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Activation of dendritic cell function by soypeptide lunasin as a novel vaccine adjuvant

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ABSTRACT

The addition of an appropriate adjuvant that activates the innate immunity is essential to subsequent development of the adaptive immunity specific to the vaccine antigens. Thus, any innovation capable of improving the immune responses may lead to a more efficacious vaccine. We recently identified a novel immune modulator using a naturally occurring seed peptide called lunasin. Lunasin was originally isolated from soybeans, and it is a small peptide containing 43 amino acids. Our studies revealed stimulatory effects of lunasin on innate immune cells by regulating expression of a number of genes that are important for immune responses. The objective was to define the effectiveness of lunasin as an adjuvant that enhances immune responses. The immune modulating functions of lunasin were characterized in dendritic cells (DCs) from human peripheral blood mononuclear cells (PBMCs). Lunasin-treated conventional DCs (cDCs) not only expressed elevated levels of co-stimulatory molecules (CD86, CD40) but also exhibited up-regulation of cytokines (*IL1B*, *IL6*) and chemokines (*CCL3*, *CCL4*). Lunasin-treated cDCs induced higher proliferation of allogeneic CD4+ T cells when comparing with medium control treatment in the mixed leukocyte reaction (MLR). Immunization of mice with ovalbumin (OVA) and lunasin inhibited the growth of OVA-expressing A20 B-lymphomas, which was correlated with OVA-specific CD8+ T cells. In addition, lunasin was an effective adjuvant for immunization with OVA, which together improved animal survival against lethal challenge with influenza virus expressing the MHC class I OVA peptide SIINFEKL (PR8-OTI). These results suggest that lunasin may function as a vaccine adjuvant by promoting DC maturation, which in turn enhances the development of protective immune responses to the vaccine antigens.

1. Introduction

Dendritic cells (DCs) comprise an integral part of the innate immunity that induces the activation of naïve T lymphocytes essential for the adaptive immune responses. DCs process antigens to yield peptides that are presented to T cell receptor (TCR) in the context of major histocompatibility complex (MHC) molecules. Successful priming of naïve T cells also requires strong stimulation upon binding to co-stimulatory molecules expressed by matured DCs. DC maturation can be accomplished by a variety of stimulating agents including microbial products, which bind to various pattern recognition receptors (PRRs) [1]. This binding stimulates the signaling pathways that regulate expression of target genes involved in DC maturation. Thus, an immune stimulating agent or adjuvant that induces DC maturation will ultimately lead to activation of antigen-specific T cells in vaccination.

Conventional DCs (cDCs) express Toll-Like Receptor 4 (TLR4) that responds to lipopolysaccharide (LPS)-based adjuvants [2]. However, TLR4 is expressed at variable levels among different individuals [3]. Individuals with low TLR4 expression may not

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be efficiently primed following vaccination with the adjuvant Monophosphoryl Lipid A (MPL), a derivative of LPS. In addition, patients with immune dysfunctions or in a disease setting may be refractory to stimulation with TLR agonists due to down-regulation of these receptors [4–6]. Despite the effectiveness of current adjuvant with the TLR4 agonist MPL [7], development of novel immunostimulatory agents that overcome the above limitations may offer alternatives to activate DCs for improved vaccine efficacy.

We recently identified a novel function of soypeptide lunasin as an immune modulating agent that exerts potent synergistic effects with IL-12 or IL-2 on augmenting IFNγ and granzyme B expression by natural killer (NK) cells [8]. Lunasin was originally isolated from soybeans, and is a naturally occurring peptide containing 43 amino acids. This peptide exhibits properties that have multiple health benefits, and is a promising chemopreventive agent [9–12]. To further define lunasin's potential as an adjuvant, the immune modulating functions of lunasin were established with DCs. Using purified human DCs from peripheral blood mononuclear cells (PBMCs), lunasin's stimulatory effects on the expression of genes important for DC maturation such as cytokines, chemokines, and co-stimulatory molecules were demonstrated. The in vivo effectiveness of lunasin as an adjuvant for a model antigen ovalbumin (OVA) was assessed in the prevention model against the A20-OVA B-lymphoma as well as the PR8-OTI influenza virus. Together, these studies demonstrate the immune modulating effects of lunasin on DC maturation, suggesting its potential as a vaccine adjuvant to enhance the immune responses against the vaccine antigens.

