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Passive Immunization with Anti-Glucosaminidase Monoclonal Antibodies Protects Mice from Implant-Associated Osteomyelitis by Mediating Opsonophagocytosis of *Staphylococcus aureus* Megaclusters

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Abstract

Towards development of a methicillin-resistant *S. aureus* (MRSA) vaccine we evaluated a neutralizing anti-glucosaminidase (Gmd) monoclonal antibody (1C11) in a murine model of implant-associated osteomyelitis, and compared its effects on LAC USA300 MRSA versus placebo (alpha-T2m) and a Gmd-deficient isogenic strain (delta-Gmd). 1C11 significantly reduced infection severity, as determined by bioluminescent imaging of bacteria, micro-CT assessment of osteolysis and histomorphometry of abscess numbers ($p < 0.05$). Histology also revealed infiltrating macrophages, and the complete lack of staphylococcal abscess communities (SAC), in marrow abscesses of 1C11 treated mice. *In vitro*, 1C11 had no direct effects on proliferation, but electron microscopy demonstrated that 1C11 treatment phenocopies delta-Gmd defects in binary fission. Moreover, addition of 1C11 to MRSA cultures induced the formation of large bacterial aggregates (*megaclusters*) that sedimented out of solution, which was not observed in delta-Gmd cultures or 1C11 treated cultures of a protein A-deficient strain (delta-Spa), suggesting that the combined effects of Gmd inhibition and antibody-mediated agglutination are required. Finally, we demonstrated that macrophage opsonophagocytosis of MRSA and megaclusters is significantly increased by 1C11 ($p < 0.01$). Collectively, these results suggest that the primary mechanism of anti-Gmd humoral immunity against MRSA osteomyelitis is macrophage invasion of SAC and opsonophagocytosis of megaclusters.

Keywords

Osteomyelitis; Methicillin-Resistant *Staphylococcus aureus* (MRSA); Passive Immunization; Opsonophagocytosis; Electron Microscopy

Introduction

Staphylococcus aureus is the primary pathogen isolated from infected orthopaedic implants¹, approximately 50% of which is methicillin-resistant *S. aureus* (MRSA) acquired in both hospital and community settings^{2,3}. Although the number of primary infections following total joint replacement (TJR) is low (<5%), reinfection rates from MRSA are very high (15-40%) and often require a two-stage exchange arthroplasty to remedy the problem⁴⁻⁹. Given that MRSA has surpassed HIV as the most deadly pathogen in the United States, accounting for 94,360 hospitalizations and 18,650 deaths each year^{10,11}, and there is an emergence of multidrug resistant *S. aureus* strains that cannot be treated with available agents^{12,13}, alternatives to antibiotic therapies are now in great demand as concluded by the first international consensus meeting on periprosthetic joint infection¹⁴.

Despite tremendous efforts, an effective vaccine against *S. aureus* remains elusive, as several clinical trials have failed¹⁵⁻¹⁸. As pointed out in a recent review on the current state of *S. aureus* vaccines, it is possible that the correct antigen or combination of antigens has not yet been discovered, or that the antibodies used in these failed clinical trials were fully capable of opsonizing the bacteria but could not induce their lysis or phagocytosis, and eventual death¹⁹. Therefore, the recent push has been towards immunization against multiple targets with essential functions for *S. aureus* colonization, growth, and survival in the host. This immunization may act by directly promoting humoral immunity, or by increasing bacterial susceptibility to antibiotics through their use as a complementary treatment^{20,21}.

An additional challenge in developing an effective vaccine to prevent and treat *S. aureus* TJR infection is the known immunodeficiency in this susceptible patient population due to aging, autoimmunity, obesity and diabetes²²⁻²⁵. Thus, passive immunization via infusion of neutralizing antibodies against specific *S. aureus* surface proteins (i.e., ClfA²⁶) and virulence factors (i.e., alpha-toxin²⁷, coagulases²⁸) is a more attractive option. Ideally, the passive immunization would comprise a dual-acting monoclonal antibody (mAb) that has direct antimicrobial effects by inhibiting a critical *S. aureus* molecule, and also have immunomodulatory effects to enhance the host response and bacterial clearance. For these reasons we have focused our efforts on the glucosaminidase (Gmd) subunit of autolysin (Atl), which several groups have identified as an immunodominant antigen^{29,30}.

