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14. ABSTRACT

This project extended over a three year period and focused on the use of the MIMIC® system to assess the immunogenicity/efficacy of commercial influenza vaccine and a plant-produced recombinant hemagglutin (HA) influenza vaccine, from the HA sequence of the pandemic A/California H1N1 strain, produced by the Fraunhofer Center for Molecular Biology. Initially the task was to evaluate Fraunhofer’s vaccine for efficacy in vitro either formulated with adjuvant (alum) or with no adjuvant and to evaluate the cross-reactivity and A/California H1N1

15. SUBJECT TERMS

MIMIC®; Fraunhofer H1 (California); elderly; LTE; cell sparing; cross-reactivity; cross-neutralization; seasonal influenza; H1N1 California; hemagglutin (HA); antigen; vaccine; antibody; functionality

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Report Title

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Number of Papers published in peer-reviewed journals:

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(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

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"Coupling sensitive in vitro and in silico techniques to assess cross-reactive CD4+ T cells against the swine-origin H1N1 influenza virus" Authors: Brian C. Schanen, Anne S. De Groot, L. Moise, Matt Ardito, Elizabeth McClaine, William Martin, Vaughan Wittman, William L. Warren, Donald R. Drake III

"In Vitro Biomimetic Model of the Human Immune System for Predictive Vaccine Assessments"; Authors: Donald R. Drake III, Inderpal Singh, Michael Nguyen, Anatoly Kachurin, Vaughan Wittman, Robert Parkhill, Olga Kachurina, Janice Moser, Anthony Byers, Vipra Dhir, Tenekua Tapia, Charlotte Vernhes, Jyoti Gangur, Tirumalai Kamalaand, Nithya Swaminathan, and
William L. Warren

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Immune Analysis of Brisbane and California H1N1 in Human Sera and the MIMIC® System

Correlating a H1N1 Pandemic Influenza Clinical Trial with a "Clinical Trial in a Test Tube"

Contract # W911NF-09-C-0119
BAA09-31 & BAA-10-55

Final Report

Date of Proposal:

Prepared by Sanofi Pasteur VaxDesign Campus

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Director, Innovative Methods

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Contents

1. Executive Summary

This project extended over a three year period and focused on the use of the MIMIC® system to assess the immunogenicity/efficacy of commercial influenza vaccine and a plant-produced recombinant hemagglutin (HA) influenza vaccine, from the HA sequence of the pandemic A/California H1N1 strain, produced by the Fraunhofer Center for Molecular Biology. Initially the task was to evaluate Fraunhofer's vaccine for efficacy *in vitro* either formulated with adjuvant (alum) or with no adjuvant and to evaluate the cross-reactivity and A/California H1N1 strain. The second section of the project focused on the coupling of the MIMIC to a clinical trial involving the Fraunhofer antigen and a commercial vaccine and the characterization and comparison of the *in vivo* and *in vitro* responses. During the contract period methods were developed to evaluate the relationship between the level of total anti-HA antibody and the level of functional antibody as well as alternative methods for conducting MIMIC® evaluations with smaller starting numbers of peripheral blood mononuclear cells (PBMC). MIMIC® was also shown to appropriately reflect segmentation in adult and elderly populations following influenza vaccination and to identify differences in immunogenicity between various commercial vaccine formulations. Finally, the system was used to evaluate recombinant hemagglutinin supplied by Fraunhofer to see if constructs encoding various molecular forms (trimeric or VLP) were more immunogenic than monomeric vaccine in the MIMIC® system.

The report is organized by year, 2010-2012. More detailed descriptions of the various points outlined in this report can be found in the progress reports submitted each year.

2010

- Sensitive assays were developed to measure the magnitiude of the antibody response against hemagglutinins from various influenza strains.
    - Antibody magnitude was measured by our antibody forensics (AF) method in which recombinant hemagglutins (HA) are coupled to luminex beads. Each HA is coupled to a bead with a unique fluorescent signature and multiple bead/HA combinations can be evaluated in a single well (multiplex analysis) using a minimal volume of sample.
    - Antibody functionality was measured by surface assisted hemagglutination inhibition (SA-HAI), a method that enabled the evaluation of functional antibodies present in low levels such as those found in MIMIC® culture supernatants.
- Response profiles of sera and MIMIC® were generated in a pre/post immunization study with seasonal vaccine and found to show very similar trends in when AF values were compared.
    - Donors received the 2009-10 seasonal vaccine containing the A/H1N1 strain Brisbane and were evaluated by AF. The sera and MIMIC responses showed that donors responded strongly to H1 HA from previous year's vaccines but poorly to the newly emerged pandemic A/California strain.
    - Cross-reactivity profiles generated from the AF results demonstrated that there were few antibodies present in serum that cross-reacted with A/California HA from donors immunized with A/Brisbane but that MIMIC® supernatant contained more cross reactive antibodies. This indicated that there may be repertoire available in the memory B cells in circulation that is larger than that represented in the serum.

    - SA-HAI profiles of MIMIC® supernatants and sera confirmed that MIMIC® supernatants contained proportionally more functional antibodies capable of binding the A/California virus than serum.
- T cell responses to recombinant HA from seasonal (Brisbane) or pandemic (California) H1 strains showed a high degree of cross-reactivity.
- Unformulated Fraunhofer HA (Fh) was evaluated for anti-HA antibody production in MIMIC® and compared to responses generated by recombinant HA from Protein Sciences (PS), seasonal vaccine (FV) and inactivated influenza virus (BPL). The magnitude of response was as follows: BPL>FV>Fh>PS.
    - Alum formulated Fh was roughly equivalent to unformulated Fh for generation of anti-A/California HA antibodies.

2011

- Sera and MIMIC® culture anti-HA (AF) values were compared following vaccination with the monovalent pandemic H1N1 vaccine and found to generate essentially equivalent stimulation ratios on the population level when the post response value for vaccine recipients was divided by the pre response value.
    - For functional antibodies (SA-HAI) the MIMIC® cultures yielded substantially lower levels of inhibitory antibodies than sera but the profiles, as evaluated demonstrated an increase in functional antibodies at 3 week followed by a decrease at 12 weeks, resembled that found in serum.
- One of the primary tasks for this part of the contract was to "assess *ex vivo* and *in vitro* immune responses to AMP Vaccine H1N1 (California) after each inoculation in the clinic."
    - Logistics for sample collection and processing were developed and technical staff was deployed to WRAIR during the study. 232 of 240 planned samples were successfully processed.
    - All donors were pre-screened for HAI titers to A/California at VaxDesign and those with titers < 40 were selected for the study.
    - The MIMIC® culture system was adapted to work with small numbers of input cells.
    - Samples were collected from 8 groups of 10 donors each. The study was set up as an escalating dose study for Fraunhofer's vaccine at 15, 45 and 90 µg/dose, with or without alum adjuvant and included a group which received a commercial monovalent pandemic vaccine (containing A/California) and a saline control group.
    - *In vivo* samples - antibody profiles, sera analysis by Antibody Forensics:
        - Anti-A/California hemagglutinin levels of IgM were 100-200 times lower than those for IgG indicating that the IgM probably played a minor role in the response to the pandemic strain.
        - Vaccination with Fraunhofer's recombinant A/California HA generated a high level of antibodies that were cross-reactive with the hemagglutinins from previous years' seasonal strains while vaccination with the commercial pandemic vaccine did not.
    - *In vivo* samples - functional antibody profiles, sera evaluation using the Surface Assisted Hemagglutination Assay (SA-HAI):
        - SA-HAI results for each donor were compared with traditional HAI tests run at the Centers for Disease controls and correlated extremely well (Kcorr = 0.92)

indicating that the modifications made to enhance the sensitivity of the SA-HAI did not compromise the assay's accuracy.
        - Maximum levels of functional antibody for the Fraunhofer vaccine were achieved after a booster dose at the 45 μg level for alum formulated material and the 90 μg level for the unformulated material.
        - A single dose of the commercial vaccine generated a higher level of functional antibody which remained roughly twofold higher than the highest level achieved by the Fraunhofer material after boost when measured at day 56.
    - Results from the sera analyses indicated that there was a higher ratio of functional anti-HA antibodies produced by the commercial pandemic vaccine than by the Fraunhofer vaccine as determined by calculating the SA-HAI (functional antibodies)/AF (total anti-HA antibodies) ratio.
    - In vitro samples - MIMIC® results:
        - Comparison of the population geometric mean titers for the MIMIC® and sera results showed similar trends in total anti-HA production indicating that the *in vitro* assay tracked closely with the *in vivo* response.
        - CD4 T cell frequency was evaluated by IFNγ ELISPOT and was found to be maintained at a relatively constant level during the vaccination series with unadjuvanted Fraunhofer material and commercial pandemic vaccine but to increase when alum formulated Fraunhofer material was used.
            - Very high $CD4^+$ T cell responses tended to be at the demise of influenza-specific Ab responses.
        - Evaluation of the reactogenicity of the Fraunhofer formulations was done in the MIMIC® PTE module by evaluating alterations in cell phenotype and cytokine response.
            - Phenotyping analysis - The majority of markers show very minor changes between no antigen, +rHA and alum+rHA within a indicating there is little, if any, reactogenicity associated with the rHA antigen.
            - The Fraunhofer formulations do not stimulate the production of any inflammatory cytokines to a level that significantly exceeds that of the no Ag baseline control.

