RECOMBINANT YEAST THERAPEUTIC VACCINES EXPRESSING HEPATITIS B VIRUS (HBV) X, S, AND CORE ANTIGENS GENERATE ANTIGEN SPECIFIC T CELL RESPONSES AND TUMOR PROTECTION IN MICE

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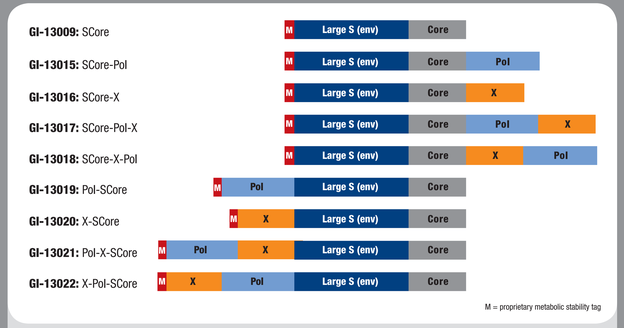
Introduction

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.

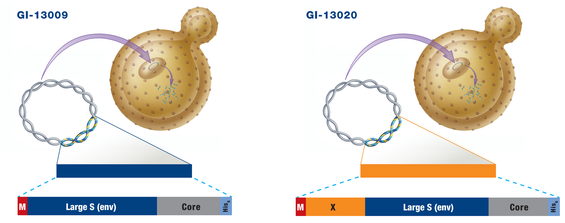
Chronic HBV is characterized by suboptimal T cell responses against viral antigens (Boni *et al*, Gastroenterology 2012). A therapeutic vaccine capable of generating an HBV-specific T cell immune response that is administered concomitantly in patients whose disease is under virologic control with a direct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

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 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 HBV Tarmogens expressing variations of HBV X, S, Pol and Core antigens were engineered and evaluated. Two product candidates were chosen for functional testing on the basis of favorable antigen expression and growth rate. 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 a panel of assays.



Evaluation of the first ten Tarmogens revealed favorable protein expression, immunogenicity and growth advantages in GI-13009, a Tarmogen expressing an S-Core chimera, "SCore". Eight additional constructs were created using SCore as a base, aiming to expand the antigen repertoire of the product by attaching Pol and X variants. When these new constructs were evaluated, GI-13020, a Tarmogen expressing a chimera of HBV X, S and Core ("X-SCore") offered promising initial results. These Tarmogens were optimized to include a broad range of immunogenic epitopes from multiple HBV antigens to provide pan-genotypic HBV coverage for patients.



Murine *in vitro* immunogenicity – BALB/c mice

The results in Figures 1 and 2 show that both GI-13009 and GI-13020 elicit S and/or Core specific T cell ELISpot responses (IFNγ and IL-2) with stronger effects observed for GI-13020. Both *E. coli* and *Pichia*-expressed recombinant antigens elicited responses *in vitro* and HBV Tarmogen vaccination produced stronger signals than the empty vector yeast (Yvec) control demonstrating that the T cell responses were HBV antigen-specific.

Figure 1: IFNγ ELISpot response of HBV Tarmogen-immunized BALB/C mice to IVS with recombinant HBV antigens (lymph node cells)

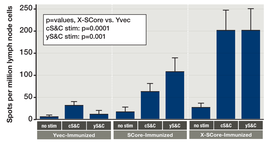
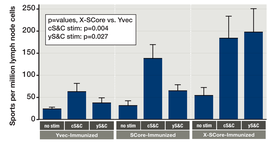


Figure 2: IL-2 ELISpot of HBV Tarmogen-Immunized BALB/C mice to IVS with recombinant HBV antigens (lymph node cells)



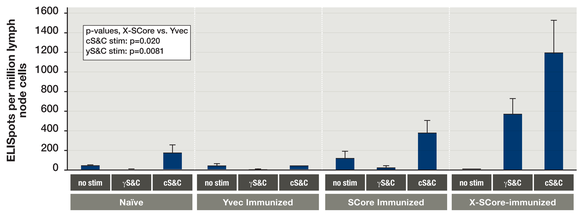
IVS stimulants: cS&C, yS&C: *E coli* and yeast (*Pichia pastoris*)-expressed recombinant antigens, respectively, 3 ug/mL each; "no stim": growth media without HBV antigen.

