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***Listeria*-derived ActA is an Effective Adjuvant for Primary and Metastatic Tumor Immunotherapy**

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Abstract

Tumor immunotherapy is currently at the cusp of becoming an important aspect of comprehensive cancer treatment in the clinic. However, the need for improved adjuvants to augment immune responses against tumor antigens is always present. In this paper, we characterize the *Listeria monocytogenes*-derived actin-nucleating protein, ActA, as a novel adjuvant for use in tumor immunotherapy. ActA is a virulence factor that is expressed on the cell surface of *Listeria monocytogenes* and facilitates the production of actin tails that propel *Listeria* throughout the cytosol of an infected host cell. It is believed that this ActA-dependent cytosolic motility allows *Listeria* to evade adaptive host cell defenses and facilitates its invasion into a proximal uninfected host cell. However, there is evidence that ActA fused to a tumor antigen and delivered by *Listeria monocytogenes* can perform a beneficial function in tumor immunotherapy as an adjuvant. Our investigation of this adjuvant activity demonstrates that ActA either fused to or administered as a mixture with a tumor antigen can augment anti-tumor immune responses, break immune tolerance, and facilitate tumor eradication, which suggests that ActA is not only an effective adjuvant in tumor immunotherapy but can also be applied in a number of therapeutic settings.

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Conflict of Interest Yvonne Paterson wishes to disclose that she has a financial interest in Advaxis, a vaccine and therapeutic company that has licensed or has an option to license all patents from the University of Pennsylvania that concern the use of *Listeria monocytogenes* or listerial products as vaccines.

Keywords

ActA; Adjuvant; Cancer; Vaccine; *Listeria monocytogenes*; Immunotherapy

Introduction

It has been over a century since the first reports by Dr. William Coley of the utility of bacteria and bacterial products in the treatment of cancer [1,2]. While the initial work using *Streptococcus pyogenes* to treat patients' malignancies met with some success, the introduction of radiation therapy marginalized the use of bacteria in cancer treatment [2]. Nevertheless the utility of bacteria in cancer treatment persisted as evidenced by the use of an attenuated mycobacterium, *Bacillus Calmette-Guerin* (BCG), as a frontline treatment in bladder cancer [3]. Interestingly, the mechanism of the therapeutic action of BCG is believed to be due to non-specific stimulation of the immune system and not due to direct killing of the cancer cells by the bacterium [4].

Like most pathogens, bacteria stimulate both a strong antigen-specific and non-specific immune response. While BCG treatment demonstrates the utility of the non-specific immune response, many are now harnessing the strong specific adaptive immune response induced by a bacterial infection to fight cancer [5]. There is now growing evidence that recombinant bacteria engineered to express tumor specific antigens are able to stimulate a specific anti-tumor adaptive immune response that culminates in tumor destruction [5, 6]. Specifically, attenuated recombinant *Listeria monocytogenes* (Lm) has proven to be a uniquely adept vaccine vector for stimulation of a strong cytotoxic T lymphocyte (CTL) response and establishment of long-term central memory T cells against specific tumor antigens [7, 8].

While Lm engineered to produce tumor antigens does lead to a specific CTL response, anti-tumor efficacy is greatly augmented by fusing the tumor antigen of interest to the endogenous Lm virulence factor Listeriolysin O (LLO) [7,8]. LLO is an endogenous pore-forming toxin of Lm that is a well-characterized adjuvant when fused to a tumor antigen and secreted by recombinant Lm [9,10]. In some instances, the fusion of adjuvant proteins to tumor antigens is believed to facilitate their ubiquitin-mediated degradation and processing for eventual presentation on MHC Class I molecules [11, 12]. Since LLO is an N-end rule regulated protein and degraded by the ubiquitin-proteasome system, this appears to be a likely mechanism of adjuvancy [12,13]. Interestingly, it is also reported that delivery of LLO unfused to a tumor antigen can also augment an adaptive immune response to the tumor antigen [10]. While this mechanism of adjuvancy is uncharacterized as of yet, there is evidence that LLO can induce signaling that leads to chromatin remodeling and cytokine production independent of its pore-forming ability [14,15]. This evidence suggests that LLO may be acting as a pathogen-associated molecular pattern (PAMP) much like a number of other adjuvants used in tumor immunotherapy [16].