2012

- Alternative setup conditions for MIMIC® cultures were established to minimize the required number of peripheral blood mononuclear (PBMC) needed and still maintain reasonable response levels to influenza antigens. These adjustments were investigated to facilitate analysis of low volume or low yield samples such as those encountered in a clinical study or a study involving the elderly.
- A study conducted with adult and elderly donors in which PBMC from the pre-vaccination were used to set up the MIMIC® culture and sera was collected pre and post vaccination with a trivalent seasonal vaccine. The MIMIC® culture received either the seasonal vaccine ('in vitro vaccination') or no antigen (representing the pre vaccine status) and the supernatant was evaluated for the presence of antibodies which bound the HA representing the 3 influenza strains present in the vaccine. The MIMIC® response profiles, the *in vitro* vaccination profiles, closely paralleled the profiles found in sera for each of the 3 influenza strains and the adult and elderly population segmentation was also reliably predicted by the *in vitro* responses.

- The ability of the MIMIC® system to differentiate between different formulations of a commercial influenza vaccine was demonstrated using four different formulations of quadravalent influenza vaccine. The donors were evaluated by AF and SA-HAI and both assay formats showed the differential between formulations. There was a close correlation between the functional antibody responses (SA-HAI) and the magnitude of anti-HA antibodies measured by AF. The similarity in trends between the AF and SA-HAI assay indicate that the AF may be a reasonable way to monitor vaccine potency for commercial vaccines. The use of both assays allows the assessment of the efficacy of a formulation to drive the production of functional antibodies.
- As a follow up to the observation that Fraunhofer's vaccine generated a lower functional antibody ratio as measured by the SA-HAI/AF ratio we developed an assay to evaluate antibodies capable of binding the conserved stem region of the hemagglutinin. The rationale was that Fraunhofer's vaccine may be less effective at generating antibodies capable of binding the head region of HA, which is involved in binding red blood cells and detected by the SA-HAI assay, but may effectively drive the production of anti-stem antibodies which would neutralize virus but not be evident in the SA-HAI. This assay was a modification of the AF that involved competition of serum antibodies with monoclonal antibodies known to bind the stem region of HA. Using this assay it was found that the Fraunhofer vaccine did not direct the production of anti-stem antibodies at a rate higher than that of the commercial vaccine.
- Two studies were conducted with other forms of the A/California recombinant HA vaccine from Fraunhofer. Fraunhofer genetically modified the HA construct to produce HA that would be made as a trimer (the native form of the molecule) or as a VLP (a virus-like particle form of the molecule which would be multivalent).
    - The first study was conducted using the Fraunhofer's monomeric HA, which was used in the clinical study, their trimer format, a recombinant A/California HA made in baculovirus, and various commercial vaccines. The trimer material was not found to be any more effective at generating an immune response than the monomer form using either the AF or SA-HAI assays. The Fraunhofer material was used in the study at a final HA concentration of 500 ng/ml while the commercial vaccines were adjusted to a final concentration of 3 ng/ml of A/California HA. The Fraunhofer material generated anti-HA antibody levels (AF) equivalent to two of the commercial control vaccines but yielded lower SA-HAI levels than any of the control vaccines.
    - A final study was conducted with a VLP construct that Fraunhofer sent as the project was closing out. The study was designed to compare the monomer, trimer and VLP construct at different doses against the commercial vaccine control. The study had to be terminated due to contamination of the VLP mix that was sent.

2. Results

2.1. 2010 Summary

The purpose of the project is to enumerate and characterize cross-reactive and cross-protective immune responses between seasonal and pandemic H1N1 influenza strains using both commercial and novel plant expressed vaccine candidates. The study was designed to address some basic questions with respect to the immunological relationship of these strains. The report is organized to reflect the Tasks defined in the original proposal. Briefly, the goals of these tasks were to: [1] evaluate cross-reactivity and cross-neutralization relationships between seasonal influenza and H1N1 California using sera and MIMIC® cultures (Task 3), [2]. assess immune profiles of unformulated Fraunhofer H1 (California) in the MIMIC® system (Task 1) and [3]. assess AMP (Fraunhofer HA) and, optionally, BARDA H1N1 (California) vaccines (Task 2). The work, completed within the first year, allowed us to address some fundamental questions regarding the immune relationships between seasonal and pandemic influenza and, additionally, has provided insight into basic immune function.

One of the overriding questions facing public health officials when the H1N1 pandemic influenza strain appeared was whether vaccination with the existing seasonal vaccine would protect or ameliorate the morbidity and mortality of infection with the emerging virus. We addressed the question of cross-reactivity between the strains in Task 3 by performing a pre/post analysis of sera and MIMIC® samples from the same donors. This analysis employed a set of sensitive analytical tools to determine the quantity and quality of hemagglutinin reactive antibodies (Figures 1 & 2).

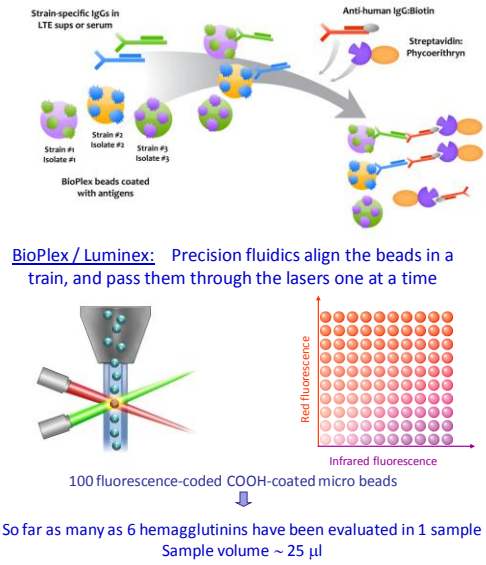


Figure 1. Antibody Forensics process for evaluation of anti-hemagglutinin antibody levels. This cartoon outlines several features of the process used to evaluate anti-hemagglutinin antibody levels using the BioPlex system. A flow scheme of the interaction of labeled beads and antibodies is pictured at the top. The principle involved in the acquisition of signal is explained below. Analysis of antibody reactivity towards several different hemagglutinins can be carried out in a single well with a small volume of sample.

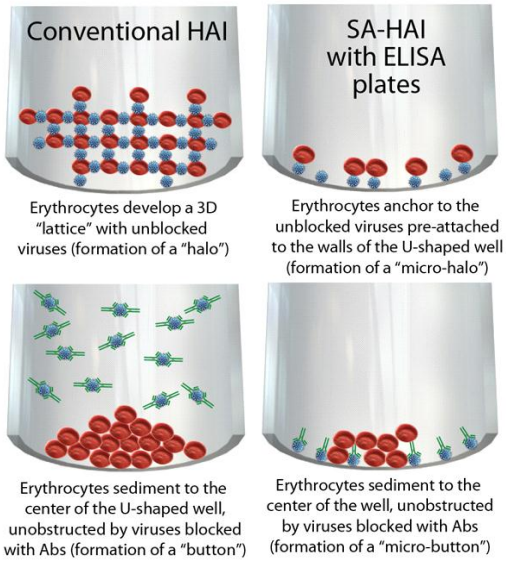
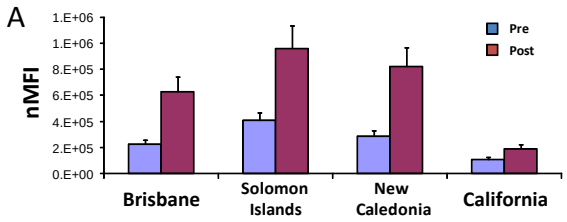


Figure 2. Concepts behind the traditional HAI and the more sensitive SA-HAI. The conventional HAI depends upon the formation of a three dimensional lattice composed of cross-linked erythrocytes and virus to detect the presence of virus (the absence of blocking antibodies) SA-HAI is dependent upon the formation of a two dimensional layer of virus-erythrocyte to detect the presence of virus. The addition of antibodies capable of blocking viral binding to erythrocytes results in the settling of the erythrocytes into a "button" at the bottom of a round-bottomed well.