Functionally, Atl has been shown to be essential for cell wall biosynthesis and degradation during binary fission³¹⁻³³. Atl has also been shown to function as an adhesin³⁴, a biofilm enzyme³⁰, was identified as a potential molecular target of vancomycin³⁵, and has been reported to interfere with the production of antibodies in mice³⁶.

Recently, we have generated recombinant histidine-tagged Gmd protein (His-Gmd), and used it as an active vaccine to generate a pool of neutralizing anti-Gmd mAb³⁷. Of the 36 anti-Gmd producing hybridomas screened as candidates for the passive immunization, we identified one IgG1 mAb (1C11) with superior *in vitro* characteristics in terms of affinity ($k_f = 3.1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$; $k_r = 5.0 \times 10^{-5} \text{ s}^{-1}$; and $K_D = 1.6 \times 10^{-9} \text{ M}$) and His-Gmd neutralizing activity (Suppl. Fig. 1). We also used it to demonstrate the potential of circulating anti-Gmd antibodies as a serum biomarker of protective immunity against *S. aureus* in patients with

orthopaedic infections³⁸. Here we formally evaluate its potential as a passive vaccine by testing the hypothesis that anti-Gmd passive immunization protects mice from implant-associated osteomyelitis by inhibiting *S. aureus* growth and binary fission, and facilitating opsonophagocytosis.

Methods

Preparation of monoclonal antibodies and *S. aureus* strains

Murine IgG1 mAb 1C11 was produced as previously described³⁸. A control IgG1 mAb (mouse anti-T2 mycotoxin; alpha-T2m) was purchased from Southern Biotech (Birmingham, AL). Gmd neutralizing activity of the mAb was determined via cell wall digestion assay as previously described³⁸, and the percent inhibition was calculated as $100 \times (1 - (\text{delta-60A}_{490} \text{ inhibitor} / \text{delta-60A}_{490} \text{ no inhibitor control}))$ (Suppl. Fig. 1B).

The *S. aureus* strains UAMS-1³⁹, UAMS-1 protein A-deficient mutant (delta-Spa), and LAC USA300⁴⁰ (MRSA), were gifts from Dr. Paul Dunman. We generated a LAC USA300 Gmd-deficient isogenic mutant (LAC delta-Gmd) strain via homologous recombination using a gene knockout shuttle vector (pWedge) provided by Dr. Steven Gill, as previously described⁴¹. Genomic *Gmd* deletion was confirmed with PCR, and the absence of Gmd protein was confirmed by Western blotting (Suppl. Fig. 1C). The bioluminescent *S. aureus* strain Xen29 was purchased from Caliper Life Sciences (Hopkinton, MA).

Passive immunization and assessment of implant-associated osteomyelitis in mice

All *in vivo* experiments were performed on protocols approved by the University of Rochester Committee on Animal Resources. In the bioluminescent imaging (BLI) experiment, forty 8-10 week old female BALB/cJ mice (The Jackson Laboratory, Bar Harbor, ME) were randomized to placebo (PBS) or 1 mg of 1C11 mAb (40 mg/kg i.p.) one day prior to the surgery. Implant-associated osteomyelitis was induced in the right tibia of the mice by surgically implanting a stainless steel pin contaminated with Xen29 as previously described⁴². Mice were removed from either group if they died of anesthesia following surgery, during longitudinal BLI, or if a mouse removed its pin during the course of the 14-day experiment, leaving the placebo and 1C11 treatment groups at n=15 and n=17, respectively. BLI of all mice was performed on days 0, 3, 5, 7, 10 and 14 using the Xenogen IVIS Spectrum imaging system (Caliper Life Sciences, Hopkinton, MA), and the peak BLI on day 3 was quantified as previously described⁴². The effects of Gmd inhibition on the establishment of MRSA chronic osteomyelitis were assessed radiographic and histologically 14 days after infection using three cohorts. Mice (n= 5) were treated with PBS (Group 1 placebo) or with 40mg/kg of 1C11 (Group 2 anti-Gmd), and 24h later received a USA300 LAC infected transtibial pin. A third group of mice received an infected transtibial pin with delta-Gmd USA300 LAC (Group 3 delta-Gmd). The mice were euthanized on day 14 post-infection, and the tibiae were assessed by micro-CT, and processed for alcian blue hematoxylin /orange G (ABH/OG) and Brown and Brenn (Gram) staining and light microscopy, as previously described⁴³.

