Supplementary Information

Retargeting pre-existing human antibodies to a bacterial pathogen with an alpha-Gal conjugated aptamer

Sascha A. Kristian, John H. Hwang, Bradley Hall, Emma Leire, John Iacomini, Robert Old, Uri Galili, Charles Roberts, Kary B. Mullis , Mike Westby, Victor Nizet

Supplementary Methods

Aptamer preparation for assays. Aptamer/alphamer stocks in nuclease-free water or Dulbecco's phosphate buffered saline (DPBS) were stored at -20°C in low retention microreaction tubes. Measuring their absorbance at 260 nm and taking into account their extinction coefficient spectrophotometrically determined aptamer concentrations. Before every experiment, the respective vehicle, aptamers, and alphamers in H_2O or DPBS were pre-heated for 15 min at 72-80°C and then cooled to room temperature to allow for re-folding.

Bacterial strains and culture conditions. Several GAS strains representing clinically relevant M serotypes were used: 5488 (Serotype: M1T1)[1], SF370 (M1; ATCC®-700294™), 20224 (M3; provided by Malak Kotb), 4063_05 (M4; provided by Bernard Beall), NS414 (M11) and NS488 (M12)[2], NZ131 (M49T14; ATCC®-BAA-1633™), NS236 (M77)[2]. Isogenic 5448 mutants deficient in M1 protein [3], capsule [4], SpeB [5], and DNAse SdaI [6] and an animal-passaged strain [7] were used for determining the target molecule of the GAS aptamer 20A24P. For most experiments, bacteria were grown in Todd Hewitt broth (THB) to late exponential or early stationary phase or overnight to stationary phase. Subsequently, the bacteria were washed twice in cell-culture tested Dulbecco's phosphate buffered saline (DPBS) or Hank's Balanced Salt Solution with calcium and magnesium (HBSS+/+) and then spectrophometrically adjusted to the desired colony forming units (CFU) concentrations in the respective assay buffers. The GAS 5448 M1-deficient mutant strain carrying the M1 protein gene *in trans* on a plasmid [3] was grown in THB, 2 μg/mL erythromycin (Erm). To determine the bacterial growth phase during

the aptamer 20A24P bound best to GAS 5448 cells, bacteria were grown in THB and samples taken at various stages of growth and bacteria further processed for FACS binding assays as indicated below.

Aptamer/alphamer and M protein antiserum IgG binding to live bacteria. The assays were carried out in polystyrene 96-well plates (Costar). In some experiments, the 96-well plates were pre-blocked with casein blocking buffer (Thermo Scientific) and then washed three times with assay buffer before adding aptamers/alphamers and GAS cells to the wells. For aptamer/alphamer binding assays, 10^8 CFU/mL of washed GAS in a total volume of 25-100 μL were incubated for 30 to 45 min at 37°C with 5'- or 3'-FAM labeled aptamers, alphamers, or vehicle in 10% [v/v] nuclease-free H_2O or DPBS (vehicle), 80% [v/v] aptamer binding buffer (50 mM Tris-HCl, pH 7.4, 5 mM KCl, 100 mM NaCl, 1 mM $MgCl_2$)[8] or other specified buffers, such as HBSS+/+, and 10% [v/v] 1 mg/mL DNA from salmon testes (Calbiochem) in nuclease-free H_2O. Subsequently, the bacteria were washed twice with ice-cold assay buffer. To correlate aptamer binding with M protein detectability by anti-M protein antibodies, the bacteria were incubated with buffer only, or negative control or non-immune or M protein specific rabbit or mouse hyper-immune sera. Anti-M protein IgG binding was detected with secondary Alexa Fluor® 488-labeled antibodies. Then, the green fluorescence intensities of 10,000-20,000 bacterial particles showing gated by forward and side scatters were measured using the FL-1 channel of a BD Biosciences FACSCalibur™ flow cytometer. Data analysis was done with CellQuest™ (BD Biosciences) or FlowJo™ (Tree Star Inc.).