Our study revealed that there are modest levels of antibodies that recognize the pandemic H1N1 strain in sera samples collected pre- and post-vaccination with the seasonal vaccine and that the levels of functional antibody were low (Figure 3).



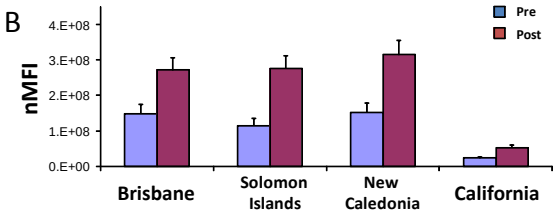


Figure 3. Average AF IgG values for California, Brisbane, New Caledonia and Solomon Islands hemagglutinin-linked beads from MIMIC supernatant and sera samples. Bar graph representations of the magnitude of the IgG response of donor's vaccine-stimulated MIMIC samples tested in multiplex analysis with beads liked with the various H1 hemagglutinins. Values are represented as normalized mean fluorescence intensity (nMFI) and Y axis is in linear scale on these graphs. A. MIMIC samples B. Serum samples

In contrast, parallel analysis of the same subjects in the MIMIC® system revealed a substantial increase in the relative proportion of antibodies that recognized the pandemic strain (Table 1 and Figure 4). This observation suggested that the memory cell subsets which produce antibody in the MIMIC® have a different spectrum of specificity than that seen in serum. This profile may allow them respond to infection by pandemic virus and potentially reduce disease severity.

Sample	Brisbane x California	Brisbane x New Caledonia	Brisbane x Solomon Islands
MIMIC®	0.628	0.945	0.982
Sera	0.265	0.831	0.729

Table 1. Cross-reactivity values for MIMIC® and sera antibodies reactive with H1 hemagglutinins. Correlation coefficients were calculated by comparing IgG values obtained on beads linked to Brisbane hemagglutinin with beads linked to California, New Caledonia or Solomon Islands hemagglutinins. The correlation between Brisbane, New Caledonia and Solomon Islands is high in both MIMIC® and sera. There is a reasonable correlation between Brisbane and California in MIMIC® which is not evident in the sera.

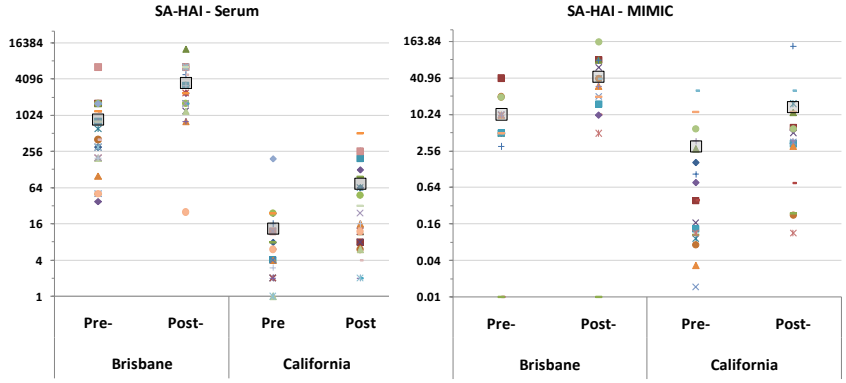


Figure 4. SA-HAI values for A/California H1N1 and A/Brisbane H1N1 from serum and MIMIC samples. Donor's values represented by unique points in each condition for serum or MIMIC supernatant samples. Color coding and marker shape are consistent for each donor for each graph but are not shared between graphs. The average value for each condition is represented by a black square with a grey center

The question of cross-reactivity of the $CD4^+$ T cell population's response to H1 hemagglutinin from pandemic and seasonal virus was addressed in Task 2. MIMIC® cultures were grown in the presence of influenza antigens containing hemagglutinin from the pandemic or seasonal influenza strains and restimulated with either the pandemic or seasonal hemagglutinin. Results indicated that there was a

significant degree of response to target cells containing the seasonal hemagglutinin by cells cultured in the presence of pandemic hemagglutinin (Figure 5). This suggests a degree of conservation in the $CD4^+$ T cell epitopes present on the two hemagglutinins leading to substantial cross-reactivity between the strains. These results also imply a strong probability of the presence of pre-existing immunity that may ameliorate disease severity.

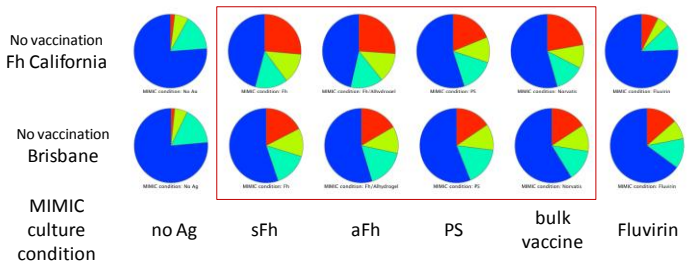


Figure 5. Boolean evaluation of the CD4 T cell response to target cells pulsed with California or Brisbane hemagglutinin. Individual intracellular staining results for the unvaccinated donors were combined and averaged. Donors were grown in MIMIC culture with the antigens indicated before being exposed to target cells which had been pulsed with California or Brisbane hemagglutinin. The red box indicates growth conditions containing the various California hemagglutinins. Colors indicate the proportion of the CD4+ T cell population producing 4 (red), 3 (yellow), 2 (aqua) or 1 (blue) of the following indicators of activation: CD154, IFN-γ, TNF-α or IL-2

The focus of Tasks 1 and 2 was the evaluation of the Fraunhofer's plant derived recombinant pandemic (California) hemagglutinin as an antigen in the MIMIC® system. For this purpose, we selected 18 donors, nine of whom were previously vaccinated with the seasonal vaccine containing the H1 A/Brisbane hemagglutinin and nine donors with no vaccination history. Task 1 was designed to evaluate the ability of Fraunhofer hemagglutinin to stimulate production of antibodies reactive with Brisbane and California hemagglutinins. Here, we also enumerated the affinity of resultant antibodies and their capacity to block viral binding. In this study we compared Fraunhofer material with recombinant California hemagglutinin made in baculovirus (Protein Sciences), seasonal vaccine containing Brisbane H1 hemagglutinin (Fluvirin) and inactivated whole virus (BPL) (Figure 6). The ability of the various antigens to induce anti-hemagglutinin antibodies in MIMIC® revealed that whole virus was the strongest antigen followed by seasonal vaccine, Fraunhofer and then Protein Sciences. It is not surprising that the virus and vaccine formulations were more potent antigens than purified soluble protein. Whole virus and vaccine possess more components (such as neuraminidase) that could be stimulatory and are either not purified or partially purified away from the egg proteins in which they were produced.

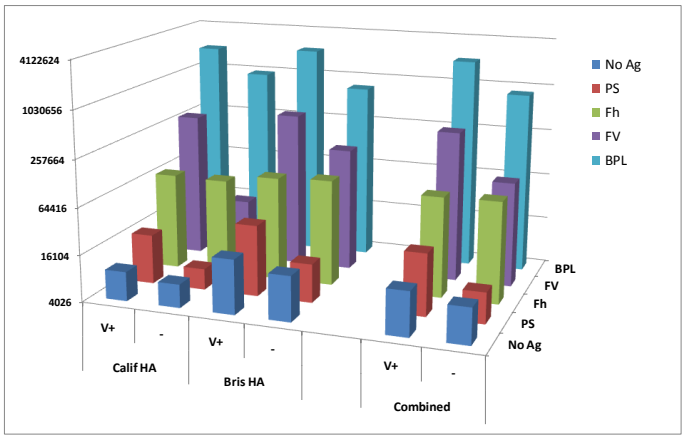


Figure 6. Average IgG readings from California and Brisbane hemagglutinin beads. MIMIC cultures were incubated with California hemagglutinin from Protein Sciences (PS)or Fraunhofer (Fh), seasonal vaccine (Fv), inactivated whole virus (BPL) or no added antigen (no Ag) and supernatants were evaluated by antibody forensics for reactivity with beads linked to either Fh California or PS Brisbane hemagglutinins. The bars represent the average value of each set of donors. V"+" indicates culture was with cells from vaccinated donor,"- " indicates culture was with cells from donor with no history of vaccination prior to PBMC collection.

The affinity of the antibodies generated in MIMIC® in response to the Fraunhofer material were roughly equivalent to that of the virus and vaccine and higher than that of the Protein Sciences material. Cross-reactivity, as measured by binding to Brisbane and California hemagglutinin-linked beads, of the antibodies produced in Fraunhofer-stimulated MIMIC® was high whereas samples from sera from the same donors was not (Figure 7).

