Development of Therapeutic Antibodies Against Quorum Sensing Receptor of Methicillin Resistant *Staphylococcus aureus* (MRSA-252) : A New Approach to Stop Bacteria and Bacterial Resistance to Antibiotic

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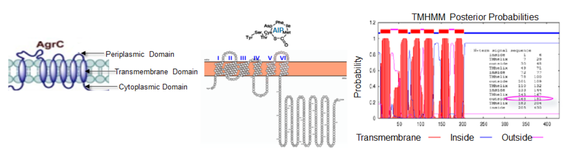
BACKGROUND

Methicillin-resistant *Staphylococcus aureus* (MRSA) is considered a "superbug" and responsible for serious nosocomial and community-acquired infections to life-threatening diseases worldwide. In the USA, community-acquired MRSA is often detected at military training units. The frequency ranges between 27 and 32 MRSA infections per thousand soldiers. Here, we report an antibody therapeutic approach against MRSA by effectively blocking the surface-associated quorum sensing receptor (AgrC) with a high affinity anti-AgrC monoclonal antibody (MA-01), and a genetically engineered recombinant version of a tiny-body termed [MA-01(scFv-CH2)]. The AgrC is a histidine kinase receptor on the cell surface of MRSA, whose sensor domain is activated by trans autophosphorylation upon recognition of autoinducer peptide (AIP) stimulus, resulting in the expression of a large arsenal of virulence factors. A high affinity mAb (MA-01) was obtained through animal immunization and hybridoma technology. The MA-01 was characterized by immunoassays (ELISA, Western blot) and isotyping. The MA-01 was sequenced and genetically engineered to a single-chain variable fragment with the Fc-CH2 domain [MA-01(scFv-CH2)] and coined "Tiny-body." The tiny-body plasmid was utilized to express recombinant "Tiny-body" protein (r-Tiny-body) in Expi293F cells. Subsequently, the binding abilities of MA-01 and the r-Tiny-body to MRSA-252 surface receptor (AgrC) were assessed using both *in vitro* and *in vivo* assays, including the receptor blocking assay, prophylactic assay, therapeutic assay, and animal survival assay. The biodistribution of the antibodies was evaluated in blood and different organs. Overall, these results show that the r-Tiny-body represents a superior and effective therapeutic agent as it demonstrated significant specificity for the AgrC receptor of MRSA-252 and conferred effector functions such as receptor-ligand blockade.

IDENTIFICATION OF MRSA252 AgrC RECEPTOR PEPTIDE

The fully sequenced *Staphylococcus aureus*-MRSA252 (BAA-1720™ ATCC) was used in this study. Based on *in-silico* analysis of the sequence identity of "AgrC" receptor transmembrane domains (I-VI), a 16 amino acid (AA) peptide hot-spot (AgrC-AIP docking site) was identified on AgrC domain VI (Panel A). The 16 AA peptide was synthesized with N-terminus Cystine (C) to conjugate with KLH (Panel B) and generated murine monoclonal antibodies (mAbs) by somatic fusion of mouse B lymphocytes of the spleen with immortal mouse myeloma cells.

Panel A



Sequence	Peptide
Staphylococcus aureus (MRSA252) : AgrC (430 AA) MEALNDYNYVLFVIVQVSLMFFISAFISGIRYKKSDYIYIIGIVLSSVYFFDKIGSISLVVITIFIII FLYFKRLYSVFLVMVTQINLYCANFVYIIFSYIITISHSVFIVLPIFLVVYVSISYALAVYLNRILK RINGTYLSLNKKFLTVITIVIVITFSLLFAYSQIDASDASTIKQYSLLFLGIIILLSILIFIYSQFTLK EMKYKRNQEEIETYYEYTLKIEAINNEMRKFRHDYVNILTTLSYIREDDMTGLRDYFNKNI VPMKDNLQMNALKLNGIENLKVREIKGLLTAKILRAQEMNIPISIEIPDEVTRINLNMIDLSR SIGILDNAIEASSEIDPIRVAFIESENSVTFIVMNKCADDIPRIHELFQESFSTKGEGRGL GLSTLKEIADNADNVLLDTIIENGFFIQKVEIINN	YSQIDASDASTIKQYS KLH conjugated Peptide: KLH-(C)YSQIDASDASTIKQYS

Figure 1. *MRSA252 Quorum sensing receptor hot-spot (AgrC-AIP docking site) in silico analysis (Panel A). The AgrC receptor protein sequence, AIP binding peptide selection and KLH conjugated synthetic peptide (Panel B).*

MONOCLONAL ANTIBODY DISCOVERY PLATFORM

Several hybridomas were generated with the fusion of splenocytes from mice immunized with KLH conjugated AgrC receptor peptide and mouse myeloma cells (Panel A). The monoclonal antibody-producing hybridomas were screened by ELISA. A high-affinity and desired isotype monoclonal antibody (MA-01) was identified and characterized by immunoassays. The MA-01 was produced in bioreactors, ~~and~~ purified, and sequenced. The DNA from a single chain variable fragment (scFv) with Fc-CH2 domain was cloned in a mammalian expression plasmid (pcDNA 3.4) to make a single chain fragment variable Tiny-body plasmid (AgMA-01scFv-CH2-pcDNA3.4) consisting of variable regions of heavy (*VH*) and light (*VL*) chains, joining together by a flexible GS linker, including the CH2-domain of Fc and 6xHis (Panel B).