ELISA with recombinant M1 protein variants to determine GAS aptamer target. Recombinant his_6-tagged full-length or truncated M1 protein variants and negative control protein were cloned, expressed and purified as described [9]; bovine serum albumin (BSA; Sigma-Aldrich) served as additional negative control protein. 1-2 μg of each of the proteins in PBS were immobilized to polystyrene Microplate Immulon 4HBX 96-well flat bottom plates (Fisher Scientific) by overnight incubation at 4°C. Subsequently, remaining protein and DNA binding sites were blocked by incubation for 2 h at 37°C with casein blocking buffer (Thermo Scientific) containing 2.5-5 mg salmon testes DNA. After washing the wells three times with HBSS without calcium and magnesium (HBSS-/-), the tested 3' biotinylated aptamers or vehicle

diluted in HBSS-/- were added to the wells and the plates incubated for 1 h at 37°C. For detection of aptamer binding, the wells were washed thrice with HBSS-/-, then HRP-conjugated streptavidin (R&D Systems) diluted 1/200 in HBSS-/- was added to each well, the plates were incubated for 1-2 h at 37°C. Then, the wells were washed three times with HBSS-/- and 50 μL TMB substrate solution (eBioscience) was added to each well. After 5-30 minutes of incubation at room temperature in the dark, 50 μL of 0.2N sulfuric acid was added to each well to stop the TMB substrate conversion by HRP. Finally, the absorbance of each well at 450 nm was read and recorded using a microplate reader and SoftMax® Pro software. The higher the absorbance values at 450 nm were, the higher the degree of streptavidin-HRP and thus biotinylated aptamer binding to immobilized proteins in the wells. Pictures of the ELISA plates were taken immediately after absorbance measurements with an iPhone 5 camera (Apple Inc.) using default settings and flash. Photos were imported into Microsoft® Powerpoint® for Mac 2011 and the sharpness corrected for the whole pictures in the program. Then, representative of duplicate wells of a single experiment were cropped and used for the photographic illustration of the ELISA results.

Purification of IgG and IgM antibodies from transgenic mice and measurement of anti-Gal antibody titers mouse and human antibodies. Transgenic $GT^{-/-}$ mice expressing the variable region of the heavy chain (V_H) of the anti-Gal monoclonal antibody M86 produce α-Gal-reactive IgM and IgG antibodies [10]. Serum from several animals was pooled and IgG separated from other immunoglobulins by Protein A chromatography as described [11]; the initial flow-through was collected to obtain IgM antibodies. IgG was eluted with 0.2 M glycine in PBS, pH 2.8, and pH neutralized by addition of NaOH. Antibodies were stored at -80°C until use. The anti-Gal titers in mouse polyclonal IgG and IgM and human polyclonal IgG (Human IVIG (hIVIG); Gamunex-C, Talecris Biotherapeutics) was determined by ELISA. To that aim, human serum albumin (HSA)-conjugated alpha-Gal (V-Labs, Inc.) was immobilized to polystyrene high binding Costar® EIA/RIA 96-well plates. HSA (Sigma-Aldrich) immobilized in equimolar amounts served as negative control protein. After immobilization, the wells were washed twice with DPBS. Then, the wells were blocked by incubation for 2 h at 37°C with casein blocking buffer (Thermo Scientific). Then, serial antibody dilutions in casein blocking buffer were added to triplicate wells. Wells with blocking buffer alone served as no antibody controls. After 1-h

incubation at 37°C, the wells were washed 3x. Then, HRP-conjugated goat anti-mouse IgG or IgM (both SouthernBiotech) or anti-human IgG (Bio-Rad) secondary antibodies in blocking buffer was added to appropriate wells and plates incubated for 1 h at 37°C. After washing the wells 3x, TMB substrate was added to each well. After 5-30 minutes of incubation at room temperature in the dark, 0.2N sulfuric acid was added. Finally, the well absorbances at 450 nm were recorded using a microplate reader and SoftMax® Pro software.

