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Immunogenicity and protective efficacy induced by self-amplifying mRNA vaccines encoding bacterial antigens

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ABSTRACT

Nucleic acid vaccines represent an attractive approach to vaccination, combining the positive attributes of both viral vectors and live-attenuated vaccines, without the inherent limitations of each technology. We have developed a novel technology, the Self-Amplifying mRNA (SAM) platform, which is based on the synthesis of self-amplifying mRNA formulated and delivered as a vaccine. SAM vaccines have been shown to stimulate robust innate and adaptive immune responses in small animals and non-human primates against a variety of viral antigens, thus representing a safe and versatile tool against viral infections. To assess whether the SAM technology could be used for a broader range of targets, we investigated the immunogenicity and efficacy of SAM vaccines expressing antigens from Group A (GAS) and Group B (GBS) Streptococci, as models of bacterial pathogens. Two prototype bacterial antigens (the double-mutated GAS Streptolysin-O (SLOdm) and the GBS pilus 2a backbone protein (BP-2a)) were successfully expressed by SAM vectors. Mice immunized with both vaccines produced significant amounts of fully functional serum antibodies. The antibody responses generated by SAM vaccines were capable of conferring consistent protection in murine models of GAS and GBS infections. Inclusion of a eukaryotic secretion signal or boosting with the recombinant protein resulted in higher specific-antibody levels and protection. Our results support the concept of using SAM vaccines as potential solution for a wide range of both viral and bacterial pathogens, due to the versatility of the manufacturing processes and the broad spectrum of elicited protective immune response.

1. Introduction

During the past three decades, nucleic acid vaccines have been gaining attention as a means to combine the positive attributes of live-attenuated vaccines, viral vectors and subunit vaccines [1,2]. Nucleic acid-based vaccines, including viral vectors, plasmid DNA (pDNA) and RNA, have significant advantages over live-attenuated vaccines, including: (i) induction of both B and T-cell responses (among which cytotoxic T lymphocytes); (ii) specificity; (iii) relatively low production cost; (iv) high stability; (v) the possibility of expressing complex antigens; (vi) absence of anti-vector immunity; (vii) lower reactogenicity [1,3,4]. Moreover they induce more efficient and strong CD8 responses compared to subunit vaccines [5].

To date, the majority of preclinical and clinical studies using nucleic acid-based vaccines have been conducted with plasmid DNA [6,7] and DNA-based viral vectors [4], that have been shown to be safe, well-tolerated and immunogenic, but with suboptimal potency [8]. Recently, enhanced delivery technologies, such as electroporation, have increased the efficacy of DNA vaccines in clinical trials [9].

RNA-based vaccines, both messenger RNA (mRNA) and self-amplifying replicons, have emerged as an increasingly promising alternative to pDNA for gene vaccination [1,10,11]. RNA vaccines were shown to elicit antigen specific antibody and cellular immune responses against several viral pathogens [12–15], with some clear advantages over pDNA. Unlike DNA, RNA needs to be delivered into the host cytoplasm to be translated and cannot be integrated into the host cell's genome, which reduces the risk of gene dysregulation [4,16]. Moreover, mRNA is produced using a cell-free

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enzymatic transcription reaction, increasing production yields and avoiding safety concerns associated with the use of living organisms or anti-vector immunity associated with the use of viral vectors [12,17].

We have previously described the SAM vaccine platform [13]. This platform is based on a synthetic, self-amplifying mRNA, delivered by synthetic lipid nanoparticles or by cationic nanoemulsions [13,18]. The 9-kb self-amplifying mRNA is based on an engineered alphavirus genome containing the genes encoding the alphavirus RNA replication machinery [19] whereas the structural protein sequences are replaced with the gene of interest. The resulting RNAs are capable of directing their self-replication, generating multiple copies of the antigen-encoding mRNA, and express high levels of the heterologous gene when they are introduced into the cytoplasm of host cells, in a way that mimics production of antigens *in vivo* by viral pathogens, triggering both humoral and cellular immune responses [19,20]. Initial testing of SAM vaccines has shown non-viral delivery to be capable of inducing neutralizing antibodies and polyfunctional CD4+ and CD8+ T cell responses in various animal species, including non-human primates [13,18,21,22], and against numerous viral pathogens [14,18,21,22].

In the current study, we explored the potential of SAM vaccines to be used against bacterial pathogens, which would make of this platform a robust and broadly applicable vaccine technology.

Here we evaluated the potency of SAM vaccines expressing antigens from Group A (GAS, *Streptococcus pyogenes*) and Group B (GBS, *Streptococcus agalactiae*) streptococci in mice and compared their immunogenicity and efficacy to those of formulated recombinant proteins. We selected two prototype bacterial antigens, the double-mutated Streptolysin-O (SLOdm) and the backbone protein of pilus island 2a (BP-2a) from GAS and GBS respectively, that are well-known to have a key role in bacterial virulence and pathogenesis and to be capable of eliciting protective antibodies in mouse models of infection [23,24].

A cationic nanoemulsion (CNE) that binds to self-amplifying mRNAs [18] was selected to formulate synthetic SAM vectors expressing bacterial antigens. CNE is based on the oil-in-water emulsion adjuvant MF59, which has an established clinical safety profile and is well tolerated in children, adults, and the elderly [25,26].