We recently found that fusion of another Lm virulence factor, ActA, to a tumor antigen and secreted by a recombinant Lm can also facilitate an augmented anti-tumor immune response [17,18]. The normal role of ActA in the life cycle of Lm is to function as an actin nucleating

protein, much like WASP [19,20]. Upon entering the host cell cytosol, Lm produces and secretes ActA which induces rapid polymerization of filamentous actin through interaction with the Arp2/3 complex [21]. This action allows Lm to propel itself through the host cell cytoplasm to avoid host cell defenses and allow transit to neighboring cells [22]. Interestingly, the host cell rapidly degrades ActA through the ubiquitin-proteasome system in an N-end rule dependent manner, much like LLO [23]. When ActA is produced and secreted by Lm as a fusion protein, along with a tumor antigen, it demonstrates a similar profile of adjuvancy as LLO fusion [17,18]. In studies by Sewell et al, administration of a recombinant Lm that secretes a fusion protein of ActA and HPV 16 E7 leads to regression of tumors expressing E7 [17]. Additional work by Souders et al, demonstrates that this vaccine, Lm-ActAE7, can even lead to strong anti-tumor responses in an E7-tolerized host [18]. The mechanism of adjuvancy by ActA and whether it depends on fusion to the tumor antigen and secretion specifically by Lm has, as of yet, not been characterized.

In this study, we characterize the properties of adjuvancy by ActA in tumor immunotherapy. We employ purified ActA protein along with the tumor antigen HPV-16 E7 to determine if this is sufficient to provide an effective anti-tumor immune response. In addition, we demonstrate that ActA does not need to be covalently conjugated to the tumor antigen for its adjuvancy. We believe these studies provide insight into the mechanism and efficacy of ActA as an adjuvant and highlight an additional tool for clinicians to utilize in tumor immunotherapy.

Methods

Mice

Six to eight week-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). The HPV16 E6/E7 transgenic mouse on the C57BL/6 background was bred and utilized for tolerance studies and is described elsewhere [18, 24].

Cell Lines

The C57BL/6 syngeneic TC-1 tumor cell line has been immortalized with HPV-16 E6 and E7 and transformed with the *H-ras* oncogene [25]. TC-1 tumor cells express low levels of E6 and E7 and are highly tumorigenic. TC-1 cells were grown in RPMI 1640 medium, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 mM non-essential amino acids, 1 mM sodium pyruvate, 50 mM 2-ME at 37°C with 7% CO₂. The J774A.1 immortalized murine macrophage-like cell line was purchased from ATCC and cultured as recommended by the supplier.

Construction and purification of protein vaccines

The gene encoding for ActA(amino acid 25-421) protein was amplified using chromosomal material from *L. monocytogenes*, strain 10403S, using PCR primers: Adv347 (5'-TATCGGATCCGCGACAGATAGCGAAGATTCT-3') and Adv102 (5'-CGTGCTCGAGAGCTAGGCGATCAATTTCTTC-3'). ActA E7 was constructed by fusing ActA to HPV16 E7 in the open reading frame using oligos Adv58 (5'-GAAGAAGAAATTGATCGCCTAGCTCTCGAGCATGGAGATACACCTACATTGC

