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2 **Immunogenicity in mice and non-human primates of the Group A Streptococcal J8**
3 **peptide vaccine candidate conjugated to CRM197**

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10 **Keywords:** Group A streptococcus vaccine, immunogenicity in mice and non-human
11 primates

12 **Abbreviations:** amorphous aluminum hydrophosphate sulfate adjuvant, AAHSA;
13 ISCOMATRIX™ adjuvant; bovine serum albumin BSA, Group A streptococcus, GAS

14 **Abstract:** Vaccine development for Group A streptococcal (GAS) infection has been
15 extensively focused on the N-terminal hypervariable or the C-terminal conserved regions
16 of the M protein, a major virulence factor of GAS. We evaluated the immunogenicity and
17 functional activity of the conserved C-terminal peptide vaccine candidate, J8, conjugated
18 to CRM197, in two mouse strains: C3H (H2^k) and Balb/c (H2^d), and in Rhesus macaques.
19 Mice were immunized with J8-CRM197 formulated with Amorphous Aluminum
20 Hydrophosphate Sulfate Adjuvant (AAHSA), and non-human primates were immunized
21 with J8-CRM197 formulated with AAHSA, ISCOMATRIX® adjuvant, or
22 AAHSA/ISCOMATRIX adjuvant. J8-CRM197 was immunogenic in mice from both H2^k
23 and H2^d backgrounds, and the antibodies generated bound to the surface of four different
24 GAS serotypes and had functional bacterial opsonic activity. Mice immunized with J8-
25 CRM197/AAHSA demonstrated varying degrees of protection from lethal challenge. We
26 also demonstrated that J8-CRM197 is immunogenic in non human primates. Our data
27 confirm the utility of J8 as a potential GAS vaccine candidate and demonstrate that
28 CRM197 is an acceptable protein carrier for this peptide.
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Introduction

Group A Streptococcus (GAS) remains a leading cause of several diseases with global impact, ranging from mild (pharyngitis and impetigo) to invasive (cellulitis, bacteremia, pneumonia, necrotizing fasciitis and streptococcal toxic shock syndrome) infections, as well as non suppurative sequelae (acute rheumatic fever and post-streptococcal glomerulonephritis) which are prominent in developing countries¹.

M protein has been described as the major surface-expressed virulence factor and the determinant of naturally acquired immunity against GAS^{2, 3}. Until recently, M protein has been the major focus of vaccine design, and two strategies targeting this antigen have been extensively studied. The most advanced work to date has focused on the highly immunogenic but hypervariable N-terminal region of M protein as the basis for a multi-epitopic vaccine approach. A 26-valent vaccine has been evaluated in human clinical trials and demonstrated to induce opsonic antibodies to several representative GAS serotypes⁴. However, the utility of such an approach has been questioned since serotype distribution of strains causing disease differs based upon geographical region studied and is complex and poorly understood in many parts of the world where vaccines would be of the greatest benefit; however, inclusion of representative N-terminal epitopes for specific geographical locations may be one approach to overcome these restrictions⁵⁻⁸. A second approach is based on the more highly conserved C-terminal region of the M protein⁹⁻¹¹. This approach is expected to be advantageous since sequence conservation would imply broader strain coverage with fewer vaccine components; although, safety concerns exist since this region of the protein has been shown to induce T cell responses that are cross reactive with human cardiac myosin¹². Good et al. defined a peptide sequence (p145) located in this region, which was recognized by antibodies in the sera of most adults living in areas of endemic streptococcal disease^{11, 13}. A minimal B cell epitope and non-host reactive peptide derived from p145, termed J8, has been shown to be immunogenic and protective in mice, either administered as a peptide alone formulated with Freund's adjuvant in B10.BR (H2^k) mice¹⁴ or conjugated to Diphtheria Toxoid (DT) and formulated on an aluminum phosphate adjuvant^{15, 16}.

Here we describe conjugation of J8 to an alternate protein carrier, CRM197, a non-toxic analog of DT which has been used as the basis for multiple bacterial polysaccharide conjugate vaccines¹⁷. We show that J8-CRM197 is immunogenic in inbred mice from two genetic backgrounds, H2^k and H2^d, and, for the first time, demonstrate immunogenicity of this conjugate vaccine in non-human primates. Our results indicate that J8-CRM197 performs similarly to the previously reported J8-DT conjugate in mice, confirming that CRM197 can serve as an alternative carrier protein to DT for J8 conjugate vaccines.

Results

J8- CRM197 conjugate vaccine is immunogenic in Balb/c and C3H mice. Initial experiments were designed to assess the immunogenicity of J8-CRM197 formulated with AAHSA at a single concentration of 12.5 μg in inbred Balb/c (H2^{d}) and C3H (H2^{k}) mice. Immunogenicity was comparable in both mouse strains with total IgG titers of $\sim 10,000$ elicited after a single dose of vaccine and a subsequent 10-fold increase in titer following the second dose. No further increase in titer was observed after the third immunization (**Fig. 1A**). No significant difference in response to the vaccine was observed between these genetically distinct inbred mice. ($p = 0.8$). We next performed a dose-ranging study by immunizing a smaller cohort of Balb/c mice ($n=3$) with 12.5, 5, 1 or 0.1 μg of J8 peptide conjugated to CRM197. We observed no differences in J8-specific antibody titers across all four doses (**Fig. 1B**), although there was a trend towards lower antibody responses at the 0.1 μg dose. No J8-specific antibodies were detected in control animals immunized with CRM197/AAHSA or Saline/AAHSA (data not shown).

J8-specific antibodies bind to M-protein on the GAS surface. We examined the ability of antibodies elicited by immunization of mice with J8-CRM197 to recognize the corresponding M protein epitope on intact bacterial cells. We evaluated binding of J8-CRM197/AAHSA, CRM197/AAHSA, and AAHSA immune serum to four different GAS serotypes (M1, M3, M6 and M97) and found that of the groups tested, the only serum that contained antibodies which bound to the surface of GAS was from mice immunized with J8-CRM197/AAHSA. One representative experiment using serotype M3, is shown in **Figure 2**. Similar results were obtained with other GAS serotypes (data not shown).