CNE formulated SAM vaccines encoding BP-2a or SLOdm were tested for immunogenicity and for their capacity to confer protection from infection in animal models, using formulated recombinant proteins as benchmarks. The addition of a eukaryotic secretion signal fused to the antigen was also explored to allow antigen presentation in a fashion similar to a subunit vaccine and to increase production of protective antibodies. Finally, a SAM prime/protein boost regimen was shown to elicit immune responses of greater breadth and protection.

2. Results

2.1. BP-2a and SLOdm as model antigens to assess the potency of SAM vaccine against bacterial pathogens

As a proof-of concept for application of SAM platform against bacterial pathogens, we selected GBS BP-2a and GAS SLOdm antigens, which are promising candidates for the development of broadly-protective vaccines against GBS and GAS respectively [23,24].

We generated two RNA vectors expressing each one of the selected antigens (Fig. 1A). The corresponding RNAs were synthesized *in vitro* by an enzymatic transcription reaction from a linear plasmid DNA template using a T7 RNA polymerase and RNA integrity was evaluated by agarose gel electrophoresis (data not shown).

The ability of the replicon constructs to express BP-2a and SLOdm despite the non-native codon preference of these bacterial genes in a eukaryotic system was tested *in vitro* after transfection of BHK-V cells. Intracellular antigen expression by transfected BHK-V cells was characterized by Western Blot. Fig. 1B and C demonstrates that BP-2a and SLOdm respectively expressed by SAM constructs are abundant, and display the same electrophoretic mobility of the full-length native proteins loaded as size control.

2.2. Immunization with SAM BP-2a and SAM SLOdm vaccines in mice induces functional antibodies

To assess immunogenicity elicited by SAM vaccines against BP-2a and SLOdm, RNA vectors expressing the antigens were formulated with CNE and used to immunize mice. Recombinant BP-2a and SLOdm proteins were used as benchmarks. All groups of mice were immunized three times and sera were collected 2 weeks after the 2nd and 3rd vaccination (2wp2, 2wp3).

SAM vaccines elicited antigen specific IgG responses, even if lower in magnitude as compared to recombinant proteins. Interestingly, while SAM SLOdm peaked already after the second immunization, SAM BP-2a needed a third dose to boost the immune response (Table 1).

Antibody subclass distribution was compared between RNA and protein formulations. Table 1 reports the ratio between IgG1and IgG2a titers, showing that SAM based vaccines induce a major T_H1 (IgG2a > IgG1) immune response, while recombinant protein vaccines a major T_H2 (IgG1 > IgG2a) response.

It has been well established that the investigated protein antigens induce functional antibodies that mediate opsonophagocytosis (BP-2a) [27] or inhibit protein-mediated cytotoxicity (SLOdm) *in vitro* [23,28]. We therefore measured *in vitro* functional activity of antibodies raised against SAM vaccines. Sera sampled 2wp3 from mice immunized with SAM BP-2a vaccine were incubated with a serotype V BP-2a-expressing GBS strain and bacterial killing was measured in the presence of differentiated HL-60 cells and rabbit complement, using as positive control sera raised against the alum formulated recombinant protein. As shown in Fig. 2A, SAM vaccine was able to elicit opsonophagocytic antibodies against GBS expressing BP-2a that mediated *in vitro* bacteria killing. Further, 2-fold dilutions of 2wp3 sera raised against SAM SLOdm were pre-incubated with wild type SLO and then a red blood cells suspension was added as previously reported [23,28]. As benchmark, we used 2wp3 sera obtained immunizing mice against the MF59 adjuvanted recombinant SLOdm. As shown in Fig. 2B, significant levels of inhibitory antibodies were induced immunizing mice with the SAM vaccine, confirming the ability of this strategy of vaccination to induce functional antibodies also against bacterial antigens.

2.3. Immunization with SAM BP-2a or SAM SLOdm provides protection in mouse models of bacterial infection

To evaluate the *in vivo* protective efficacy of the designed SAM vaccines, we took advantage of well-established animal models of GBS or GAS infection [28,29].

Female mice immunized with SAM BP-2a vaccine were mated and the resulting offspring were intraperitoneally challenged with a BP-2a-expressing serotype V GBS strain. As positive control we used rBP-2a/alum vaccine. As shown in Fig. 2C, SAM BP-2a active immunization of the mothers resulted in a partial protection of the offspring against a lethal challenge with GBS.

The protective potential of SAM SLOdm was determined in a mouse model of GAS infection. At the end of the immunization schedule described above, vaccinated mice were intraperitoneally