ATG-3'), Adv 59 (5'-CATGCAATGTAGGTGTATCTCCATGCTCGAGAGCTAGGCGATCAATTTCTTCTTC-3'), Adv347 and Adv125 (5'-GTGCTCGAGTGGTTTCTGAGAACAGATGG-3) by the gene-SOEing PCR method. Both ActA and ActAE7 were cloned into pET30a in the open reading frame with the N and C terminal His tags at BamHI and XhoI sites, respectively. The sequences of the target genes in these plasmids, pAdv 155.2 (ActAE7/pET30a) and 157.1 (ActA/pET30a) were confirmed by DNA sequencing (Genewiz, Plainfield, NJ). BL21-DE3 *E. coli* strain (Invitrogen) was transformed with each ActA plasmid. The *E. coli* strain expressing HPV16 E7 was obtained as a gift from Dr. Drew Pardoll, Johns Hopkins University. Briefly, the HPV16 E7 ORF was cloned into pOE30 (Qiagen) and placed into the M15pREP4 *Escherichia coli* (*E. coli*) strain (Qiagen). The E7 synthesized in this strain has a histidine motif at the amino terminus to allow for purification over a Ni-NTA column. Proteins were purified using a Ni-NTA column (Qiagen) according to the manufacturer's instructions. The purity of the proteins was verified by separating the proteins by SDS-PAGE followed by Coomassie staining. Endotoxins were removed from the purified protein preparations with the Norgen Proteospin Endotoxin Removal Maxi kit as per manufacturer's instructions (Norgen Biotek Corporation). Endotoxin removal was confirmed by Western blotting with anti-LPS core antibody (HyCult biotechnology).

Phagocytosis Assay

The protocol for the Vybrant Phagocytosis Assay Kit (Invitrogen) was followed according to the manufacturer instructions. Briefly, the J774A.1 macrophage-like cell line was cultured for 4 days and then plated in a 96-well plate at 10^5 cells/well. The cells were then left untreated (positive control) or coincubated with the indicated amounts of purified ActA protein in a volume of 150 μ L of media (DMEM with 10% FBS for 4 hrs). Additionally, as a negative control, some wells were left cell-free and filled only with 150 μ L of media. After 4 hrs of incubation, fluorescein-labeled *E. coli* (K-12 strain) BioParticles were added to the wells and phagocytic uptake was allowed to proceed for 2 hrs. Trypan blue was then added to each well to quench the fluorescence of any extracellular non-phagocytosed bioparticles. Phagocytic uptake was then measured with a fluorescent microplate reader at an absorbance at O.D. 535 nm.

Tumor Load Study

TC-1 tumor cells (2×10^5) were injected subcutaneously (s.c.) in the flanks of either wild type C57BL/6 mice or C57BL/6 mice transgenic for HPV-16, E6 and E7 (8 mice/group). After allowing the tumors to establish for 7 days at which time they are palpable and roughly 5mm in diameter, protein vaccines were administered s.c. at equimolar amounts (3.3 μ moles) as follows: 50 μ g of E7, 200 μ g of ActA, 250 μ g of ActA genetically fused to E7 (ActAE7), or 50 μ g of E7 mixed with 200 μ g of ActA (ActA+E7) per mouse. Mice were immunized again on Day 14 and tumor growth was measured every 3 days with calipers. Tumors with a volume of less than 9 mm³ could not be measured and the mice were classified as tumor-free. Mice were sacrificed when the tumor volume reached > 4000 mm³. Data are shown as the percentage of mice tumor free for each vaccine group at various time points after tumor challenge. Tumor volume was calculated as (tumor diameter)³/2.

Metastatic Tumor Study

TC-1 tumor cells (2×10^5) were injected intravenously in the tail vein of C57BL/6 mice (5-6 mice/group). On days 5 and 12 post injection, protein vaccines were administered s.c. at equimolar amounts (3.3 μ moles) as described above. Mice were then sacrificed on day 30 and lungs isolated and perfused with PBS. Lung surface metastatic nodules per lung were then counted with a Nikon SMZ1B Zoom Stereomicroscope attached to a Fostec 8375 Illuminator and Ringlight.