J8-CRM197 mouse antibodies have opsonophagocytic activity. The functional activity of antibodies raised by immunization of mice with J8-CRM197 was evaluated in an opsonophagocytic assay using GAS 88/30 M97. Naïve mouse serum was tested in the same experiments to serve as a non-immune control for determination of percent opsonization. Results from four different immunization experiments are shown in Figure 3; two using Balb/c mice (Experiment-1 and Experiment-2), and two using C3H mice (Experiment-3 and Experiment-4). From each of the four experiments only J8-CRM197/AAHSA immune serum promoted killing of the bacteria (activity ranging from 57-93%) which was statistically significant compared to the negative controls (CRM197/AAHSA or AAHSA immune serum [$P<0.001$]) (**Fig. 3**). Serum from mice immunized with GASM97 was used as a positive control and in all experiments caused between 90-100% killing (Data not shown).

Immunization with J8-CRM197 induces limited protection in mice following systemic and intranasal challenge. Groups of Balb/c and C3H mice were immunized with J8-CRM197/AAHSA, CRM197/AAHSA, AAHSA, heat-killed GAS M1, or recombinant M protein (strain M3). Balb/c mice were challenged (i.p) with GAS pM1 SR in mucin. Ninety percent of the J8-CRM197/AAHSA immunized group and 100% of the heat-killed M1 immunized groups (positive control) survived challenge ($P = 0.0001$ relative to CRM197/AAHSA and AAHSA groups) (**Fig. 4A**). In order to demonstrate protection in a second model system, C3H mice were challenged (i.n) with a GAS M3 SR strain. Seventy percent of the J8-CRM197/AAHSA immunized group and 100% of the recombinant M protein immunized group (positive control) were protected from

challenge, while 60% of the CRM197/MAA group also survived challenge ($P = 0.001$ relative to the AAHSA group) (**Fig. 4B**).

J8-CRM197 is immunogenic in non-human primates. We evaluated immunogenicity of J8-CRM197 formulated with three adjuvant combinations, AAHSA, ISCOMATRIX adjuvant, and AAHSA + ISCOMATRIX adjuvant, in rhesus macaques. Figure 5 shows the time course of the antibody responses to J8 as determined by ELISA. Pre-immune sera were tested as pooled samples while post-immunization titers were assessed from individual animals. IgG titers were lowest overall in animals immunized with J8-CRM197/AAHSA with titers of only ~6,000 and ~9,000 following one and two doses of conjugate, respectively. In contrast, J8-CRM197 formulated in ISCOMATRIX adjuvant alone or with an AAHSA/ISCOMATRIX adjuvant mixture induced IgG titers of > 20,000 after a single dose and >60,000 following a second dose. Moreover, the post-dose two titers were sustained after a third dose. J8-specific IgG titers were statistically significantly different ($P < 0.05$) between groups immunized with J8-CRM197/AAHSA-ISCOMATRIX adjuvant or J8-CRM197/ISCOMATRIX adjuvant vs. the group immunized with J8-CRM197/AAHSA at both post-dose two and post-dose three time points. We attempted to evaluate sera from NHPs in both the OPK and IFA assays; unfortunately the pre-immune sera contained high levels of background antibodies that led to both high killing activity in the OPK as well as background fluorescence in the IFA which made the data very difficult to interpret.

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Discussion

2 An efficacious vaccine for prevention of Group A streptococcal infection would be an
4 important tool for reducing rheumatic fever and rheumatic heart disease in the developing
6 world as well as for reducing recurrent pharyngitis in both the developing and developed
8 world¹⁸. Recent reports detailing an increase in global expansion of macrolide resistant
strains of *Streptococcus pyogenes* adds further impetus to the desire to develop such a
vaccine^{19, 20}. Although *S. pyogenes* is susceptible to penicillin, macrolides are the
antibiotics of choice for individuals who are allergic to β -lactams²¹.

10 M-protein, the major surface protein of GAS, is a coiled-coil protein composed of a
highly variable N-terminal region, which is the focus of serotyping (M typing) and
12 genotyping (*emm* typing), A-repeat and B-repeat domains which do not induce opsonic
antibodies²², and a C-repeat domain, which is the most conserved of the three repeat
14 regions and contains the J8 peptide. Previously described vaccine approaches utilizing the
M protein conserved C-repeat region include: (1) a recombinant protein domain
16 encompassing the C-terminal region of strain M6⁹; (2) selected B and T cell epitopes
from strain M5 presented as either synthetic peptides or recombinant protein²³; and (3)
18 the 12-amino acid minimal B cell epitope J8 presented as a synthetic peptide¹⁵. Murine
studies evaluating J8 conjugated to DT and formulated with aluminum hydroxide
20 established that this potential vaccine candidate induced opsonic antibodies which were
protective in a lethal challenge model^{24, 25}.

22 The objectives of the current study were, first to assess the immunogenicity and
protective efficacy in mice of a vaccine candidate consisting of the J8 peptide covalently
24 conjugated to the non-toxic DT analog, CRM197, and secondly to demonstrate that
immunogenicity of this vaccine candidate was extendable to non-human primates. The
26 use of CRM197 as an alternate carrier protein to Diphtheria toxin offers several potential
advantages in terms of vaccine manufacturability and safety. CRM197 is a component of
28 several licensed vaccines including PREVNAR7®, PREVNAR13®, and HibTITER®¹⁷.
As such, protocols for cGMP supply and release of the protein have been established.
30 Importantly, a substantial safety profile for use of this protein as a carrier for bacterial
polysaccharides has been established in humans, including infants. It is reasonable to
32 expect that this safety profile would extend to its use as a carrier for peptide vaccine
antigens as well. CRM197 has been evaluated pre-clinically for additional investigational
34 conjugate vaccines including studies with GAS polysaccharides²⁶.

