Optimization of a Granzyme B ELISPOT Assay to Measure Cytotoxic T Lymphocyte Responses in a Herpes Simplex 2 Vaccine Clinical Trial

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ABSTRACT

Herpes simplex virus 2 (HSV-2) is the main cause of genital ulcers worldwide with no cure; and, despite the availability of antiviral drugs, continues to spread. A vaccine that stimulates both humoral and cell-mediated immune responses has the potential to reduce herpes lesions and recurrences. We have developed an immunotherapy, GEN-003, consisting of recombinant ICP4.2 and gDΔTMR adjuvanted with Matrix-M2 that is currently in Phase 2 clinical studies. To examine vaccine-induced T cell responses, we have developed a Granzyme B (GrB) enzyme-linked immunospot (ELISPOT) assay to measure cytotoxic T cell responses in peripheral blood mononuclear cells (PBMC). ELISPOT assays measure the frequency of T cell responses on a single cell basis and are used to evaluate T cell functionality. GrB is a serine protease secreted primarily by cytotoxic T cells that induces apoptosis in target cells therefore providing a direct correlation to T cell killing activity. To optimize this assay for use in our clinical trials, we assessed different parameters including incubation times and cell concentrations. PBMC from a subset of HSV-2-infected subjects participating in a randomized, double-blind, placebo controlled study of GEN-003 were tested in the optimized GrB ELISPOT pre-vaccination, and one week after each of 3 doses. After the first dose, mean increases in GrB responses were greater than 25-fold in vaccinated subjects; placebo subjects had a mean fold change of less than 1.8. Following the third dose of vaccine, GrB responses remained significantly above both baseline and responses among placebo recipients. These results demonstrate that GEN-003 induces cytotoxic T lymphocyte responses in HSV-2 infected subjects. Additional studies are underway to evaluate the durability of response at 6- and 12 months and determine if $CD8^+$ T cells are mediators of response. The GrB ELISPOT is a useful biomarker assay to quantify cell-mediated immune responses in vaccinated subjects.

INTRODUCTION

- ELISPOT assays are a highly sensitive method used to measure individual antigen-specific T cell functionality.[1]
- The IFNɣ ELISPOT is commonly used as a measure of T cell activation, however, it does not directly assess cell-mediated cytotoxicity.
- Granzyme B (GrB) can mediate apoptosis in virus-infected cells and is a more specific indicator of cytotoxic ability.[2]

GEN-003 is an immunotherapy vaccine comprised of

- A large fragment of the immediate early protein infected cell polypeptide 4 (ICP4.2): a truncated form of a T cell antigen
- A transmembrane deletion mutant of glycoprotein D2 (gD2ΔTMR): a surface-expressed HSV antigen and a primary target for antibody generation and $CD4^+$ T cell activity
- Matrix-M2: a saponin derived adjuvant, (Novavax, Gaithersburg, MD)

Here, we developed a GrB ELISPOT assay using human PBMC from subjects participating in a Phase 2 clinical trial, GEN-003-002.[3]

STUDY DESIGN

Samples	• PBMC samples were isolated and cryopreserved from whole blood collected from healthy subjects (Research Blood Components, Boston, Ma)
	• PBMC samples from 39 subjects enrolled in a herpes immunotherapeutic vaccine study, GEN-003-002. [3]
	  • 10 placebo inoculated subjects
	  • 29 subjects who received 3 doses of GEN-003

Stimulation	• Negative control: AIM-V media • Overlapping peptides spanning the GEN-003 antigens: ICP4.2 (GB208) and gD2 (GB217) • Positive control: Phytohemagglutinin (PHA-L) (Sigma-Aldrich, St. Louis, MO) • Peptide positive controls: CEF (32 peptides from HLA class I restricted T cell epitopes) and CEFT (27 peptides from HLA class I and II restricted T cell epitopes) (JPT, Berlin, Germany)
ELISPOT Reagents	• Commercially available human GrB kit, (Mabtech, Nacka Strand, Sweden)

METHODS

Day 1: Cells were resuspended at $2x10^6$ cells/mL and rested overnight at 37°C. PVDF membrane plates were treated with 35% EtOH before coating with capture antibody and stored overnight at 4°C.

Day 2: Rested PBMC were counted and viable cells were plated with appropriate stimuli. Plates were then incubated for 48 hours in 37ºC incubator.

Day 4: Plates were developed using a biotinylated detection antibody and spots were visualized using streptavidin-ALP or substrate solution BCIP/NBT.

PBMC Secrete GrB in Response to Antigen Stimulation

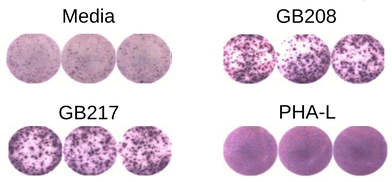

Figure 1: Representative GrB ELISPOT wells. PBMC from GEN-003 immunized subjects (2 x 10^5 cells/well) were stimulated in triplicate and incubated for 48 hours before development. Spot forming units (SFUs) were quantified using the CTL Immunospot Micro analyzer plate reader. One representative donor is shown.