Recognition of alphamers bound to GAS by anti-Gal antibodies. 10^8 CFU/mL GAS 5448 M1T1 bacteria were incubated with 1.0-2.5 μM 5'-α-Gal, 3'-FAM GAS alphamer α20A24P or negative control alphamer αRAND-80 or 5'-α-Gal GAS alphamer α20A24P.A3 for 45 min at 37°C in buffer consisting of 80% HBSS+/+ (v/v), 10% DPBS (v/v), and 10% nuclease free water (v/v) and containing 100 μg/mL salmon testes DNA. 3'-FAM GAS aptamers with no α-Gal and vehicle only were used as additional negative controls. After pre-incubation of bacteria with alphamers, aptamers or vehicle, the bacteria were washed twice in refrigerated HBSS+/+. Then, the bacteria were resuspended in refrigerator-cold HBSS+/+ (no primary antibody control) or purified mouse IgG or IgM (from the above described transgenic mice) diluted in HBSS+/+. After 1-h co-incubation of the bacteria with primary antibody, the cells were washed twice with cold HBSS+/+ and then incubated with secondary Alexa Fluor® 647 goat anti-mouse IgG or IgM antibodies (both Invitrogen) for 1 h at 4°C. After washing the bacteria twice with cold HBSS+/+, the bacteria were resuspended in HBSS+/+ and then subjected to flow cytometry to simultaneously analyze for aptamer and alphamer binding in FL-1 channel (green fluorescence) and mouse IgG or IgM primary antibody binding in FL-4 channel (red fluorescence). The instrument settings were adjusted as such, that FL-1 and FL-4 signals would not interfere with each other as controlled by no aptamer/alphamer (vehicle control) and no primary antibody control samples.

Purification of human blood neutrophils. Heparinized human vein blood was obtained from healthy volunteers and neutrophils purified using Polymorphprep™ (Fresenius Kabi Norge AS) according to manufacturer's instructions. The viability of the phagocytes after each purification exceeded 90-95% as determined by trypan blue exclusion.

Phagocytosis studies. To obtain green fluorescent bacteria, GAS M1T1 5448 was grown overnight in THB containing 20 μg/mL calcein-AM (Invitrogen) and then washed four times with DPBS. Then, the bacteria were centrifuged, resuspended in HBSS+/+ and spectrophotometrically adjusted to the desired cell concentration. Subsequently, the bacteria were incubated at 37˚C for 10-30 min with 5 μM alphamers, aptamers, or vehicle in 25 μL samples in 96-well plates in 90% HBSS+/+ [v/v], 10% DPBS [v/v]. Then, 25 μL of polyclonal mouse IgG or hIVIG in HBSS+/+ as anti-Gal antibody sources were added and samples incubated for an additional 20 min at 37°C. Subsequently, the bacterial suspensions were mixed with 50 μL of freshly purified human blood neutrophils at a bacteria to phagocyte end ratio of 2:1 to 3:1. After a 20-min incubation with shaking at 225 rpm, the phagocytosis reactions were stopped by addition of 150 μL ice-cold HBSS+/+. Neutrophils were then separated from non-cell-associated bacteria by differential centrifugation (520 x *g*, 5 min), phagocytes resuspended in 10 μL ice-cold HBSS+/+ and samples placed on ice until analysis. The percentage of neutrophils with ingested bacteria was determined as previously described [12]. Briefly, 100-200 human cells per sample analyzed for phagocytosis of bacteria by fluorescence microscopy. To that aim, samples were mixed 1:1 with 1 mg/mL ethidium bromide. Ethidium bromide causes extracellular calcein-labeled GAS to fluoresce red-orange, whereas phagocytosed, intracellular GAS remain green because live neutrophils do not take up the dye. This process allowed for simultaneous visualization of intracellular and extracellular bacteria by using a dichroic fluorescence filter.