Depletion Experiments

CD8⁺ cells were depleted in TC-1 tumor-bearing mice by injecting the mice with 0.5 mg of anti-CD8 antibody (clone 2.43) on days 6, 7, 8, 10, 12, and 14 post-tumor challenge. To control for non-specific effects, a group of mice were treated under the same conditions with an isotype matched, control antibody specific for beta-galactosidase. The concurrent tumor load study was adhered to as described in "Tumor Load Study" in this methods section.

ELISpot Analysis

The 96-well filtration plates (Millipore, Bedford, MA) were coated with 15 μ g/ml rat anti-mouse IFN- γ antibody (clone AN18, MABTECH, Mariemont, OH) in 100 μ l of PBS. After overnight incubation at 4°C, the wells were washed and blocked with culture medium containing 10% fetal bovine serum. Splenocytes from each experimental group (1×10^5 or 2×10^4 per well) were added to the wells along with E7 protein (5 μ g/ml) or E7-specific H-2Db CTL epitope, RAHYNIVTF (5 μ g/ml) [26] plus IL-2 (5 U/ml). Cells were incubated at 37°C for 24 h. The plate was washed followed by incubation with 1 μ g/ml biotinylated IFN- γ antibody (clone R4-6A2, MABTECH, Mariemont, OH) in 100 μ l PBS at 4°C overnight. After washing, 1:100 streptavidin-horseradish peroxidase in 100 μ l PBS were added and incubated for 1 h at room temperature. Spots were developed by adding 100 μ l of substrate after washing and incubated at room temperature for 15 min. Color development was stopped by washing extensively in tap water and spot-forming cells (SFC) were counted on an ELISpot reader. The ability of each ActA vaccination to enhance IFN- γ secretion responses was statistically compared against the E7 vaccine alone as described below.

Detection of E7-specific Tumor Infiltrating Lymphocytes (TILs)

C57BL/6 mice were implanted with TC-1 tumors and immunized s.c. with E7, ActA, ActA + E7, or ActAE7 and boosted 7 days later as described above. Tumors were harvested 9 days after boosting and manually dissociated into a single-cell suspension. The tumor cell suspension was then Ficoll-purified to remove dead cells and cellular debris by excluding the low density fraction after centrifugation. The remaining tumor cells were then subjected to three-color flow cytometry for CD8 (53-6.7, FITC conjugated), CD62 ligand (CD62L; MEL-14, APC conjugated), and E7 H-2Db tetramer-PE conjugated (RAHYNIVTF) using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, Mountain View, CA). Tetramers were provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility and used at a 1/200 dilution. Results from three separate experiments were combined and analyzed as described above to compare the ability of each ActA

vaccination to induce tetramer⁺, CD8⁺, CD62L⁺, E7-specific TILs with E7 vaccination alone.

Statistical analyses

One-tailed student's *t*-tests were performed comparing E7 vaccination to either ActA+E7 or ActAE7 vaccination for all data except for Fig. 1C where the Positive Control was compared to ActA(100ng, 10ng, and 1ng) treatment using GraphPad Prism version 4.0a for Macintosh (San Diego, CA, www.graphpad.com). Significant p-values for all comparisons are depicted in figures as follows: *=*p*<0.05, **=*p*<0.01, and ***=*p*<0.001. Non-significant p-values are depicted as ns.

Results

Characterization of ActA Vaccines

To determine the adjuvant properties of ActA, the pET expression system was utilized for the production and purification of E7, ActA, and ActAE7 (Fig. 1). Proper purification of each protein was determined by SDS-PAGE analysis followed by Coomassie staining (Fig. 1A). While all preparations appear to be very pure, the ActAE7 preparation has a faint band corresponding to non-fused ActA. We have previously observed a similar phenomenon where similar fusion proteins partially dissociate upon secretion by *Listeria* [data not shown].

Since the nature of the experiments necessitated that most endotoxins were eliminated, purified proteins were detoxified at least twice with Norgen ProteoSpin columns (Norgen Biotek Corporation). Detoxified proteins were then subjected to SDS-PAGE analysis and Western blotting with anti-LPS core antibody. As shown, in Figure 1B, LPS is detected in both ActA preparations prior to detoxification but is completely undetectable after detoxification.

