RECOMBINANT YEAST THERAPEUTIC VACCINES EXPRESSING (HBV) X, S AND CORE ANTIGENS GENERATE ANTIGEN SPECIFIC T CELL RESPONSES IN IMMUNE SAMPLES FROM HEALTHY AND CHRONIC HBV DONORS

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Introduction

Hepatitis B virus (HBV) is the leading cause of chronic infection of the liver in the world. Worldwide, approximately 350 million people are chronic carriers of HBV, of whom more than 620,000 die from liver-related disease each year. In the United States, approximately 800,000 to 1.4 million Americans are chronic hepatitis B virus carriers, resulting in approximately 3,000 deaths annually.

Chronic HBV is characterized by suboptimal T cell responses against viral antigens (Boni et al, Gastroenterology 2012). A therapeutic vaccine capable of generating an HBV-specific T cell immune response that is administered concomitantly in patients whose disease is under virologic control with a direct acting antiviral therapy, could be an attractive clinical option. The goal of the combination therapy would be to improve the cure rate in patients with chronic HBV infection as measured by S antigen seroconversion.

Tarmogens® (targeted molecular immunogens) are recombinant, intact *Saccharomyces cerevisiae* yeast engineered to express high quantities of disease-related proteins inside the yeast cell. Tarmogens have been shown to generate robust, antigen-specific CD4⁺ and CD8⁺ T cell responses in cancer and chronic infectious diseases. Tarmogens have also demonstrated a good tolerability profile to date in numerous preclinical pharmacology studies, GLP toxicology studies, and in over 300 trial subjects in multiple oncology and hepatitis C clinical programs.

Eighteen separate HBV Tarmogen products expressing variations of HBV X, S, Pol and Core antigens were engineered and evaluated. Two lead product candidates were tested using human *ex vivo* assays on the basis of favorable antigen expression and growth rate/and or morphology. One Tarmogen contains a chimera of HBV S and Core (GI-13009 or "SCore"), and a second contains a chimera of HBV X, S and Core (GI-13020 or "X-SCore"). These Tarmogens were tested in panel of assays that were specifically designed to serve as a proxy for the immune response that would be expected in human clinical trials. The assays featured donor cells from both S antigen vaccinated healthy volunteers and a subject chronically infected with HBV.

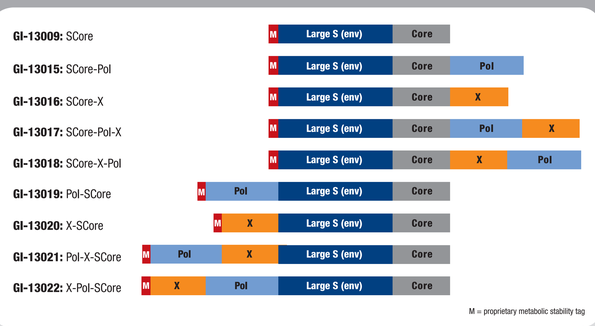
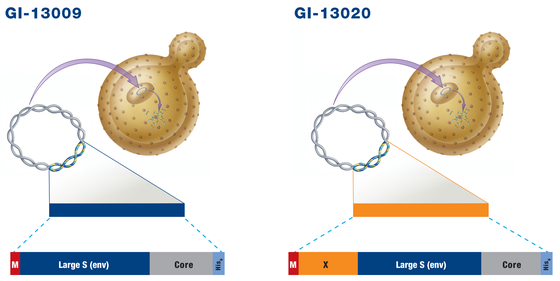


Figure 1: Evaluation of the first ten Tarmogen constructs revealed favorable protein expression, immunogenicity and growth characteristic advantages in GI-13009, a Tarmogen expressing a particular chimera of S and Core proteins "SCore". Eight additional constructs were created using the SCore chimera protein as a foundation, in order to expand the antigen repertoire of the product by attaching multiple variations of Pol and X protein. When the eight new constructs were evaluated, GI-13020, a Tarmogen expressing a chimera of HBV X, S and Core ("X-SCore") offered promising initial results. GI-13020 and GI-13009 were evaluated in multiple model systems described herein to establish proof of HBV antigen-specific immunogenicity using harvested cells from human donors.

