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Candida skin test reagent as a novel adjuvant for a human papillomavirus peptide-based therapeutic vaccine

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ABSTRACT

A vaccine adjuvant that can effectively promote cell-mediated immunity is currently not available. Because of the ability of a *Candida* skin test reagent injection to induce common wart regression, our group is using it as a novel adjuvant in a clinical trial of a peptide-based human papillomavirus therapeutic vaccine. The goal of this current study was to investigate the mechanisms of how *Candida* enhances the vaccine immune responses. Maturation effects on Langerhans cells, capacity to proliferate T-cells, expression of cytokines and pattern recognition receptors by Langerhans cells, and ability to induce Th1, Th2, and Th17 responses were investigated in healthy subjects. The vaccine, human papillomavirus peptides with *Candida*, demonstrated partial maturation effects on Langerhans cells indicated by significantly up-regulated CD40 ($p = 0.00007$) and CD80 ($p < 0.00001$) levels, and showed T-cell proliferative capacity ($p < 0.00001$) when presented by Langerhans cells in vitro. Interestingly, the maturation effects were due to the peptides while *Candida* was responsible for the T-cell proliferation. The cytokine profile (IL-1β, IL-6, IL-8, IL-10, IL-12p40, IL-23Ap19, IFN-γ and TNF-α) of Langerhans cells treated with the vaccine or *Candida* alone showed that IL-12p40 mRNA was most frequently induced, and IL-12p70 protein was detected in the supernatants. The presence of pattern recognition receptors known to associate with *Candida albicans* (DC-SIGN, dectin-1, dectin-2, galectin-3, mincle, mannose receptor, Toll-like receptors-1, 2, 4, 6 and 9) were demonstrated in all subjects. On the other hand, the induction of Th1 response demonstrated by IFN-γ secretion by CD4 cells stimulated with the vaccine or *Candida* pulsed Langerhans cells was demonstrated only in one subject. In summary, the Langerhans cell maturation effects of the vaccine were due to the peptides while the T-cell proliferative capacity was derived from *Candida*, and the most frequently induced cytokine was IL-12.

1. Introduction

The most widely used adjuvant in approved human vaccines is an alum-based adjuvant that has been shown to elicit a predominantly Th2 immune response [1]. Therefore, the alum-based adjuvant would be useful in a vaccine designed to boost antibody responses, but not for a vaccine designed to stimulate cellular immune responses. Since successful clearance of human papillomavirus (HPV) infection is believed to be induced by cell-mediated immunity [2,3], an adjuvant that would promote such an immunity is necessary, but not available.

Our group and others have shown that serial intra-lesional injections of common warts with skin testing reagents such as *Candida*, mumps, and/or *Trichophyton* can induce regression not only of treated warts but also of distant untreated warts [4–9]. In a Phase I clinical trial (NCT00569231), our group used Candin®

Abbreviations: APCs, antigen presenting cells; HPV, human papillomavirus; LCs, Langerhans cells; MFI, mean fluorescence intensity; PAMPs, pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; qRT-PCR, quantitative real-time PCR; PRRs, pattern recognition receptors.

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(Allermed, San Diego, CA), a colorless extract of *Candida albicans*, to treat common warts. Resolution of treated warts occurred in 82% of the subjects, and anti-HPV T-cell responses were demonstrated [8]. Given that Candin is derived from *C. albicans*, it should contain numerous pathogen-associated molecular patterns (PAMPs). We hypothesized that Candin would be an effective vaccine adjuvant which would stimulate multiple pattern recognition receptors (PRRs) and induce innate as well as adaptive immunity.

Cervical cancer is almost always caused by high-risk HPV infection, and is the 2nd most common cancer among women in the world. Two very effective prophylactic HPV vaccines, Gardasil® (Merck, NJ, USA) and Cervarix® (GlaxoSmithKline, Middlesex, UK), are available, and they work by inducing high titers of neutralizing antibody [10–12]. However, they are not effective for women with pre-existing HPV infection [10,12,13]. Therefore, a therapeutic HPV vaccine that can be used for those already infected with HPV and/or have developed HPV-associated neoplasia is not available. Our group studied naturally induced immunity in women with HPV infection and/or cervical lesions, and have found that the ability to induce T-cell responses against E6, one of the oncoproteins of high-risk HPVs, is associated with HPV clearance and regression of cervical lesions [3,14,15]. Therefore, we designed an HPV therapeutic vaccine which consists of four HPV type 16 E6 peptides and Candin, and are conducting a Phase I clinical trial (NCT01653249).

In the current study, we examined the immune enhancing effects of Candin as a vaccine adjuvant. Surprisingly, the E6 peptides were responsible for the partial maturation of Langerhans cells (LCs) while Candin was responsible for the T-cell proliferative effects. The most commonly induced cytokine by the LCs was IL-12.