Listeria-secreted ActA is highly efficient at nucleating filamentous actin in the cytosol of infected host cells, it was also important to determine if our purified ActA protein would affect an important actin-dependent cellular process, phagocytosis. Phagocytosis is the process in which foreign antigens are engulfed and processed by antigen-presenting cells and its impairment would likely inhibit uptake of a target antigen and, subsequently, anti-tumor immune responses. However, as seen in Figure 1C, incubation of purified ActA protein along with the J774A.1 murine macrophage-like cell line did not significantly affect the ability of the cells to phagocytose fluorescein-labeled bioparticles in comparison to untreated cells.

ActA enhances anti-tumor efficacy against a target antigen

The efficacy of ActA as an adjuvant was assayed against a widely used tumor model for HPV16-associated cancer, TC-1 which is a transformed lung epithelial cell line immortalized by HPV16 E6 and E7 followed by transformation with the *H-ras* oncogene. The constitutive expression of HPV16 E7 by TC-1 allows for effective and targeted tumor immunotherapy in both a therapeutic and preventive model of HPV-associated tumors. To

assay for the ability of ActA to enhance anti-tumor immune responses, TC-1 tumor cells were implanted s.c. in the hind flank and purified ActA and E7 proteins were administered on day 7 post-implantation either alone, mixed, or genetically fused. Vaccinated mice were then boosted on day 14 post-implantation and tumor volume measured throughout. Shortly after vaccination with either the ActA + E7 mix or the ActAE7 fusion protein, reduced growth or regression of tumors was observed. However, vaccination with either E7 protein or ActA protein alone had little effect on tumor growth. Additionally, over half of the mice in the regimens that included both ActA and E7 were tumor-free by completion of the experiment, whereas no mice treated with E7 alone and only one mouse treated with ActA alone were tumor free. In addition, mice tolerated the vaccines well with no observable adverse symptoms. To assess whether ActA + E7 regimens lead to the creation of central memory T cells and long-term immunity, tumor-free mice from the previous experiment were re-challenged two months after initial tumor implantation with TC-1 tumor cells and tumor outgrowth was monitored. All TC-1 rechallenged mice remained tumor-free while unvaccinated and tumor-naïve mice, implanted with the same TC-1 cells, grew tumors as expected with the same TC-1 cells (data not shown), suggesting that ActA can stimulate an effective long-lasting immune response to a targeted antigen. In addition to augmenting therapeutic responses against a primary tumor, ActA was also an effective adjuvant in a model of metastatic disease. After intravenous tail vein injection of TC-1 cells, mice were subsequently vaccinated twice and lungs removed on day 30 post-implantation. Examination of the lungs for TC-1 revealed that vaccination with ActA+E7 and ActAE7 significantly reduced ($p=0.001$ and $p=0.0002$, respectively) the number of surface lung metastases (1.6 ± 0.5 metastases/lung and 0.6 ± 0.2 metastases/lung, respectively) detectable at day 30 in comparison to vaccination with E7 alone (7.4 ± 1 metastases/lung) (Fig. 2C).

ActA facilitates a CD8⁺ cell dependent anti-tumor immune response

Evidence suggests that the most effective anti-tumor immune responses are dependent upon a strong CD8⁺ T cell response against the tumor [as reviewed in 27]. To determine the importance of the CD8⁺ T cell response after ActA + E7 vaccination, mice were implanted with TC-1 tumor cells and depleted of CD8⁺ cells. Subsequently, depleted and non-depleted control mice were vaccinated with either of the ActA + E7 vaccine regimens. While vaccination with ActA protein along with E7 protein led to complete tumor regression in 62.5% of mice 42 days after tumor implantation, depletion of CD8⁺ cells resulted in rapid tumor outgrowth in all mice. Similar results were observed after vaccination with ActA fused to E7, wherein, CD8⁺ cell depletion resulted in the rapid outgrowth of tumors (Fig. 3).