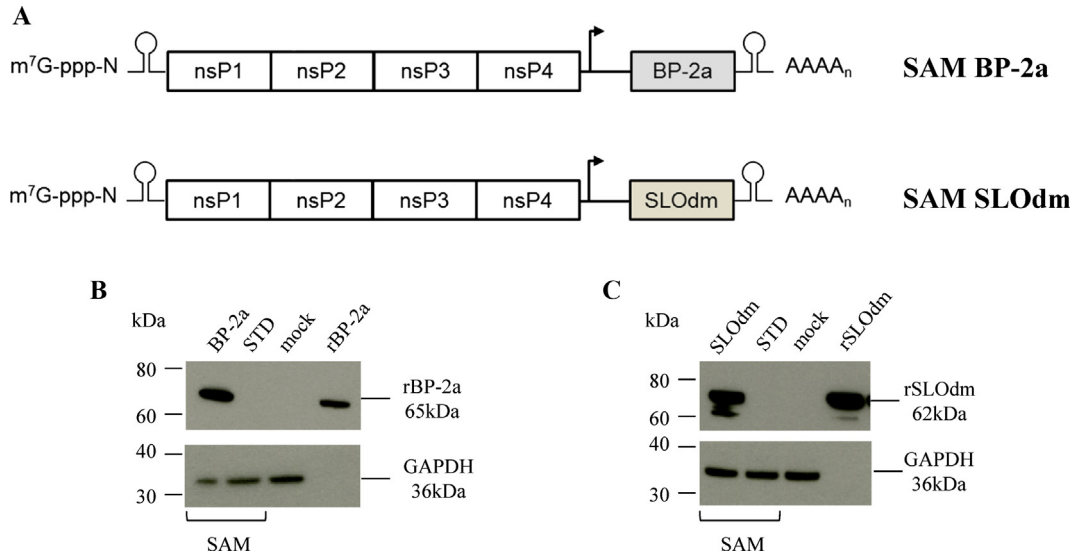

Fig. 1. Schematic representation of self-amplifying RNA replicons and their characterization *in vitro*. (A) SAM BP-2a and SAM SLOdm construct showing a 5′ cap, four non-structural genes (nsp1-4), a 26S subgenomic promoter (black arrow), the vaccine antigen(s), and a 3′ polyadenylated tail. (B and C) Western blots showing expression of BP-2a (B) or SLOdm (C) in cell lysates from transfected BHK-V cells. Purified recombinant proteins (rBP-2a/rSLOdm) were added as positive controls. Mock, non-transfected cells. Numbers to the left of the blots mark the relative mobilities of molecular size markers kDa. Blots were probed with mouse anti-BP-2a or anti-SLOdm polyclonal sera. Probing with mouse monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control.

Table 1
Immunogenicity of SAM BP-2a and SAM SLOdm vaccines in mice[a].

Antigen	Vaccine	Total IgG 2wp2 (GMT)	Total IgG 2wp3 (GMT)	Ratio IgG1/IgG2a
BP-2a	SAM BP-2a	4.7×10^2	4.7×10^3	0.13
	rBP2a	1.5×10^4	3.6×10^4	39.14
	Mock	<100	<100	–
SLOdm	SAM SLOdm	1.25×10^4	2.5×10^4	0.17
	rSLOdm	4×10^5	6.25×10^5	10.55
	Mock	<100	<100	–

[a] Groups of 6/8 mice received three doses of SAM vectors formulated with CNE, recombinant proteins adjuvanted either with alum (rBP-2a) or MF59 (rSLOdm) or adjuvant alone (Mock). Total IgG titers 2 weeks after the 2nd (2wp2) and the 3rd (2wp3) immunizations were measured and geometric mean titers (GMTs) are represented. IgG1 and IgG2a titers were also measured at 2wp3 from pooled sera and IgG1/IgG2a ratios are reported.

infected with GAS M1T1-3348 strain and survival was followed up to 7 days post infection. rSLOdm/MF59 formulation was used as positive control. Consistent protection against M1T1-3348 strain with respect to the negative-control group was obtained (Fig. 2D) underlining the potential of SAM vaccines to induce a protective immune response in preclinical models of bacterial infection.

2.4. In vivo antigen secretion and protein boosting enhance protection induced by SAM vaccines

To investigate whether a higher protective response could be reached using the SAM vaccines, we decided to explore alternative antigen presentation routes through antigen engineering and vaccination regimens, using BP-2a as prototype antigen. In order to allow antigen presentation in a fashion similar to the recombinant protein vaccine, we looked at the possibility of redirecting the antigen to the extracellular compartment. An additional SAM vector was therefore designed to target the antigen to the secretion pathway of eukaryotic cells by fusing a signal peptide (the murine Ig κ-chain leader sequence) to the N-terminus of BP-2a (Fig. 3A). Self-amplification in BHK-V cells after RNA transfection was initially confirmed to occur with the new construct (data not shown). We demonstrated that while BP-2a expressed by SAM without signal sequence (SAM BP-2a) was only present in the cell pellet (Fig. 3B), the presence of the signal peptide (SAM LS-BP-2a) allowed also the secretion of the protein (Fig. 3C).

We reasoned that redirecting the antigen to the secretory pathway through the use of a signal peptide could expose the protein to the glycosylation machinery, as glycosylation in eukaryotes normally occurs during the process of export through the endoplasmic reticulum [30]. *In silico* analysis of LS-BP-2a sequence predicted the presence of eight asparagines with a glycosylation potential >0.5. To test whether the predicted sites were targeted by the N-linked glycosylation machinery *in vivo*, the most widely distributed form of glycosylation in eukaryotes, lysates from BHK-V cells transfected with SAM LS-BP-2a were exposed to Peptide-N-Glycosidase F (PNGase F) and then subjected to Western blotting. As shown in Fig. 3D, PNGase F treatment did not affect electrophoretic mobility, indicating that the protein was not subjected to glycosylation.

In parallel, we evaluated the possibility of boosting SAM vaccines induced immune response with the rBP-2a protein antigen.