2. Materials and methods

2.1. Generation of monocytes-derived LCs

Mononuclear cells were collected from healthy blood donors ($n = 10$) by apheresis (Key Biologics, LLC, Memphis, TN). The subjects were numbered in a chronological order. Peripheral blood mononuclear cells (PBMCs) were purified using the ficoll gradient centrifugation method. Monocytes were negatively isolated from PBMC using Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, CA), and were converted to LCs using granulocyte-macrophage colony-stimulating factor, IL-4, and transforming growth factor β-1 as described by Fahey et al. [17]. The effectiveness of conversion to LCs was demonstrated by detecting CD1a (eBioscience, San Diego, CA), Langerin (Beckman-Coulter, Brea, CA), and E-cadherin (eBioscience) using FACS Fortessa (University of Arkansas for Medical Sciences Microbiology and Immunology Flow Cytometry Core Laboratory) and CellQuest Pro software (BD Biosciences, San Jose, CA) in selected experiments (Fig. 1). Sufficient number of cells were available from all subjects except for subject 1 in whom the LC maturation experiment could not be performed.

2.2. Maturation analysis of LCs treated with Candin and/or HPV peptides

Candin was dialyzed before use to remove a small amount of solvent (0.4% phenol) using Slide-A-Lyzer G2 Dialysis Cassette (Thermo Scientific, Rockford, IL). LCs were prepared as described above, and one million LCs each were treated with Candin (150 μl/ml), four current good manufacturing practice-grade HPV16 E6 peptides [E6 1-45, E6 46-80, E6 81-115, and E6 116-158 (referred to as "peptides" hereafter); 10 μg/ml/peptide; made by CPC Scientific, Sunnyvale, CA and vialed by Integrity Bio, Camarillo, CA], or Candin/"peptides". Zymosan (10 μg/ml, InvivoGen, San Diego, CA), a yeast cell wall particle containing many polysaccharides including β-glucan and mannan [18], was used as a positive control. After 48 h incubation, cells were stained with anti-human CD40 phycoerythrin (PE)-Cy5.5, CD80 fluorescein isothiocyanate, CD86 PE-Cy5 and HLA-DR PE (eBioscience, San Diego, CA). Ten thousand events were acquired, and the data were analyzed using Flowjo software (BD Biosciences).

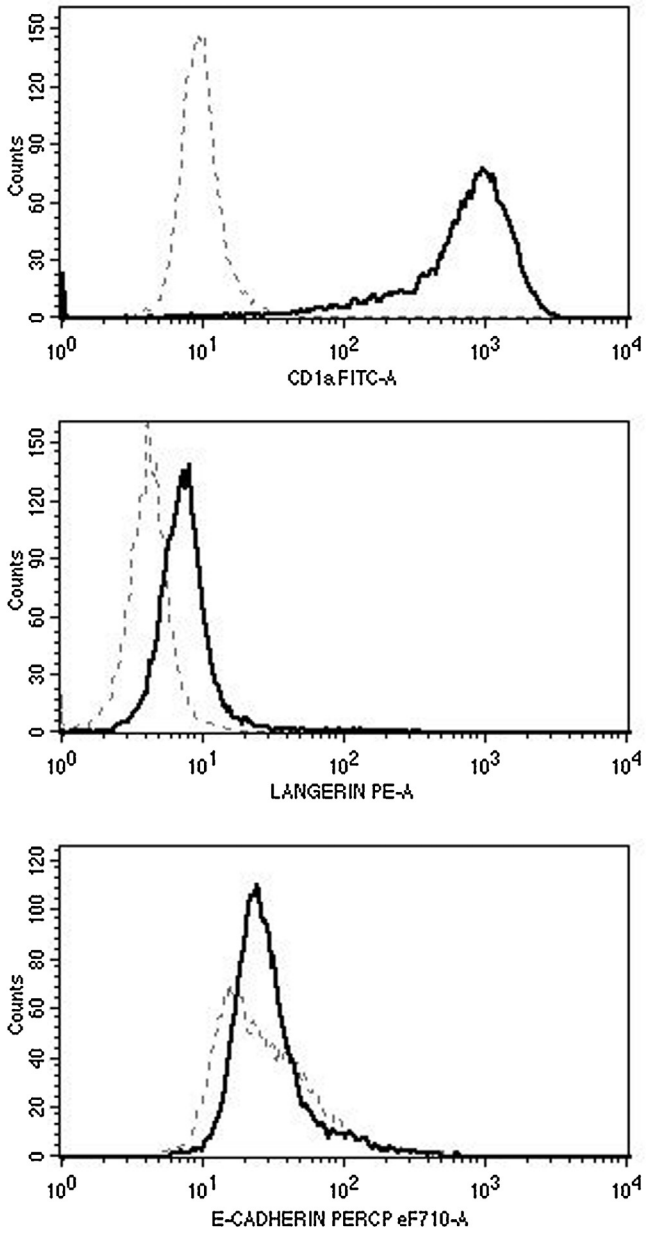

Fig. 1. Surface expressions of CD1a (top), Langerin (middle), and E-cadherin (bottom) show successful conversion to LCs (solid lines). The dotted lines represent the relevant isotype controls.

2.3. Analysis of T cell proliferation induced by LCs treated with Candin and/or "peptides"

On day 7 of LCs conversion, CD3 T cells from the same subjects were negatively isolated from PBMCs using Pan T-Cell Isolation Kit II (Miltenyi Biotec). To remove CD25 regulatory T cells, human CD25 Antibody-Biotin (Miltenyi Biotec) was added. T cell proliferation assay was performed in 6 replicate wells by co-culturing T cells (1.5×10^6 cells/ml) with autologous LCs (3×10^4 cells/ml) in