Abbreviation	Full Name
APC	Antigen Presenting Cell
CD40L	Cluster of differentiation 40 Ligand (DC activator)
DC	Dendritic cell
ELISpot	Enzyme-linked immunosorbent spot
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
ICCS	Intracellular Cytokine Staining
IFNγ	Interferon gamma
IL prefix (eg, IL-4, IL-2)	Interleukin
LAMP1	Lysosomal-associated membrane protein 1
Peripheral Blood Mononuclear Cells	PBMCs



Three-round DC stimulation method for PBMCs

To simulate the immune processing of Tarmogen by dendritic cells (DC) and subsequent presentation to T cells via activated DCs, *ex vivo* immune experiments were performed using immune cells harvested from both healthy and chronic HBV donors.

DCs were prepared by treatment with GM-CSF & IL-4 for 5 days. Tarmogen was incubated with DCs for 48h, and the DCs were combined with T cells for 1 week to complete 1 round of stimulation; a total of 3 stimulation rounds was performed. On day 6 of the 3rd round, an additional stimulation was sometimes performed using recombinant HBV antigen, as part of ELISpot and ICCS assays.

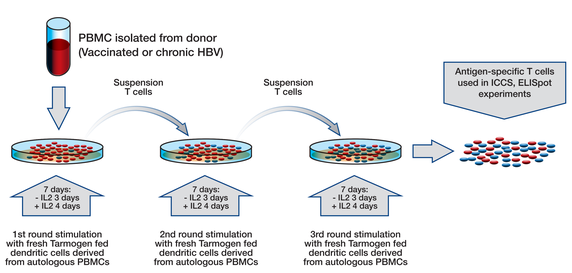


Figure 2: Tarmogen-pulsed DCs expand IFNγ⁺, S antigen-specific T cells from an Engerix-B vaccinated donor

Individuals vaccinated with the S antigen-based vaccine Engerix-B should possess S antigen-specific memory T cells. As a test of the Tarmogens' ability to activate these memory T cells, we fed DCs with SCore, X-SCore or Yvec and then used the fed ('pulsed') DCs to stimulate donor PBMCs over 3 rounds. The effector T cells were then quantified by IFNγ ELISpot assay. The results showed that S antigen-specific, IFNγ-producing T cells were indeed elicited in the assay (Fig. 2). The final stimulation with recombinant S antigen was not essential, as cultures with only the 3 round DC conditioning ("no stim") also produced the cytokine. Subsequent ICCS revealed that both CD8⁺ and CD4⁺ T cells produced IFNγ (not shown).

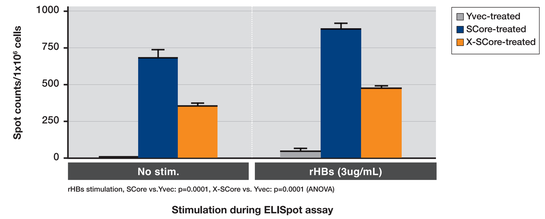
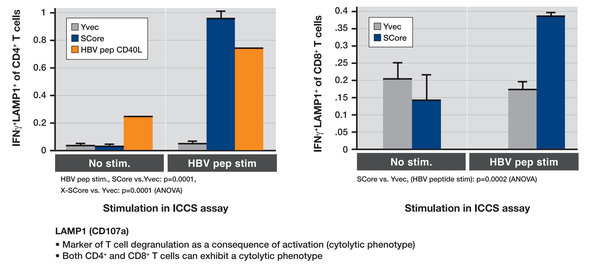


Figure 3: Tarmogen pulsed dendritic cells elicit S antigen specific cytotoxic T cells

A key goal of Tarmogen immunization is to generate HBV-specific cytotoxic T lymphocytes (CTLs) that can clear infected hepatocytes. To determine if the DC-stimulated T cells possess this cytotoxic phenotype, T cell populations were evaluated for expression of a degranulation marker (LAMP1). DC-stimulated T cells were incubated with HBV peptides, stained with dye-coupled antibodies recognizing CD4, CD8, and LAMP1, and IFNγ, and evaluated by flow cytometry. The result showed that the T cells are LAMP1 positive, indicating that SCore-pulsed DCs indeed elicit S antigen-specific CTLs. The Tarmogen effect for CD4⁺ T cells is on par with that of a known activator of DCs, CD40L.