Opsonophagocytic killing assays with GAS and neutrophils. ~6-7x10^7 colony forming units (CFU)/mL of late exponential phase GAS SF370 were pre-incubated in a 96-well round bottom plate at 37°C with 1-3 μM GAS alphamer α20A24P or control alphamer αRAND-80 in 15 μL Hank's Balanced Salt Solution with calcium and magnesium (HBSS+/+). After 20 min, freshly purified neutrophils in HBSS+/+ and human IgG (hIVIG) in 45 μL were added and the plate incubated at 37°C with 220 rpm shaking. The endconcentration of the phagocytes in the 60-μL samples was 2.5x10^6 cells/mL, the hIVIG endconcentration 1 mg/mL. Samples to which vehicle (HBSS+/+) and hIVIG instead of neutrophils were added served as no phagocyte controls. At various time points, 5 μL samples were taken and diluted 1/10 in sterile water (to lyse

phagocytes) and then further to 1/100, 1/1,000, and 1/10,000 in HBSS+/+. Then, 5 μL of each dilution was dropped on agar plates and incubated overnight. The next day, colonies were counted and CFU concentrations were calculated. Quadruplicate to hexuplicate samples were run for each condition and average bacterial percentage values as compared to the initial bacterial concentrations ± SD plotted.

Human whole blood killing assays. Late exponential phase GAS M1T1 5448 cells were washed twice with HBSS+/+ and then pre-incubated for 15 min at 37°C with 5'-α-Gal, 3'-FAM GAS alphamer α20A24P or the control alphamer αRAND-80 in a total volume of 30 μL of 80% HBSS+/+, 20% DPBS in a round bottom 96-well plate. Then, 120 μL of heparinized human whole blood was added, the plate sealed with a silicon lid and rotated overhead at 37°C. At various time points, 10 μL samples were taken and serially diluted in ddH_2O to lyse the blood cells. 5-μL of each dilution was spotted on THA plates. After 24-h incubation at 37°C, colonies on the agar plates were counted and the bacterial concentrations in a given sample calculated. Each sample was run in quadruplicate.

Supplementary References

1. Kansal RG, McGeer A, Low DE, Norrby-Teglund A, Kotb M (2000) Inverse relation between disease severity and expression of the streptococcal cysteine protease, SpeB, among clonal M1T1 isolates recovered from invasive group A streptococcal infection cases. Infection and immunity 68: 6362-6369

2. McKay FC, McArthur JD, Sanderson-Smith ML, Gardam S, Currie BJ, Sriprakash KS, Fagan PK, Towers RJ, Batzloff MR, Chhatwal GS, Ranson M, Walker MJ (2004) Plasminogen binding by group A streptococcal isolates from a region of hyperendemicity for streptococcal skin infection and a high incidence of invasive infection. Infection and immunity 72: 364-370

3. Lauth X, von Kockritz-Blickwede M, McNamara CW, Myskowski S, Zinkernagel AS, Beall B, Ghosh P, Gallo RL, Nizet V (2009) M1 protein allows Group A streptococcal survival in phagocyte extracellular traps through cathelicidin inhibition. Journal of innate immunity 1: 202-214. DOI 10.1159/000203645

4. Hollands A, Pence MA, Timmer AM, Osvath SR, Turnbull L, Whitchurch CB, Walker MJ, Nizet V (2010) Genetic switch to hypervirulence reduces colonization phenotypes of the globally disseminated group A streptococcus M1T1 clone. The Journal of infectious diseases 202: 11-19. DOI 10.1086/653124

5. Kansal RG, Nizet V, Jeng A, Chuang WJ, Kotb M (2003) Selective modulation of superantigen-induced responses by streptococcal cysteine protease. The Journal of infectious diseases 187: 398-407. DOI 10.1086/368022

6. Buchanan JT, Simpson AJ, Aziz RK, Liu GY, Kristian SA, Kotb M, Feramisco J, Nizet V (2006) DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. Current biology : CB 16: 396-400. DOI 10.1016/j.cub.2005.12.039

7. Walker MJ, Hollands A, Sanderson-Smith ML, Cole JN, Kirk JK, Henningham A, McArthur JD, Dinkla K, Aziz RK, Kansal RG, Simpson AJ, Buchanan JT, Chhatwal GS, Kotb M, Nizet V (2007) DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. Nature medicine 13: 981-985. DOI 10.1038/nm1612

8. Hamula CL, Le XC, Li XF (2011) DNA aptamers binding to multiple prevalent M-types of Streptococcus pyogenes. Analytical chemistry 83: 3640-3647. DOI 10.1021/ac200575e