Mice received either three doses of the 2 SAM vaccines with or without the leader sequence (SAM BP-2a/CNE or SAM LS-BP-2a/CNE), or 2 doses of these formulations followed by a last dose of protein adjuvanted with alum. No statistically significant differences were observed in term of total IgG production (Table 2) and functionality (data not shown) of the antibodies when the leader sequence was added and compared to SAM BP-2a vaccine.

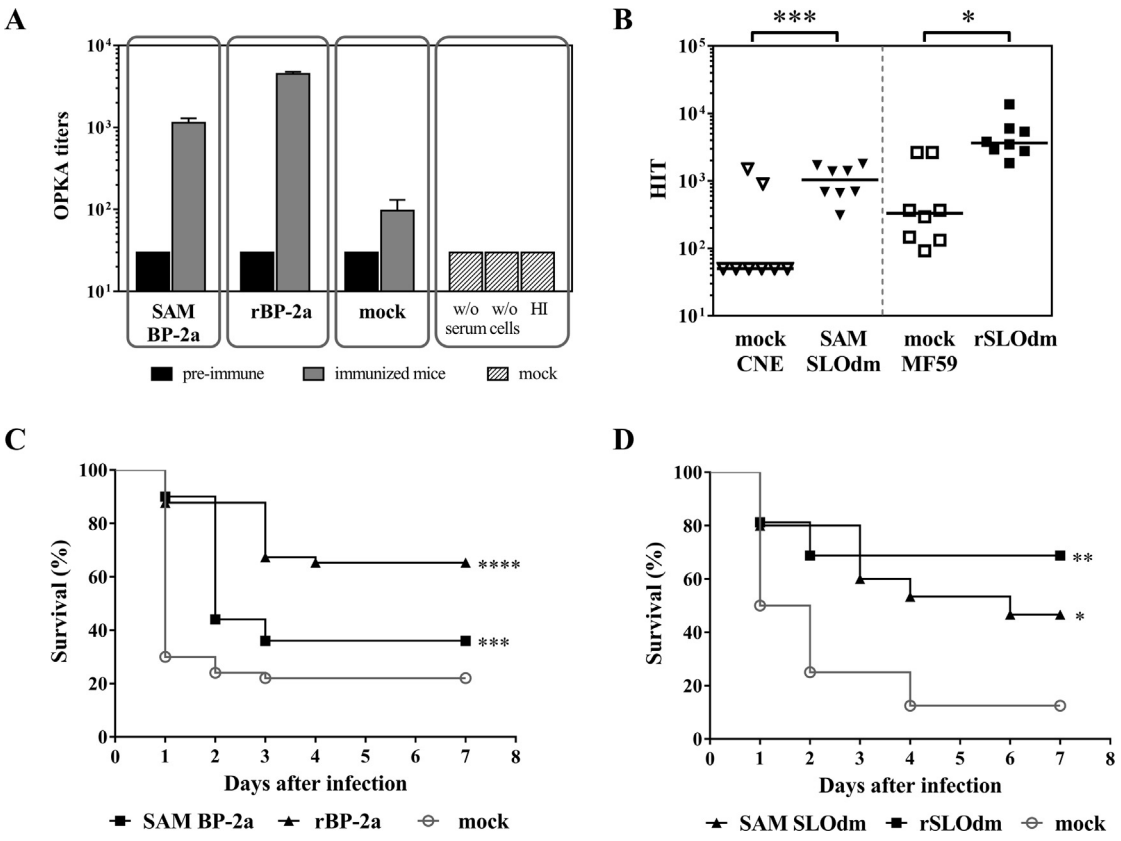

Fig. 2. Immunogenicity and efficacy of SAM vaccines. (A and B) Functional activity of antibodies elicited by immunization with SAM vaccines. (A) Opsonophagocytic activity of pooled sera from mice immunized with 3 doses of SAM BP-2a, rBP-2a or adjuvant alone as negative control (mock). OPKA titers, expressed as the reciprocal serum dilution leading to 50% killing of bacteria, are shown. Killing in the absence of serum (w/o serum) or cells (w/o cells) or with heat inactivated (HI) complement were used as negative controls of the assay. Error bars indicate SD from two independent experiments. (B) Sheep blood cells hemolysis inhibition titers (HIT) of individual sera from mice immunized with 3 doses of SAM SLOdm or rSLOdm or adjuvants alone (CNE or MF59). HI titers, expressed as the lowest serum dilution factor that completely inhibited SLO induced hemolysis, are represented. Asterisks indicate statistically significant differences between vaccine and adjuvant immunized mice, as measured by two-tailed Mann-Whitney *U* test. $^{***}p<0.0005$; $^{*}p = 0.01$. (C and D) *In vivo* protective efficacy of BP-2a and SLOdm SAM vaccines. (C) Survival curves of puppies intraperitoneally infected with a GBS serotype V strain expressing BP-2a. 50–60 puppies/group from mothers immunized with SAM BP-2a, rBP-2a or adjuvant alone were treated and protection was reported up to 7 days after infection. (D) Kaplan-Meier survival curves of CD1 mice immunized with SAM SLOdm, rSLOdm or adjuvant alone and intraperitoneally infected with GAS M1T1 3348 strain. Survival was followed for 7 days after infection and data from 16 mice/group from 2 independent experiments are shown. Asterisks indicate statistically significant protection, as calculated using the log-rank (Mantel-Cox) test ($^{****}P < 0.0001$; $^{***}p < 0.0005$; $^{**}P < 0.005$; $^{*}P < 0.05$).