Figure 3a: LAMP1-positivity in the CD4⁺ subset.

Figure 3b: LAMP1 positivity in the CD8⁺ subset.



LAMP1 (CD107a)
- Marker of T cell degranulation as a consequence of activation (cytolytic phenotype)
- Both CD4⁺ and CD8⁺ T cells can exhibit a cytolytic phenotype

HBV Tarmogen pulsed DCs stimulate IL-4 and IL-17 production by PBMCs of an Engerix-B-vaccinated donor

Flow cytometry based-ICCS was performed on T cells stimulated by Tarmogen-pulsed DCs to evaluate cytokine production in CD4⁺ cells. IL-4 indicates the presence of a Th2 phenotype that may promote generation of HBV-specific antibodies. IL-17 is a hallmark of Th17 cell generation, which is accompanied by a reduction in the number of regulatory T cells.

Tarmogen	% IL-4⁺ of CD4⁺ T cells	% IL-17⁺ of CD4⁺ T cells
Yvec	4.2	2.2
SCore	8.2	3.4
X-SCore	6.7	3.2

SCore vs. Yvec (IL-4): p=0.0001, X-SCore vs. Yvec (IL-4): p=0.0001, SCore vs. Yvec (IL-17): p=0.0449 (ANOVA)

Figure 4: SCore-pulsed DCs expand IFNγ⁺, HBV antigen-specific T cells from a chronic HBV donor

To test Tarmogen performance on a clinically relevant sample, the DC assay was conducted with PBMCs from a chronic HBV infected donor. Following the 3 round stimulation, cultures were incubated with recombinant S and Core antigens in an IFNγ ELISpot assay. IFNγ production was observed even without the addition of S and Core antigens, indicating the Tarmogen/DC stimulation alone drives the HBV-specific responses.

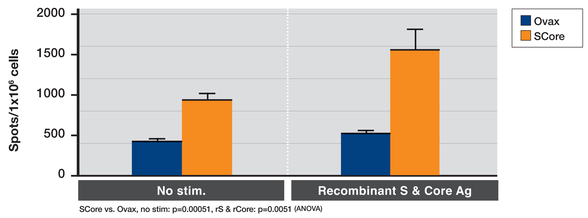


Figure 5: SCore-pulsed DCs elicit IFNγ-producing, HBV-specific CD4⁺ and CD8⁺ T cells from a chronic HBV donor

To evaluate whether the IFNγ ELISpot response of the chronic HBV donor arose from CD4⁺ or CD8⁺ T cells or both, ICCS was conducted with probes for these markers. Following 3 round DC stimulation of PBMCs with Tarmogen-pulsed DCs, T cells were stained with dye-coupled CD4 and CD8 probes and assayed for IFNγ production as in Figure 3. Both subsets produced IFNγ, consistent with the mechanism of action of Tarmogens which involves antigen presentation with class I and class II MHC.