9. McNamara C, Zinkernagel AS, Macheboeuf P, Cunningham MW, Nizet V, Ghosh P (2008) Coiled-coil irregularities and instabilities in group A Streptococcus M1 are required for virulence. Science 319: 1405-1408. DOI 10.1126/science.1154470

10. Cretin N, Iacomini J (2002) Immunoglobulin heavy chain transgenic mice expressing Galalpha(1,3)Gal-reactive antibodies. Transplantation 73: 1558-1564

11. Galili U, Anaraki F (1995) alpha-galactosyl (Galalpha1-3Galbeta1-4GlcNAc-R) epitopes on human cells: synthesis of the epitope on human red cells by recombinant primate alpha1,3galactosyltransferase expressed in E.coli. Glycobiology 5: 775-782

12. Kristian SA, Datta V, Weidenmaier C, Kansal R, Fedtke I, Peschel A, Gallo RL, Nizet V (2005) D-alanylation of teichoic acids promotes group a streptococcus antimicrobial peptide resistance, neutrophil survival, and epithelial cell invasion. Journal of bacteriology 187: 6719-6725. DOI 10.1128/JB.187.19.6719-6725.2005

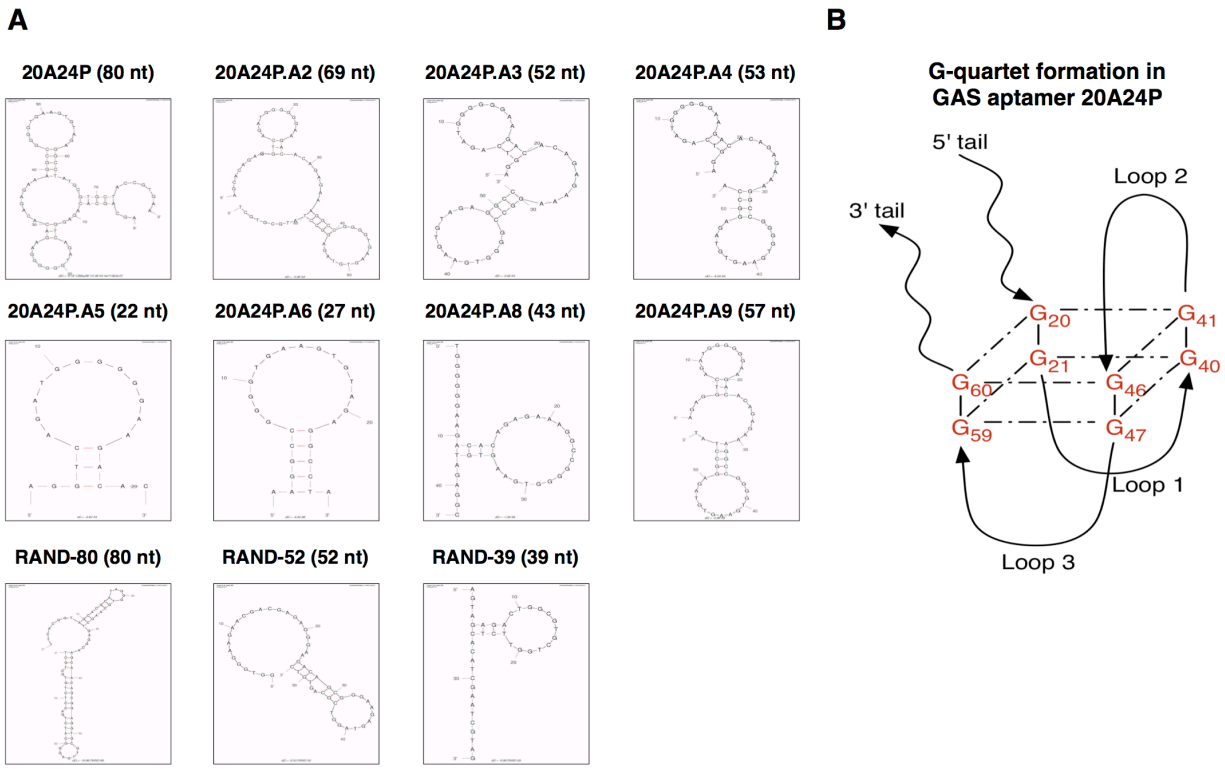
13. Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic acids research 31: 3406-3415