MIMIC® challenge	Brisbane x California	Serum
PS Calif. HA (PS)	0.94	
Fh Calif. HA (Fh)	0.88	
Seasonal vaccine (Fv)	0.88	0.34
Whole virus (BPL)	0.95	

Figure 7. IgGs produced in response to various influenza antigens MIMIC® cross-react with Brisbane and California hemagglutinins. Correlation coefficients were calculated by comparing IgG values of MIMIC® cultures which had been challenged with various influenza antigens. Antibody binding to Brisbane beads was compared with the levels bound to California and the values are shown in the table. Sera value was calculated by combining the values of both vaccinated and unvaccinated donors.

The production of functional antibodies capable of blocking viral binding was not enhanced by either of the recombinant proteins as compared to the no antigen control while the vaccine and whole virus raised the average value more than four fold in both vaccinated and unvaccinated donors.

In Task 2 the effects of formulation of the Fraunhofer recombinant California hemagglutinin in alhydrogel, an adjuvant approved for use in humans, was evaluated. The impact of the alhydrogel-formulated material (aFh) on antibody production in MIMIC® was compared with unformulated Fraunhofer protein (sFh), Protein Sciences California heagglutinin (PS) and two vaccines, seasonal trivalent vaccine containing Brisbane hemaggluininin (Fv) and H1 California monovalent (bulk formulation) pandemic vaccine (Nov). The average anti-hemagglutinin levels produced by the recombinant hemagglutinins aFh, sFh and PS were roughly equivalent with the Fraunhofer materials showing a trend to be more effective in the unvaccinated donors (Figure 8). The antibody levels produced in the vaccine stimulated MIMIC® cultures was essentially equivalent to that of aFh. These findings indicated that there was little effect on the production of anti-hemagglutinin antibodies when the Fraunhofer material was formulated in alhydrogel.

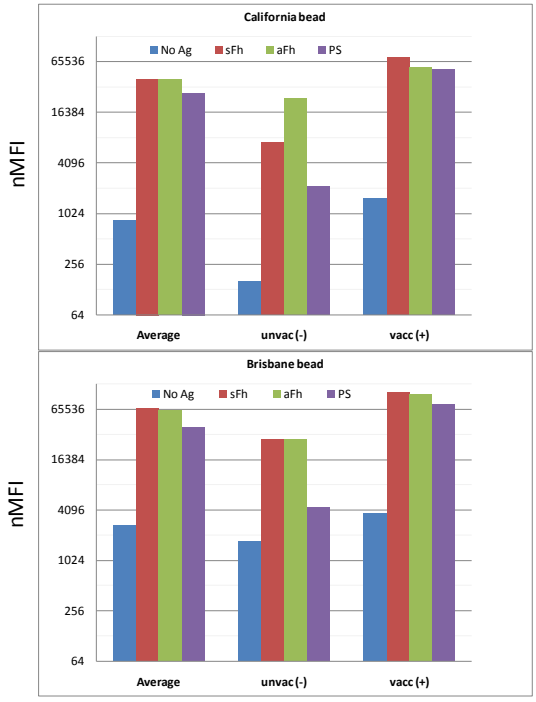


Figure 8. Effect of various formulations of recombinant California hemagglutinins on IgG from California and Brisbane beads. MIMIC cultures were incubated with California hemagglutinin from Protein Sciences (PS), Fraunhofer (aFh), Fraunhofer formulated in alhydrogel (aFh) or no added antigen (no Ag) and supernatants were evaluated by antibody forensics for reactivity with beads linked to either California or Brisbane hemagglutinins. Vacc(+) indicates culture was with cells from vaccinated donor, unvac(-) indicates culture was with cells from donor with no history of vaccination prior to PBMC collection. Average values for 9 donors in each category are shown.

Analysis of antibody affinity from the various sample conditions indicated that the antibodies produced in response to the Fraunhofer materials were of roughly equal affinity which was similar to that generated by the vaccine formulations. The production of functional antibodies was low in all conditions and rendering interpretation of the data difficult. In addition, MIMIC® cultures designed to evaluate the response of $CD4^+$ T cells to the various formulations were carried out on the donors in this task and are described above. In summary, there was a significant amount of cross-reactivity in T cell response to hemagglutinins from Brisbane and California but there was little difference in the response between alhydrogel-formulated and soluble forms of the Fraunhofer

material. Overall our MIMIC results indicate little or no benefit in formulation of the Fraunhofer hemagglutinin with alhydrogel.

In summary, this project has demonstrated that the plant-derived California hemagglutinin from Fraunhofer is capable of stimulating a fully human in vitro system to produce antibodies that recognize recombinant hemagglutinin from both the pandemic and seasonal H1 viral strains. In terms of its ability to drive the production of specific immunoglobulin, the Fraunhofer material is weaker than more complex antigens found such as whole virus or in vaccines containing complex antigens. However, the plant derived material did appear to outperform HA produced in baculovirus. Plant derived HA was observed to generate antibodies with an affinity for hemagglutinin comparable to those stimulated by whole virus or vaccine. Generation of functional antibodies in the MIMIC® system by whole virus or vaccine was substantially higher than for the Fraunhofer material. This may reflect the purity of the protein that Fraunhofer produces or, possibly, relate to the specific post-translational differences inherent to a plant-based expression system. The question of cross-reactivity between the seasonal and pandemic hemagglutinins was addressed in a pre/post study with donors that received seasonal vaccine and in an investigation of the $CD4^+$ T cell response to both. Here, we observed a considerable level of cross-reactivity between the proteins at the T cell level. Examination of antibodies produced in MIMIC® from the donors that received the seasonal vaccine revealed that there was a higher proportion of cross-reactive functional antibody produced by memory cells than there was in sera. This cross-reactivity implies that there is immune potential to react to the pandemic strain that is not visualized by considering the serum alone. Indeed, sera is a reflection of the presence of antibody secreting cells whereas the MIMIC system relies upon activation of memory B cells, a scenario that may account for differences in antibody profiles.

2.2. 2011 Summary

The overall scope of this project, which has been expanded upon this year, is to enumerate and characterize (1) cross-reactive and cross-protective immune responses between seasonal and pandemic H1N1 influenza strains using both commercial and novel plant expressed vaccine candidates and (2) to assess the predictability of an *in vitro* "clinical trial in a test tube" (the MIMIC® system) in the context of a prospective pandemic H1N1 clinical trial.

For this study, we coupled the MIMIC® system to a Phase I clinical trial to evaluate the safety and immunogenicity of a recombinant, plant-expressed hemagglutinin from the pandemic H1N1 strain A/California/7/2009 (rHA) produced by Fraunhofer CMB USA. The study was designed to address both basic and applied questions with respect to the immunological quality of the HA species present in the two vaccines by assessing their ability to produce antibodies that recognize HA (magnitude of response) and functional antibodies that are active in the hemagglutination inhibition assay (HAI). The report is organized to reflect the Tasks defined in an extension of the original proposal and to report our assessment of the Phase 1 clinical trial study using the MIMIC® system. Briefly, the goals of these tasks were to: [1] conduct a dosing study with rHA in MIMIC® cultures (Task 3), [2]. assess the effect of vaccination with trivalent seasonal vaccine on the $CD4^+$ T cell response to California H1 hemagglutinin (HA) in the MIMIC® system (Task 3) [3] assess the *ex vivo* and *in vitro* immune responses to a commercial pandemic H1N1 vaccine (new Task) and [4] couple the MIMIC® system with an H1N1 pandemic influenza vaccine Phase 1 clinical trial. During the past year we have been able to address some fundamental questions regarding the immune relationships between seasonal and pandemic influenza, gaining insight into basic immune functions relating to anti-influenza $CD4^+$ T cell responses, and thoroughly characterizing immune responses generated to a this novel vaccine formulation in a clinical trial.
We discuss each of the four main tasks below in more detail:
[1] In preparation for our participation in the clinical trial using the rHA vaccine from Fraunhofer, we conducted dosing studies in the MIMIC® system to evaluate the rHA protein's effect on induction of anti-California HA antibodies (Task 2 extension). These studies were set up to reflect, as closely as possible, co-culture conditions used in the MIMIC® LTE (lymphoid tissue equivalent module) for evaluation during the clinical trial. Three trials were performed using 4-5 donors each at varied doses of rHA, formulated with or without alhydrogel adjuvant. A single subset of donors evaluated the effects of transformation of the LTE cultures infected with Epstein Barr virus (EBV) to enhance antibody production. Results suggested:

- Doses of 0.54 μg/ml or above stimulated approximately the same response from most of the donors in the study.
- There appeared to be little effect on either DC or B cell viability over the dose range we studied indicating that the rHA had little toxicity.
- EBV transformation offered no apparent advantage in these cultures.
- As a result of these studies we decided to use the following stimulation conditions for the clinical trial: 1.6 μg/ml of rHA as a dose in the PTE (peripheral tissue equivalent), 0.8 μg/ml rHA to pulse the B cells and the standard MIMIC® culture condition without EBV addition.
- Since the clinical trial study was blinded we had to use the same conditions to evaluate each of the trial arms.