J8-CRM197 formulated with AAHSA was shown to be highly immunogenic in
36 Balb/c (H2^d) and C3H (H2^k) mice at a peptide dose as low as 0.1 μ g, however we choose
the 12.5 μ g dose since opsonophagocytic (OPK) activity was reduced at lower doses (data
38 not shown). Antibodies elicited by immunization with J8-CRM197/AAHSA bound to the
surface of four different GAS strains (M1, M3, M6 and M97), confirming that the J8
40 epitope is conserved across these strains as well as demonstrating that the conjugation
chemistry employed here does not compromise presentation of the J8 epitope.
42 Furthermore, we demonstrated that J8-CRM197/AAHSA induces functional antibodies
which mediate opsonophagocytosis of GAS 88/30 M97 *in vitro* and lead to killing of
44 bacteria by human phagocytic cells, a major pathway for bacterial clearance.
Unfortunately, we were unable to expand upon this opsonophagocytosis data with
46 additional serotypes of GAS as suitable human blood donors for phagocytic cells could

not be identified for these types. This is likely due to the fact that most adults have been exposed to multiple GAS serotypes through their life and therefore have pre-existing M-protein based immunity. The opsonic activity of J8-CRM197 immune serum in four different mouse immunization experiments (two using Balb/c mice and two using C3H mice), ranged from 53% to 97%, but no opsonic activity was observed in the sera of control animals immunized with CRM197 or adjuvant alone.

J8-CRM197/AAHSA induced protective immunity in mice against two serotypes of GAS in two separate challenge models suggesting that protection mediated by this vaccine is not restricted to a single M-type, but rather may be broadly effective. In a systemic challenge study performed with GAS pM1 SR in Balb/c mice, the bacteria were mixed with mucin in order to decrease the bacterial dose required for lethality in the model as observed in experiments with other bacterial pathogens²⁷, 90% of J8-CRM197/AAHSA immunized animals were protected, while CRM197/AAHSA and AAHSA immunized mice did not survive. This study was repeated a total of three times, and J8-CRM197/AAHSA immunized mice were protected in two out of three experiments. In an intranasal challenge study performed with GAS M3 SR in C3H mice, 70% of J8-CRM197/AAHSA immunized mice were protected, in contrast to 10% survival observed in mice immunized with AAHSA alone. Unexpectedly, 60% of animals immunized with CRM197/AAHSA also survived. It has been previously reported that DT formulated with adjuvant also induced non-specific protection¹⁵. We attempted to overcome this issue by increasing the time between the last immunization and subsequent bacterial challenge; however some experiments continued to show non-specific protection by the CRM197 conjugate control (data not shown). This study was repeated a total of five times, and J8-CRM197/AAHSA immunized mice were protected in three of the experiments. However, since significant protection was not obtained in 100% of either the i.p. or i.n. challenge studies, the results of the mouse challenge models must be interpreted with caution. Since mouse models of infection for many bacterial pathogens can be quite variable, and GAS is not a natural mouse pathogen, non-human primates may serve as a more relevant model system for pre-clinical GAS vaccine evaluation²⁸.

It has been previously suggested that antibodies generated by immunization with the C-terminal region of GAS M protein exhibit cross-reactive binding to human serum albumin (HSA), and, therefore, are not expected to exhibit any specific functional activity²⁹. Our data showing opsonophagocytic activity of J8-directed antibodies appears to contradict this assumption. Additionally, we performed ELISA assays to directly assess binding of sera from J8-immunized animals to HSA, and we failed to detect any binding even at antigen coating concentrations as high as 10 µg/ml (data not shown). This suggests that the J8 epitope is located outside of the HSA binding site localized to the C-terminal portion of M protein. Alternatively, J8 may form a portion of the HSA binding site, but binding to HSA is insufficient to abolish its specific opsonophagocytic activity.

To our knowledge, the current study describes for the first time immunogenicity of the J8 peptide conjugated to CRM197 in mice and non human primates. J8-CRM197 formulated on any of the three adjuvants tested in rhesus macaques was well-tolerated and no adverse effects were observed in any vaccinated animals throughout the time course of the study. All vaccinees sero-converted following a single immunization with 10 µg of J8-CRM197 conjugate, achieving serum IgG titers of 6,000 to 20,000.

2 Following a second administration, titers rose to > 65,000 in animals immunized with J8-
CRM197/ISCOMATRIX adjuvant or J8-CRM197/AAHSA/ISCOMATRIX adjuvant.
4 Interestingly, animals immunized with J8-CRM197/AAHSA elicited lower IgG titers of
~10,000 after the second dose. Statistical comparisons indicated that the saponin-based
6 ISCOMATRIX adjuvant is significantly more potent than AAHSA in non-human
primates. No additional enhancement in IgG levels was observed in any group following
8 a third immunization, suggesting that further enhancement will require additional
evaluation of adjuvant, dose and immunization regimens. Our data demonstrating robust
10 immunogenicity of J8-CRM197 formulated with ISCOMATRIX adjuvant in non human
primates establishes this immunogen as a viable vaccine candidate for further
12 optimization and testing by itself or combined with other GAS antigens. We were unable
to evaluate the functional activity of these sera due to high pre-existing antibodies to
14 GAS in non-human primates. This may be due to previous exposure of the animals to this
organism as has been described ^{30, 31}. In conclusion, we report that J8 conjugated to
CRM197 is immunogenic and confers variable degrees of protection in mouse models.
16 We also demonstrated that J8-CRM197 formulated with the human compatible adjuvants
AAHSA, AAHSA + ISCOMATRIX adjuvant and ISCOMATRIX adjuvant is safe and
18 immunogenic in non human primates which further supports pre-clinical development of
this promising GAS vaccine candidate.