References

[1] Malyguine et al. Cells 2012
[2] Weaver et al. Journal of Translational Medicine 2003
[3] Van Wagoner et al, GEN-003 Phase 2 results: Therapeutic vaccine for genital herpes significantly reduces viral shedding and lesions, presented at IUSTI2016, Marrakesh, Morocco

Increased PBMC Concentration Correlates to Higher GrB Response to CEF Peptide Pools

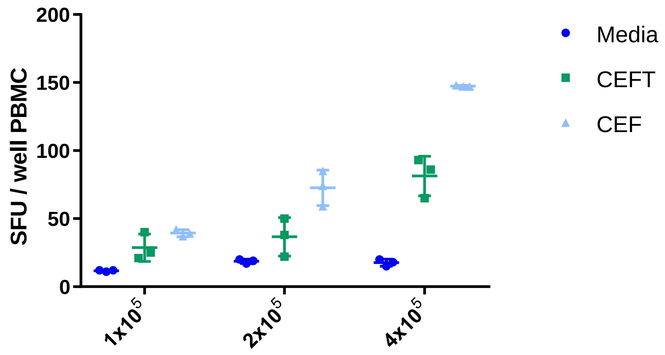

Figure 2: Cell titration optimization. PBMC from a healthy donor were titrated as shown on the x-axis and stimulated in triplicate with CEF peptide pools to determine optimal cell concentration.

48 Hour Incubation Increases GrB Response Without Increasing Background

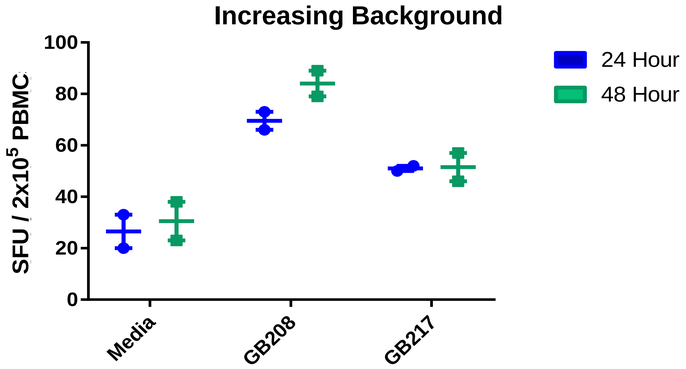

Figure 3: Incubation time optimization. PBMC from a GEN-003 vaccinated donor were plated in duplicate and stimulated with GEN-003 antigens as shown on x-axis to determine the optimal stimulation time.

Inclusion of Tween in Wash Buffer Reduces GrB Background Response

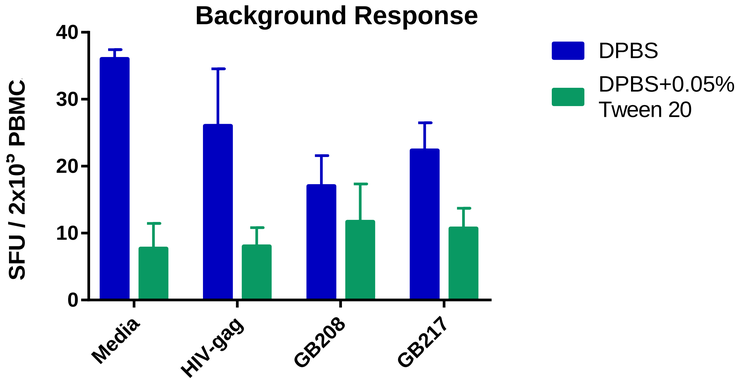

Figure 4: Addition of Tween 20 in wash buffer reduces background. PBMC from a naïve donor were evaluated on two separate plates for non-specific responses.

GrB Secretion is Specific to GEN-003 Antigens

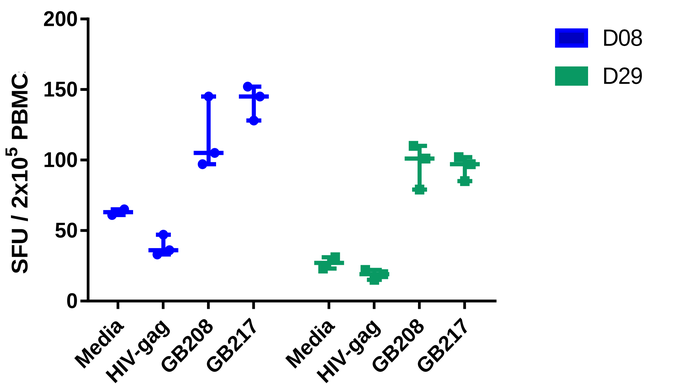

Figure 5: Antigen specificity confirmed using Human Immunodeficiency Virus-group specific antigen (HIV-gag) as irrelevant antigen. PBMC from an HIV negative HSV-2 positive donor who had been vaccinated with GEN-003 were evaluated at two time points. PBMC were plated in either duplicate or triplicate.