Growth inhibition assays

Dose-dependent effects (0.05-50 mg/ml) of 1C11 vs. alpha-T2m on LAC USA300 growth in tryptic soy broth (TSB) supplemented with erythromycin (10 µg/ml) were determined by: traditional CFU assay; longitudinal O.D. at 490 nm in 96-well plates; and tritiated-thymidine incorporation as previously described⁴⁴.

Scanning Electron Microscopy (SEM)

For *in vitro* cluster analysis, LAC USA300 or LAC delta-Gmd was grown at 37°C for 12 hours in TSB, then diluted and incubated with PBS, or 50 mg/ml of αT2m or 1C11, for 4 hours. Samples were plated onto glass coverslips, fixed in 4% paraformaldehyde/2.5% glutaraldehyde in 0.1 M Cacodylate buffer, dehydrated, and coated with gold for SEM. Three SEM micrographs per sample group were randomly chosen for the analysis. Each micrograph was divided into a 3×3 grid using Adobe Photoshop CS3, and the number and size of clusters was counted within each grid. The total number and size of clusters was then calculated for each treatment group.

Transmission Electron Microscopy (TEM) and phagocytosis assays

The mouse macrophage cell line RAW 264.7 (ATCC, Manassas, VA) was used in all of our phagocytosis assays. For analysis by TEM, UAMS-1 *S. aureus* was treated with 50 mg/ml 1C11 for 30 minutes or 4 hours (assay dependent), and then the bacteria were exposed to complement-rich mouse serum (Innovative Research, Novi, MI) and RAW macrophages in DMEM media (Invitrogen, Carlsbad, CA) for a period of 0, 15, or 30 minutes before overnight fixation in 4% paraformaldehyde/2.5% glutaraldehyde in 0.1 M Cacodylate buffer at 4°C. Samples were trapped in agarose, post-fixed in 1% osmium tetroxide, dehydrated, and embedded in EPON/Araldite epoxy resin. The epoxy blocks were then ultra thin sectioned (70 nm) onto grids, stained with uranyl acetate and lead citrate, and imaged using a Hitachi 7650 TEM with attached Gatan 11 megapixel digital camera. The mean number of macrophages containing internalized bacteria from three random images was calculated as the mean +/- SD.

Statistical analysis

Data are presented as mean +/- one standard deviation. In our 1C11 passive immunization experiments, the lowest BLI value of the placebo group at each time point was used as the objective cutoff threshold for analysis by Fisher's Exact Test (FET). For *in vitro* statistical analysis, five replicates were performed for each experiment and significance was determined via two-tailed unpaired student's t-test, for which $p < 0.05$ was considered statistically significant.

Results

In order to assess the effects of 1C11 on the establishment of implant-associated osteomyelitis, we administered the mAb or placebo to mice one day before the Xen29 *S. aureus* transtibial challenge, and monitored the infection via longitudinal BLI. Consistent with prior studies⁴², the placebo treated mice uniformly displayed a peak BLI on day 3 (Fig. 1A top). In contrast, the 1C11 treated group displayed a bimodal response in which half of

the mice displayed a similar response to the placebo mice, and the other half had a very low BLI signal (Fig. 1A bottom). To quantify this outcome, we performed a threshold analysis using the lowest BLI value in the placebo group as the cutoff for infection as previously described⁴³. The results demonstrated that the 1C11 passive immunization significantly ($p=0.016$) reduced the severity of infection in ~50% of the mice at day 3 post-infection (Fig. 1B).

S. aureus establishes chronic osteomyelitis via colonization of necrotic bone with concomitant osteolysis and reactive bone formation^{42,43}, and generation of coagulase-mediated staphylococcal abscess communities (SAC) in soft tissues^{28,45}. To assess the effects of 1C11 and Gmd loss of function on these processes, we repeated the *in vivo* experiments with mice passively immunized with placebo or 1C11 prior to MRSA infection, and mice challenged with delta-Gmd, and analyzed the infected tibiae on Day 14 via micro-CT and histology (Fig. 2). The results showed that immunologic inhibition and genetic ablation of Gmd results in a significant decrease in osteolysis and abscess formation ($p<0.05$ vs. PBS). Additionally, while MRSA was able to establish biofilms in necrotic bone fragments in all the mice, it could only generate SAC in PBS and delta-Gmd treated mice. Most impressively, macrophages that are inhibited from entering SAC by *S. aureus* nuclease and adenosine synthase⁴⁵, could be readily seen in abscesses void of Gram-positive bacteria in all of the 1C11 treated mice, suggesting that anti-Gmd mAb has immunomodulatory effects.