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Methods

Ethics statement. All animal work was performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Merck Research Labs, West Point, PA

Synthesis of J8 peptide. The peptide (Ac-Gln-Ala-Glu-Asp-Lys-Val-Lys-Gln-Ser-Arg-Glu-Ala-Lys-Lys-Gln-Val-Glu-Lys-Ala-Leu-Lys-Gln-Leu-Glu-Asp-Lys-Val-Gln-Aha-Cys-NH₂, Aha= 6-aminohexanoic acid, MW=3541.1) was prepared by standard solid-phase peptide synthesis with Fmoc/t-Bu chemistry. The N-terminus was acetylated and the C-terminus was amidated. Briefly, the peptide was synthesized by solid phase chemistry on a NovaPEG Rink Amide resin (0.62 mmeq/g, EMD Biosciences) using a Symphony Synthesizer (Protein Technologies, Inc). Acylations were performed with double couplings for 30 min with a 5-fold excess of amino acids activated with equimolar amounts of HBTU [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] and HOBT (N-hydroxybenzotriazole), and a 2-fold molar excess of DIEA (N,N-diisopropylethylamine). At the end of the assembly, the peptide was acetylated by treatment with a 10-fold molar excess of acetic anhydride. The dry peptide resin was then treated with cleavage mixture (82.5% trifluoroacetic acid, 5% phenol, 5% thioanisole, 5% water, 2.5% ethanedithiol) for 2h at room temperature. The filtered peptide solution was precipitated with cold diethyl ether, isolated by centrifugation, washed twice with cold diethyl ether, dried, resuspended, and lyophilized. The peptide was purified by Reverse Phase HPLC on a C18 Jupiter Column (Phenomenex Jupiter 10 μ , C18, 300A) and eluted with an appropriate linear gradient of an increasing concentration of acetonitrile containing 0.1% TFA. The purified peptide was characterized by electrospray mass spectrometry.

Synthesis of biotinylated J8 peptide. The peptide (Ac-Gln-Ala-Glu-Asp-Lys-Val-Lys-Gln-Ser-Arg-Glu-Ala-Lys-Lys-Gln-Val-Glu-Lys-Ala-Leu-Lys-Gln-Leu-Glu-Asp-Lys-Val-Gln-Aha-Cys (PEG-Biotin)-NH₂, Aha= 6-aminohexanoic acid, PEG= polyethylene glycol, MW=4066.8) was prepared by a reaction of purified J8 peptide with a 2-fold molar excess of EZ-Link maleimide-PEG2-Biotin (Thermo Fisher Scientific, Rockford, IL) in 0.1M ammonium acetate buffer, pH 7.0 for 1 hour. The peptide was purified by Reverse Phase HPLC on a C18 Jupiter Column (Phenomenex Jupiter 10 μ , C18, 300A) and eluted with an appropriate linear gradient of an increasing concentration of acetonitrile containing 0.1% TFA. The purified peptide was characterized by electrospray mass spectrometry.

Preparation of J8-CRM197 conjugate. Purified CRM197 carrier protein was obtained from Merck Research Laboratories (West Point, PA) and activated for conjugation by addition of maleimide groups to surface-accessible lysine residues using the heterobifunctional cross-linking reagent succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, Thermo Fisher Scientific, Rockford, IL)³². Briefly, a concentrated stock of CRM197 in phosphate buffer was diluted in 20mM HEPES, 0.15M NaCl, 0.005M EDTA, pH 7.3 to a total protein concentration of 1 mg/ml. Nine mg of SMCC (10-fold molar ratio to theoretical CRM197 lysine content) was dissolved in 1 ml of dry dimethyl sulfoxide (DMSO) and added to the CRM197 solution while it was being gently vortexed. The reaction mixture

2 was incubated in the dark at room temperature for 3 hours. The sample was then reduced
3 in volume to 5 ml using an Amicon Ultra 4 centrifugal concentrator and desalted over a
4 Hi Prep 26/10 desalting column (GE Healthcare Life Sciences) equilibrated in 20mM
5 HEPES, 0.15M NaCl, 5mM EDTA, pH 7.3. The amount of maleimide incorporated into
6 the CRM197 was determined using a 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) -based
7 assay which relies upon the consumption of a fixed amount of N-acetyl cysteine from a
8 standard solution. CRM197 total protein was determined by the bicinchoninic acid
9 (BCA) protein assay. Conjugation of thiolated J8 peptide to maleimide-activated
10 CRM197 was performed at a 2 to1 molar ratio of peptide thiol to CRM197 maleimide.
11 The required amount of thiolated J8 peptide was weighed out and placed in a 20 ml glass
12 vial to which the activated CRM197 solution was added. The solution was mixed to
13 ensure that the peptide was completely dissolved and then incubated overnight at room
14 temperature. The conjugate was purified of un-reacted peptide by size exclusion
15 chromatography over a 2.5 by 45 cm Superdex 30 column equilibrated and run in 20mM
16 HEPES, 0.15M NaCl, pH 7.3. The molar loading ratio of peptide to carrier was
17 determined by quantitative amino acid analysis using least squares linear regression
18 analysis and quantitation of unique amino acid residues generated during the conjugation
19 reaction³³.

20 The adjuvants used for formulation were amorphous aluminum hydroxyphosphate
21 sulfate (AAHSA) and ISCOMATRIX adjuvant. AAHSA was obtained from the Merck
22 Manufacturing Division, West Point, PA., and ISCOMATRIX adjuvant was obtained
23 from CSL Biotherapies Inc., King of Prussia, PA.

24 **Immunization of mice and non-human primates.** Mouse experiments were
25 approved by the Institutional Animal Care and Use Committee at Merck & Co., Inc. Five
26 to seven week old female C3H or Balb/c mice were purchased from Taconic Farms
27 (Hudson, NY) and housed in microisolator cages in the animal facility at Merck Research
28 Laboratories (MRL), West Point, PA. Mice were intramuscularly (i.m) immunized with
29 J8-CRM197 (doses ranged from 0.1 to 12.5 µg based on peptide content), formulated
30 with amorphous aluminum hydroxyphosphate sulfate adjuvant (AAHSA). Negative
31 control animals received CRM197/AAHSA or AAHSA, while positive control animals
32 received 10 µg of recombinant, full length M3 protein (rM3)²⁸ or 10⁹ CFU of heat-killed
33 GAS, serotype M1. For generation of high-titered immune sera for use as a positive
34 control in OPK assays, mice were immunized intraperitoneally with 10⁷ CFU of heat-
35 killed GAS 88/30 strain (serotype M97)³⁴. All immunizations were performed on days 0,
36 21 and 42. Blood samples were collected on days -1, 20, 41 and 50. Samples from all
37 dates were tested in ELISA and samples from day 50 were tested in immunofluorescence
38 and bactericidal assays. The aluminum concentration in each of the vaccine formulations
39 was 45 µg/dose.