14. Kikin O, D'Antonio L, Bagga PS (2006) QGRS Mapper: a web-based server for predicting G-quadruplexes in nucleotide sequences. Nucleic Acids Res 34: W676-682. DOI 10.1093/nar/gkl253

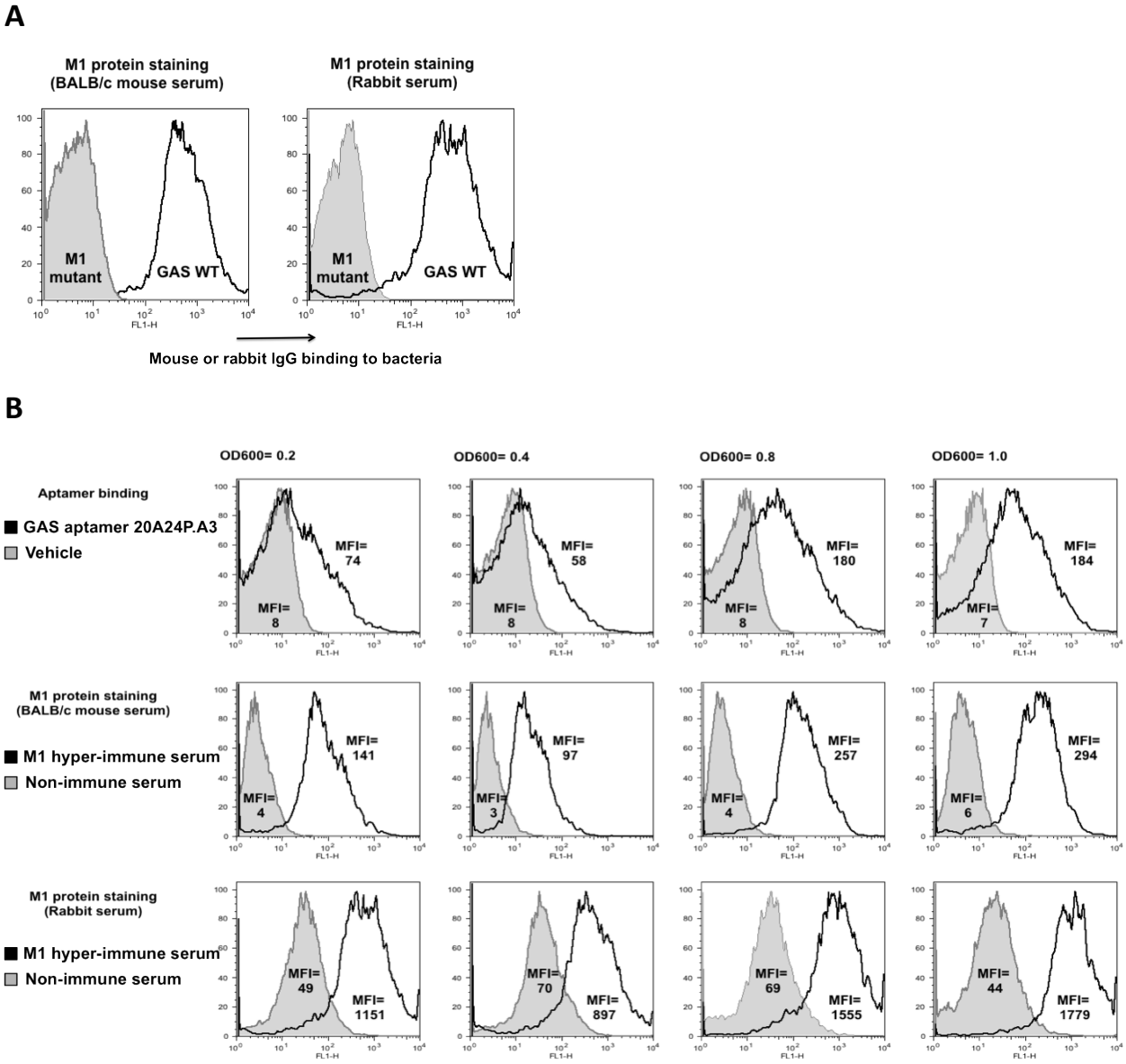
Supplementary Table 1: Sequences of truncated variants of GAS aptamer 20A24P