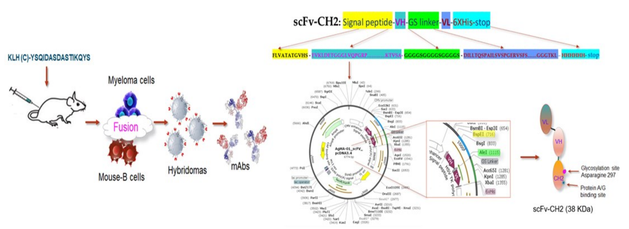


Figure 2. *Using the rational design approach, 16 amino acid sequences of MRSA252 AgrC receptor as the potential AIP binding epitopes on the outer surface were synthesized, KLH conjugated, and mouse hybridoma/monoclonal antibody were successfully generated (Panel A). The mAb (MA-01) was sequenced, and genetically engineered to make a sc-Fv–CH2 recombinant "Tiny body" as immunotherapy candidate (Panel B).*

TINY BODY PROTEIN EXPRESSION, PURIFICATION

The recombinant protein of "Tiny body" (sc-Fv-CH2)" was expressed in Expi293F cells (Fig.3A). The expression of the recombinant protein was confirmed by SDS-PAGE (Fig.3B). The recombinant protein was purified, and the purity was verified by Western blot (Fig.3C). The specificity of the Tiny body (sc-Fv-CH2) recombinant protein was also analyzed using MRSA252 culture supernatant protein and purified AgrC protein along with M. tb protein as control (Fig.3D). The endotoxin level was measured by the Limulus Amebocyte Lysate (LAL) test (Charles River Laboratories).

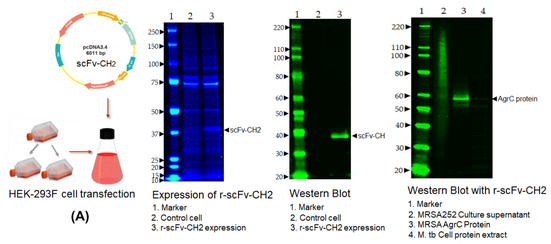


Figure 3. *Rational design of the "Tiny body" plasmid (AgMA-01scFv-CH2-pcDNA3.4) and HEK-293F cell transfection (Fig. A). Expression of "Tiny body" recombinant protein verified in SDS-PAGE (Fig. B). Western blot analysis of the purified "Tiny body" recombinant by probing with anti-His mouse mAb (Fig. C). The binding specificity of the "Tiny body" recombinant protein was evaluated in Wester blot using MRSA252 culture supernatant protein and purified AgrC protein along with M. tb protein (control).*

RECEPTOR BLOCKING ASSAY WITH MA-01 & r-TINYBODY

MRSA-252 AgrC receptor blocking/opsonization assay using MA-01 and r-Tiny body: MRSA252 cell suspension 5 x10^6 CFU ml^{-1} was incubated with 200 µg and 100 µg of MA-01 and r-Tiny body and isotype control antibody. After 12hr of incubation at 37 °C, the receptor binding activity was observed under phase contrast microscope (100X) and photographed.

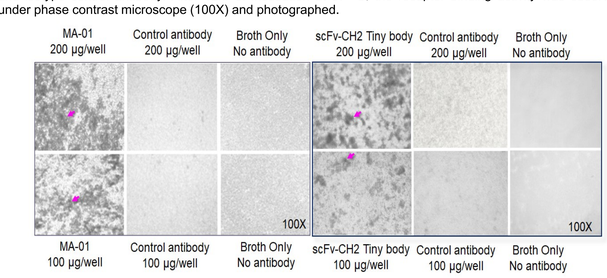


Figure 4. *MRSA252 Quorum sensing receptor "AgrC" blocking assay using MA-01 (Panel A) and the r-Tiny body (Panel B). The colored arrows show the opsonized MRSA252 cells by MA-01and r-Tiny body. No aggregation observed in isotype control antibody and broth only wells*

RECEPTOR BLOCKING ASSAY WITH MA-01 & r-TINYBODY

The AgrC receptor blocking assay was performed using Resazurin Microtiter Assay (REMA) in 96-well plates. The MRSA252 cell 5 x10^5 CFU ml^{-1} was incubated with different concentrations of MA-01, r-Tiny body and isotype control antibody. After incubation for 24 h at 37 °C, resazurin added to all wells and further incubated for 4hr for the observation of color change. The kinetics of resazurin metabolization, i.e., its reduction to fluorescent resorufin, was evaluated fluorometrically (λ_{ex}520/λ_{em}590 nm).