40 Non-human primate experiments were approved by the Institutional Animal Care and
41 Use Committee at both Merck & Co., Inc. and the University of Louisiana, New Iberia
42 Research Center (NIRC, Lafayette, LA). A group of 12 rhesus macaques were included
43 in the study. Selected animals were matched by age, sex and weight, housed in social
44 settings and randomly assigned to three experimental groups of four individuals each that
45 received different vaccine formulations. Four monkeys per group were immunized i.m
46 with J8-CRM197 with various adjuvant formulations. Monkeys received a vaccine dose
47 on days 0, 56 and 112. Vaccine antigen/adjuvant concentrations were: 20 µg/ml of J8

peptide formulated with AAHSA (450 µg/ml) and/or ISCOMATRIX™ (120 µg/ml). A vaccine dose of 0.5 ml was injected per animal. Serum samples were collected on days 0 (pre-immunization), 28, 56, 70, 84, 126 and 140. Only samples collected on days 0, and weeks 4, 10 and 20 were tested in ELISA.

Bacterial strains and culture conditions. For bacterial designations, M type and origin see Table 1. All the previously mentioned strains and a GAS serotype M6-12348 obtained from ATCC were grown in Todd Hewitt broth (THB) (Becton Dickinson) overnight and processed for use in immunofluorescence or bactericidal assays.

ELISA assays. Immulon 2 HB (Thermo Labsystems, Catalog # 3455) 96 well plates coated with J8 peptide were used for testing mouse sera samples and Neutravidin (Pierce, Cat # 15129) 96-well plates coated with biotinylated J8 were used to test non-human primate sera samples. In both experiments the plates were coated with 0.25 µg/well of J8 peptide in coating solution (KPL, Cat # 50-84-00) overnight at 4°C. Wells were washed with PBS containing 0.05% Tween 20 (PBS-T), and unbound sites blocked with Blocking/Dilution Solution (KPL, Cat # 50-61-00) by incubation for 2 h at room temperature. Mouse or non-human primate sera were 5-fold serially diluted on the plates and incubated at room temperature for 1 h. Plates were washed with PBS-T and incubated with goat anti-mouse (Jackson Immuno Research, Cat # 115-035-071) or anti-rhesus (Rockland Immunochemicals Inc, Cat # 617-103-012) IgG HRP secondary antibody for 1 h at room temperature. Following an additional wash, ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate (KPL, Cat # 50-62-00) was added and after 30 min, the reaction was stopped with ABTS Stop Solution (KPL, Cat # 50-85-01) and absorbance measured at 405nm.

Immunofluorescence Assay. Antibodies elicited after three immunizations with J8-CRM197 were tested in indirect immunofluorescence assays (IFA) against four different GAS strains³⁵. Strains 88/30 M97, 315, M6 and M1 were grown overnight, centrifuged at 3000 rpm and the pellet resuspended in PBS to an O.D₆₀₀ ~0.1. Ten µl of bacterial suspension per well were applied to 12-well glass microscope slides, dried overnight at room temperature, fixed with 3% formaldehyde, rinsed with PBS and stored at -20°C until used. Slides were brought to room temperature, washed in PBS and blocked with 5% skim milk in PBS-T in a humidified chamber at 37°C for 30 min, rinsed with PBS and incubated with a 1:200 dilution of purified non-specific human IgG (Serotec, Cat # PHP-001) for 1h at 37°C followed by incubation with J8 immune mouse sera (1:50 dilution). A rabbit serum with specificity for GAS (Statens Serum Institut, Cat # 22433) or serum from mice immunized with individual serotypes M1 or M3 or M97 heat killed GAS were included as a positive control. Slides were then washed and incubated with FITC-conjugated F(ab)₂ goat anti mouse IgG (Invitrogen, Cat # 62-6311) diluted 1:50 in Evans blue (MP Biomedicals) and incubated for 45 min in the dark. Goat anti-rabbit IgG (H+L) secondary antibody if the rabbit positive serum was used (Invitrogen, Cat # A10526) was used for detection of the positive control antiserum. Wells were washed three times with PBS for 10 min. Mounting solution was added and stained bacteria were visualized at 100X magnification using a fluorescence microscope.

Whole Blood Bactericidal Assay. Murine antibodies were assayed for opsonic activity against GAS M97 as previously described^{3, 25, 36}. Briefly, GAS were incubated end-over-end rotation at 37°C for 3h in the presence of non opsonic human blood and either mouse immune or control naïve mouse sera. Antisera to heat killed GAS strain

2 M97 was used as a positive control. Bacteria were plated in triplicate on sheep blood agar
plates (Remel, Cat # 01202) and colony forming units (cfu) were counted after 24h
incubation. The percentage of bacteria killed was determined using the following
4 equation: $[(\text{mean cfu}_{\text{negative control}}) - (\text{mean cfu}_{\text{test sample}}) / \text{mean cfu}_{\text{negative control}}] \times 100$.

