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The choice of linker for conjugating R848 to inactivated influenza virus determines the stimulatory capacity for innate immune cells

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ABSTRACT

Inactivated influenza vaccines are not approved for use in infants less than 6 months of age due to poor immunogenicity in that population. While the live attenuated influenza vaccine has the potential to be more immunogenic, it is not an option for infants and other vulnerable populations, including the elderly and immunocompromised individuals due to safety concerns. In an effort to improve the immunogenicity of the inactivated vaccine for use in vulnerable populations, we have used an approach of chemically crosslinking the Toll-like receptor (TLR) 7/8 agonist R848 directly to virus particles. We have reported previously that an R848-conjugated, inactivated vaccine is more effective at inducing adaptive immune responses and protecting against lung pathology in influenza challenged neonatal African green monkeys than is the unmodified counterpart. In the current study, we describe a second generation vaccine that utilizes an amide-sulfhydryl crosslinker with different spacer chemistry and length to couple R848 to virions. The new vaccine has significantly enhanced immunostimulatory activity for murine macrophages and importantly for monocyte derived human dendritic cells. Demonstration of the significant differences in stimulatory activity afforded by modest changes in linker impacts our fundamental view of the design of TLR agonist-antigen vaccines.

1. Introduction

Inactivated vaccines against viral and bacterial pathogens are generally not as immunogenic as their live, attenuated counterparts. Nevertheless, they remain an important part of the armament of vaccines currently in use due to their relative safety, especially in vulnerable populations such as infants, the elderly, and other immunocompromised individuals. The most highly utilized adjuvant alum can significantly increase the immunogenicity of killed and component vaccines [1]; however, its propensity to elicit Th2 responses, limited ability to promote $CD8^+$ T cell responses, and capacity to stimulate local inflammation [2] provide strong rationale for the development of new adjuvants. In recent years, it has been demonstrated in a variety of systems that small molecule Toll-like receptor (TLR) agonists are effective adjuvants (for review see [3,4]). TLR belong to a family of pattern recognition receptors that bind molecules derived from viral, bacterial, and fungal pathogens as well as endogenous molecules that signal danger [5,6]. TLR are broadly distributed on immune cells and their engagement promotes robust cellular activation/maturation following microbial infection. Thus, multiple cell types including T cells, B cells, and dendritic cells (DC) can be modulated by these ligands. The majority of TLR are expressed at the cell surface, but a subset (TLR3, TLR7, TLR8, and TLR9) are contained within endosomes [7]. This subset of TLR recognize nucleic acids structures; i.e. dsRNA (TLR3), ssRNA (TLR7 and TLR8), and unmethylated CpG (TLR9).

Complications from influenza virus infection kill approximately 20–30,000 individuals per year in the U.S. alone, and most of these deaths occur in immunocompromised individuals including the very young and the elderly. The increased susceptibility to severe disease in these groups is compounded by the poor immunogenicity of the inactivated vaccine [8,9]. With this knowledge, we have focused our efforts on developing a safe and effective influenza vaccine for these difficult-to-vaccinate, vulnerable populations. To this end, we have explored the TLR7/8 agonist resiquimod (R848) as an adjuvant for an inactivated influenza vaccine. In experimental settings, R848 (or its closely related analog 3M-012) has been shown to promote multiple arms of the adaptive

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immune response including antibody, $CD8^+$ T cells, and Th1 responses [10–14]. In addition, R848 engagement of TLR8 has been reported to inhibit the suppressive activity of Tregs [15]. Thus, R848 has immunoregulatory properties that make it very attractive as an adjuvant. TLR7 has been reported to play an important role in the immune response to influenza virus [16–18]. While the role of TLR8 is relatively unknown, given the demonstrated TLR8 signaling mediated by influenza viral RNA [19], it seems likely that this sensor contributes to immunity following infection.

We have employed the principle of physically associating adjuvant and antigen given the studies showing physical association of TLR agonists with antigen promotes more robust adaptive immune responses in comparison to a mixture of adjuvant and antigen (for review see [4]). Association has been achieved by a variety of approaches including chemical linkage and synthesis of fusion proteins [4,11,20–22]. The proposed advantage of direct linkage lies in targeting of the immunostimulatory component and the antigen to the same cell resulting in optimal maturation of the antigen presenting cell.

We have stably conjugated R848 to influenza virus particles followed by formalin-inactivation. This approach has the advantage that all viral proteins present in the vaccine can serve as potential targets for the immune response. Our published studies show that R848-conjugated inactivated influenza virus is a potent inducer of antibody and cell mediated immunity in a nonhuman primate neonate model [23]. Here we describe a second generation construct that has markedly improved potency for maturation of human DC.