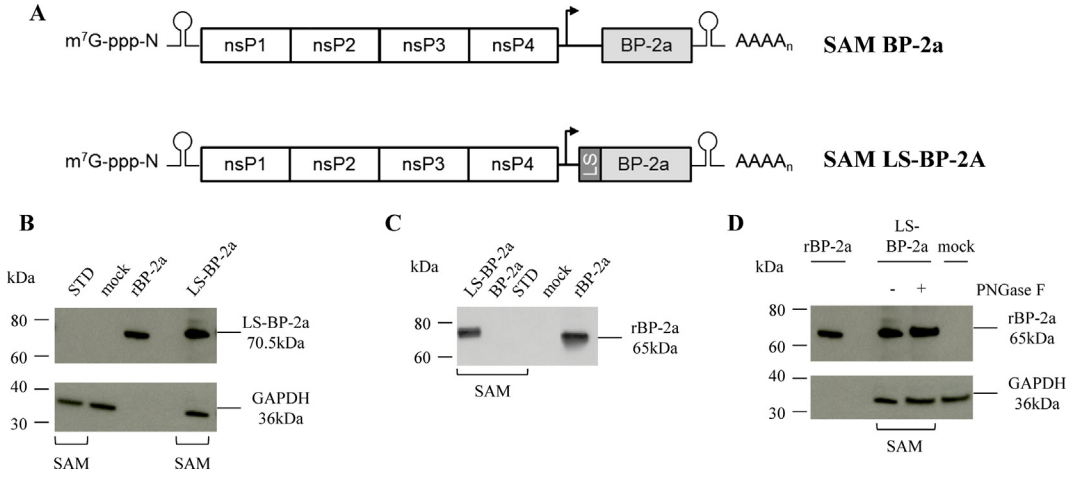

Fig. 3. Antigen expression from SAM vectors expressing intracellular or secreted BP-2a protein. (A) SAM BP-2a and SAM LS-BP-2a construct showing a 5′ cap, four non-structural genes (nsp1-4), a 26S subgenomic promoter (black arrow), the vaccine antigen(s), and a 3′ polyadenylated tail. BP-2a expression was assessed by Western blot of cell lysates (B) or supernatants (C) collected from BHK-V cells transfected with SAM vectors expressing BP-2a, LS-BP-2a or STD RNA, as negative control. (D) Glycosylation of LS-BP-2a expressed by SAM transfected cells was evaluated by western blot on PNGase F treated lysates. Blots were probed with mouse anti-BP-2a polyclonal sera. Probing with mouse monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Purified recombinant protein (rBP-2a) was added as positive control. Numbers to the left of the blots mark the relative mobilities of molecular size markers in kDa (B–D). mock, non-transfected cells; PNGase F, Peptide-N-Glycosidase F.

Table 2
Effect of antigen secretion and SAM prime/protein boost regimen on immunogenicity.[a]

Antigen	Vaccine	Total IgG 2wp3 (GMT)	IgG1/IgG2a 2wp3 (ratio)	Statistical significance vs rBP-2a 3x[*]	Statistical significance vs SAM BP-2a 3x[*]
BP-2a	SAM 3x	4.7×10^3	0.13	$p < 0.005$	–
	SAM 2x + Protein 1x	1.5×10^4	0.09	$p = 0.09$	$p < 0.05$
LS-BP-2a	SAM 3x	2.3×10^3	0.27	$p < 0.005$	$p = 0.33$
	SAM 2x + Protein 1x	1.5×10^4	0.14	$p = 0.47$	$p < 0.05$
rBP2a	Protein 3x	3.6×10^4	39.14	–	$p < 0.005$
n/a	Mock	<100	n/a		

[a] Groups of 6 mice received three doses of each vaccine and total IgG titers 2 weeks after the 3rd immunization (2wp3) were measured from individual mice. Geometric mean titers (GMTs) are reported. IgG1 and IgG2a titers were also measured at 2wp3 from pooled sera for each group and IgG1/IgG2a ratios are represented.
[*] P value, as calculated by two-tailed Mann-Whitney *U* test.

Vaccination with SAM followed by a protein boost, instead, resulted in a significant 3-fold increase in antigen specific IgG titers (Table 2).

Inclusion of the eukaryotic secretion signal or boosting with the recombinant protein did not change the IgG1/IgG2a ratio compared to SAM BP-2a vaccine, consistent with a T_H1 helper phenotype stimulated by SAM vaccination.

Remarkably, both of the strategies followed to improve the outcome of the vaccination resulted in higher mouse protection levels as compared to SAM BP-2a vaccine. Indeed, the percentage of puppies protected against GBS intraperitoneal challenge reached 50–60%, the same levels obtained with our protein benchmark (Table 3).

3. Discussion

RNA-based vaccines hold great promise as vaccine delivery vehicles since they offer the advantage of being fully synthetic combining therefore the positive attributes of live attenuated vaccines while avoiding many of their limitations [1,2].

We recently showed Self-Amplifying mRNA (SAM) vaccines to be safe, efficacious and versatile tools against viral pathogens, with the major advantages being represented by the relatively low production cost, the high stability and the very short time required for their development [14,18,21].