6 **Challenge experiments.** Mice were challenged 50-55 days after the primary
immunization regimen. Both the pM1 and M3 strains used for challenge experiments had
been serially passaged in mouse spleen to enhance virulence and made streptomycin
8 resistant (SR)²⁵, then labeled (pM1 SR) and (M3 SR)

10 For systemic challenge: the pM1 SR strain was grown overnight in 10 ml aliquots of
THBN. The cultures were combined and bacteria were collected by centrifugation at
3000 rpm for 10 minutes, the pellet was resuspended in cold THBN and serially diluted,
12 cfu were determined by plating on sheep blood agar and the undiluted culture was held at
4°C overnight. The following day, the culture was resuspended to 1×10^8 cfu/ml in THBN,
14 then serially diluted in THBN plus 5% mucin type II (Sigma, St. Louis, MO). The 1×10^5
cfu/ml dilution was further diluted to the inoculum challenge dose of 7.5×10^3 cfu/ml in
16 mucin and injected intraperitoneally (i.p) into Balb/c mice at 0.4 ml/mouse. The mice
were observed daily for survival for 15 days. Inoculum concentration was confirmed by
18 plating on blood agar plates.

20 For intranasal (i.n) challenge: 48 hours before challenge, mice were given
streptomycin (200 µg/ml) in their drinking water. This strain was grown as described
previously³⁷. Briefly, a frozen stock of GAS M3 SR was cultured overnight in THB
22 supplemented with 0.5% yeast extract (THYE) containing 200 µg/ml streptomycin
(Teknova, Cat # S6550) at 37°C in 5% CO₂. The overnight culture was subcultured and
24 grown for 4.5 h at 37°C with 5% CO₂ to an A₆₀₀ between 0.9 and 1.0. The bacteria were
collected by centrifugation, washed and resuspended in THYE containing 25% glycerol
26 (final concentration) and frozen at -70°C. The concentration of viable bacteria ($\sim 1.7 \times 10^9$
cfu/ml), was confirmed by plating on sheep blood agar plates containing streptomycin
28 (Teknova, Cat # B0143). On the day of the challenge the frozen GAS M3 SR was
thawed and used undiluted ($\sim 1.7 \times 10^9$ cfu/ml). The mice were sedated with 100mg/kg
30 ketamine (Fort Dodge Animal Health) and 10mg/kg xylazine (AnaSed®) injected
intraperitoneally. The bacterial suspension (15 µl/nare) was administered to the sedated
32 mice. The mice were observed daily for survival for 15 days.

34 **Statistical analysis.** Antibody responses were log transformed and multiple
comparisons were done using Student's *t*-test. Opsonophagocytosis analysis was
performed using Anova followed by Bonferroni's multiple comparison test. A log-rank
36 (Mantel-Cox) test was used to determine the differences in protection between vaccine
immunized and negative control groups.

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List of Figures:

2

4 **Figure 1.** Serum IgG antibody responses to J8 in Balb/c and C3H mice over time. Titers
6 from C3H and Balb/c mice immunized with J8 -CRM197/AAHSA (n=10) at 12.5µg/dose
8 based on specific peptide content (A). Dose titration of J8-CRM197/AAHSA in Balb/c
mice (n=3), mice were immunized with 12.5, 5, 1 and 0.1 µg /dose, based on J8 peptide
content (B).

10 **Figure 2.** Photomicrographs (representative of the slide) of immunofluorescent
12 (fluorescein isothiocyanate) - stained group A streptococci (GAS, serotype M3) viewed
under the same conditions, using a microscope with a 100X objective. Bacteria were
14 stained with pre-immune mouse sera (A), CRM197 immune serum (B), J8-CRM197
immune serum (C) and type M3 immune serum as positive control (D). All sera were
taken after the last boost and diluted 1:50.

16

18 **Figure 3.** Average percent opsonization of GAS M97 strain by sera taken one week after
the last boost (Day 50). Filled symbols (●▲) represent two independent experiments
20 using mouse sera taken from C3H immunized mice and open symbols (○Δ) represent two
independent experiments using sera taken from Balb/c mice. In all four experiments only
J8-CRM197/AAHSA immune serum promoted killing of the bacteria (activity ranging
22 from 57-93%) which was statistically significant compared to the negative controls
(CRM197/AAHSA or AAHSA) immune serum $p < 0.001$.

24

26 **Fig. 4.** Survival of C3H and Balb/c mice after challenge. Balb/c mice challenged
intraperitoneally (i.p) with GAS pM1 SR in mucin (A) 90% of the J8-CRM197/AAHSA
immunized group and 100% of the heat-killed M1 immunized groups (positive control)
28 survived challenge $p < 0.0001$ relative to CRM197/AAHSA and AAHSA groups. C3H
mice challenged intranasally (i.n) with GAS M3 SR. (B) 70% percent of the J8-
30 CRM197/AAHSA immunized group and 100% of the recombinant M protein immunized
group (positive control) were protected from challenge, while 60% of the CRM197/MAA
32 group also survived challenge $p < 0.001$ relative to the AAHSA group.

34 **Fig 5.** J8-specific serum IgG titers in rhesus macaques immunized with J8-CRM197
formulated with three different adjuvants. Antibody titers were evaluated by ELISA.
36 Note that after the second immunization, animals that received J8-
CRM197/ISCOMATRIX adjuvant (IMX) had higher anti-J8 IgG titers statistically
38 significant compared to the group that only received J8-CRM197/AAHSA $p = 0.0331^*$,
and the group that received J8-CRM197/ISCOMATRIX adjuvant (IMX) $p = 0.0001^{***}$.