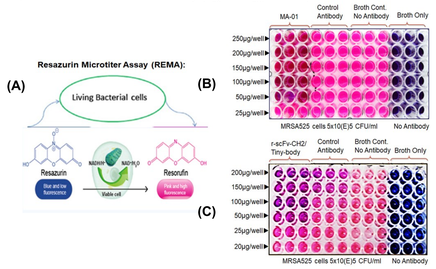


Figure 5. *Resazurin viability assay to detect metabolically active cells, the blue non-fluorescent dye is reduced to the pink and highly fluorescent compound resorufin allowing for a quantitative measurement of cell viability (Fig. A). The MA-01 capable of blocking MRSA252 AgrC receptor with as low 50µg of protein (Fig. A) and the r-Tiny body capable of blocking MRSA252 AgrC receptor with as low 25µg of protein (Fig. B)*

PROPHYLACTIC ASSAY WITH MA-01 & r-TINYBODY

Prophylactic assay and bacterial renal quantification : To investigate the *in vivo* protection of MA-01 and r-Tiny body by binding to its target (AgrC) and eliciting an antibody-blocking effect, two groups (n=7) of 8-week-old Balb/c mice were injected intraperitoneally with 20mg/kg of body wight purified MA-01, r-Tiny body and control mock mAb. On the second day, mice were inoculated IP with a sublethal dose (2×10^8 CFU/mL) of MRSA252 and observed until the sixth day. On the seventh day, animals were euthanized, the kidneys were aseptically removed, homogenized in sterile Luria Broth (LB) and plated on LB plates. The resulting colonies were counted in order to calculate the concentration of bacteria in the kidneys (Fig 6).

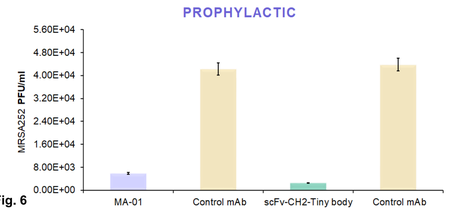


Fig. 6

Figure 6. *Bacterial load in kidneys was assessed after systemic infection with MRSA 252 and treated with MA-01, r-Tiny body and isotype control antibody. The extent of protection conferred by MA-01 and r-Tiny body treatment in a murine model resulted in significant bacterial load reduction in mice as compared to control mAb. There was 3408 PFU/ml (3.5 log) reduction of MRSA 252 by r-Tiny body as compared to MA-01.*

THERAPEUTIC ASSAY WITH MA-01 AND r-TINYBODY

Therapeutic potential of MA-01 and r-Tiny body: was evaluated in a mouse model of infection. Two groups (n=7) of 8-week-old Balb/c mice administered intraperitoneally (IP) with infective dose of (5×10^6 CFU/mL) and after 6 h of the administration, 20mg/kg of body wight purified MA-01, r-Tiny body and mock mAb inoculated IP and continue every day for three days. Animals were euthanized on the fourth day, the kidneys were aseptically removed, homogenized in of sterile Luria Broth (LB) and plated on LB plates. The resulting colonies were counted in order to calculate the concentration of bacteria in the kidneys.

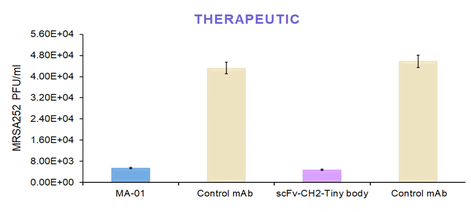


Fig. 7

Figure 7. *Bacterial load was assessed in mice kidneys after inoculation with MRSA 252 and treated with MA-01, r-Tiny body (sc-Fv-CH2) and isotype control antibody. The extent of protection conferred by MA-01 and r-Tiny body treatment in a murine model resulted in significant bacterial load reduction in mice as compared to control mAb. There was 600 PFU/ml (2.8 log) reduction of MRSA 252 by r-Tiny body as compared to MA-01.*

SURVIVAL ASSAY WITH MA-01 AND r-TINY BODY

Assessment of the protection conferred by MA-01 and r-Tiny body: in Balb/c mice after challenge with MRSA252. Briefly, on day one, two groups (n=7) of 8-week-old female BALB/c mice were injected IP with 20mg/Kg body weight of MA-01 or r-tiny-body and mock mAb. The next day, mice were injected (IP) with a sublethal dose (2×10^8 CFU/ml) of MRSA-252 and observed until the day 10^{th}. The survivors were subsequently euthanized.