BALB/c mice were immunized SQ once/week for 3 weeks with 5YU (1YU=10^7 yeast) of either GI-13009, GI-13020 or empty vector yeast (Yvec) control. 2.5YU was administered at two sites: flank and scruff. Two weeks following the third immunization, mice were sacrificed and inguinal lymph nodes were removed and placed into *in vitro* stimulation (IVS) with HBV peptides and recombinant antigens for 4 days. Cells were transferred to dual-color ELISpot plates for 24h and the assay processed to detect cytokines. Error bars: s.e. P values calculated by ANOVA throughout poster, except for Kaplan-Meier analyses.

Murine *in vitro* immunogenicity – C57BL/6 mice

To evaluate whether the IFNγ ELISpot response found in BALB/C mice extends to other murine genetic backgrounds, C57BL/6 mice were immunized, IVS treated, and assayed by ELISpot as described previously. X-SCore vaccination elicited robust IFNγ ELISpot responses in lymph node cells of these mice. HBV-antigen specificity was demonstrated by a 19-fold greater response for X-SCore than Yvec-immunized mice.

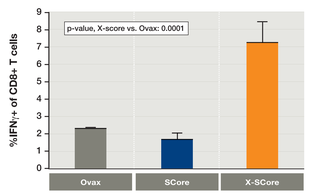
Figure 3: IFNγ ELISpot response of HBV Tarmogen-immunized C57BL/6 mice to IVS with rHBV antigens



X-SCore elicits S Ag-specific $CD8^+$ T cell responses

To further delineate the immune response to HBV Tarmogens, splenocytes from vaccinated mice were stimulated with peptide VWLSVIWM, a class I MHC restricted SAg epitope, and stained with antibodies to CD8, TNFα, IL-2 and IFNγ. The results showed that X-SCore vaccination elicits SAg-specific $CD8^+$ T cells producing these cytokines.

Figure 4: IFNγ production by CD8 T cells from Tarmogen vaccinated C57BL/6 mice



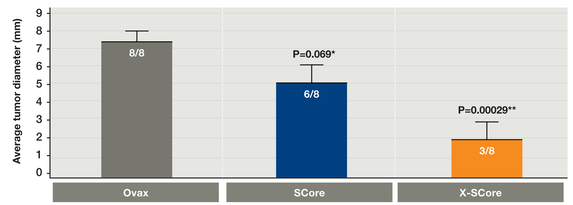
Mice vaccinated with:

- TNFα and IL-2 production were observed by CD8+IFNγ+ T cells from X-SCore vaccinated mice (results not shown).
- Ovax: Ovalbumin-expressing Tarmogen

Adoptive transfer tumor protection

Tumor challenge studies were conducted to determine if HBV Tarmogens generate protective responses *in vivo*. In the first model, splenocytes from HBV Tarmogen immunized mice were transferred to SCID mice, which were then challenged with 300,000 EL4 tumor cells expressing the SCore antigen. The results showed that immune cells from X-SCore vaccinated mice significantly inhibited growth of the EL4 tumors relative to Ovax. This demonstrates HBV antigen specific, immune-mediated protection *in vivo*. P values by ANOVA: *, SCore/Ovax; ** X-SCore/Ovax.

Figure 5: HBV Tarmogen vaccination inhibits growth of EL4-S-Core tumors in SCID mice (day 7 post challenge)

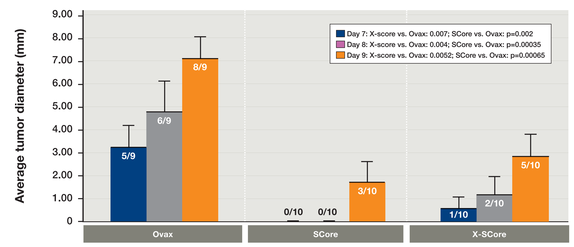


Donor mice vaccinated with either Ovax, SCore or X-SCore. Fraction (X/Y) represents the number of animals with measurable tumors on day 7.