Here we have applied the SAM technology to bacterial antigens demonstrating that mRNA based vaccines might be a powerful tool to potentially face bacterial infectious diseases. Two different prototypes of bacterial proteins have been chosen for our purposes, a detoxified version of the GAS toxin Streptolysin O (SLOdm) and the GBS pilus 2a backbone protein (BP-2a). Our choice was dictated by several factors. First, these two antigens are quite different in their structure and function. Indeed, SLO is a secreted toxin which exerts its toxicity mainly against the cells of the immune system [31] and GBS BP-2a is a surface exposed protein component of the GBS pili, long protruding structures involved in cellular adhesion and invasion [32,33]. Second, both proteins induce strong antibody responses that mediate protection in well-established models of mouse infection [28,29,34]. Last but not least, robust *in vitro* assays measuring the functional activity of the exerted antibody responses have been already set up [27,35].

Despite the remarkable differences between these antigens, both constructs were easily obtained and large amount of proteins were produced in the transfected eukaryotic cells (Fig. 1B and C). The proteins produced by SAM transfected eukaryotic cells were similar in their electrophoretic mobility to the prokaryotic expressed antigens [36], as demonstrated by the double band obtained for SLOdm (Fig. 1C) [36]. Bacterial antigen expression *in vivo* can mimic a true infection, but for bacterial derived sequences it might be detrimental if the expressed antigens undergo post-translational modifications, especially if targeted to the secretory pathway. Addition of N-linked sugars, in particular, might alter the epitopes on the protein compared to the native bacterial protein affecting peptide presentation by MHC II molecules [37,38]. The BP-2a sequence contained potential N-glycosylation motifs, as predicted by *in silico* analysis, but the protein expressed by SAM was not glycosylated in transfected cells when redirected to the extracellular compartment (Fig. 3D).

After characterizing the ability of the constructs to be expressed by eukaryotic cells, mice were immunized and humoral responses investigated. Both SAM vaccines were able to induce a strong immune response which was demonstrated to be functional *in vitro* (Table 1 and Fig. 2 A and B). Antibodies directed against the host-expressed antigen were indeed functional in inhibiting SLO mediated red blood cells lysis and in mediating bacterial opsonophagocytic killing (BP-2a), the major mechanisms of defense against streptococcal pathogens [35,39]. Moreover, as previously demonstrated for viral proteins, also SAM delivered bacterial antigens exerted mainly T_H1-like immune response (Table 1) and this could be an added value for these vaccine formulations, since T_H1 response has been demonstrated to contribute to protection against bacterial infections [40–43].

Since functional antibodies have been shown to be predictive of *in vivo* protection [23,24], immunized animals were systemically infected and SAM vaccines were shown to partially protect mice through passive and active immunization (Fig. 2C and D). These results are relevant, as this is the first time that an mRNA based vaccine was demonstrated to be protective against bacteria in preclinical animal models of infection. Furthermore, protection was also transferred to newborns, making SAM delivered vaccines suitable for maternal immunization.

Despite the promising results achieved, the overall protection induced by SAM vaccines was inferior in magnitude to what obtained with the recombinant proteins, especially for BP-2a (Fig. 2C and D) and this could be at least partially due to a weaker antibody response (Table 1 and Fig. 2A and B). Two main strategies were attempted to improve vaccine efficacy, using GBS-BP-2a as a model antigen. The first one was the addition of a signal peptide to redirect the protein to the secretory pathway mimicking the features of a subunit vaccine. Second, SAM vaccine administration was followed by a protein boost, resulting in a significant increment in antibody titers, which might account for the higher protection rate. The combination of the two strategies led to the highest and most significant improvement in protection reaching the efficacy rate obtained with our protein benchmark (Tables 2 and 3). Altogether these data suggest that *in vivo* antigen expression followed by subunit boost can potentiate immune responses. Furthermore, they support the versatility of SAM vaccines, showing

Table 3
Protection conferred by BP-2a delivered by SAM or as a recombinant protein, assessed by active maternal mouse immunization/neonatal pup challenge model.[a]

Antigen	Vaccine	Protected/treated (N°puppies)	Protection (%)	Statistical significance vs Mock group[*]	Statistical significance vs SAM BP-2a 3x group[*]
BP-2a	SAM 3x	16/50	32	$p < 0.0005$	
	SAM 2x + Protein 1x	31/60	52	$p < 0.0001$	$p = 0.186$
LS-BP-2a	SAM 3x	34/57	60	$p < 0.0001$	$p < 0.05$
	SAM 2x + Protein 1x	39/60	65	$p < 0.0001$	$p < 0.005$
rBP-2a	Protein 3x	32/49	65	$p < 0.0001$	$p < 0.005$
n/a	Mock	7/50	14		

[a] Groups of 6 female mice received three doses of each vaccine were then mated and their offspring were challenged with LD_{90} of a serotype V GBS strain (CJB111 strain).
[*] P value, as calculated by the log-rank (Mantel-Cox) test.

that, depending on the nature of the pathogen and the antigens, sequence engineering and combination with other vaccine strategies can be used to identify optimal regimens for achieving protective immunity. Thanks to their fully synthetic nature and to the versatility of the manufacturing processes, SAM vaccines can be easily produced in large amounts within few days from the release of an antigen sequence, therefore representing a rapid-response platform in case of pandemic infectious diseases or newly emerging pathogens [21,44].

