Immunization with genetically fused SP2108, SP1912 and SP0148 proteins primes T_H17 responses that are protective in a mouse colonization model

M.Skoberne[1], K.Moffitt[2], C.Gavrilescu[1], B.Dixit[1], S.Munzer[1], P.Gouveia[1], S.Pluskey[1], N.Siddall[1], D.Turkington[1], P.Giannasca[1], M.Alderson[3], R.Malley[2], J.Flechtner[1]

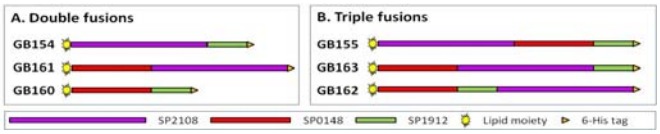
[1]Genocea Biosciences, Inc, [2]Boston Children's Hospital, [3]PATH

Introduction

The development of affordable pneumococcal vaccines is a global health priority. Prevention of colonization is essential in eradication of *S. pneumoniae*-mediated disease since it will not only protect an individual but may also contribute to herd immunity. A vaccine that targets conserved *S. pneumoniae* antigens and reduces colonization will complement current antibody-targeting vaccines by providing an additional mechanism of action and addressing serotype replacement. We have previously identified three protein antigens SP2108, SP0148 and SP1912, that were consistently recognized by T_H17 cells from healthy human donors, presumably exposed to *S. pneumoniae*. Of these, two proteins, SP2108 and SP0148, are naturally lipidated, membrane transport associated proteins and the third, SP1912 is a non-lipidated hypothetical protein. When the antigens are adsorbed to Alhydrogel® and administered to mice subcutaneously either individually or in combination, they elicit $CD4^+$ T cell immunity that significantly decreases colonization of the nasopharynx, the initial step in pathogenesis of *S. pneumoniae*, in an IL-17A-dependent manner. Our goal was to create a more cost-effective vaccine by combining multiple antigens into genetic fusion constructs, while preserving the immunogenicity and efficacy of the individual antigens.

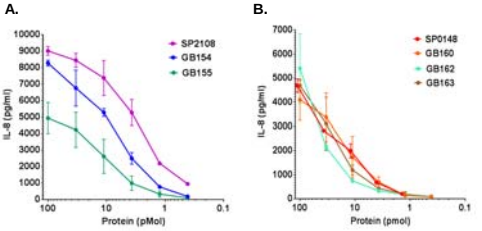
Results

6 fusion proteins were produced



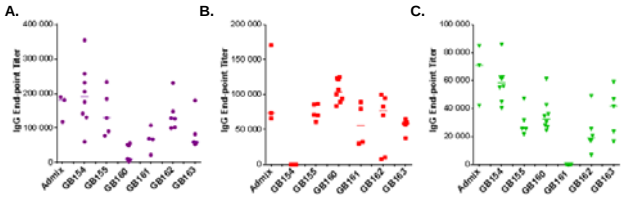
Each fusion protein comprised of a lipoprotein (either SP2108 or SP0148) at the N-terminus in order to achieve proper post-translational processing. All constructs contained a C-terminal 6-His tag for affinity purification via IMAC.

Fusion proteins stimulate TLR2



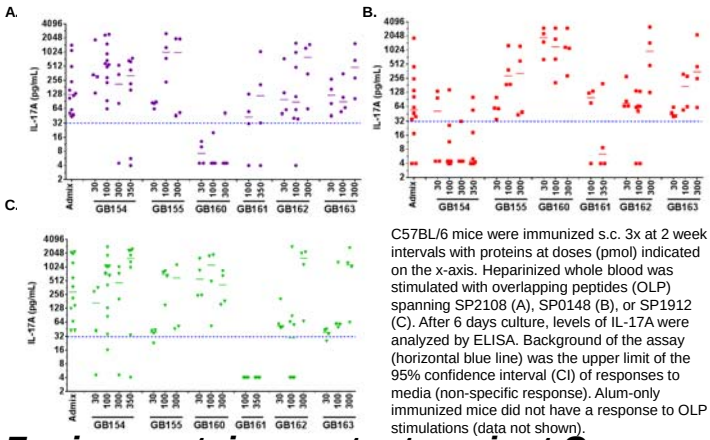
HEK293xTLR2 cells were grown to 75% confluency and stimulated overnight with the indicated concentrations of fusion or single proteins. Culture supernatants (S/N) were tested by ELISA for the concentration of the reporter cytokine IL-8. The positive control Pam3CSK4 at the highest concentration (1µg/mL) induced 8000pg/mL of IL-8 (data not shown); no IL-8 was detected in S/N from unstimulated or non-lipidated protein stimulated cell cultures (data not shown).

Fusion proteins prime antibodies



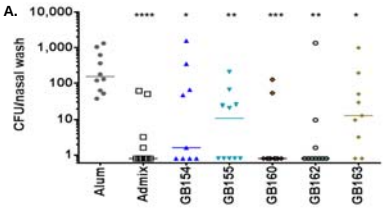
C57BL/6 mice were immunized s.c. 3x at 2 week intervals with an admix of SP2108, SP0148 and SP1912 or 100-350 pmol fusion constructs GB154, GB155, GB161, GB162 and GB163. All formulations contained 250µg of Alhydrogel® per dose. Three weeks post last immunization, heparinized whole blood was collected and plasma was isolated. Plasma of up to 5 mice per treatment group were pooled for each experiment and evaluated by ELISA for endpoint titer against SP2108 (A), SP0148 (B) or SP1912 (C). Each dot represents mean endpoint titer from a separate experiment.