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2 **Table 1.** Groups of rhesus macaques immunized with J8-CRM197 using different adjuvant formulations

Group Code	Vaccine ^a	~J8 Peptide Dose
1	J8-CRM197	10 µg
2	J8-CRM197/AAHSA	10 µg
3	J8-CRM197/IMX ^b	10 µg
4	J8-CRM197/AAHSA-IMX ^b	10 µg

*n = 4 rhesus macaques per group

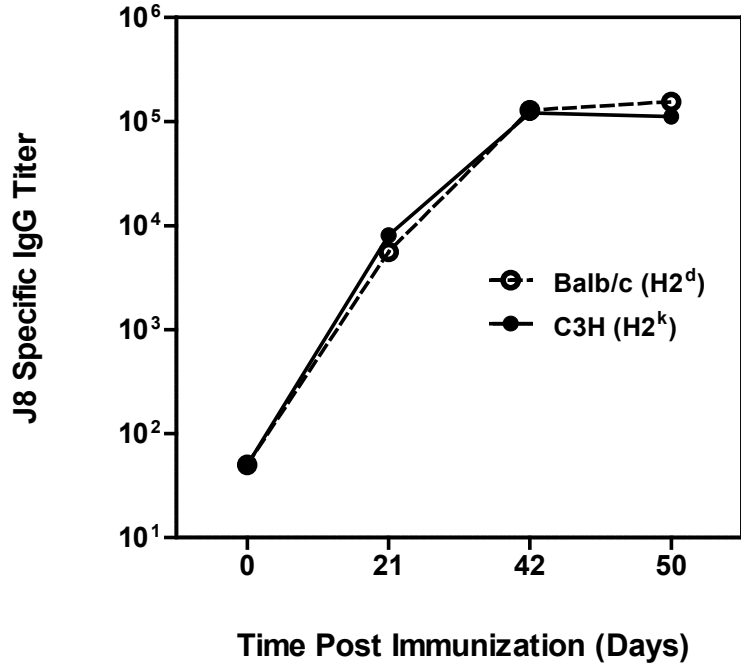
^a Administered intramuscularly in a final volume of 500 µl

4 ^b Iscomatrix (IMX) dose 60 µg

6

Figure 1.

A.



B.

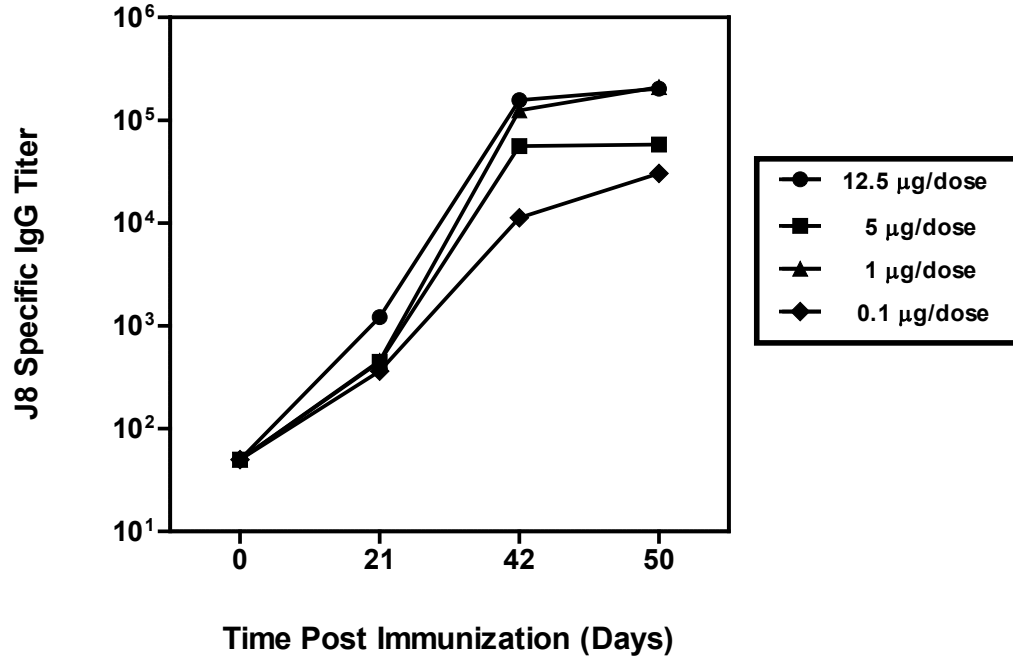


Figure 2.

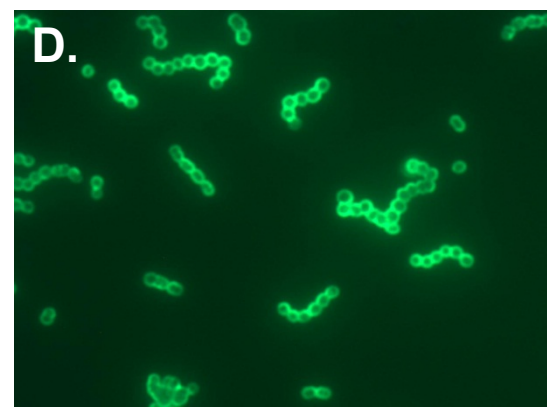
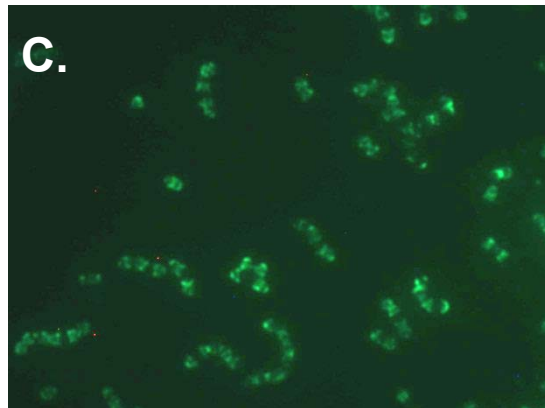
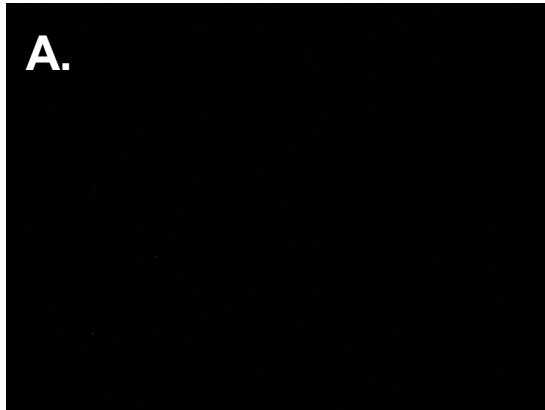


Figure 3.