4. Materials and methods

4.1. Cell cultures

BHK-V cells were maintained in Dulbecco minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine, and penicillin–streptomycin at 37 °C with 5% CO_2. HL-60, a promyelocytic leukemia cell line used for opsonophagocytosis assay, were obtained from the American Type Culture Collection (CCL-240) and were maintained in RPMI 1640 Glutamax (Life technologies), supplemented with 10% heat inactivated Fetal Bovine Serum (FBS, HyClone). Cells were grown and differentiated to neutrophils in growth medium supplemented with 0.78% Dimethyl Formamide (DMF, Sigma), according to Romero-Steiner et al. [45].

4.2. RNA synthesis of SAM BP-2a and SLOdm vectors

DNA plasmids encoding the self-amplifying RNAs were constructed using standard molecular biology techniques. Briefly, cDNA encoding the BP-2a antigen was obtained from GBS CJB111 strain (serotype V) [24], while cDNA encoding SLOdm antigen (P427L/W535F SLO double mutant) was obtained from GAS SF370 strain [23]. To direct BP-2a to the secretory pathway the murine Ig κ-chain leader sequence from pDisplay™ vector (Life Technologies) was added at the N-terminus of BP-2a sequence. To predict potential glycosylation motifs the prediction algorithm of NetNGlyc (version 1.0; Center for Biological Sequence Analysis, Technical University of Denmark [http://www.cbs.dtu.dk/services/NetNGlyc/]) was used. Threshold was set at 0.5. cDNAs were cloned into *Sal*I and *Xba*I sites of a modified replicon construct [19]. RNA was prepared as previously reported [13].

4.3. BHK-V cell transfection and Western blot analysis of expressed BP-2a, LS-BP-2a and SLOdm proteins

Expression of BP-2a, LS-BP-2a and SLOdm proteins from SAM vectors was confirmed by transient transfection of BHK-V cells. 10^6 BHK-V cells were electroporated (120 V, 25 ms pulse) with 200 ng of RNA and incubated for 16–18 h at 37 °C and 5% CO2. At 16 h post transfection supernatants were collected from transfected BHK-V and concentrated by 10-folds on 10 kDa Amicon Ultra-14 Centrifugal Filter Units (Millipore), in accordance with the manufacturer's instructions. Transfected cells were lysed as previously described [21]. Lysates and supernatants were separated under reducing conditions as previously reported [21]. BP-2a and SLOdm were visualized using mouse polyclonal BP-2a or SLO antisera, produced by immunizing CD1 mice with the purified rBP-2a or rSLO protein, at 1:1000 dilution. Membranes were probed with a mouse monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control. For glycosylation analysis, N-linked oligosaccharides removal was carried out using recombinant PNGase F (New England BioLabs) according to the manufacturer's instruction. Briefly, 10 μg of BHK lysates were combined with 1 μl of 10X Glycoprotein Denaturing Buffer and then denaturated by heating reaction at 95 °C for 5 min. Proteins were chilled on ice and centrifuged for 10 s before adding 2 μl of 10X G7 Reaction Buffer, 2 μl 10% NP40, 6 μl H_2O and 1 μl PNGase F to achieve a final volume of reaction of 20 μl. Samples were gently mixed and incubated at 37 °C for 1 h. To assess the extent of deglycosylation, treated lysates were separated under reducing conditions, subjected to Western blotting, and detected as described above.

4.4. Preparation of CNE

CNE was prepared as previously described [18,25]. Briefly, Briefly, squalene, DOTAP, and sorbitan trioleate were combined and heated to 37 °C. The resulting oil phase was then combined with an aqueous phase consisting of polysorbate 80 in 10 mM citrate buffer at pH 6.5. The final weight by weight percentages of squalene, DOTAP, sorbitan trioleate, and polysorbate 80 were 4.3%, 0.4%, 0.5%, and 0.5%, respectively. This mixture was homogenized using a T25 homogenizer with a 13.4 mm diameter rotor (IKA) at 24,000 RPM to produce a primary emulsion. This was then passed through a M-110P Microfluidizer (Microfluidics) with an ice bath cooling coil at a homogenization pressure of 137 Mpa approximately 8 times. The formulation was stored at 4 °C before use. The 100-nm CNE had a positive surface charge, which was used to adsorb the RNA to the surface of the oil droplet through an electrostatic interaction with the negatively charge phosphate backbone. RNA was diluted to 200 μg/mL and was added to an equal volume of CNE, mixed, and allowed to equilibrate on ice for 30 min to 2 h. Endotoxin levels were measured by the gel clot LAL assay per the manufacturer's instructions (Cape Cod Associates).

4.5. Recombinant protein expression, purification and formulation

The recombinant tagless full-length BP-2a and SLOdm proteins, corresponding to GBS CJB111 and GAS M1-SF370 strains respectively, were expressed, purified, and further analyzed for purity and endotoxin levels before immunization studies, as previously described [23,24,46]. Shortly before immunization, the SLOdm

protein was mixed with an equal volume of MF59 while BP-2a protein was adsorbed to aluminum hydroxide (alum). MF59 and alum formulations were prepared as described elsewhere [47].

4.6. Ethical statements

All animal studies were carried out in compliance with current Italian legislation on the care and use of animals in experimentation (Legislative Decree 26/2014) and with the Novartis (a GSK company) Animal Welfare Policy and Standards. Protocols were approved by the Italian Ministry of Health (authorizations 230/2011-B and 110/2012-B) and by the local Novartis (a GSK company) Animal Welfare Body (authorization AWB 201105 and AWB 201114).