[2] Task 3 extension. The outbreak of the novel swine-origin H1N1 influenza in the spring of 2009 galvanized a massive worldwide effort to produce millions of vaccine doses to protect against this single virus strain. Of particular concern was the apparent lack of pre-existing antibody capable of eliciting

cross-protective immunity against this novel influenza virus. This fueled fears that this strain would trigger a particularly far-reaching and lethal pandemic. Given that disease caused by the swine-origin virus was far less severe than anticipated, we hypothesized that cellular immunity to cross-conserved T cell epitopes might have played a significant role in protecting against the pandemic H1N1 in the absence of cross-reactive humoral immunity. To address this question, we collaborated with Epivax Inc. to evaluate a series of peptide epitopes shared between the 2008-09 seasonal H1N1 vaccine strain and the pandemic H1N1 A/California/04/2009 using the MIMIC® system (*1*).

- PBMCs from human donors not exposed to the pandemic virus demonstrated that pre-existing $CD4^+$ T cells can elicit cross-reactive effector responses against the pandemic H1N1 virus.
- Computational tools were 80–90% accurate in predicting $CD4^+$ T cell epitopes and their HLA-DRB1-dependent response profiles in donors that were chosen at random for HLA haplotype.
- Results confirm the power of coupling immunoinformatics with biological assays to define broadly reactive $CD4^+$ T cell epitopes with highly sensitive *in vitro* biological assays to verify these in silico predictions as a means to understand human cellular immunity, including cross-protective responses.
- Results also indicated the ability of immunoinformatics to define $CD4^+$ T cell epitopes for potential vaccination efforts against future influenza viruses and other pathogens.

[3] Task 3 extension. As part of our continued efforts using the MIMIC® system to characterize the anti-influenza response of donors following vaccination with seasonal vaccines we conducted a 20 donor study with a commercial monovalent pandemic H1N1 vaccine from Novartis. It was anticipated that this study would help benchmark donor's response to a commercial pandemic vaccine in anticipation of the upcoming clinical trial. The study was structured to allow evaluation of pre- and post-immunization responses in serum and MIMIC® lymphoid tissue equivalent (LTE) supernatants to the following H1 hemagglutinin (HA) types using Antibody Forensics (AF): New Caledonia-1999; Solomon Islands-2006; Brisbane-2007, California-2009 (swine flu). AF allowed us to investigate the magnitude of the antibody response to the various HAs as well as characteristics such as antibody affinity and cross-reactivity. In addition to evaluating the antibody's HA binding we also assessed their functional (neutralizing) capabilities. To this end we used a highly sensitive variation of the traditional hemagglutinination inhibition assay. This assay, the surface assisted hemagglutination inhibition assay (SA-HAI), was used to evaluate the level of functional antibodies generated by vaccination. An adaptation of this assay, designated the functional avidity index (FAI), was developed to estimate the contribution of high avidity antibodies to the functional humoral response. The general findings are illustrated below:

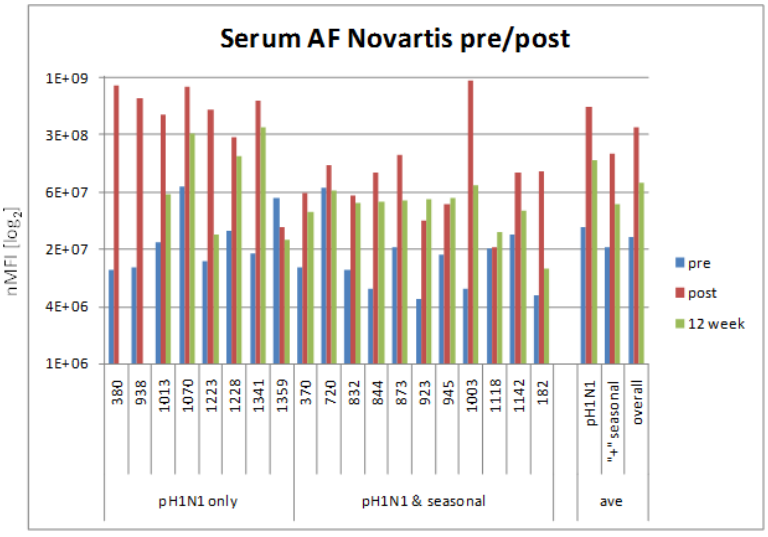


Figure 9. Anti-California HA IgG levels in sera. Donors (X-axis) were separated into two groups based on their prior exposure to seasonal vaccine. Bars represent normalized mean fluorescence intensity, nMFI (bead fluorescence x dilution).

- Sera AF analysis indicated that the average anti-California HA antibody levels for donors who had previously received the seasonal vaccine versus those

who had not did not vary substantially (Figure 9). This observation is consistent with other reports indicating a lack of pre-existing antibody response to the pandemic strain.

- Sera functional analysis indicated a high degree of seroconversion post vaccination with the commercial pandemic vaccine. The SA-HAI analysis is roughly 6 fold more sensitive than traditional HAI. This factor was used to estimate a sero-threshold limit that would equate with the 40 HAI unit level considered protective for the traditional assay. (This is indicated on Figure 10.) When this threshold is applied it is evident that 19 -20 of the 22 donors achieved a level of antibody indicative of protection after vaccination with the commercial pandemic vaccine.

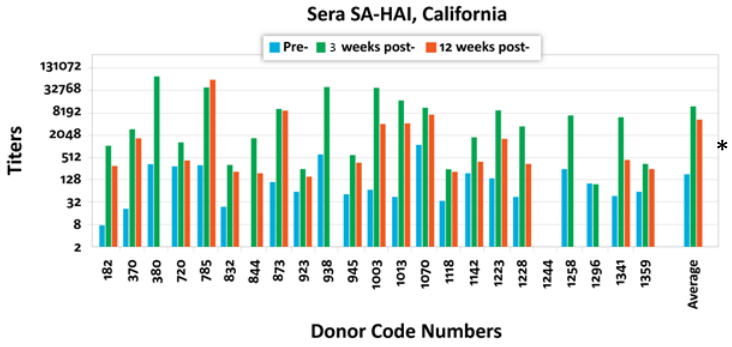


Figure 10. Functional analysis of donor serum at 3 and 12 weeks post-vaccination. Donor's serum was evaluated using the SA-HAI assay at three time points, pre vaccination and 3 or 12 weeks post vaccination. The SA-HAI is roughly 6 fold more sensitive than traditional HAI. A serothreshold level, equivalent to a response of 40 HAI in the traditional assay, is shown (*).

- Measurement of the functional affinity index FAI indicated that, on average, the contribution of high avidity antibodies is 50% of the overall functional reactivity.
- The differential response in MIMIC® pre samples between the No Ag and H1N1 condition (*in vitro* immunization) is approximately of the same magnitude as the differential in the serum between the pre and post 3 week samples (Figure 11) indicating that MIMIC® responses were predictive of human serological findings.

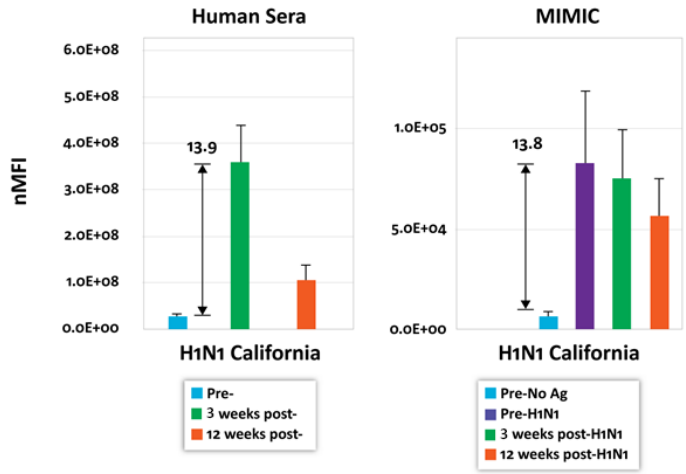


Figure 11. Comparison of Sera and MIMIC supernatant antibody by AF pre and 3 and 12 weeks post-vaccination. Population averages are shown for supernatants from 14 day MIMIC cultures or serum evaluated for the presence of anti-California antibodies using AF. Results of analysis of the "Pre" MIMIC supernatant from co-cultures containing DC and B cells that had been pulsed with vaccine (H1N1) or had not (No Ag) are shown. The 3 and 12 week post supernatants were from cultures pulsed with vaccine. The fold differential between the pre and 3 week serum sample and the No Ag and H1N1 MIMIC are indicated.

