room temperature. The plate was incubated with serum from mock or immunized mice serially diluted 10-fold in TBS-T and incubated for 1 h at room temperature. Antigen-specific IgG was detected with HRP-conjugated secondary antibody and the plates were developed with TMB-Plus (Thermo Scientific) followed by addition of stop solution. Absorbance was measured at 450 nm, and the titer was determined as the dilution of immunized serum that was 3 times above the reading of the control (mock) serum.

2.17. GAS Challenge Studies

Immunized and control mice (n = 20) were challenged intraperitoneally with 5 × 107 CFU of GAS 2 or 5 weeks after the last immunization and monitored for near mortality using the parameters described in Supplemental Experimental Procedures. Results were graphically displayed as a Kaplan-Meier survival curve and analyzed using the log-rank test.

2.18. Detection of Human Antibodies Against AdcA

Blood was collected from patients with invasive GAS infections or no known recent GAS exposure (Streptolysin O negative). Samples were collected from patients with necrotizing fasciitis at the time of presentation to our hospital. Sera were separated by centrifugation and transferred to sterile cryovials for storage at −80 °C. This study was approved by the Institutional Review Board at Houston Methodist Hospital and Houston Methodist Research Institute.

Purified AdcA-NTD was resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membrane was incubated with individual human serum sample at 1:50,000 dilution in TBS-T for 1 h at room temperature followed by additional 1 h incubation with goat anti-human HRP–conjugated secondary antibodies (Thermo scientific) diluted at 1:10,000. Antibody-antigen interaction was visualized by chemiluminescence.

3. Results

3.1. GAS Encounters Calprotectin During Infection

To investigate the role of CP in host defense against GAS infection, we assessed the presence of CP at GAS infection sites using two mouse models of invasive disease. Mice were infected either intramuscularly or subcutaneously with serotype M3 GAS or saline control and total protein extracts from the lesions were probed for CP by immunoblotting. CP was detected in intramuscular lesions from GAS-infected mice as early as day 1 post infection (pi) and this response persisted until day 4 pi (Fig. 1A). Furthermore, quantification of CP levels in the GAS-infected tissues by ELISA revealed that CP was present at an average concentration of ~61 μg/ml at day 1 pi and the levels increased significantly to 155 μg/ml at day 4 pi (Fig. 1B). Since GAS infects different host niches and the nutritional immune mechanisms vary with tissue microenvironment (Botella et al., 2011; Mason and Skaar, 2009; Ong et al., 2014), we also investigated the role of CP in a subcutaneous mouse model of invasive infection. As observed in intramuscular infection, CP was detected in GAS-infected tissues at an average concentration of 138 μg/ml at day 3 pi (Fig. 1C and D). Collectively, these results demonstrate that CP is present at GAS infection sites in the host and likely participates in host defense against GAS infection.
3.2. Zinc Sequestration by Calprotectin Contributes to GAS Growth Inhibition

To elucidate the role of CP in host defense against GAS infection, we purified recombinant human CP (CP) (Fig. S1) and tested bacterial growth at varying concentrations of CP. A CP concentration-dependent GAS growth inhibition was observed. GAS growth was comparable to untreated sample at the lowest CP concentration tested, whereas growth was either retarded (250 μg/ml) or inhibited at higher concentrations (500 μg/ml; Fig. 1E). The anti-streptococcal activity of CP was more evident in the colony-forming unit (CFU) analysis of GAS growth: it was drastically reduced at higher CP concentrations compared to the untreated control (Fig. 1F). However, GAS displayed a marked ability to sustain growth at 125 μg/ml and to a lesser extent at 250 μg/ml CP, suggesting that the pathogen possesses potent adaptive mechanisms to overcome CP-mediated bacteriostatic effect.

