Keyhole Limpet Hemocyanin (KLH) is a heavily glycosylated protein that can exist as 8MDa cylinders composed of 20 copies (didecamer) of a 400KDa subunit, or as separate subunits. KLH is a well-established immune stimulant, hapten carrier and immunotherapy vaccine component. However, literature reports cite dramatically different immune responses to different preparations of KLH.

We are developing functional assays to investigate the immunogenicity of different forms and preparations of KLH with the goal of improving the predictive outcome of its use as vaccine carrier or diagnostic agent for both research and clinical studies.

Dendritic cells (DCs) derived from mouse bone marrow mononuclear cells, human peripheral blood mononuclear cells, mouse DC line JAW9, as well as human leukemia lines THP-1 and KG-1, were treated with different forms of KLH. To compare the efficiency of DCs to stimulate KLH-specific adaptive immune responses, KLH treated primary mouse DCs or JAWSII cells were used to activate T cells extracted from spleens of mice that were immunized with KLH. DC antigen uptake efficiency, DC maturation, T cell proliferation and cytokine secretion were compared. Ovalbumin was conjugated to different forms of KLH. DCs derived from mouse bone marrow mononuclear cells were treated with the KLH-Ovalbumin conjugates for antigen uptake efficiency and ovalbumin-specific T cell activation studies.

We found that didecameric KLH and subunit KLH have significantly different properties. We report here the results of these studies which suggest the need for methods to identify appropriate forms of KLH when used as hapten carrier or immunogen.

### Methods

**KLH-FITC and KLH-SMPH-OVA-FITC Preparation**

KLH-FITC was prepared by reacting KLH directly with FITC (Brevi, USA). Antigen-ovalbumin (OVA), was conjugated to KLH using heterofunctional linker (SMPH) through two-step procedure. Final concentration of the conjugates was determined by Bradford protein assay.

**Dendritic Cell Uptake Assay with KLH-FITC and KLH-SMPH-OVA-FITC**

Interleukin-1α stained DC line JAWSII cells and immature DCs of DC-like cells derived from human PBMC and human leukemia lines KG-1 and THP-1 were pulsed with KLH-FITC and KLH-SMPH-OVA-FITC for 24 h. The uptake of KLH-FITC and KLH-SMPH-OVA-FITC taken up by cells was determined by measuring the fluorescence intensity of KLH conjugates in cell culture supernatants and standard curve built with known concentrations of the conjugates.

**Stellar Subunit KLH Induces Mild Secretion of Inflammatory Cytokines by Dendritic Cells**

KLH-SMPH-OVA-FITC was pulsed with BSA (negative control), BSA-Antigen X conjugate and KLH respectively for 24 h before mixing with DC4 T cells. Cytokine amount in cell culture supernatant was determined with MILLIPLEX Map kit using a mouse TNF cytokine panel on a Luminex Multiplexing instrument with MAGPIX system.

**Stellar Subunit KLH is a Good Carrier for Carbohydrate Antigen**

SuKLH was conjugated with a carbohydrate antigen (Antigen X). CD4 T cells extracted from spleens of mice vaccinated with SuKLH-Antigen X were reacted with DCs generated from bone marrow harvested from immune-history-free mice. DCs were pulsed with BSA (negative control), BSA-Antigen X conjugate and SuKLH respectively for 24 h before mixing with CD4 T cells. Cytokine amount in cell culture supernatant was determined with MILLIPLEX Map kit using a mouse TNF cytokine panel on a Luminex Multiplexing instrument with MAGPIX system.

### Results

Four different KLH (from three commercial sources) were evaluated – Stellar Biotechnologies’ Subunit KLH (Stellar SuKLH), Stellar Biotechnologies’ HMW KLH (Stellar HMW KLH), Source 2 Subunit KLH (Source 2 SuKLH), and Source 3 HMW KLH. All experiments were performed three times in triplicates. Standard errors are shown.

### Abstract

Keyhole Limpet Hemocyanin (KLH) is a heavily glycosylated protein that can exist as 8MDa cylinders composed of 20 copies (didecamer) of a 400KDa subunit, or as separate subunits. KLH is a well-established immune stimulant, hapten carrier and immunotherapy vaccine component. However, literature reports cite dramatically different immune responses to different preparations of KLH. We are developing functional assays to investigate the immunogenicity of different forms and preparations of KLH with the goal of improving the predictive outcome of its use as vaccine carrier or diagnostic agent for both research and clinical studies.

Dendritic cells (DCs) derived from mouse bone marrow mononuclear cells, human peripheral blood mononuclear cells, mouse DC line JAWSII, as well as human leukemia lines THP-1 and KG-1, were treated with different forms of KLH. To compare the efficiency of DCs to stimulate KLH-specific adaptive immune responses, KLH treated primary mouse DCs or JAWSII cells were used to activate T cells extracted from spleens of mice that were immunized with KLH. DC antigen uptake efficiency, DC maturation, T cell proliferation and cytokine secretion were compared. Ovalbumin was conjugated to different forms of KLH. DCs derived from mouse bone marrow mononuclear cells were treated with the KLH-Ovalbumin conjugates for antigen uptake efficiency and ovalbumin-specific T cell activation studies.

We found that didecameric KLH and subunit KLH have significantly different properties. We report here the results of these studies which suggest the need for methods to identify appropriate forms of KLH when used as hapten carrier or immunogen.

### Summary

1. Both subunit KLH (SuKLH) and high molecular weight KLH (HMW KLH) can be easily conjugated with antigens, suggesting that both have the potential for being good antigen carriers.
2. All types of DCs (mouse DC line, human leukemia line-derived DC-like cells and human PBMC-derived DC) uptake antigen conjugates built with Stellar suKLH and HMW KLH more efficiently than that built with KLH from other sources. It is worthy to mention that only limited lots of KLH samples from other sources were tested.
3. When compared to LPS, KLH only induced mild inflammatory cytokine release, demonstrating the safety of KLH as a potential vaccine carrier.
4. Besides its well accepted role as a good protein antigen carrier for vaccine development, Stellar suKLH might be a good carrier for carbohydrate antigens for vaccine development.

### About KLH

Keyhole Limpet Hemocyanin (KLH) exists as cylinder-shape didecamer (20-mer) (side and top view below), which can dissociate into monomers (KLH subunits or SuKLH). The subunit isoforms (approx. 360-400 KDa monomeric molecular weight) are each composed of 7 or 8 functional units. This complex molecular structure can be used to generate multiple product configurations.

The Giant Keyhole Limpet (M. californica) is a scarce marine mollusk and the sole source for KLH protein. It naturally lives in the rocky shallows only along a limited stretch of Pacific Ocean coastline. Stellar Biotechnologies is the leader in sustainable manufacture of KLH.