Figure 5: Stim. of cHBV PBMCs with SCore-pulsed DCs elicits IFNg production by CD4⁺ and CD8⁺ T cells

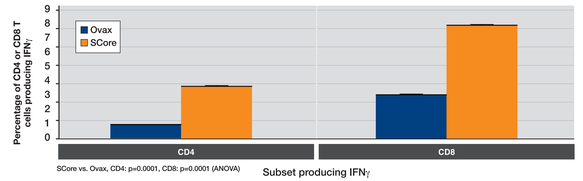


Figure 6: SCore-pulsed DCs elicit HBV antigen specific CD4⁺ and CD8⁺ T cells that exhibit a cytotoxic phenotype in chronic HBV donor cells

It is anticipated that Tarmogen-pulsed DCs will create a cytotoxic phenotype in chronic HBV T cell cultures. To test this hypothesis, ICCS was performed using HBV peptide pools. Following 3 round stimulation of T cells with Tarmogen-pulsed DCs, the cultures were incubated with HBV peptides, stained for CD4, CD8, LAMP1 and IFNγ markers, and subjected to flow cytometry. The results show that CD4⁺ (Fig. 6a) and CD8⁺ (Fig. 6b) antigen-specific T cells possess a cytotoxic phenotype, indicating that Tarmogens elicit functional responses *ex vivo* in a clinically relevant chronic HBV donor.

Figure 6a: LAMP1 staining of cHBV donor PBMCs after 3 round stimulation with SCore-pulsed DCs -CD4⁺

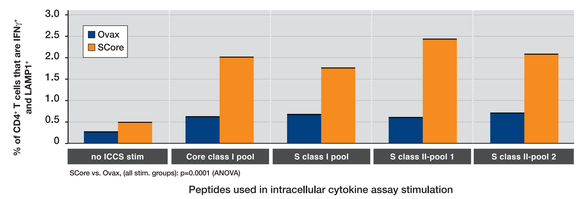
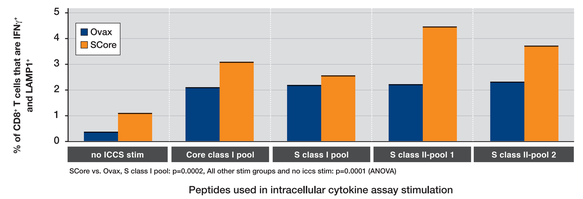


Figure 6b: LAMP1 staining of cHBV donor PBMCs after 3 round stimulation with SCore-pulsed DCs -CD8⁺



Conclusions

- Tarmogens elicit HBV-specific T cell responses in immune cells from healthy and chronic HBV volunteer donors. These data may serve as a proxy for the immune response that could be elicited in chronic HBV patients.
- DCs pulsed with HBV Tarmogens elicit effector CD4⁺ and CD8⁺ T cells following *ex vivo* stimulation of healthy and chronic HBV donor samples. A subset of these T cells possesses a cytotoxic phenotype, consistent with the anticipated effect of Tarmogen administration *in vivo*.

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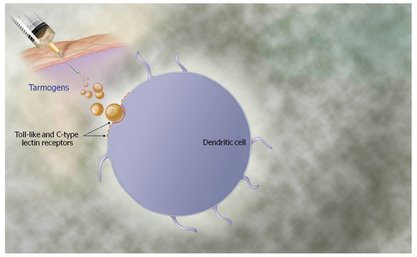
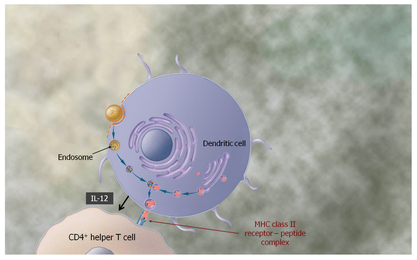
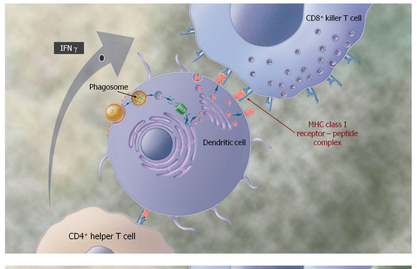
Active immunotherapy with yeast-based Tarmogens



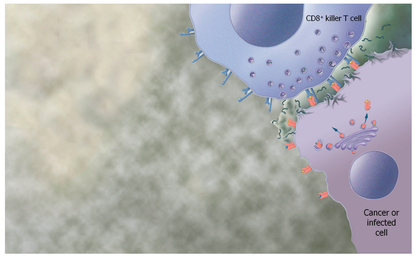
Administration of Tarmogens initially results in binding of the yeast to antigen-presenting cells, the most important of which are dendritic cells, near the injection site. The dendritic cells are activated as a result of the Tarmogens binding to Toll-like receptors and other receptor molecules on the surface of the dendritic cell, resulting in the activation of cytokine immune signaling molecules. The dendritic cell also engulfs the Tarmogen. Multiple Tarmogens may be taken up by the same dendritic cell.