To elucidate the mechanism of growth inhibition by CP, we pursued two lines of investigation. To test whether CP imposes zinc starvation on GAS, we monitored the upregulation of AdcR-controlled genes as a marker for bacterial sensing of zinc limitation. Cells grown in the presence of 125 and 250 μg/ml of CP exhibited a dose-dependent induction of zinc acquisition systems (Fig. 1G). Transcript levels of the \(lmb\) gene were induced by ~146- and 228-fold at 125 and 250 μg/ml CP, respectively, relative to untreated control (Fig. 1G). A similar upregulation profile in concert with increasing CP concentration was observed for \(adcA\) and \(adcC\) genes (Fig. 1G). Subsequently, we measured zinc content in growth media treated with CP by ICP-MS. As expected, CP treatment significantly reduced zinc, manganese, and iron concentrations in the growth medium compared to untreated samples (Figs. 1H and S1E). Together, these results indicate that CP sequesters metal ions from GAS and induces a known zinc limitation response.

Fig. 1. Calprotectin is present at the GAS colonization surface in the host. a) Lesions from mice infected intramuscularly at days 1 and 4 post-infection with either saline (mock) \((n = 4)\) or wild type GAS \((n = 4)\) were probed for S100A8, S100A9, and loading control GAPDH by immunoblotting. b) Quantification of calprotectin in the infected muscular tissue lesions by ELISA. Data graphed are mean ± standard deviation. Statistical significance between the mock and GAS-infected samples is indicated by * \((P < 0.05)\). P values were derived from two-sample t-test. c) and d) Calprotectin levels in the subcutaneous abscesses of mice infected with either saline (mock) \((n = 4)\) or wild type GAS \((n = 4)\) were analyzed by immunoblotting (c) and quantified by ELISA (d) at day 3 post infection. Data graphed are mean ± standard deviation. Statistical significance between the mock and GAS-infected samples is indicated by * \((P < 0.05)\). P values were derived from two-sample t-test. e) GAS were grown in triplicate in THY broth supplemented with the indicated concentrations of CP. Growth was monitored by absorption at A600 in a microplate reader at the indicated time points. f) Comparison of the number of CFUs of GAS grown in the indicated concentrations of CP. Triplicate cultures were grown at the indicated concentrations. Using the optical density of the untreated GAS growth, cells were collected at the start point \((t = 0)\), early exponential \((EE, A600 = 0.125)\), mid-exponential \((ME, A600 = 0.25)\), late-exponential \((LE, A600 = 0.375)\), and stationary \((STAT, A600 = 0.5)\). Statistical significance between the untreated and calprotectin treated samples is indicated by ** \((P < 0.0001)\). Data graphed are mean ± standard deviation. h) Metal content analysis of THY broth treated with the indicated concentrations of CP. Metal concentration \((M)\) is denoted in the y-axis (in μM). Samples were analyzed in triplicate and data graphed are mean ± standard deviation. Statistical significance between the untreated and calprotectin treated samples is indicated by ** \((P < 0.0001)\). P values were derived from two-sample t-test.
3.3. Zinc Uptake is Critical for GAS Defense Against CP and Pathogenesis

To understand the contribution of zinc importers to bacterial defense against host-mediated zinc sequestration, we constructed an isogenic ΔadcC mutant with an inactive cytoplasmic ATPase component of the tripartite zinc importer AdcABC (Fig. S2). Although ΔadcC mutant exhibited growth comparable to wild type (WT) in nutrient-rich medium, it displayed significant growth defects in either medium unamended with zinc or medium pretreated with zinc chelator, TPEN (Fig. 2A–C). Growth defect of the ΔadcC mutant was rescued by the trans-complementation plasmid pDC-adcCB (Figs. 2A–C, and S2). Furthermore, growth experiments with CP revealed an inability of ΔadcC to compete against CP-mediated zinc sequestration. Mutant growth was arrested at 125 μg/ml CP compared to 500 μg/ml CP required to cause similar effects on WT growth (Fig. 2D). Finally, metal content analysis of ΔadcC grown in rich laboratory medium revealed significant reduction in cytosolic zinc levels in the ΔadcC compared to WT and trans-complemented strains, indicating its critical role in zinc uptake by GAS (Fig. 2E). Collectively, these results underscore the significance of zinc uptake system in GAS defense against CP-mediated zinc limitation.