ActA vaccines augment E7-specific IFN- γ production by CD8⁺ T cells in spleens and tumors

Previous work demonstrated that a *Listeria*-based vaccine secreting ActA fused to E7 can lead to elevated levels of E7-specific CTLs in the spleen and tumor [17,18]. As seen in Figure 4A and 4B, the ActA+E7 and ActAE7 fusion protein vaccinations both resulted in an increased percentage of E7-specific CTLs in the spleen. In addition, as seen in Figure 5B, the mean percentage of tumor infiltrating activated E7-specific CD8⁺ T cells was highly elevated in the tumors of ActAE7 and ActA plus E7 vaccinated mice in comparison to control vaccinated mice. While the increased presence of E7-specific CD8⁺ T cells have

been a good prognostic for vaccine efficacy in previous studies [8], we wanted to examine the functionality of these antigen-specific T cell by secretion of IFN- γ upon antigenic stimulation. To determine the number of E7-specific IFN- γ secreting CD8⁺ T cells from the spleens and tumors of vaccinated mice, ELISpots on single cell suspensions from spleens or tumors were performed with or without an E7-specific H2b-restricted CTL peptide, RAHYNIVTF, to induce IFN- γ secretion. A significant increase in the amount of E7-specific IFN- γ secreting CD8⁺ splenocytes was observed after vaccination with E7 (76 ± 4 SFC/ 5×10^5 cells), ActA + E7 (196 ± 7.5 SFC/ 5×10^5 cells), and ActAE7 (211 ± 4 SFC/ 5×10^5 cells) in comparison to naïve (62 ± 7.2 SFC/ 5×10^5 cells) or ActA (39 ± 1.5 SFC/ 5×10^5 cells) vaccinated mice (Fig. 4C). As expected, depletion of CD4⁺ splenocytes has little effect on E7-peptide specific IFN- γ secretion suggesting that this response is mainly due to CD8⁺ splenocytes. However, vaccination with ActA along with E7, either mixed or genetically fused, resulted in approximately double the amount of E7-specific IFN- γ secreting CD8⁺ splenocytes compared to E7 alone. Similar results were observed in the tumors of vaccinated mice (Fig. 5C).

Overcoming immune tolerance to tumor antigens by ActA

Since most tumor antigens are actually self-proteins, the importance of an adjuvant to facilitate the breaking of immune tolerance is paramount [28]. In order to assay for the ability of ActA to overcome immune tolerance, we utilized the E6/E7 transgenic mouse on the C57BL/6 background along with s.c. implanted TC-1 tumors. As in the wild-type mouse, vaccination of mice with ActA+E7 and ActAE7 was able to facilitate an effective therapeutic anti-tumor response against implanted TC-1 tumors in the E6/E7 transgenic mouse. While the E7 vaccination alone reduced tumor growth slightly over the course of the experiment, effective tumor regression was only observed in the ActA+E7 and ActAE7 vaccinated mice. In fact, the ActA+E7 and ActAE7 vaccine regimens led to complete regression of TC-1 tumors in 62.5% of mice and significant tumor reduction in the rest of the immunized mice. In addition, these therapeutic responses correlated with increased E7-specific responses in the spleen. The ActA+E7 and ActAE7 vaccine regimens induced a significant increase in numbers of E7-specific IFN- γ secreting splenocytes (68.5 ± 2 SFC/ 10^6 cells and 53.5 ± 1.9 SFC/ 10^6 cells, respectively) in comparison to E7 vaccination alone (6.5 ± 0.3 SFC/ 10^6 cells) as measured by ELISpot assay. We also observed significantly elevated percentages of activated (CD62L-) E7-tetramer positive CTLs in the spleens of ActA+E7 and ActAE7 vaccinated mice (5 ± 0.9 and 10.7 ± 1 , respectively) in comparison to E7 vaccinated mice (0.8 ± 0.2). (Fig. 6).