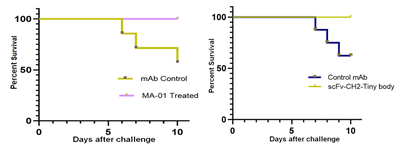


Figure 8. *Survival analysis of mice with the MA-01 and r-Tint body (sc-Fv-CH2) was performed in vivo. None of the animals died in MA-01 treated group (Panel A) and r-Tiny body treated group (Panel B), three animals in each control groups were died in different time point (6-10 days).*

BIODISTRIBUTION ASSAY OF MA-01 AND r-TINYBODY

Biodistribution of MA-01 and r-Tiny-body was evaluated by ELISA in serum, lung, spleen and kidney of Balb/c mice at different time points. Briefly, on day one, two groups of 8-week-old female BALB/c mice (n = 15) administrated IP with 20mg/Kg of purified MA-01, and r-Tiny-body protein. After inoculation samples of serum, lungs, spleens, and kidneys were collected at different time points (12, 24, 48, 72, and 96 h; and three mice per time point). The diluted serum samples and the homogenized organ samples were used to perform ELISA in AgrC protein coated plate (Fig. 9A & B).

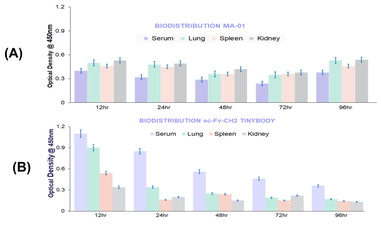


Figure 9. *Biodistribution of the MA-01 and r-Tiny body (sc-Fv-CH2) in vivo, the MA-01 and r-Tiny body (sc-Fv-CH2) reached serum and different tissues (Fig. 9A & B) within twelve hours after the administration and detected in serum and all organs as late as 96h.*

RESULTS

- To develop MRSA252 anti-AgrC receptor mAb, BALB/c mice were immunized with KHL conjugated synthetic peptide (16+1 AA) of the extracellular region of AgrC receptor domain VI.
- Monoclonal antibody (MA-01), which showed the highest binding activity for receptor protein was selected and further characterized.
- The MA-01 was engineered into "Tiny body", which is comprised of the VH and VL chains and CH2 domain of the parental IgG and retain the antigen binding properties of the intact IgG.
- Specific binding of MA-01 and Tiny body to AgrC receptor protein was also confirmed by Western blot analysis. MA-01 bound to the 55 kDa protein band corresponding to the dimeric forms of AgrC receptor.
- The prophylactic studies in a murine model demonstrated a significant bacterial load reduction in the kidneys of the groups treated with either the MA-01 or r-tiny-body compared to the isotype mAb control group.
- The therapeutic studies in murine model demonstrated, the MA-01 or r-tiny-body treatments resulted in reduction in kidney bacterial load in treated group, as compared to the isotype mAb control group.
- Survival analysis of mice with the MA-01 and r-Tint body (sc-Fv-CH2) was performed in vivo. None of the animals died in MA-01 treated group (100%) and r-Tiny body treated group, three animals in each control groups were died in different time point (6-10 days).
- Biodistribution was evaluated at different time points (12–96 h) after MA-01 or r-Tiny-body administration, both the antibodies reached in blood and different tissues after 12 h. The r-tiny-body predominantly remained in the serum, lungs at high levels as compared to MA-01, but these antibodies were also detectable in the kidneys, lungs, and spleen at low concentrations at all observed time points.

CONCLUSION

- We have sought to elucidate the consequences of quorum sensing signaling using highly specific anti-AgrC receptor monoclonal antibody (MA-01) elicited against a 16 amino acid peptide of MARS AgrC receptor domain-VI.
- The "Tiny body" (scFv-CH2) against *MRSA252* was successfully constructed and is a good candidate for the development of future immunotherapy for severe *MRSA* infections.
- The *in vitro* and *in vivo* data indicate that the jamming the docking site of AIP on AgrC receptor domain-VI by MA-01 or r-Tiny-body is sufficient to suppress QS-system of MRSA-252.
- In contrast, the r-Tiny-body engaged very effectively in blocking the AgrC receptor for autoinducer (AIP) docking and inhibiting the growth of MRSA-252, because of its smaller in size, higher tissue penetration capability (biodistribution), greater avidity, ability to bind complement.
- The r-Tiny-body represent a new and valuable set of immunological tool for both the study of QS-controlled processes and potentially an alternative strategy engaging immunochemotherapy for the prevention or treatment of MRSA infections in which QS signaling contributes to bacterial pathogenesis.
- The other benefit is that antibodies, once administered, provide immediate protection to individuals who have been exposed to an infectious organism and could represent an effective approach to combat infections associated with emergency interventions.

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