- AF comparison of the cross-reactivity profiles of antibodies present in serum and MIMIC® supernatant differed substantially (see Table 2). The MIMIC cultures generated antibody profiles that were far more cross-reactive than those seen for serum.

- An explanation for this difference in profile may be explained by the cell types that are responsible for antibody production. It might be anticipated that the memory population (MIMIC®) would harbor B cells with broader repertoire of antigen recognition than those that had recently responded to challenge by a vaccine and gone through affinity maturation in response to a specific antigen (serum).

MIMIC: cross-reactivity correlations Post-Vaccine IgG Data

	California	New Caledonia	Brisbane	Solomon Islands
California	X	0.98	0.98	0.87
New Caledonia	X	X	0.98	0.87
Brisbane	X	X	X	0.86

Serology: cross-reactivity correlations: Post-Vaccine IgG Data

	California	New Caledonia	Brisbane	Solomon Islands
California	X	0.13	0.14	0.33
New Caledonia	X	X	0.82	0.82
Brisbane	X	X	X	0.85

Table 2. Cross-reactivity of antibodies present in serum and MIMIC® supernatants assessed by AF. Supernatant from 14 day MIMIC® cultures, grown in the presence of vaccine-pulsed DC and B cells, was evaluated for the presence of anti-California or seasonal (New Caledonia, Brisbane or Solomon Islands) anti-HA antibodies using AF. Serum samples from the 3 week draw were compared against the same HAs (see Table 3.3.1). The presence of antibodies in each serum which reacted with the different HAs was evaluated using the correl function in Excel.

- Functional antibody responses were evaluated by SA-HAI. While MIMIC® cultures yielded substantially lower levels of inhibitory antibodies as would be anticipated for an *in vitro* system, the profile, characterized by an increase in functional antibodies at 3 weeks followed by a decrease by 12 weeks, closely resembled trends found in serum (Figure 12).

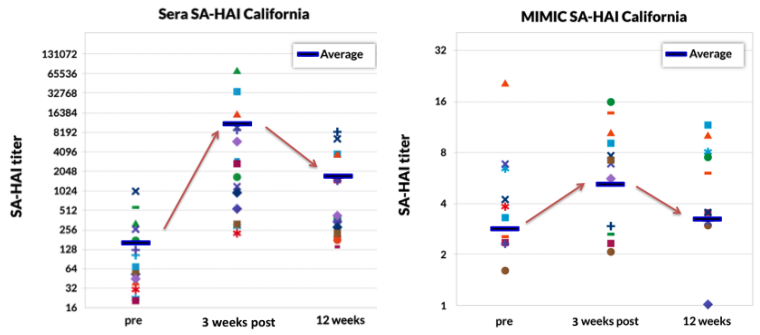


Figure 12. Anti-California virus SA-HAI reactivity of MIMIC supernatants and serum. Supernatant from 14 day MIMIC cultures, grown in the presence of vaccine-pulsed DC and B cells, or serum samples were evaluated for the presence of antibodies capable of inhibiting hemagglutination of RBC by A/California by SA-HAI. Individual donor's responses are shown. Average response is indicated by a heavy blue bar. Samples were acquired pre vaccination or 3 or 12 weeks post vaccination.

[4] Couple the MIMIC® system with a H1N1 pandemic influenza clinical trial. Our goals for the clinical trial, conducted in collaboration with the Walter Reed Army Institute of Research (WRAIR) and Fraunhofer CBM USA, were to provide a systematic immunological assessment of the candidate pandemic influenza vaccine, with specific attention to the recombinant, plant-derived hemagglutinin from H1 A/California (rHA). Here we anticipated that the clinical trial would further refine the accuracy, utility, and predictive powers of the MIMIC® construct for influenza vaccines by comparing *ex vivo* and *in vitro* immune responses to those observed in a prospective clinical trial. Our systems immunological approach takes into account pre-existing influenza exposure, safety, and the quantity, quality, and functionality of influenza-specific immune responses to the rHA vaccine candidate. Since the tasks we defined were so inter-related, the results are simpler to explain if they are presented based on the analysis performed rather by task. The data was also separated to clearly define whether the results were from serum or MIMIC® samples. However, for Option 2 – "To explore the presence of IgM

responses to the California H1 strain using Antibody Forensics"- the data is covered in this Tasks section. Options 1 and 3 will be covered individually as they are not directly linked to the analysis conducted for the Tasks.

In summary, the IgG relationships between the MIMIC® system and the clinical trial sera results for general "trends" were found to be excellent, thus supporting the predictive nature of the MIMIC® system as an "*in vitro* clinical trial in a test tube." This was found for both the rHA and control vaccine samples. We also performed correspondence tests between our advanced immunoassays (SA-HAI) and traditional HAI and found the correlations to be excellent as well.
Our assessment of the clinical study samples has provided insights into the nature of the response to the rHA vaccine. We found little reactogenicity associated with the rHA material. A limited donor study involving the enumeration of precursor $CD4^+$ T cells indicated that there appeared to be an inverse relationship between high levels of T cells and the anti-HA antibody level. AF studies indicated that there is a high level of cross-reactivity in the serum antibodies between the hemagglutinins of the H1 strains California, Brisbane, Solomon Islands and New Caledonia in donors that received the rHA vaccine but not the control commercial pandemic vaccine. The SA-HAI assay indicates that the rHA vaccine is capable of generating functional antibodies but that the efficiency of production of these antibodies differs from that of the commercial vaccine

- Logistics – Donor screening and sample collections
    - Selection of study donors was based on identifying applicants with a HAI titer against A/California (H1N1) of ≤ 40. VaxDesign tested 221 samples of which 112 were qualified for participation in the trial.
    - Collection and processing of whole blood samples was performed on site at WRAIR. VaxDesign established a remote processing laboratory on site to process samples immediately after collection.
    - A total of 232 clinical trial samples were processed out of the 240 slated for processing.
    - Whole blood sample collected as part of the clinical trial were processed using a standardized format to produce a serum sample and a peripheral blood mononuclear cell (PBMC) product.
- The MIMIC® LTE module was adapted specifically for the clinical trial to deal with the reduced cell numbers recovered from the 100-150 ml whole blood donations.
- Antibody Forensics (AF) comparisons of MIMIC® and human sera from the clinical trial.
    - Samples were taken at 3 time points, pre-vaccination and at day 21 and 56 post-vaccination. The post-vaccination time points were scheduled to occur 3 weeks after the initial (prime) vaccination and after the subsequent boost with the rHA vaccine.
    - Immunization with rHA or control commercial pandemic vaccine (California) significantly increased the California-specific IgG levels for both the human sera and the MIMIC® cultures.
    - MIMIC® and sera show similar trends in anti-California HA antibody response (Figure13).