Increased GrB Secretion in GEN-003 Vaccinated Subjects Compared to Subjects Receiving Placebo

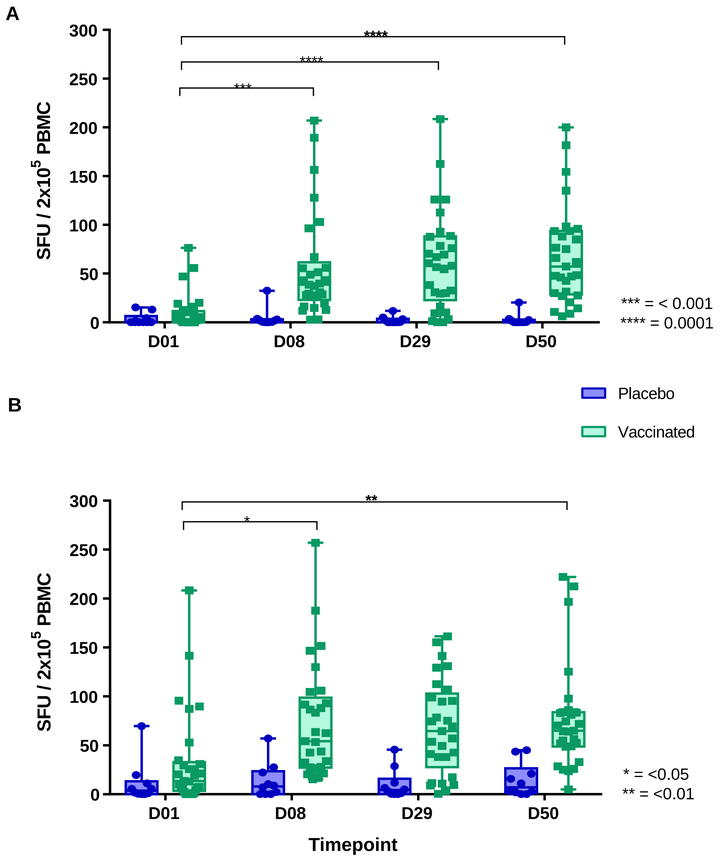

Figure 6: Antigen specific GrB response in GEN-003 clinical trial samples. PBMC were stimulated with GB208 (A) or GB217 (B) OLPs. Mean GrB response is shown pre–vaccination (D01) and seven days post each vaccine dose (Day 08, Day 29, Day 50) for all 39 subjects. Media subtracted values shown. Statistical analysis with one-way ANOVA. Placebo was normal saline, vaccinated were multiple GEN-003 dose levels combined.

Majority of GEN-003 Vaccinated Subjects have a >3-fold Change in GrB Secretion for all Timepoints

		Placebo				Vaccinated			
Stimuli	**Time point**	**Mean FC from D1**	**Median FC from D1**	**SD**	**% Subjects FC > 3**	**Mean FC from D1**	**Median FC from D1**	**SD**	**% Subjects FC > 3**
GB208	D08	0.7	0.6	0.7	0%	25.9	13.6	37.1	66%
	D29	0.5	0.1	0.7	0%	20.9	9.6	23.5	72%
	D50	0.5	0.1	0.8	0%	38.8	12.1	52.9	79%
GB217	D08	1.8	1.0	2.3	20%	16.1	5.5	29.9	65%
	D29	2.0	1.1	2.4	30%	13.3	3.1	25.6	52%
	D50	3.5	1.9	4.9	30%	23.1	3.9	49.2	62%

Table 1: Fold change over baseline. The mean and median fold-change (FC) over baseline are shown for each antigen at Day 08, Day 29 and Day 50 for placebo and GEN-003 vaccinated subjects. The % of subjects with FC greater than 3 are also shown.

SUMMARY

- Granzyme B ELISPOT is a functional biomarker assay that can be used to measure cell-mediated immune responses in vaccinated subjects.
- Optimization of the GrB ELISPOT:
  - PBMC concentration: 2 $x10^5$ cells/well
  - Stimulation time: 48 hours
  - Wash buffer: DPBS/Tween 20
  - Stimuli: AIM-V Media, GB208 OLP, GB217 OLP, PHA-L
- Immune response of subjects following GEN-003 immunotherapy:
  - After the first dose, GrB responses were significantly increased in vaccinated subjects compared to subjects receiving placebo.
  - Following the third dose, GrB responses in vaccinated subjects remained significantly increased above baseline and compared to subjects receiving placebo.
  - Cytotoxic T lymphocyte responses were observed in most subjects after GEN-003 immunization.
- Further tests are underway to determine the durability of response and to determine the respective role of $CD4^+$ or $CD8^+$ T cells in response to GEN-003.