Prophylactic tumor protection in immunocompetent mice

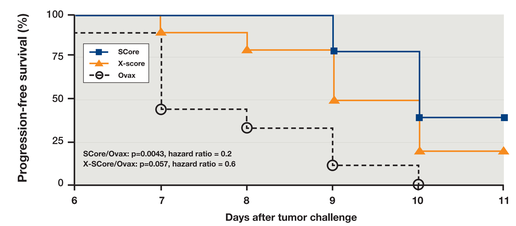
In a second model C57BL/6 mice were vaccinated with three weekly HBV Tarmogen vaccinations, challenged one week later with 300,000 syngeneic EL4-SCore tumor cells, and tumors were caliper-measured daily. The results showed that both Tarmogens significantly inhibited tumor growth relative to the Ovax control (Fig 6a, tumor size; 6b, Kaplan Meier survival analysis) again showing SCore antigen-specific protection *in vivo*.

Figure 6a: Tumor size in Tarmogen-vaccinated C57BL/6 mice. Fractions at the top of each bar represent the number of mice with measurable tumors, and day 7-9 represents the day post-tumor challenge that measurements were taken. P values calculated by ANOVA.



Donor mice vaccinated with either Ovax, SCore or X-SCore. Fraction (X/Y) represents the number of animals with measurable tumors.

Figure 6b: Survival curve for HBV Tarmogen-vaccinated mice. Progression free survival defined as tumor diameter of below 5 mm, for this analysis.



- 300,000 SCore tumor cells used for challenge; n=10 mice/group p value and hazard ratio from Cox proportional hazards analysis

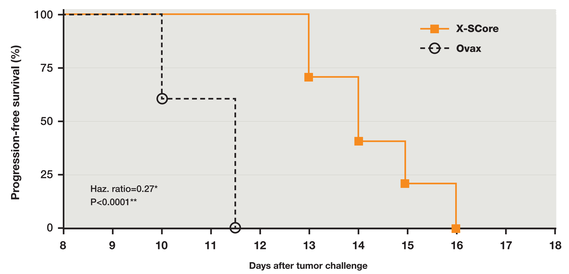
Tumor protection studies targeting X and Core antigens

To evaluate the individual immunogenicity of X and Core antigens, several improvements were made to the immunocompetent model in an effort to stabilize expression of HBV antigen expressed in tumor cells over time (see methods bullets). The results show that the X and Core antigen components of the X-SCore are immunogenic and vaccination with X-SCore significantly slows the growth of EL4-X (Figure 7a) and EL4-Core (Figure 7b) tumors.

Lentiviral model

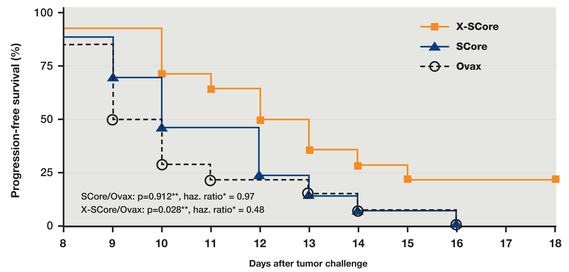
- EL-4 tumors were engineered with a lentiviral vector to stably express HBV X or Core antigens
- Mice were immunized at 4 sites instead of 2 (Wansley *et al*, 2008)
- Immunizations were conducted both pre-challenge (3 weekly vaccinations) and one post challenge vaccination
- Tumor cell dose was reduced from 300,000 to 90,000 (X) or 30,000 (Core) cells based on dosing optimization studies

Figure 7a: X-SCore significantly slows growth of EL4-X tumors



90,000 EL4/lenti-X tumor cells used for challenge n=10 mice/group
* DG Altman "Practical statistics for medical research" 1991
** Log rank test from Kaplan-Meier analysis

Figure 7b: X-SCore significantly slows growth of EL4-core tumors



30,000 EL4/lenti-core tumor cells used for challenge, n=14 mice/group
* DG Altman "Practical statistics for medical research" 1991
** Log rank test from Kaplan-Meier analysis

Conclusions

- X-SCore (GI-13020) is immunogenic, generating antigen-specific T cell responses to X, S and Core antigens
- X-SCore (GI-13020) was shown to be substantially more immunogenic than SCore (GI-13009) in most experiments, based on antigen-specific responses relative to control Tarmogen (Yvec or Ovax).
- X-SCore (GI-13020) vaccination elicits X- and Core specific tumor protection, providing further rationale for inclusion of these antigens along with S antigen in the Tarmogen.