To elucidate the mechanism by which 1C11 reduced the severity of *S. aureus* osteomyelitis in our murine model, we first evaluated the direct effects of the mAb on MRSA growth *in vitro*. Consistent with previous reports on the growth of Gmd- and Atl-deficient strains⁴⁶⁻⁴⁸, we found that addition of 1C11 to cultures of LAC USA300 inhibited complete separation of daughter cells undergoing binary fission in a manner that appeared to phenocopy the delta-Gmd control (Fig. 3A). When we measured the turbidity of the suspension cultures over time we found that 1C11 significantly reduced the peak optical density, whereas genomic deletion of Gmd had no effect on optical density at any time point in this assay (Fig. 3B). Coincident with this decrease in O.D. at 4 hours was the appearance of sedimented bacteria at the bottom of 1C11 treated cultures, which SEM confirmed to be megacusters (>25 bacteria) of MRSA (Fig. 3C). A quantitative analysis of MRSA clusters in suspension culture at 4 hours revealed that loss of Gmd function leads to significantly larger clusters of bacteria (Fig. 3D), as predicted due to defective binary fission (Fig. 3A). Interestingly, the control mAb also induced significantly more small clusters (7-24 bacteria) vs. a PBS control, but did not induce megacusters, suggesting limited aggregation via non-specific mAb binding to MRSA and Spa crosslinking. We also found that addition of 1C11 to delta-Spa MRSA did not significantly change the growth pattern of suspension culture (Suppl. Fig. 2). Thus, the observations that megacusters are only found in 1C11 treated LAC USA300 MRSA cultures suggests that both inhibition of Gmd-mediated binary fission and bacterial agglutination are required for this to occur.

To test if 1C11 inhibition of binary fission and clustering had any direct effects on MRSA proliferation, we assayed tritiated thymidine incorporation into the DNA of actively dividing log phase bacteria. No difference in proliferation rate was observed between the placebo and

1C11 treatment groups (Suppl. Fig. 3A). This finding that Gmd function is not required for normal MRSA DNA replication was confirmed in tritiated thymidine incorporation studies with the delta-Gmd strain (Suppl. Fig. 3B), and in traditional CFU assays that failed to demonstrate significant effects of 1C11 and delta-Gmd on proliferation (Suppl. Fig. 4).

To determine if 1C11 induced clustering promotes opsonophagocytosis by macrophages *in vitro*, we first demonstrated that naïve RAW cells are capable of efficiently internalizing antibody-opsonized *S. aureus* in a time-dependent manner (Suppl. Fig. 5). Though not yet confirmed, these internalized bacteria appear to be contained within vacuoles as previously reported.⁴⁹ Interestingly, TEM revealed that the 1C11 opsonophagocytosed bacteria were mostly retained in the cytoplasm as large clusters held together by a matrix (Fig. 4A), while the internalized *S. aureus* in delta-T2m treated cultures resided in the cytoplasm as individual bacterium (Fig. 4B). Quantification confirmed that 1C11 significantly induced the % of internalized MRSA vs. α T2m (88.5 $\pm$ 7.4 vs. 58.7 $\pm$ 3.3; $p < 0.006$). Moreover, SEM of the pins from 1C11 immunized and MRSA challenged mice provided the first evidence that anti-Gmd facilitates macrophage opsonophagocytosis and internalization of megaclusters *in vivo* (Fig. 4C&D).

Discussion

With the intent of developing a passive vaccine to prevent *S. aureus* infection in patients undergoing TJR, and treating patients with MRSA infected implants, here we evaluated the effects of a candidate anti-Gmd mAb (1C11) *in vivo* and *in vitro*. Our demonstration that 1C11 significantly reduced the severity of *S. aureus* implant-associated osteomyelitis as assessed by BLI, micro-CT and histology, is remarkable considering the overwhelming bacterial inoculum ($\sim 5 \times 10^5$ CFU) used in this model. As it is known that mice protect themselves from this infection via a Th1-driven immune response that leads to IgG2b dominated humoral immunity^{42,50}, we believe that this efficacy could be enhanced by *in vitro* conversion of the 1C11 IgG1 heavy chain to enhance mAb effector function. In support of this, one descriptive study from 1979 found that a heterogeneous IgG2 antibody fraction from mouse serum, but not fractions containing mouse IgM or IgG1 isotypes, possessed the capability to efficiently activate the classical and alternative complement pathways in their *in vitro* assay⁵¹. To this end, we have recently generated IgG2a and IgG2b isotypes of 1C11 that will be used in future studies to evaluate the effects of antibody heavy chain class switching *in vitro* and *in vivo*.