To determine the interplay between CP and bacterial zinc acquisition system in vivo, we compared the virulence of WT, ΔadcC, and trans-complemented strains using mouse models of necrotizing myositis and skin and soft tissue infection. In both models of invasive infection, virulence of ΔadcC was significantly attenuated compared to WT and trans-complemented strains (Fig. 2F–K). Histopathological analysis of...
intramuscular lesions revealed that the ΔadcC caused markedly smaller lesions with less tissue destruction compared to WT and trans-complemented strains (Fig. 2G). Since adcC is critical for GAS survival in vitro, we assessed bacterial load in the infected muscular lesions by measuring CFUs. At day 1 pi, all 3 strains exhibited similar bacterial burden. However, consistent with in vitro findings, growth of ΔadcC was significantly reduced at day 3 pi, suggesting that the mutant has a decreased ability to survive in vivo (Fig. 2H). Measurement of CP levels revealed that lesions from mice infected with WT and trans-complemented strains had elevated levels of CP at both time points, whereas lesions caused by ΔadcC strain had much lower CP levels at day 1 pi and were significantly reduced at day 3 pi (Fig. 2I and J). Similar observations were made in subcutaneous lesions (Fig. 2L and M), indicating that GAS adaptive responses to host-induced zinc limitation are critical for GAS pathogenesis at multiple host anatomic sites. These results also suggest that infection caused by ΔadcC mutant strain is resolved by host defenses earlier than WT and trans-complemented strains, leading to reduced inflammation and CP levels in lesions caused by ΔadcC. Together, these results emphasize the importance of the zinc uptake system to bacterial survival in vivo and GAS virulence.

3.4. Molecular Mechanism of Varied Gene Regulation by AdcR and Its Impact on GAS Virulence

GAS exhibited a dose-dependent induction of zinc acquisition genes in response to increasing CP concentration, which resembles our earlier findings that AdcR mediates gradual derepression of its target genes in response to decreasing zinc availability (Sanson et al., 2015). To deduce the molecular basis for varied gene regulation, we determined the crystal structure of AdcR. The AdcR dimer has a triangular-shaped fold, in which the DNA-binding and dimerization domains are located at the base and apex of the triangle, respectively (Figs. 3A and S3). Each AdcR subunit has one zinc atom bound at the interdomain region (Fig. 3A and B). Consistent with our previous genetic studies (Sanson et al., 2015), the zinc is coordinated by the side chains of H42 from helix α2, and H108 and H112 from helix α5 (Figs. 3B and S3). Comparative structural analyses with the crystal structure of the related pneumococcal AdcR (AdcRspn) (Guerra et al., 2011; Reyes-Caballero et al., 2010) revealed that the side chain of E24 from the loop connecting helices α1 and α2 also participate in zinc coordination. Due to the lack of well-defined electron density, we could not unambiguously locate the main chain and side chain atoms of E24 in AdcRGAS structure. Given that E24 is conserved between the two proteins, we hypothesized that the side chain of E24 is involved in zinc binding in AdcRGAS. Furthermore, our X-ray analysis of AdcR crystals at the zinc absorption edge indicated that there are two zinc atoms per AdcR subunit within the asymmetric unit of AdcR crystal. Interestingly, the crystal structure of the AdcRspn has 2 zinc atoms per subunit (Guerra et al., 2011). The high affinity zinc site of AdcRspn, site 1, corresponds to the site identified in AdcRGAS structure (Guerra et al., 2011). The second site in AdcRspn, site 2, is coordinated by C30 from the loop connecting helices α1 and α2, E41 from helix α2, and E107 from helix α5 (Guerra et al., 2011; Reyes-Caballero et al., 2010) (Fig. S3). Importantly, the metal ligands identified at site 2 of AdcRspn are conserved in AdcRGAS, suggesting that AdcRGAS may contain the analogous second zinc-sensing site. However, the second zinc could not be traced in the structure due to missing electron density for the loop connecting the helices α1 and α2 (Fig. S3).