GAS Aptamers	**Size**	**Aptamer Sequences ***
20A24P (Parental)	80 nt	5'-AGCAGCACAGAGGTCAGATGGGGGGAAGACACAGAGAAA GGCCGGGGTGAAGTGTAGAGGCCTATGCGTGCTACCGTGAA-3'
20A24P.A2	69 nt	5'-AGCACAGAGGTCAGATGGGGGGAAGACACAGAGAAAGGCC GGGGTGAAGTGTAGAGGCCTATGCGTGCT-3'
20A24P.A3	52 nt	5'-AGGTCAGATGGGGGGAAGACACAGAGAAAGGCCGGGGTGA AGTGTAGAGGCC-3'
20A24P.A4	53 nt	5'-AGGTCAGATGGGGGGAAGACACAGAGAAAGGCCGGGGTGA AGTGTAGAGGCCA-3'
20A24P.A5	22 nt	5'-AGGTCAGATGGGGGGAAGACAC-3'
20A24P.A6	27 nt	5'-AAGGCCGGGGTGAAGTGTAGAGGCCTA-3'
20A24P.A8	43 nt	5'-TGGGGGGAAGACACAGAGAAAGGCCGGGGTGA AGTGTAGAGGC-3'
20A24P.A9	57 nt	5'-AGAGGTCAGATGGGGGGAAGACACAGAGAA AGGCCGGGGTGAAGTGTAGAGGCCTAT-3'
Negative Control Aptamer	**Size**	**Aptamer Sequence ***
RAND-52	52 nt	5'-AGGGAGAAGGTGAAGAGGGGATGTGCCCGGGTAGAGAGGA AGACACACGTGC-3'

*, all GAS aptamers were labeled with 6-Carboxyfluorescein (FAM) at the 5'- or 3'- end