There are several limitations to our *in vivo* studies that warrant further investigation. The first is a better understanding of the BLI data, and what the biphasic response on day 3 means in term of host protection from acute and chronic infection. The second is our finding that loss of Gmd function significantly decreases osteolysis during the establishment of MRSA osteomyelitis, but only anti-Gmd therapy eradicates SAC. Our interpretation of these results is that defective binary fission leads to decreased motility and subsequent osteolysis. However, unopsonized MRSA clusters can still induce SAC, as their coagulase and deoxyadenosine activities, and proliferation potential are not significantly affected. In contrast, opsonized megaclusters are efficiently cleared by macrophages, whose invasion into MRSA abscesses is not inhibited. Lastly, our finding of biofilm containing necrotic

bone sequestrum in all MRSA challenged mice highlights this component of osteomyelitis as the most challenging to prevent and treat.

Equally important to the efficacy of a passive immunization for MRSA osteomyelitis is the elucidation of its mechanism of action, such that its limitations can be overcome by modifications to the mAb and co-administration of complementary treatments. To facilitate this for 1C11, we generated a Gmd-deficient isogenic mutant of LAC USA300 MRSA, and used it as a positive control in our studies. Although we could not generate a bioluminescent version of DGmd in this strain for longitudinal BLI in the timeframe of this study, as the antibiotic resistance genes for selection were already present in LAC: *lux*⁵², we were able to compare the effects of 1C11 administration vs. genomic deletion of Gmd in all of the other assays used in this study. Remarkably, 1C11 treatment phenocopied DGmd defects in binary fission and cell separation. Interestingly, 1C11 induced the formation of megaclusters (>25 bacteria) that sedimented out of solution, which was not observed with the DGmd strain, suggesting an additional antibody effect over Gmd inhibition. Collectively, these data provide complementary evidence that Gmd is required for *S. aureus* binary fission, but does not have a direct effect on proliferation. Deductively, our failure to observe megacluster formation in: 1) MRSA cultures treated with α T2m, 2) DGmd cultures treated with 1C11, or 3) Spa cultures treated with 1C11, strongly suggests that megaclusters are formed by the combined effects of Gmd inhibition and antibody-mediated agglutination. This conclusion was further substantiated by the TEM images of the 1C11-induced phagocytosis of megaclusters by macrophages (Fig. 4). Interestingly, we observed that 1C11 opsonophagocytosed megaclusters contain live and dead bacteria that are tethered by a thick matrix, which was absent in our control samples (Fig. 4). Therefore, these results suggest that the primary mechanism of anti-Gmd humoral immunity against MRSA osteomyelitis is opsonophagocytosis of large bacterial clusters. As we are unaware of prior reports of this antibody-induced matrix, further studies are warranted to determine its molecular composition and potential bactericidal function following phagocytosis.

As a final point, we must acknowledge the identification of autolysin as a potential target of vancomycin. A recent report identified the amidase portion of autolysin as a potential protein target of vancomycin, and suggested that inhibition of amidase may enhance *S. aureus* tolerance to low concentrations of the antibiotic³⁵. However, as this has not been proven *in vitro* or *in vivo*, and because of numerous contrasting reports regarding autolysin upregulation or downregulation in the presence of vancomycin⁵³⁻⁵⁵, we do not believe that vancomycin resistant *S. aureus* (VRSA) will be resistant to our anti-Gmd mAb therapy. To test this, future experiments will focus on the complementary use of 1C11 with vancomycin to eradicate multidrug-resistant *S. aureus* infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Passive immunization with 1C11 reduces the severity of *S. aureus* implant-associated osteomyelitis

(A) Mice were immunized and challenged as described in Methods. All placebo-treated mice displayed a robust BLI signal (top), while half of the 1C11-treated mice had a dramatically reduced BLI signal (bottom). (B) The Day 3 BLI data for each mouse and mean for each group (black line) are presented. The lowest BLI value in the placebo group was used as the threshold value for infection (dashed red line), which revealed a significant protective effect of 1C11 as determined by Fisher's Exact Test ($p=0.016$).