Fig. 3. Molecular mechanism of metal sensing and gene regulation by AdcR and its contribution to GAS pathogenesis. a) Ribbon representation of the crystal structure of AdcR dimer; individual subunits of a AdcR dimer are color-coded. The zinc atom bound to each AdcR subunit are shown as spheres and colored in yellow. The water molecules that participate in zinc coordination are shown as cyan spheres. The secondary structural elements of one subunit are labeled and the amino- and carboxy-terminus of the molecule are labeled as N and C, respectively. The DNA-binding and dimerization domains of AdcR are marked and labeled. b) A close up view of the metal-binding site (boxed in panel A) with the metal binding ligands labeled. Zinc bound to the primary metal sensing site in the structure of AdcR is shown as yellow sphere and the side chains of metal coordinating amino acids are depicted in stick representation. Water molecules are shown as cyan spheres. c) Metal content analysis of AdcR by ICP-MS. Measurements are reported as average from triplicate studies. Data shown are mean ± standard deviation. d) The indicated strains were grown to late exponential phase (A600 ~ 1.0) in zinc-replete chemically defined growth medium supplemented with 15 μM ZnOAc and transcript levels of adcA were measured by qRT-PCR. Three biological replicates were used. Data graphed are mean ± standard deviation. Average values for wild type strain grown in zinc-rich condition were used as reference and the fold changes in the transcript levels in the indicated strains relative to reference sample are shown above the bars. Statistical significance of P < 0.0001 between the reference and indicated mutant strains is indicated by **. P values were derived from two-sample t-test. e) Fifteen CD-1 mice were infected with each indicated strain intraperitoneally and the near mortality was presented on a Kaplan-Meier survival curve with P-values derived by log rank test. Statistical significance of P < 0.0001 is indicated by **.
To elucidate the regulatory roles of individual sites, we introduced single or double alanine substitutions at site 1 and site 2 metal ligands of AdcR. Since we have characterized the alanine mutants of metal ligand H42 and H108A at site 1 in our previous studies for DNA binding, gene regulation, and their contribution to GAS virulence (Sanson et al., 2015), we included the uncharacterized E24A mutant in this study. Similarly, to investigate the metal binding and gene regulation properties of site 2, we included the single alanine mutants of metal ligands C30 and E41 of site 2. To determine the metal content of AdcR, we purified recombinant WT AdcR and the alanine mutants of either site 1 (H42A/H108A) or 2 metal ligands (C30A). The purified WT and mutant AdcR proteins remained soluble and eluted as a dimer in the size exclusion chromatography, suggesting that the alanine substitutions did not cause major protein misfolding (data not shown). Next, we assessed the metal content of purified WT and mutant derivatives of AdcR by ICP-MS. The WT AdcR contains two zinc atoms per subunit (Fig. 3C), indicating the presence of two metal-sensing sites similar to AdcRspn. However, the site 1 or 2 mutants have closer to one zinc per subunit (Fig. 3C), suggesting that AdcR can bind zinc independently at two different sites.

To delineate the functional roles of AdcR metal binding sites, site 1 mutants (E24A and H108A), and site 2 mutants (C30A and E41A) were characterized for their regulatory function by qRT-PCR. In the absence of zinc, all tested strains displayed maximal induction of adcA expression (data not shown). However, 3 different phenotypes were observed in the presence of zinc. The fully metallated WT strain displayed the highest levels of repression, and site 1 mutants (E24A and H108A) and ΔadcR mutant exhibited a lack of repression. In contrast, site 2 mutants (C30A and E41A) displayed an intermediate phenotype, in which partial derepression was observed (Fig. 3D). These data indicate that zinc occupancy at site 2 has a role, albeit a lesser one, in regulating GAS adaptive response to zinc limitation. Since metal occupancy at site 1 is essential for gene regulation by AdcR but unnecessary for protein folding or dimerization (Guerre et al., 2011; Reyes-Caballero et al., 2010; Sanson et al., 2015), we designated this site as the primary metal-sensing site and site 2 as the secondary site. To corroborate the findings at the level of gene regulation to GAS pathogenesis, we assessed the virulence of WT, representative site 1 (E24A) and 2 (C30A and E41A) mutants using an intramuscular mouse model of infection. Results showed that virulence of site 1 or 2 mutants was significantly attenuated compared to WT, suggesting that both sites have functional roles in vivo and contribute significantly to GAS virulence (Fig. 3E).