The Tarmogen is processed by the dendritic cell in two ways. First, the Tarmogen is engulfed by endosomes and the protein inside the endosome is cut into shorter peptides fragments. These peptides are presented by Class II MHC molecules on the surface of the dendritic cell. In combination with IL-12, a cytokine that is produced by the dendritic cell, these MHC-peptide complexes on the surface of the dendritic cell are recognized by and activate cells involved in viral immunity called $CD4^+$ helper T cells.



Dendritic cells also process Tarmogens by engulfing them with phagosomes. This results in presentation of peptides, including the antigen from inside the Tarmogen, to $CD8^+$ killer T cells, via Class I MHC molecules on the surface of the dendritic cell, resulting in proliferation of identical antigen specific $CD8^+$ T cells. $CD4^+$ helper T cells are so named because one of their roles is to "help" activate killer T cells by expressing interferon gamma (IFNγ).



The newly activated $CD8^+$ killer T cells move throughout the body and identify any other cell that expresses the same disease protein as the one recognized by the $CD8^+$ killer T cells. Once the $CD8^+$ killer T cell finds another cell in the body containing the target protein, it can kill the cell using multiple mechanisms.

Recombinant yeast therapeutic vaccines expressing hepatitis B virus (HBV) X, S, and Core antigens generate antigen specific T cell responses in clinical immune cell samples from healthy volunteers

Purpose: Using our therapeutic vaccine platform called Tarmogens we have targeted several important clinical indications including cancer and chronic hepC. HBV specific T cell responses should play an important role in improving HBsAg seroconversion. Here we generated Tarmogens expressing HBV X, S, and Core antigens; one version contains a chimera of HBV S and Core (SCore) and another contains a chimera of HBV X, S, and Core antigens (XSCore). These were used to stimulate human immune cell samples *ex vivo* to demonstrate HBV specific T cell responses which may serve as a proxy for the immune response expected when patients are dosed with these Tarmogens.

Methods: Peripheral blood mononuclear cells (PBMCs) were harvested from donors and stimulated over 3, 7-day rounds with autologous dendritic cells pulsed with SCore, XSCore, or empty vector (ctrl) yeast. Following this stimulation the conditioned PBMCs were incubated with HBV antigen-pulsed autologous PBMCs for 24h and evaluated by IFNγ ELISpot and by intracellular cytokine staining to measure markers of CD4 and CD8 T cell activation or function (IFNγ, IL-4, IL-17, and CD107a/LAMP1).

Results: PBMCs collected from donors up to 20 years after Engerix vaccination and stimulated *ex vivo* by SCore and XSCore (vs. ctrl yeast) demonstrated induction of HBV specific CD4 T cells producing interferon g (IFNγ; 5 to 22-fold vs. ctrl yeast), IL-4 and IL-17, and induction of HBV specific CD8 T cells producing IFNγ (2-fold vs. ctrl). IFNγ-producing T cells from a SCore-treated donor were LAMP1 positive (17-fold vs ctrl) identifying a HBV specific cytolytic T cell phenotype that is induced by *ex vivo* Tarmogen treatment.

Conclusions: SCore and XSCore are immunogenic and activate CD4 and CD8 T cells after *ex vivo* stimulation of immune samples from healthy volunteers. These data serve as a proxy for the immune response that may be observed when patients are vaccinated with SCore and XSCore, suggesting that these Tarmogens could be used to improve HBV S antigen seroconversion in chronic HBV patients.