Natural STING Agonist as an "Ideal" Adjuvant for Cutaneous Vaccination

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A potent adjuvant that induces strong protective immunity without incurring any significant skin reactogenicity is urgently needed for cutaneous vaccination. Here, we report that a natural agonist of stimulator of interferon genes (STING), 2′3′- cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), robustly augmented and prolonged the cellular and humoral immune responses provoked by H5N1 and 2009 H1N1 pandemic influenza vaccines after a single dose of intradermal, but not intramuscular, immunization. The potency of cGAMP for cutaneous vaccination was ascribed to a large number of antigen-presenting cells resident in the skin and ready for immediate activation when cGAMP was injected. However, its potency was severely compromised in the muscle, because antigen-presenting cells could not be promptly recruited to the injection site before the injected cGAMP was diffused out. The superior adjuvant effect and safety of cGAMP were also confirmed in a more clinically relevant swine model of skin. The vigorous immune responses elicited by cGAMP with no overt skin irritation was attributable to its stay in the skin, which was brief but sufficient to activate dermal dendritic cells. This small and well-characterized self-molecule holds great promise as an ideal adjuvant for cutaneous vaccination.

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INTRODUCTION

Current vaccines are mostly administered into the muscle, despite the fact that the skin is a more potent site for vaccination. Apart from inconvenience, lack of a noninflammatory, potent adjuvant remains a key issue for cutaneous vaccination (Hickling et al., 2011). As the first line of our body's defense system, epidermis and dermis contain a large number of antigen-presenting cells, making the skin effective for vaccination but also prone to severe local reactogenicity. This dilemma precludes use of many potent adjuvants for skin vaccination because of prolonged and high levels of local inflammation. For instance, the commonly used aluminum salt (Alum), water-in-oil emulsions montanide ISA 51 and ISA 720, and several Toll-like receptor (TLR) agonists (e.g., R837) provoke severe local reactions including erythema, swelling, and ulceration for weeks at the injection site (Chen et al., 2012; Ginhoux et al., 2012; Vogelbruch et al., 2000). However, a safe and effective adjuvant is indispensable for subunit or weak vaccines to enhance, shape, and broaden immune responses. The adjuvant is also crucial for antigen dose-sparing and rapid and strong protective immunity in very young and elderly populations (Reed et al., 2013). To date, only a few adjuvants have been approved for prophylactic vaccines, including alum, squalene-based emulsion, and monophosphoryl lipid A (MPL), but all of them are approved for intramuscular (IM) administration only. No adjuvant has been approved for skin immunization.

An ideal adjuvant for cutaneous vaccination should have the following properties. First, it should have natural and metabolizable components generated from humans so that a risk of inducing antibodies against the molecule can be minimized even after repeated uses. Second, the adjuvant activity should be localized and transient, thus averting unwanted adverse events in the skin while sufficiently retaining the ability to bolster vaccination. Third, the adjuvant should be potent, with its underlying mechanism well characterized. Understanding of the mechanism ensures the specificity and predictability of the immune responses in different individuals, in sharp contrast to those adjuvants empirically developed, such as alum. Recently, we reported a laser-based adjuvant that met these criteria and could potentially serve as a safe and effective adjuvant for intradermal (ID) vaccination (Chen et al., 2013; Wang et al., 2014). We used a nonablative factional laser to generate an array of micro-injuries in the skin that robustly activated sterile innate immunity. Although these micro-injuries stimulate robust innate immune responses and sufficiently augment ID vaccination, the micro-injuries can be healed, concomitant with resolution of the associated micro-inflammation, within 48 hours (Manstein et al., 2004; Wang et al., 2014). Our further investigation showed that double

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Abbreviations: alum, aluminum salt; cdGMP, cyclic di-guanosine monophosphate; cGAMP, 2′3′-cyclic guanosine monophosphate-adenosine monophosphate; GFP, green fluorescent protein; HA, hemagglutinin; HAI, hemagglutination inhibition; ID, intradermal or intradermally; IM, intramuscular or intramuscularly; MHC, major histocompatibility complex; MPL, monophosphoryl lipid A; STING, stimulator of interferon genes; Th, T helper

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stranded DNA released from laser-damaged cells was sensed by the intracellular sensor cyclic guanosine monophosphate-adenosine monophosphate synthase (Sun et al., 2013; Wang et al., 2015). Cyclic guanosine monophosphate-adenosine monophosphate synthase subsequently generated 2′3′-cyclic guanosine monophosphate−adenosine monophosphate (cGAMP) as a second messenger that binds the stimulator of interferon genes (STING), also known as TMEM173/MPYS/MITA/ERIS (Ishikawa et al., 2009; Jin et al., 2008; Wu et al., 2013; Zhong et al., 2008). Stimulation of STING then activates interferon regulatory factor 3 and NF-κB pathways, greatly increasing the transcription of type I interferons and other cytokines, and a strong T helper cell (Th)-1 immune response results (Paludan and Bowie, 2013). The finding raises an intriguing possibility that cGAMP may replace laser treatment as a safe, simple, and potent adjuvant for skin vaccination.