3.5. Zinc Acquisition Machinery as a Prophylactic Target

Since the competition for zinc between GAS and the host occurs during the early stages of infection (Figs. 1 and 2), we hypothesized that targeting the bacterial zinc acquisition machinery may provide prophylactic opportunities to control GAS infections. As AdcA is the extracellular component of the AdcABC transporter, we investigated whether AdcA might be a viable candidate for vaccine development. As antigen accessibility to the host immune system is a prerequisite for immunogenicity, we probed subcellular localization of AdcA in two GAS serotypes. AdcA was predominantly detected in the membrane and cytosolic fractions, indicating that AdcA is located on the bacterial surface (Fig. 4A). When lesions from the GAS-infected tissues were probed with anti-AdcA serum, AdcA was detected, confirming that AdcA is expressed in the host during infection (Fig. 4B). Subsequently, we tested immunogenicity of AdcA by vaccinating mice intramuscularly with purified recombinant AdcA N-terminal domain (AdcA-NTD, residues 36–320) mixed with either sigma adjuvant or clinically relevant alhydrogel, and testing the serum for reactivity (Figs. 4C and S4). Both adjuvants elicited significant antibody responses as serum from immunized mice displayed AdcA-specific reactivity and progressively higher titers after successive rounds of immunization (Figs. 4C–D, and S5). To correlate antibody titers with protection, immunized and control mice were challenged 2 weeks post final immunization by systemic GAS infection with fully sequenced clinical isolates belonging to serotypes M1, M3, M89, and M12 GAS. Mice immunized with AdcA conferred significant protection against all tested serotypes compared to the mock-treated group, indicating that AdcA is a potent vaccine antigen that affords immunity against GAS infection (Figs. 4E–H and S5). Similar protection was observed in challenge studies performed 4 weeks post final immunization with serotype M1 GAS, suggesting that AdcA vaccination confers sustained protection (Fig. 4I). Importantly, the level of protection observed with AdcA immunization is comparable to other tested GAS antigens including the leading GAS vaccine candidate, M protein (Dale et al., 2015; Kapur et al., 1994; McNamara et al., 2008; van Sorge et al., 2014; Zhang et al., 2006). Finally, we assessed immunological recognition of AdcA by human sera from patients with history of acute invasive GAS infections. Reactivity to AdcA was detected in the serum of all GAS-infected patients but not in serum from healthy controls with no recent history of GAS infection (Fig. 4J). This indicates that AdcA is accessible to host immune effectors and triggers antibody responses during natural infection in humans.

3.6. CP Inhibits the Growth of Pathogenic Streptococci

To investigate whether host-mediated zinc sequestration is a general conserved immune mechanism used against other pathogenic streptococci and Listeria monocytogenes, we performed growth studies of various streptococci and L. monocytogenes in the presence and absence of CP. As observed for GAS, CP inhibited the growth of all tested bacteria, albeit to a different extent (Fig. 5A–G). Notably, pneumococci displayed significant resistance against CP-mediated growth inhibition as the bacteria retained the ability to grow even at CP concentration of 500 μg/ml whereas CP at the same concentration caused growth arrest of other streptococci and L. monocytogenes (Fig. 5E). Given that the amino acid sequence of the zinc acquisition machinery is highly conserved among the tested pathogens, it is likely that these pathogens employ uptake systems analogous to adcABC to acquire zinc and compete with host sequestration mechanisms (Fig. 5H). Thus, prophylactic targeting of zinc import machinery, as demonstrated in GAS, may be a viable broad-spectrum vaccine strategy against these important human pathogens.

