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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