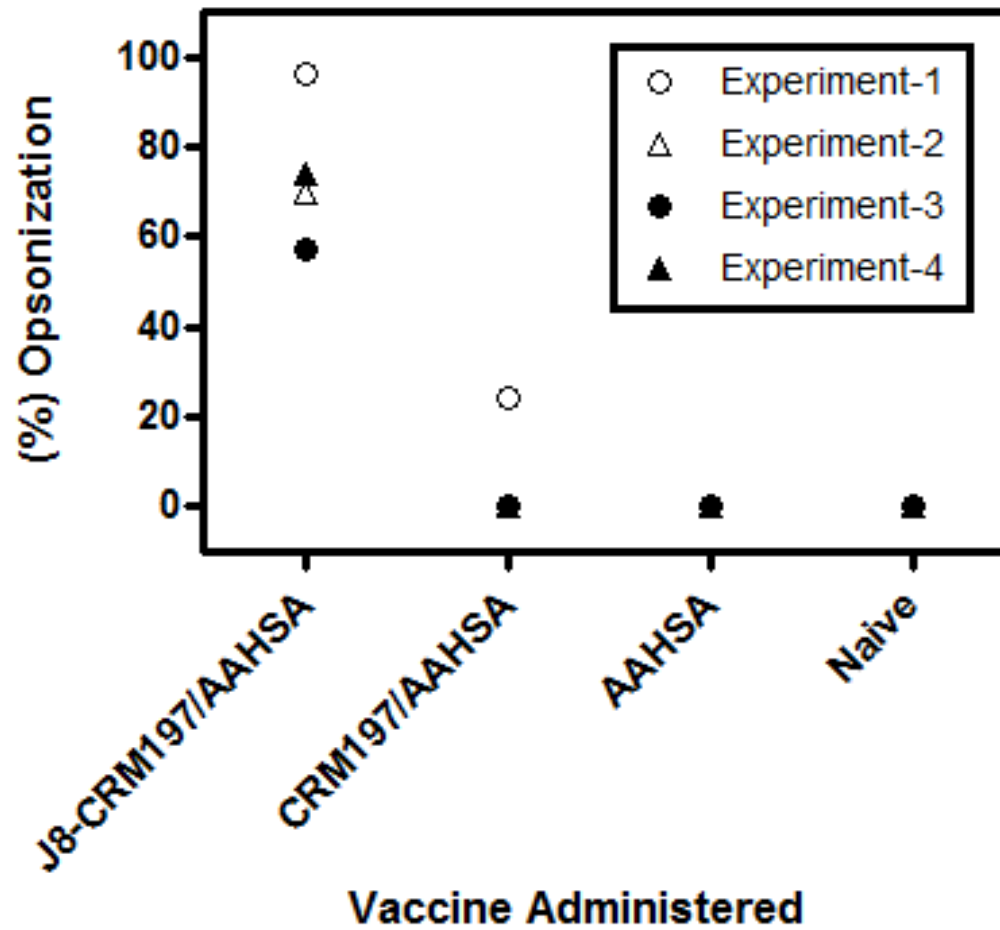
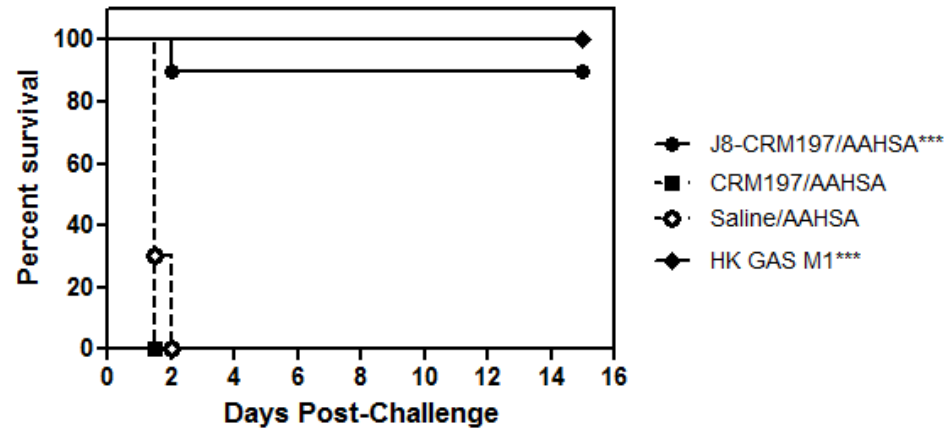


Figure 4.

A.

i.p Challenge with pM1 SR in Balb/c mice



B.

i.n Challenge with M3 SR in C3H mice

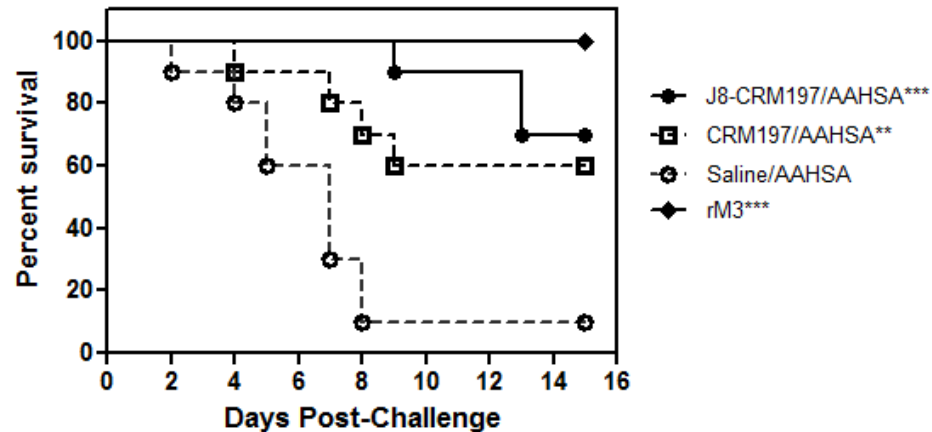


Figure 5.