Figure 2. 1C11 treatment inhibits osteolysis, decreases abscess numbers, and recruits macrophages into SAC

Mice (n= 5) were treated with PBS (A-G) or with 40mg/kg of 1C11 (H-N), and 24h later received a USA300 LAC infected transtibial pin. A third group of mice received an infected transtibial pin with delta-Gmd USA300 LAC (O-U). The mice were euthanized on day 14 post-infection, and the tibiae were harvested for micro-CT and histology. 3D renderings of representative tibiae are shown to illustrate the osteolysis and reactive bone formation (A,H,O). Representative alcian blue hematoxylin/orange G (B-E,I-L,P-S) and Brown & Brenn (F,G,M,N,T,U) stained histology from these tibiae is presented. 12.5× (B,I,P) and 40× (C,J,Q) images are shown to highlight areas that were photographed at 400×, where yellow asterisks mark abscesses (D,E,K,L,R,S); green asterisks mark SAC (F,M,T); and black asterisks mark the colonized necrotic bone fragments in sequestrum (G,N,U). Note that all groups had macrophages in the periphery of the abscess (yellow arrow heads in D,K,R), while macrophages in the center of the abscesses were only present in the 1C11 treated

group (yellow arrow heads in L). Conversely, Gram-positive SAC were only present in the marrow and soft tissues of tibiae from mice treated with PBS or delta-Gmd (F&T), as the abscesses in 1C11 treated mice did not contain any bacteria (M). Necrotic bone fragments containing Gram-positive biofilm were detected in all tibiae (G,N,U). (V) The osteolytic area on the medial side was quantified as previously described⁴², and the data for each tibia and mean for the group (bar) are presented (* $p < 0.05$ vs. PBS). (W) Abscess numbers were quantified from the ABH/OG histology as previously described²⁸, and the data for each tibia and mean for the group (bar) are presented (* $p < 0.05$ vs. PBS).

Figure 3. 1C11 induces MRSA megacluster formation *in vitro* by combined inhibition of binary fission and agglutination

(A) Representative micrographs of LAC USA300 cultures grown for 4 hours in the presence of alpha-T2m, 1C11, or PBS, and of LAC delta-Gmd. Of note is that the defective binary fission observed with Δ Gmd appears to be phenocopied by 1C11 addition to WT MRSA. (B) O.D. of parallel cultures was measured at the indicated intervals. While no differences were observed among the PBS, alpha-T2m, or delta-Gmd cultures at any time point, the O.D. of the 1C11 treated cultures was significantly lower after 4 hours (** $p < 0.01$ vs. PBS). (C) This decreased O.D. was coincident with a visible bacterial sediment, which revealed large clusters of MRSA as seen by SEM. (D) Quantification of the clusters was performed and presented as the mean $\pm$ SD (* $p < 0.05$ vs. PBS; # $p < 0.05$ vs. delta-Gmd; A: 15,000 $\times$ (bar=0.5 μ m); C: 3,000 $\times$).

Figure 4. 1C11 significantly increases internalization of *S. aureus* and megacusters

Ultrathin section TEM at 6,000 $\times$ was performed on RAW cells after 4hr of culture with MRSA opsonized with 1C11 (A) or alpha-T2m (B). Note the megacusters (arrowheads in A) are connected by a thick matrix (boxed region in A shown at 30,000 $\times$), whereas intracellular *S. aureus* in the control cultures were mostly individual bacterium (boxed region in B). Quantification confirmed that 1C11 significantly induced the % of internalized MRSA vs. alpha-T2m (88.5 $\pm$ 7.4 vs. 58.7 $\pm$ 3.3; $p < 0.006$; N=4 randomly chosen fields and the results are representative of duplicate experiments; Nu=nucleus; Cy=cytoplasm). Representative SEM of a macrophage phagocytosing a bacterium (arrow in C), and megacusters inside of a macrophage (D), on the pins harvested on day 14 from 1C11 treated mice challenged with MRSA, which were not observed on pins harvested from PBS and delta-Gmd treated mice.