Discussion

In this paper we present evidence suggesting that the *Listeria*-derived virulence factor, ActA, is a novel adjuvant that can facilitate specific tumor immunotherapy. Vaccination with ActA along with a tumor-specific antigen resulted in increased levels and generation of tumor-specific CD8⁺ T cells. Ultimately, this concluded in the eradication of tumors in a CD8⁺ T-cell dependent manner after vaccination with ActA and the tumor-specific antigen.

Previously, we have demonstrated that ActA enhances anti-tumor immunogenicity when fused to a tumor-specific antigen and secreted by recombinant Lm [17,18]. We hypothesized that fusion of ActA to the antigen may provide greater immunogenicity through increased antigen presentation. This rationale is based on a report that the ubiquitin-proteasome machinery efficiently processes ActA in an N-end rule dependent manner [23]. Fusion of a tumor-specific antigen to ActA would then likely facilitate its ubiquitin-mediated degradation and presentation of its antigenic CTL peptides on Class I MHC molecules. However, results presented in this paper suggest that even though ActA fusion may facilitate increased antigen processing and presentation, it appears to only slightly augment additional anti-tumor efficacy over the non-fused ActA vaccination regimen.

This study demonstrates that ActA does not need to be secreted by *Listeria* or fused to the tumor-specific antigen in order to gain the benefits of its adjuvant properties. Similar results were reported previously with another *Listeria*-derived virulence factor, LLO, in a tumor immunotherapy setting [8]. One likely mechanism through which ActA could be facilitating anti-tumor immune responses is by acting as a pathogen-associated molecular pattern (PAMP) like other bacterial adjuvants such as CPG and LPS [16]. PAMPs are recognized by specific pathogen recognition receptors (PRRs) that activate an immunostimulatory cascade leading to the release of proinflammatory cytokines such as TNF- α and IL-12 [29-32]. These critically important cytokines facilitate the maturation of antigen-presenting cells and the activation of antigen-specific CTLs [33]. PAMPs are used widely in tumor immunotherapy to facilitate strong CD8-mediated anti-tumor immune responses similar to those induced by ActA vaccination [16]. We are currently investigating whether ActA has PAMP properties.

While there is no evidence to suggest that actin-nucleation factors alone and/or added extracellularly can facilitate immune responses, we cannot completely eliminate that this function of ActA plays a role in our vaccines. The ActA utilized in our vaccines contains all three of the reported actin nucleation sites [34]. In addition, actin remodeling plays a critical role in dendritic cell phagocytosis and T cell activation during an immune response [35,36]. While we demonstrate no adverse or enhanced phagocytosis by a macrophage-like cell line after ActA treatment, further investigation is required to determine the importance of actin-nucleation by ActA as a mechanism for adjuvancy in tumor immunotherapy.

With the data presented in this paper, we have characterized a novel adjuvant for tumor immunotherapy, ActA. While the molecular mechanisms governing its adjuvant activity require further investigation, its ability to stimulate an effective CD8-mediated anti-tumor immune response capable of overcoming tolerance make ActA an attractive option for future therapies. In addition, the ability of ActA to function as an adjuvant without fusion demonstrates versatility in its ability to be rapidly applied in the treatment of various malignancies in the clinic.

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Fig. 1.