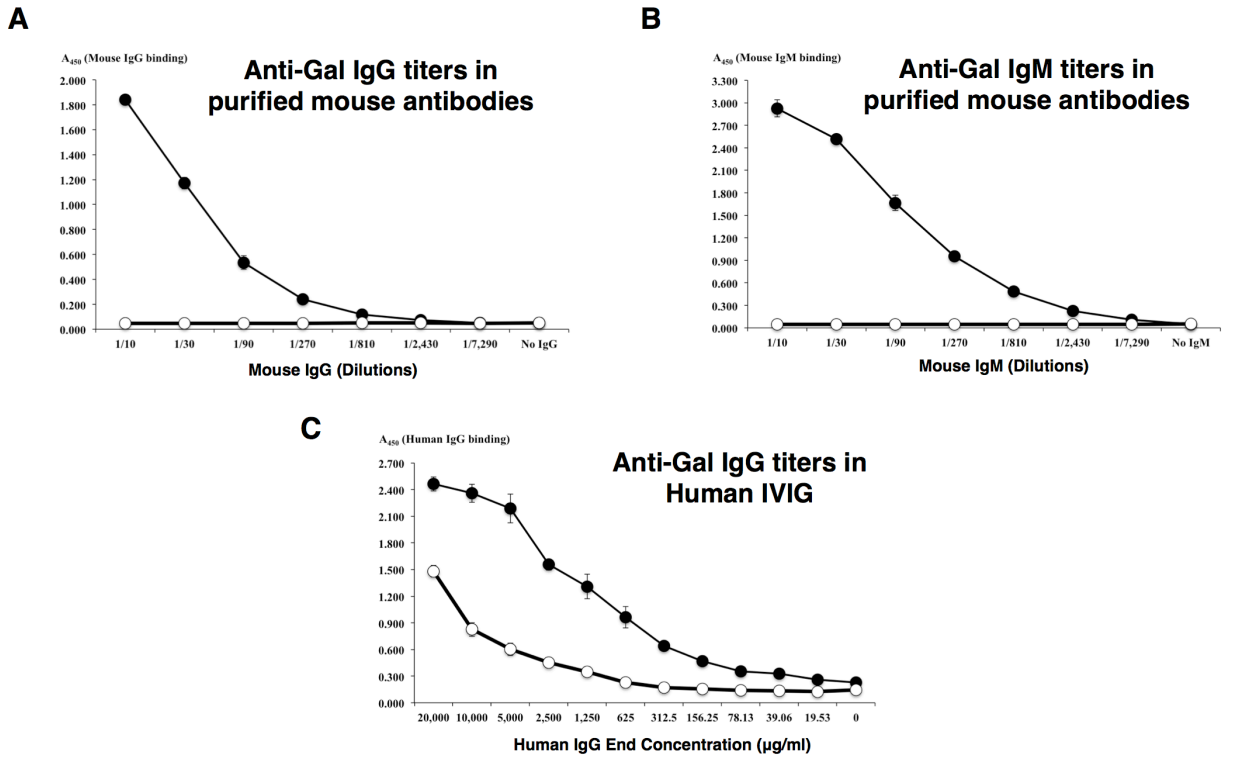


Supplemental Fig. S1. Secondary structures of truncated GAS aptamers. (A.) The secondary structures of the parental GAS aptamer, the seven truncated aptamer versions, and negative control aptamers were modeled by using the Mfold web server for nucleic acid binding prediction [13]. **(B.)** G-quartet formation in parental aptamer 20A24P was drawn based on analysis with the QGRS mapper tool [18].



Supplemental Fig. S2. Correlation of growth phase dependent M protein detection by antisera and GAS aptamer binding by flow cytometry. (A.) Stationary GAS 5448 wild-type (black lines) or an isogenic *emm1* mutant deficient in M1 protein expression (grey filled) were incubated with mouse or rabbit non-immune sera. Subsequently, IgG binding to the bacteria was tested with fluorescently labeled secondary anti-IgG antibodies. The FL-1 histograms demonstrate greatly enhanced binding of the M protein antisera to the wild-type bacteria showing M protein specificity of IgG binding. **(B.)** GAS 5448 wild-type bacteria in different growth phases (indicated by OD_{600} values) were incubated with 5'-FAM GAS aptamer 20A24P.A3 (black lines in upper histogram panel) or aptamer vehicle (grey filled in upper

histogram panel). Aptamer binding was visualized in the FL-1 flow cytometer channel and levels of aptamer binding compared by the mean fluorescence intensities (MFIs) of the samples. In parallel, bacterial samples in the different growth phases were included where the bacteria were stained with the above-mentioned mouse or rabbit M1 hyper-immune sera or non-immune sera. As in (**A.**), IgG binding was visualized with secondary antibodies. The degree of antibody binding is indicated by the respective MFI values. Overall, the data show that the M protein staining in later stages of growth correlates with improved aptamer binding.



Supplemental Fig. S3. Anti- Gal titers in mouse and human polyclonal antibodies. α-Gal-human serum albumin (HSA) or HSA were immobilized to 96-well plate wells in equimolar amounts. **(A)** Mouse IgG **(B)** mouse IgM, or **(C)** human IgG (hIVIG) containing antibody preparations at the indicated dilutions or end concentration were added. Primary antibody binding to α-Gal-HSA (Closed symbols) or control HSA (Open symbols) was detected with HRP-conjugated **(A)** anti-mouse IgG, **(B)** anti-mouse IgM, or **(C)** anti-human IgG secondary antibodies and subsequent addition of HRP substrate and stop solution and measurement of the absorbance at 450 nm (A_{450}). The graphs show the average absorbance of the respective duplicate wells ± SD. At higher antibody concentrations, significant binding of hIVIG to HSA-coated wells was observed; this was likely due to unspecific binding of hIVIG to the plate and or blocking reagents rather than to the self-antigen HSA itself; nevertheless, the enhanced binding of hIVIG to α-Gal-HSA coated wells was observed indicating the presence of anti-Gal antibodies in hIVIG.