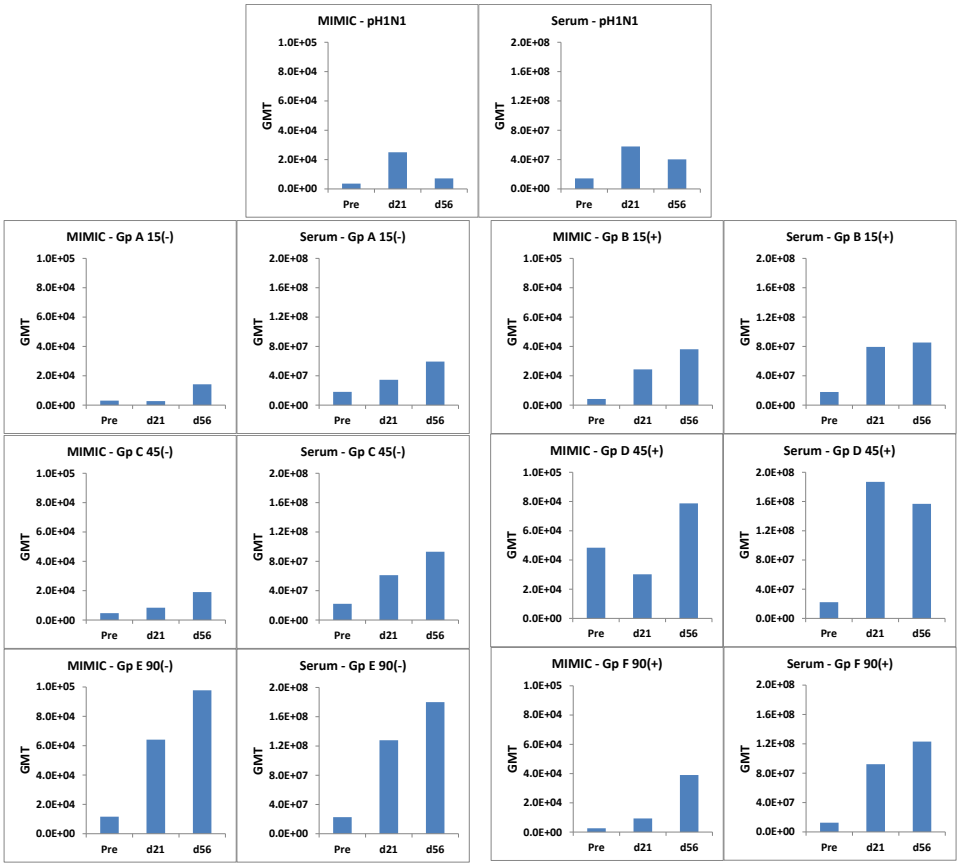


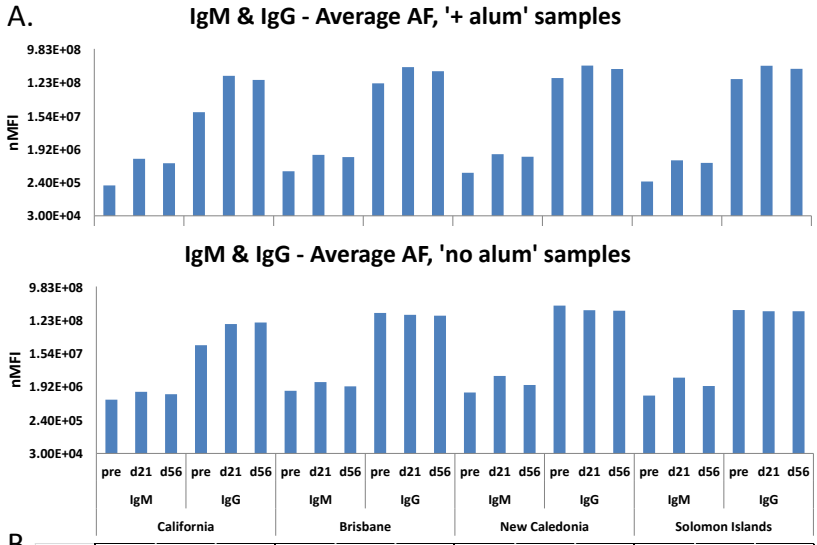
Figure 13. Comparison of geometric mean titers of Serum and MIMIC for AF with California beads. Geometric mean titers (GMT) for each of the groups were calculated for the 3 time points at which samples were taken. Graphs are paired by group and formulation.

- *In vitro* vaccination as compared to *in vivo* inoculation with FhCBM H1 vaccine candidates showed striking comparisons in the relative H1-specific IgG levels between MIMIC® and the clinical trial
- Unadjuvanted rHA vaccine groups (A, C and E) show a dose-dependent increase in titer for both MIMIC® and sera.
- Adjuvanted rHA vaccine groups show a peak response at the 45 µg dose for both MIMIC® and sera
    - Adjuvant appeared to have a dose sparing effect when the 15 and 45 µg rHA doses were compared,

- The IgG relationships between the MIMIC® system and the clinical trial on general "trends" were found to be excellent.
- The correlation between MIMIC® responses and those seen in sera indicate the ability of MIMIC® to predict a response at the population level.

- Antibody Forensics (AF) analysis of human sera. The following summary discusses of results from the serum samples collected during the clinical trial
  - The differential in antibody level between anti-California HA IgG and IgM in sera was 100 to 200 fold indicating that the IgM probably played a very minor role in the overall response to either of these viruses (Figure 14).
  - Sera AF data indicates that the cross-reactivity of antibodies generated by immunization with rHA is quite high (Table 3).
    - This result was contrary to that found for serum samples generated by the control vaccine used in the clinical study or in the study conducted in September 2010 with the Novartis monovalent pandemic vaccine.
    - This was an indication that the rHA vaccine and the control vaccine created different immunological responses.

A.



B.

	California			Brisbane			New Caledonia			Solomon Islands		
	IgG/IgM ratio			IgG/IgM ratio			IgG/IgM ratio			IgG/IgM ratio		
	pre	d21	d56	pre	d21	d56	pre	d21	d56	pre	d21	d56
+ alum	99	184	186	247	246	220	381	261	247	617	379	364
no alum	31	70	90	133	68	85	234	62	105	214	65	109
	fold increase over pre			fold increase over pre			fold increase over pre			fold increase over pre		
		d21	d56		d21	d56		d21	d56		d21	d56
+ alum	IgM	1.9	1.9	IgM	1.0	0.9	IgM	0.7	0.6	IgM	0.6	0.6
	IgG	9.8	7.5	IgG	2.8	1.8	IgG	2.2	1.8	IgG	2.3	1.9
no alum	IgM	2.3	2.9	IgM	0.5	0.6	IgM	0.3	0.5	IgM	0.3	0.5
	IgG	3.8	4.2	IgG	0.9	0.8	IgG	0.8	0.7	IgG	0.9	0.9

Figure 14. IgM and IgG antibody levels in sera samples from donors that received recombinant California HA. A. GMT values for vaccinees that received recombinant, plant-derived California HA with or without alhydrogel (alum). All donors (15, 45 and 90 mg) are included in each treatment category. B. Calculations of IgG/IgM ratios and increase in response over pre-vaccination samples.

(California H1) X (New Caledonia H1, Solomon Islands H1, Brisbane H1)

		New Caledonia	Brisbane	Solomon Islands
rec. CA HA + Alum	Day 21	0.90	0.92	0.89
	Day 56	0.83	0.84	0.81

		New Caledonia	Brisbane	Solomon Islands
rec. CA HA No Alum	Day 21	0.81	0.77	0.82
	Day 56	0.69	0.74	0.72

		New Caledonia H1N1	Brisbane H1N1	Solomon Islands H1N1
pH1N1 vaccine, control	Day 21	0.007	0.12	0.06
	Day 6	0.25	0.56	0.36

		New Caledonia H1N1	Brisbane H1N1	Solomon Islands H1N1
pH1N1, Sept. 2010	Day 21	0.13	0.14	0.33
	Day 56	0.09	0.26	0.67

Table 3. Cross-reactivity of serum samples post vaccination with recombinant California HA or commercial vaccines. Donor's serum, from 21 or 56 day timepoints, were grouped by formulation and evaluated for response to beads coated with the three hemagglutinins shown. The groups receiving the recombinant HA from A/California (Fraunhofer) were separated based on whether they

were formulated with alhydrogel (alum) or saline. There are results from two commercial vaccines shown, H1N1 vaccine control results are from the current study and the Sep t. 2010 results are from the study conducted earlier. The presence of antibodies in each serum which reacted with the different HAs was evaluated using the CORREL function in Excel.

- This high degree of cross-reactivity could be driven by two types of antibodies, those that react with HA and are not active in the protective response or those that are functional and actively protect against infection.
  - . This paradox led us to propose the alternative "best case" and "worst case" hypotheses for the recombinant HA's effects.
  - According to the "best case", the recombinant, plant-based HA vaccine contains a larger concentration of antibodies relevant to the conserved HA stem region epitopes than the response to the traditional H1 control vaccine. The "worst case" option implies that the vaccine merely drives production of antibodies that are not active in functional tests.

- Functional analysis of sera samples. The following summary discusses of results from the serum samples collected during the clinical trial comparing the SA-HAI results to those of classical HAI, evaluating the responses by group (those that received rHA vaccine or control pH1N1 vaccine) and by dose. In addition our results were compared with those obtained by the Centers for Disease Control (CDC) using traditional HAI.
  - The correspondence in titer profiles for inactivated A/California virus between SA-HAI and standard HAI run by the CDC (Figure 15) are very good, with a substantial correlation of 0.92. The strong correspondence between traditional HAI and the more sensitive SA-HAI assay run by a world-recognized influenza laboratory demonstrates the validity of our approach.