Fusion proteins prime T_H17 cells



C57BL/6 mice were immunized s.c. 3x at 2 week intervals with proteins at doses (pmol) indicated on the x-axis. Heparinized whole blood was stimulated with overlapping peptides (OLP) spanning SP2108 (A), SP0148 (B), or SP1912 (C). After 6 days culture, levels of IL-17A were analyzed by ELISA. Background of the assay (horizontal blue line) was the upper limit of the 95% confidence interval (CI) of responses to media (non-specific response). Alum-only immunized mice did not have a response to OLP stimulations (data not shown).

Fusion proteins protect against *S. pneumoniae* intranasal challenge



B.

Average log reduction compared to Alum			
	Study 1	Study 2	Study 3
admix	N/A	2.3	1.1
GB154	N/A	2	1.2
GB155	1.1	1.6	N/A
GB160	N/A	2.3	0.8
GB162	N/A	2.3	N/A
GB163	N/A	1.1	N/A

(A).Ten C57BL/6 mice/group were immunized s.c. 3x at 2 week intervals with Alum only (Alum), an admix of SP2108, SP0148 and SP1912 or with fusion proteins GB154, GB155, GB160, GB162 and GB163, all at 100pmol. All formulations contained 250µg of Alhydrogel® per dose. 25 days post last immunization, the mice were challenged intranasally with *S. pneumoniae* strain 0603. Mice were euthanized 10 days later and the nasopharynx lavaged, cultured on permissive plates, and colonies counted after overnight incubation. Each symbol represents the CFU per nasal wash of one mouse. Stars indicate statistical significance by the Mann-Whitney U test (p value: 0.01<*< 0.05; 0.001<**< 0.01; 0.0001 <***< 0.001; ****<0.0001).
(B) Summary data of 3 experiments. Data are shown as median log reduction in *S. pneumoniae* CFU relative to the Alhydrogel® (Alum) group, calculated for each vaccination group.

Methods

Protein production: All proteins were purified using immobilized metal affinity chromatography (IMAC). Purified lipoproteins were dialyzed into buffer containing 10 mM Tris-HCl, 10 mM NaCl, pH 8. SP1912 was dialyzed against 2M urea then in 5mM sodium phosphate, 5% sucrose, pH 8.
TLR2 assay: HEK293xTLR2 cells are stably transfected with the human TLR2 receptor. Cells were grown to 75% confluency and stimulated overnight with the indicated concentrations of the fusion proteins, the single proteins, medium only, or the TLR2 agonist Pam3CSK4 as a positive control. Culture supernatants were collected and stored at -80°C until evaluated by ELISA (R&D Systems, Minneapolis, MN) for the presence of IL-8, a TLR2-signaling reporter cytokine.
Immunizations: 4-6 week old C57BL/6 mice were obtained from Charles River Laboratories (C57BL/6NCrl). Mice were immunized sub-cutaneously in the nape of the neck three (3) times at two (2) week intervals, with an admix of SP2108, SP0148 and SP1912 or with fusion proteins GB154, GB155, GB160, GB161, GB162 and GB163 at indicated doses. All formulations contained 250µg of Alhydrogel® per dose. Mice were sacrificed three weeks post last immunization (d49) and blood was collected by cardiac puncture in heparinized collection tubes. All animal procedures were conducted in accordance with IACUC-approved institutional guidelines of Boston Children's Hospital, Harvard Medical School and Genocea Biosciences.
T_H17 response: Heparinized whole blood was plated in duplicates in supplemented DMEM medium, in the presence of 1 µg/ml overlapping peptides (OLP) spanning SP2108, SP0148 or SP1912, 10 µg/ml whole cell antigen (WCA) or controls. After 6 days, cell-free supernatants were collected and stored at -80°C until evaluated in an IL-17A ELISA (R&D Systems).
IgG response: Plasma collected from the heparinized whole blood of immunized mice was stored at -80°C until tested in antigen-specific endpoint titer ELISA. Briefly, plasma from mice in the group was pooled. Plates were coated overnight with 1 µg/ml SP2108, SP0148 or SP1912. After blocking the plates with 1% BSA, antibodies in diluted plasma were allowed to bind the coated proteins for 2h at RT and were detected with and AP-conjugated anti-mouse total IgG antibody and pNPP (Sigma-Aldrich, St-Louis, MO).
Colonization: 25 days following the last immunization, animals were challenged intranasally with $2x10^7$ colony-forming units (CFU) of *S. pneumoniae* strain 0603 (serotype 6B) in 20µl PBS, while gently restrained and awake. Animals were sacrificed ten days following the challenge, and nasal washes were obtained by tracheal lavage for evaluation of density of pneumococcal colonization. Dilutions of the nasal washes were plated and incubated on blood-agar plates over-night at 37°C, and colonies were counted.

Conclusions

- Multiple protein antigens can be fused to create an effective vaccine designed to induce T_H17 responses that protect against pneumococcal carriage.
- Lipidated fusion proteins triggered the TLR2 receptor *in vitro*, comparably to non-fused lipidated proteins.
- The fusions primed T_H17 and protein-specific antibody responses over a range of doses (30-350pmol), to the levels that were consistent to responses to immunization with the individual antigens.
- Vaccine formulations with any of the fusion proteins conveyed protection as measured by reduction in colonization. Colonization was reduced up to 2.3 logs compared to the Alhydrogel®-immunized control animals. The results were comparable to the admix of the three individual antigens.

Bibliography

1. Moffitt et al. T(H)17-based vaccine design for prevention of *Streptococcus pneumoniae* colonization. Cell Host Microbe. 2011 Feb 17;9(2):158-65.