4. Discussion

Molecular dialog between host and invading pathogens shapes the course of infection and often determines the disease outcome. We identified a previously unknown GAS-host interaction that occurs during infection and demonstrated that zinc is at the forefront of the host-GAS battle. Furthermore, we showed that the molecular arsenal of the pathogen involved in this host-pathogen conflict could be effectively targeted for disease prevention. Herein, we report that GAS-infected lesions are enriched with CP in two different host compartments and CP retards GAS growth by scavenging zinc from the colonization interface. Our results are consistent with the observations made in other bacterial pathogens including S. aureus, A. baumannii, S. typhimurium, and H. pylori (Gaddy et al., 2014; Hood et al., 2012; Corbin et al., 2008, Damo et al., 2013; Liu et al., 2012). In addition to zinc, CP also inhibits bacterial growth by sequestering manganese and iron (Damo et al., 2013; Diaz-Ochoa et al., 2016; Nakashige et al., 2015). Given that CP adaptive responses to manganese limitation are not fully understood, the role of CP-mediated manganese sequestration on GAS pathogenesis is yet to be elucidated. Similarly, CP binds iron and causes bacterial growth inhibition in vitro (Nakashige et al., 2015), however, the in vivo significance of CP-mediated iron limitation as a host defense remains unclear (Garcia et al., 2017).

Although the role of CP against pneumococcal infection has been investigated, the two studies provided opposing findings with varying mechanisms for CP to either inhibit or facilitate pneumococcal virulence. One study reported that CP is an anti-pneumococcal host factor
that inhibits bacterial growth during infection (De Filippo, et al., 2014), whereas a second investigation suggested that CP facilitates bacterial infection (Achouiti, et al., 2014). Thus, additional investigations are required to elucidate the precise role of CP against pneumococcal infection. In this regard, results from this study provided significant evidence that GAS encounters CP-mediated Zn limitation during invasive infection and indicated similar susceptibility of other streptococcal pathogens to CP. The observations that CP is abundant at GAS infection sites, CP imposes Zn limitation on GAS, and adaptive responses to Zn starvation are highly upregulated during GAS invasive infections lend support to our findings (Brenot et al., 2007). However, it is likely that GAS may encounter additional nutritional immune mechanisms such as zinc toxicity in other host compartments during infection (Ong et al., 2014).

The GAS arsenals to combat CP-mediated zinc withholding include the high-affinity zinc importer, AdcABC and the zinc sensor, AdcR.
Although several studies demonstrated the importance of metal importers to bacterial pathogenesis (Ammendola et al., 2007; Campoy et al., 2002; Davis et al., 2009; Bayle, et al., 2011; Sabri, et al., 2009), this study delineated the direct role of a zinc transporter in evading host nutritional immune defenses and highlighted its significant contribution to GAS survival during infection (Fig. 2). The \textit{adcCB}-inactivated strain is unable to compete against CP due to defective zinc acquisition, which likely results in growth retardation and premature bacterial clearance from the site of infection. Consistent with this, \textit{ΔadcC} mutant exhibited reduced \textit{in vivo} survival and significantly attenuated virulence compared to WT and \textit{trans}-complemented strains. Furthermore, GAS actively monitors alterations in zinc availability during infection and mounts a measured induction of adaptive responses that correlates with the degree of reduction in zinc concentration. AdcR employs two metal-sensing sites to monitor zinc availability, and each site has defined biological roles in gene regulation and GAS pathogenesis. Metallation of the primary zinc-sensing site is essential for AdcR-promoter interactions and transcription repression, and vacant site 1 results in full derepression of regulated genes. The secondary zinc-sensing site has a lesser regulatory role as zinc binding at this site causes tighter repression, and loss of zinc at site 2 leads to lower levels of target gene derepression. Such gradual gene regulation by AdcR may afford significant growth advantage as it allows the pathogen to sense and respond to a broader range of alterations in zinc concentrations. CP levels are relatively low during early stages of infection, which likely imposes milder zinc limitation on the pathogen and requires lower level induction of GAS adaptive responses. As CP concentration increases during later stages of infection, a further drop in zinc concentration may necessitate robust induction of adaptive responses. Varied target gene regulation in response to altering zinc concentration has been observed in zinc metalloregulators, Zur from \textit{B. subtilis} and \textit{Streptomyces coelicolor}, and Zap1 from \textit{Saccharomyces cerevisiae} (Ma et al., 2011; North et al., 2012; Shin et al., 2011; Shin and Helmann, 2016; Wu et al., 2008). However, this is the first study demonstrating the functional relevance of individual zinc-sensing sites.