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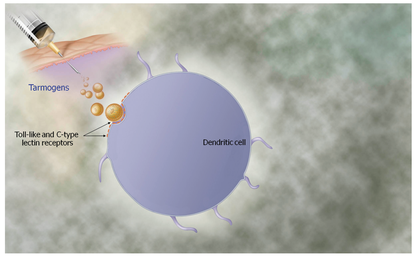
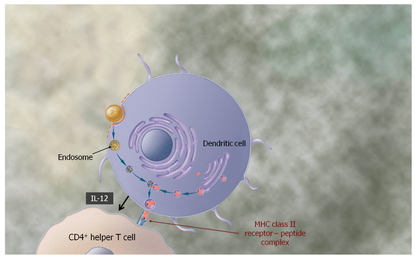
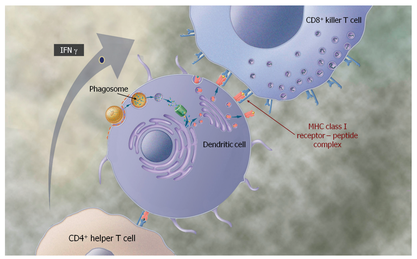
Active immunotherapy with yeast-based Tarmogens



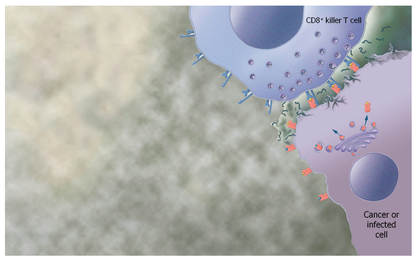
Administration of Tarmogens initially results in binding of the yeast to antigen-presenting cells, the most important of which are dendritic cells, near the injection site. The dendritic cells are activated as a result of the Tarmogens binding to Toll-like receptors and other receptor molecules on the 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 and tumor protection in mice

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Purpose: Chronic HBV is characterized by suboptimal T cell responses to viral antigens. A therapeutic vaccine capable of restoring the immune response to HBV would be an attractive clinical option. A yeast-based immunotherapy platform (Tarmogens) was used to make product candidates 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 (XSCore). These were used to elicit antigen specific T cell responses and protection from challenge with HBV S and Core expressing tumors.

Methods: Mice were subcutaneously immunized with 3 weekly doses of SCore, XSCore, or empty yeast (ctrl). One week later immune cells were evaluated by lymphocyte proliferation, interferon g (IFNγ)/IL2 ELISpot, and intracellular cytokine staining assays to assess T cell responses. Vaccinated mice were also challenged by EL4 cells expressing HBV S and Core antigens and tumor protection was evaluated with a Cox Hazards model.

Results: Mice immunized with SCore and XSCore compared to controls showed induction of lymphocyte proliferation (32 fold increase vs. ctrl), IFNγ (19-fold increase), IL2 (5-18 fold increase), and TNF-a (1.6 fold increase) when stimulated by recombinant HBV antigens and with HBV peptides associated with acute HBV clearance. CD4+ and CD8+ responses were observed. Mice vaccinated before tumor challenge showed delayed growth of EL4 cells expressing HBV antigens over an 11 day period. At day 7 post challenge, 100% of SCore and 90% of XSCore treated mice were tumor free while 44% of ctrl mice were tumor free at this time point.(eg, SCore/ctrl, hazard ratio (hr) 0.201, p=0.0043, figure). Mice receiving vaccinated donor T cells post tumor challenge showed improved survival (31% progression free for XSCore vs. 0% for control, day 18).

Conclusions: SCore and XSCore elicit HBV specific T cell activation and protection against challenge by HBV antigen expressing tumors, suggesting that these Tarmogens could be used to improve HBV S antigen seroconversion in chronic HBV patients.