4.7. Immunogenicity and in vivo protection assays

A mouse maternal immunization/pup challenge model of GBS infection was carried out as previously described [29]. Briefly, six-to-eight-week-old female CD-1 female mice were immunized on days 1, 21, and 35 with either 15 μg of CNE-formulated SAM RNA or 10 μg of purified BP-2a protein adsorbed to alum or adjuvant alone. All vaccines were injected into the quadriceps muscles of the two hind legs (50 μl per site). Sera were collected 2wp2 and 2wp3. Mice were then mated (day 35), and their offspring were challenged intraperitoneally within 48 h after delivery with a LD_{90} dose of GBS CJB111 strain. Immunization with GAS vaccines and intraperitoneal infection with GAS were carried out as previously described [28]. Briefly, 5-weeks-old CD-1 female mice were immunized on days 1, 21, and 35 with either 15 μg of CNE-formulated SAM RNA or 10 μg of purified SLOdm protein formulated in MF59 or adjuvants alone. Sera were collected 2wp2 and 2wp3. Three weeks after the third immunization, mice were infected intraperitoneally with 200 μl of a bacterial suspension (GAS M1T1 3348 strain, 1.5E+07 CFU/mouse).

In all experiments, mice were monitored on a daily basis for 7 days after treatment and euthanized when they exhibited defined humane endpoints that had been pre-established for the study in agreement with Novartis (a GSK company) Animal Welfare Policies. Statistical analysis was performed using log-rank (Mantel-Cox) test.

4.8. Enzyme-linked immunosorbent assay (ELISA) for antigen-specific serum antibody

Antigen-specific serum IgG were assayed by ELISA with individual (BP-2a) or triplicates of pooled (SLOdm) sera. ELISA plates (MaxiSorp 96-well; Nunc) were coated overnight at 4 °C with 0.1 μg/mL purified rBP-2a or rSLOdm protein in PBS and IgG, IgG1, and IgG2a titers were measured as previously reported [13]. IgG1 and IgG2a titers were measured at 2wp3 only from pooled sera. Standards, with previously defined titers, were pooled sera from internal immunogenicity studies.

4.9. Opsonophagocytosis assay

Opsonophagocytic killing assays (OPKA) using pooled sera from immunized mice was conducted as previously described [48]. Briefly, the reaction was performed in 96 well plates (Nunc), in HBSS (Hank's Balanced Salt Solution, Gibco). For each reaction mixture, heat inactivated (56 °C for 30 min; HI) test serum, GBS CJB-III strain bacteria, differentiated HL-60 cells and 10% baby rabbit complement (Cedarlane) were added. Control reactions were performed in the presence of HI complement or in the absence of antibodies or effector cells. For each serum sample, four dilutions were tested. Bacteria were prepared by directly diluting frozen aliquot stocks (OD600 = 0.45–0.5) in order to obtain a final concentration of 6 × 10^4 CFU/well. Bacteria were then diluted 1/2 in HBSS + 10% normal rabbit serum (Sigma) and dispensed in plates. The effector cells to GBS cells ratio was 25:1. The reaction plate was incubated for 1 h at 37 °C with shaking (300 rpm). T0 and T60 reactions were diluted in sterile water and 10 μL of each dilution were then plated in trypticase soy agar plate + 5% blood sheep (Particle Measuring Systems). Plates were incubated over night at 37 °C + 5% CO_2. The percent of killing was calculated as (mean CFU at T0 - mean CFU at T60)/(mean CFU at T0). OPKA titers were expressed as the reciprocal serum dilution mediating 50% bacterial killing, estimated through linear interpolation of the dilution-killing OPKA data. The Lower Limit of Detection was 1:30.

4.10. Hemolysis inhibition titration

Hemolysis inhibition assay (on individual mice) was performed using a blood cell suspension prepared washing sheep blood cells three times in PBS, then suspended in five volumes of PBS (20% in PBS sheep blood cells suspension). Serial twofold dilutions of sera were prepared in 96-well plates with U-shaped bottoms using 0.5% BSA in PBS and 10 ng of SLO toxin (diluted in BSA 0.5% in PBS) were added to each well allowing incubation at room temperature for 20 min. Following addition of 50 μl of sheep blood cells suspension, incubation was continued for additional 30 min at 37 °C. Plates were finally centrifuged for 5 min at 1000*g* and 75 μl of the supernatant were carefully transferred to 96-well flat-bottomed plates containing 100 μl/well of PBS. The absorbance was read at 540 nm. Inhibition titer was expressed as the lowest serum dilution factor that completely inhibited SLO induced hemolysis.

4.11. Statistical analyses

Statistical analysis (two-tailed Mann-Whitney *U* test, log-rank (Mantel-Cox) test) was performed using Prism 6 software (GraphPad).

5. Contributors

GM, EC, GB, DM were involved in the conception and design of the study. GM, EC, CG, SB, StB, EF acquired the data. GM, EC, GB, IM, AJG, DM analyzed and interpreted the results. All authors were involved in drafting the manuscript or revising it critically for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

Conflict of interest

This work was sponsored by Novartis Vaccines, now part of the GSK group of companies which was involved in all stages of the study conduct and analysis. All authors were employees of the Novartis group at the time the study was conducted. GM, EC, CG, SB, StB, EF, IM, GB, DM are now employees of GSK group companies.

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