cGAMP is a natural, metabolizable molecule in humans that is hydrolyzed quickly by ectonucleotide pyrophosphatase/phosphodiesterase (ENPP1) when located outside the plasma membrane (Li et al., 2014). The quick hydrolysis ensures that its adjuvant activity is transient, effectively circumventing unwanted systemic inflammation. Moreover, because cGAMP is a small, negatively charged, hydrophilic molecule, induction of antibodies against this small self-molecule is highly unlikely. The adjuvant effect of cGAMP has been shown in mice by co-injection cGAMP and ovalbumin, a model protein vaccine, into the muscle (Li et al., 2013). Its bacterial analog, cyclic di-guanosine monophosphate (cdGMP) has been studied extensively as a potential vaccine adjuvant for bacteria vaccines through IM, subcutaneous, intraperitoneal, or intranasal vaccinations (Blaauboer et al., 2015; Ebensen et al., 2007a; Ebensen et al., 2007b; Karaolis et al., 2007; Ogunniyi et al., 2008). Recently, modified non-hydrolyzable cGAMP analogs have also been shown to have potent antitumor activity when administered intratumorally (Corrales et al., 2015).

The current study evaluates the potential of cGAMP as a safe and potent adjuvant for influenza vaccines ID administered. Our results showed that cGAMP could be an ideal adjuvant for cutaneous vaccination against both seasonal and pandemic influenza. It greatly enhanced the protective humoral and cellular immune responses while evoking little local skin reaction. ID delivery of cGAMP showed superior adjuvant effects compared with IM vaccination, presumably because of the abundant antigen-presenting cells resident in the skin and the ability of the skin to better retain the small molecule than the muscle. The potency and safety of cGAMP as a cutaneous adjuvant were also confirmed in a swine model.

RESULTS

cGAMP induces superior immune responses versus ID influenza vaccines

To determine the adjuvanticity of cGAMP for ID influenza vaccines, Swiss Webster mice were ID immunized with a monovalent influenza vaccine, A/California/07/2009 H1N1 at a dose of 300 ng hemagglutinin (HA) per mouse with or without 20 μg of cGAMP. The vaccine was also IM administered either alone or with 20 μg of cGAMP or AddaVax (Invivogen, San Diego, CA) for efficacy comparison. AddaVax is a squalene-based vaccine adjuvant with similar composition to commercial adjuvant MF59 that has been used in seasonal influenza vaccine in the elderly for a decade in Europe. MF59 and another squalene-based adjuvant, AS03, were also used in the 2009 pandemic influenza vaccine in Europe and Canada. Immunization with the vaccine alone through IM or ID administration did not elevate the number of $CD4^+$ or $CD8^+$ T cells secreting IFN-γ versus unimmunized mice (Figure 1a and b). Inclusion of cGAMP or AddaVax into IM vaccination failed to augment the cellular immune responses (Figure 1a and b). However, cGAMP significantly elevated the number of IFN-γ−secreting $CD4^+$ and $CD8^+$ T cells if it was ID delivered along with the vaccine ($P < 0.05$) (Figure 1a and b). None of the immunizations tested augmented Th2 cellular responses, as suggested by a similar number of $CD4^+$ T cells producing IL-4 among unimmunized and all immunized mice (data not shown).

Humoral immune responses were next measured 4 weeks later by hemagglutination inhibition (HAI) assay, a criterion standard of influenza vaccination, in which a serum HAI titer greater than 1:40 is considered protective. As shown in Figure 1c, immunization by the vaccine alone by an ID or IM route did not give rise to protective immune responses (mean titer < 1:20), nor did IM immunization by the vaccine mixed with cGAMP or AddaVax adjuvant. In marked contrast, ID immunization by the vaccine mixed with cGAMP brought about 5−10 times higher HAI titers than any other immunization strategies tested ($P < 0.001$) (Figure 1c). The effects of cGAMP on Th1 and Th2 immune responses were subsequently assessed by measurement of influenza HA-specific IgG1 and IgG2a antibodies. Unlike AddaVax, which augmented both Th1 and Th2 immune responses similarly and modestly, cGAMP preferably strengthened Th1 immune responses, resulting in a higher IgG2a titer in both IM and ID immunizations, with a more predominant effect on the latter (Figure 1d and e). Consequently, the mice produced the highest level of IgG2a after receiving ID immunization with a mixture of cGAMP and the vaccine compared with all other vaccination procedures tested (Figure 1e). Although it augmented Th1 immune responses robustly, cGAMP displayed little influence on Th2 immune response, regardless of whether it was delivered via an ID or IM route. In accordance with the superior immune responses elicited by ID immunization, all mice (8/8) receiving the vaccine mixed with cGAMP survived a viral challenge, with only a slight body weight loss (<10%) (Figure 1f and g). In sharp contrast, all mice died within 7 days after the viral challenge in the absence of adjuvant, regardless of the route of immunization. IM vaccination in the presence of either cGAMP or AddaVax yielded only 12.5% or 25% protection, respectively (Figure 1g).

cGAMP does not evoke significant skin irritation in mice

Given the superior adjuvant effect of cGAMP for skin vaccination, we next addressed its local reactogenicity by ID injection of phosphate buffered saline (PBS), 20 μg of cGAMP, 20 μg of resiquimod and 300 ng of H1N1 vaccine or the vaccine plus 20 μg of cGAMP. cGAMP did not evoke any overt irritations from day 1 to day 5 at the inoculation site