2. Materials and methods

2.1. Antibodies, lunasin peptide, and other reagents

Fluorochrome-conjugated monoclonal antibodies to human CD1c, CD86, CD40, and to mouse CD11c, B220, CD86, were obtained from BD Biosciences (San Jose, CA). Ficoll-Paque™ PLUS was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ). The lunasin peptide with 43-amino acid was chemically synthesized with 97% purity by LifeTein (South Plainfield, NJ) as previously described [8]. Carboxyfluorescein diacetate succinimidyl ester (CFSE) was from Guava Technologies (Hayward, CA). Lipopolysaccharide (LPS from *Escherichia coli* 0111:B4) and Concanavalin A (Con A) were from Sigma–Aldrich (St. Louis, MO). Ovalbumin (OVA, chromatographically purified) was from Worthington Biochemical Corp (Lakewood, NJ). Imiquimod (R837), Alum (Alhydrogel 2%), and polymyxin B were from InvivoGen (San Diego, CA).

2.2. Human blood samples, primary immune cells, cell line, and virus

Healthy human blood samples were procured from the Indiana Blood Center (Indianapolis, IN). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque™ PLUS, and aliquots of PBMCs were cryopreserved in liquid nitrogen. Human primary conventional dendritic cells (cDCs) were negative selected from PBMCs using a CD1c (BDCA-1) Dendritic Cell Isolation Kit with ~90% of purity (Miltenyi Biotec Inc., Auburn CA). Human primary natural killer (NK) cells were isolated from PBMCs using a negative selection kit with ~95% of purity (Miltenyi Biotech). Human plasmacytoid DCs (pDCs) were positively selected from PBMCs using BDCA-4 magnetic beads with ~60% of purity (Miltenyi Biotech). Total CD4+ T cells were positively selected from PBMCs using CD4 magnetic beads with ~95% of purity (Miltenyi Biotech). A murine B-lymphoma cell line expressing OVA, A20-OVA (H-2^d) was kindly provided by Dr. Gang Zhou (Medical College of Georgia) [13]. Influenza virus expressing the MHC class I OVA peptide SIINFEKL (PR8-OTI) was generated previously [14].

2.3. Characterization of human innate immune cells following stimulation in vitro

Freshly purified human immune cells (cDCs, pDCs, and NK cells) were stimulated for 1 day as indicated. The expression levels of co-stimulatory molecules (CD86 and CD40) on cDCs were evaluated using flow cytometry with staining antibodies. For analysis of gene expression, the cell pellets following 1 day of stimulation were resuspended in Trizol Reagent for total RNA extraction. The first-strand cDNA was synthesized followed by real time qPCR using Taqman Assay with primers for IL-1β (*IL1B*), IL-6 (*IL6*), chemokine (C—C motif) ligand 3 (*CCL3*), CCL4 (*CCL4*), TNFα (*TNFA*), and *ACTB* (β-actin) as endogenous control [15]. The supernatants collected from the cultures following 1 day of stimulation were analyzed for the production of CCL3 and TNFα using ELISA [16]. In some experiments, polymyxin B or vehicle control was added into the DC cultures to block LPS-mediated effects [17].

2.4. In vivo administration of lunasin

BALB/c mice were intraperitoneally (IP) injected with PBS (–) or lunasin at 0.4 or 4 mg/kg body weight. Mice were sacrificed and spleens were collected for analysis 18 h following injection. Splenocytes from these mice were surface stained with B220, CD11c, and CD86 monoclonal antibodies. The expression levels of CD86 were analyzed on DCs gated on CD11c and B220 populations using flow cytometry.

2.5. Allogeneic mixed leukocyte reaction (MLR)

Purified cDCs were stimulated as indicated for 1 day and washed prior to co-culture with T cells. Total CD4+ T cells purified from a different donor were stained with CFSE. Treated cDCs were co-cultured with CFSE-labeled allogeneic CD4+ T cells for 5–7 days. Proliferation of alloreactive CD4+ T cells was determined from dilution of CFSE using flow cytometry.

2.6. Immunization studies

BALB/c (H-2^d) or C57BL/6 (H-2^b) mice were IP injected twice with PBS or OVA (100 μg) mixed with Alum (Alhydrogel 2%) or lunasin at the dose indicated on days 1 and 7. In the syngeneic B-lymphoma model, immunized BALB/c mice received subcutaneous challenge with 1×10^6 A20-OVA (H-2^d) 7 days following the last immunization. Tumor volumes were measured as described [8] from day 15 after tumor injection through day 22. Splenocytes processed from these mice were labeled with CFSE, and then cultured in vitro with OVA (100 μg/ml) for 5 days. Proliferation of OVA-specific CD8+ T cells was determined from diluted CFSE using flow cytometry. In the influenza model, immunized C57BL/6 mice were intranasally challenged with a lethal dose of live PR8-OTI at 700 pfu 14 days following the last immunization [14]. Animal survival and body weight was monitored daily for 18 days following challenge.

2.7. Statistical analysis

SAS/STAT (SAS Institute Inc, Cary, NC) was used to analyze the data. A mixed model was developed for analyzing the data with within-subject treatments, and the pairwise comparisons among the treatments were performed to determine the *P* values.

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