2. Materials and methods

2.1. Virus

Purified Influenza virus strain A/PR/8/34 was purchased from Charles River Avian Vaccine Services. For inactivation, virus was treated with 0.74% formaldehyde for 1 h at 37 °C, followed by extensive dialysis (20 K MWCO). Recombinant vaccinia virus (WR strain) was inactivated by the same method.

2.2. Antibodies and reagents

Antibodies for flow cytometry were as follows: APC-labeled anti-human CD11c, clone S-HCl-3 (BD Biosciences); PerCP/Cy5.5-labeled anti-human CD40, clone 5C3 (Biolegend); APC-H7-labeled anti-human CD80, clone L307.4 (BD Biosciences); BV510-labeled anti-human CD86, clone 2331; PE-labeled anti-mouse CD40, clone 3/23 (BD Biosciences). Accutase cell detachment solution was from BD Biosciences. The TLR agonists Imiquimod (R837, TLR7-specific) and TL8-506 (TLR8-specific) were from InvivoGen. R848 (TLR7/TLR8 agonist) was synthesized in house to introduce a primary amino group in place of the hydroxyl group of native R848. Compounds ML2-102-1, ML2-103-1, ML2-104-1, MAL-PEG2, MAL-PEG8, and MAL-PEG16-1 were produced by PharmAgra Labs (Brevard, NC).

2.3. Preparation of virus conjugates

R848-conjugated influenza virus was prepared by linking a derivative of R848 which is modified to contain a primary amine, directly to virions in a 2-step process using an amide to sulfhydryl crosslinker, $SM(PEG)_4$ (Thermo Scientific) as previously described [23]. This protocol was also used to generate R848-conjugated, inactivated vaccinia virus (IVV). Preparation of R848-conjugated influenza virus using a 1-step method was the same as for the 2-step method, except that the first step, covalent linkage of R848 to linker was completed by commercial production of R848 modified with 5 different amide-sulfhydryl crosslinker moieties. Free thiol content of live virus particles was measured before and after the conjugation protocol using a Measure-iT Thiol assay kit (Thermo Fisher Scientific #M30550) according to manufacturer's instructions. A reduction of virus-associated free-thiols served as an indirect measure of the amount of R848 that had covalently bound to virus particles.

2.4. Human monocyte-derived dendritic cells

Monocyte-derived dendritic cells (DC) were differentiated from $CD14^+$ human peripheral blood mononuclear cells (PMBC) as previously described using 10 ng/ml each recombinant human GMCSF and IL-4 [24]. DC purity (>95%) was determined on Day 7 by CD11c expression (flow cytometry) and morphology (cytocentrifugation and Diff-Quik staining).

2.5. Stimulation of RAW 264.7 cells or human DC

Inactivated virus preparations or TLR7/8 agonists were added to cells and cultured for 24 h. In the case of human DC, the cells were used on day 7 of culture. Activation was assessed by measuring the expression of costimulatory molecules (CD40, CD80, CD86) by flow cytometry, and by production of cytokines by ELISA (TNFα for RAW 264.7) or by inflammatory cytometric bead array (IL-12p70, TNFα, IL-10, IL-6, IL-1β, IL-8 for human DC; BD Biosciences). Samples were acquired using a BD FACS Canto II instrument. Analysis was performed using FlowJo software.

2.6. Alexafluor-680 (AF680) labeling of vaccine conjugates and measurement of association with DC

Vaccine conjugates were fluorescently labeled with AF680 NHS ester (Thermo Fisher #A37574) as follows: 50 μg of iPR8-SM $(PEG)_4$-R848 or iPR8-GMBS-R848 (50 μl of 1 mg/ml stock in PBS, pH 7.4) was mixed with 2.5 μg AF680 stock (2.5 μl of 1 mg/ml stock in DMSO), yielding 0.05 μg of AF680/μg conjugate protein. After 1 h with vigorous shaking at 23 °C, the conjugates were extensively dialyzed (20 K MWCO Slide-A Lyzer MINI dialysis units) to remove unincorporated AF680. The protein concentration was measured by the BCA protein assay, and the fluorescence units per μg of conjugate determined by measuring the AF680 signal using a POLARstar Omega instrument (BMG Labtech). To measure relative cell association of the two conjugates, human monocyte-derived DC were prepared as for activation experiments. AF680-labeled IPR8-$SM(PEG)_4$-R848 or AF680-labeled IPR8-GMBS-R848 were diluted in DC culture medium and incubated with cells for 1 h at 37 °C. The cells were then extensively washed at 4 °C to remove AF680-labeled conjugate that had not been bound or internalized, and stained for CD11c. Data was acquired using a BD Fortessa flow cytometer and analyzed using Diva software. Mean fluorescent intensities (MFI) were normalized to fluorescence units/μg protein for each conjugate.

2.7. Statistics

Experiments were performed at least 3 times and significance was determined by unpaired Student's *t* test using GraphPad Prism software.