Fig. 5. Broad-spectrum anti-streptococcal activity of calprotectin. Growth characteristics of streptococcal pathogens, a) group B streptococcus (\textit{S. agalactiae}), b) group C streptococcus (\textit{S. dysgalactiae}), c) group G streptococcus (\textit{S. dysgalactiae subsp. equisimilis}), d) \textit{S. mutans}, e) \textit{S. pneumoniae}, f) \textit{Enterococcus faecalis}, g) \textit{Listeria monocytogenes}, was assessed. Bacteria were grown in triplicate in medium supplemented with the indicated concentrations of CP. Growth was monitored by absorption at A600 in a microplate reader at the indicated time points. h) Amino acid sequence conservation of AdcA, AdcB, and AdcC proteins among different streptococcal pathogens. The respective amino acid sequence identities and chemical similarities relative to GAS sequences are shown. The protein identification number of the respective proteins is given in parentheses.
gradual gene regulation by a metalloregulator to the virulence of a human pathogen.

Since the GAS-host confrontation for zinc occurs during early stages of infection and zinc acquisition systems are upregulated to disarm host defenses (Brenot et al., 2007), we hypothesized that bacterial zinc uptake machinery could be targeted as a protective vaccine against GAS infection. To prove this, we demonstrated that the extracellular component of zinc uptake system, AdcA, is expressed during infection, accessible on the bacterial surface, antigenic, and immunization with recombinant AdcA-NTD confers protection against systemic GAS infection (Fig. 4). Importantly, as a vaccine candidate, AdcA has the potential to overcome longstanding barriers in GAS vaccine research. AdcA is highly conserved and elicited protection against different tested GAS serotypes, thus increasing the likelihood of conferring protection across multiple GAS serotypes (Figs. 4, and S6). Structural modeling indicated the lack of coiled-coil structures in AdcA, as observed in M protein structures (Kirvan et al., 2014; McNamara et al., 2008), which minimizes the lack of coiled-coil structures in AdcA, as observed in M protein structures (Kirvan et al., 2014; McNamara et al., 2008), which minimizes the risk of adhesions between the bacterial surface and the host immune system (Figs. 5, and S8), suggesting that the acquisition system that facilitates resistance to calprotectin-mediated zinc sequestration (Fig. 4). Importantly, as a vaccine candidate, AdcA has the potential to overcome longstanding barriers in GAS vaccine research. AdcA is highly conserved and elicited protection against different tested GAS serotypes, thus increasing the likelihood of conferring protection across multiple GAS serotypes (Figs. 4, and S6). Structural modeling indicated the lack of coiled-coil structures in AdcA, as observed in M protein structures (Kirvan et al., 2014; McNamara et al., 2008), which minimizes the risk of adhesions between the bacterial surface and the host immune system (Figs. 5, and S8), suggesting that the acquisition system that facilitates resistance to calprotectin-mediated zinc sequestration is resistant to bacterial infections. Mol. Microbiol. 63, 1186–1195.

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