Characterization of ActA vaccines; **a** Purified proteins used for this study were subjected to SDS-PAGE analysis on a discontinuous polyacrylamide gel and then subsequently stained with Coomassie stain. A graphical depiction of each protein utilized in this study is depicted alongside their respective band; **b** Purified proteins were subjected to SDS-PAGE analysis before and after endotoxin removal along with LPS as a positive control. A Western blot with α -LPS antibody was performed to demonstrate proper elimination of endotoxin from purified proteins; **c** Purified and detoxified ActA protein was incubated with the murine macrophage-like cell line, J774, and phagocytosis was assayed with the Vybrant Phagocytosis Assay Kit (Invitrogen). Phagocytosis of GFP-labeled heat-killed *E.coli* was measured by detection of emission at 535nm

Fig. 2. ActA enhances anti-tumor efficacy against target antigen; **a** C57BL/6 mice were implanted with TC-1 tumors and subsequently vaccinated on Day 7 and 14 with each protein vaccine (n=8). Tumor diameters were measured every 3 days and tumor volumes calculated with those values; **b** Mean percentage of mice tumor-free from three separate experiments after implantation and vaccination with the protein vaccines along with the mean percentage of tumor-free mice after TC-1 rechallenge is depicted; **c** C57BL/6 mice were injected intravenously with TC-1 tumor cells followed by vaccination. Lungs were subsequently removed and visible surface lung metastases were counted

Fig. 3.

ActA facilitates a CD8⁺ cell dependent anti-tumor immune response; **a** C57BL/6 mice were implanted with TC-1 tumors and subsequently vaccinated on Day 7 and 14 with ActA + E7 alone or along with administration of control or anti-CD8 antibody throughout the experiment (n=8). Tumor diameters were measured every 3 days and tumor volumes calculated with those values; **b** The same experiment was performed concurrently with ActAE7 alone or along with administration of control or anti-CD8 antibody throughout the experiment (n=8)

Fig. 4.

Augmentation of E7-specific IFN- γ production by CD8⁺ cells in the spleen with ActA vaccines; C57BL/6 mice were vaccinated and boosted seven days later with each protein vaccine. Subsequently, spleens were processed for E7-specific immune responses; **a** A representative FACS plot depicting E7-tetramer⁺ CD8⁺ CD62L⁻ T cells in the spleen after vaccination with E7, ActA, ActA + E7 and ActAE7; **b** The mean percentage of E7-tetramer⁺ CD8⁺ CD62L⁻ T cells in the spleen after vaccination as determined by FACS analysis (n=3); **c** Total or anti-CD4 antibody depleted splenocytes of vaccinated mice were stimulated *in vitro* with an E7-specific MHC-I peptide and the number of IFN- γ secreting cells determined by ELISpot analysis (n=3)

Fig. 5.

Increased E7-specific CD8⁺ T-cells in the tumor with ActA vaccines; C57BL/6 mice were implanted s.c. with TC-1 tumor cells and vaccinated on day 7 and 14 with each protein vaccine and subsequently tumors were processed for E7-specific immune responses by TILs; **a** A representative FACS plot depicting E7-tetramer⁺ CD8⁺ CD62L⁻ T cells in TC-1 tumors after vaccination with E7, ActA, ActA + E7 and ActAE7; **b** The mean percentage of E7-tetramer⁺ CD8⁺ CD62L⁻ T cells in TC-1 tumors after tumor-implantation and vaccination from three separate experiments as determined by FACS analysis (n=3); **c** Total or anti-CD4 antibody depleted TC-1 tumor cells of vaccinated mice were stimulated *in vitro* with an E7-specific MHC-I peptide and the number of IFN- γ secreting cells determined by ELISpot analysis (n=3)

Fig. 6.

Overcoming Tolerance in an E6/E7 Transgenic mouse with ActA vaccines; HPV16 E6/E7 transgenic mouse on the C57BL/6 background were implanted with TC-1 tumors and subsequently vaccinated on Day 7 and 14 with each protein vaccine; **a** Tumor diameters were measured every 3 days and tumor volumes calculated with those values (n=8); **b** Splenocytes from tumor-implanted and vaccinated mice were stimulated *in vitro* with an E7-specific MHC-I peptide and the number of IFN- γ secreting cells determined by ELISpot analysis (n=3); **c** The mean percentage of E7-tetramer⁺ CD8⁺ CD62L⁻ T cells in the spleens of tumor-implanted and vaccinated transgenic mice as determined by FACS analysis (n=3)