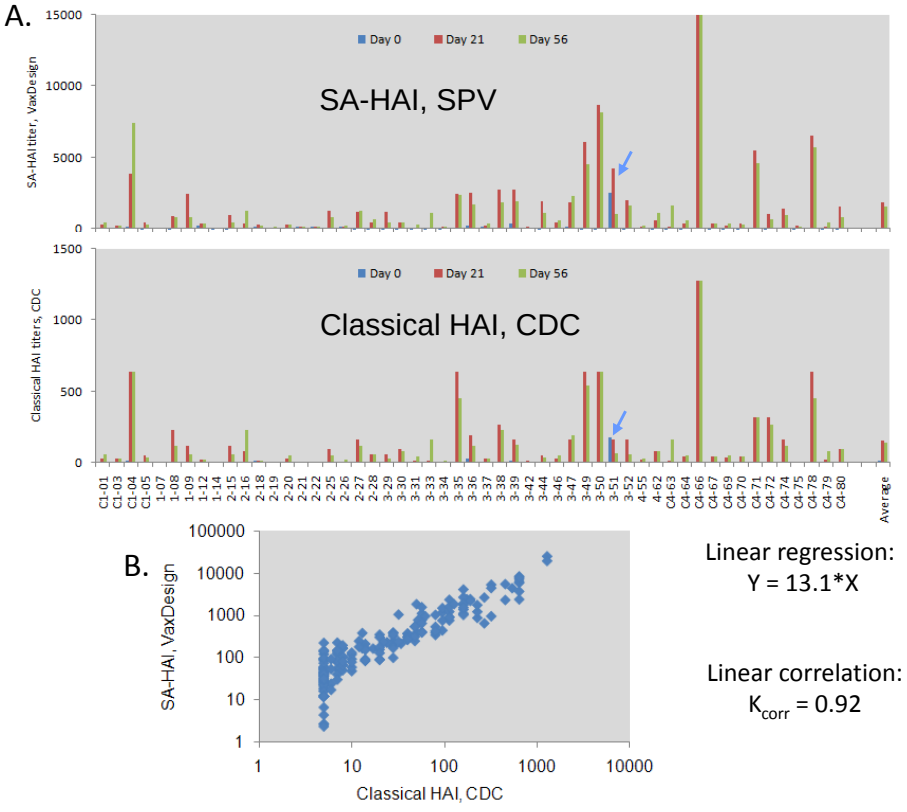


Figure 15. Comparison of classical HAI and SA-HAI reaction to inactivated A/California in sera from clinical trial. A. Results of traditional HAI and SA-HAI analyses conducted on sera from the clinical trial Arrow indicates donor that converted to seropositive prior to the start of the study**.** B. Scatter plot of SA-HAI values vs. HAI values.

- For unadjuvanted rHA, increased dose increased the SA-HAI titer.
- The formulation of recombinant HA with alhydrogel generated the highest GMT titer with the median rHA dose, 45 µg (Figure 16).
  - The 90 µg dose formulated with alhydrogel gave responses similar to the 15 µg dose.
  - Trends in SA-HAI titers closely match those seen for AF (Ab IgG levels) in serum.
- There was a striking contrast between the AF level and SA-HAI results for the control vaccine and rHA (Figure 17).

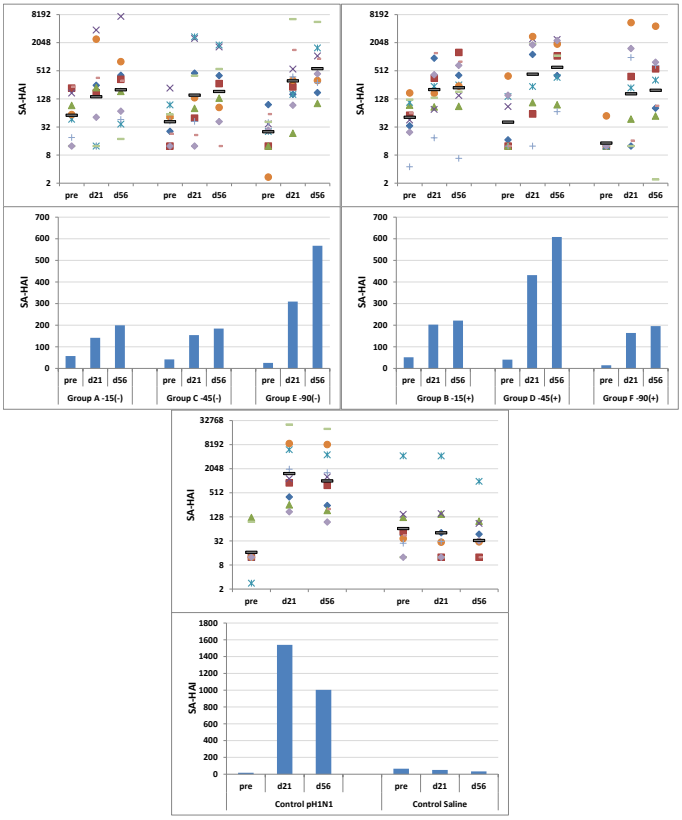


Figure 16. SA-HAI titers for A/California in sera divided by group. The groups were divided into those receiving recombinant HA without adjuvant (A, C and E), those receiving recombinant HA with adjuvant (B, D and E) and the controls (pH1N1 vaccine, saline). Results are shown by group and sample day. The top graph in each panel shows individual donor values (log 2 scale) and the bottom graph shows the group average GMT (linear scale). The maximum values for each graph are equivalent for the donors that received recombinant HA but are increased for the controls. Dark bars in the upper graphs are the GMT for the group.

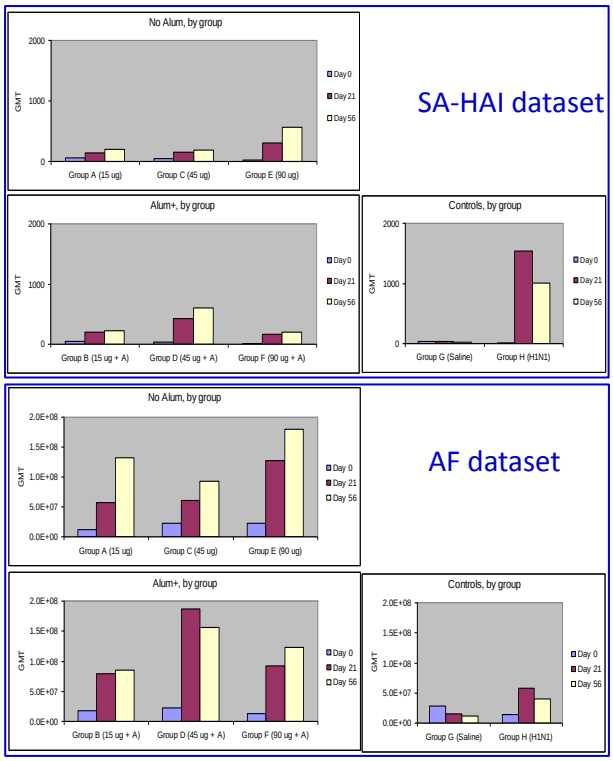


Figure 17. Comparison of the GMT values for SA-HAI and AF datasets for serum samples. GMT titers for the SA-HAI (top panels) and AF (bottom panels) of the various groups in the clinical trial are aligned to enhance comparison of the trends seen in both assay methods.

- The control vaccine samples have a higher SA-HAI/AF ratio than the rHA samples. This indicates that there is greater than a 10 fold difference in the level of functional antibody produced per unit of IgG between the

two groups. The commercial vaccine is more efficient at producing HAI functional antibodies (Figure 18).

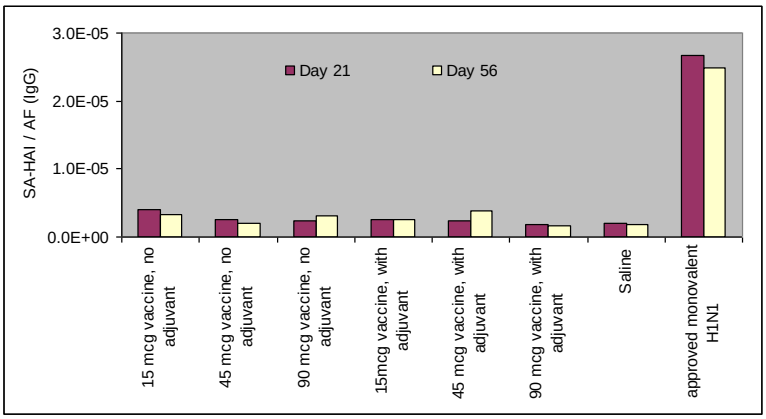


Figure 18. Relationship of magnitude to functionality of antibodies generated by vaccination with rHA (± adjuvant) and control vaccine. The ratio of functional antibody (SA-HAI) to total antibody (AF) was estimated by dividing [SA-HAI/AF]. Higher bars indicate a larger proportion of functional antibody per total antibody generated by vaccination.

- Option 1 – $CD4^+$ T cell frequency. This study examined correlations between the pre-existing $CD4^+$ T cell population and the response to rHA or the control pH1N1 vaccine.
  - An IFN-γ ELISpot assay was employed to study the $CD4^+$ T cell precursor frequency.
  - The frequency of precursors in the T cell population capable of responding to the recombinant HA varied as much as 15-fold in the various donor groups (10–150 HA-specific $CD4^+$ T cells/600,000 total $CD4^+$ T cells).
  - There was no substantial difference in the number of precursor $CD4^+$ T cells in pre, post and boost samples from donors who had received r HA formulated with saline (Figure 19). In contrast, there a noticeable increase in the precursor frequency of $CD4^+$ cells when the donors were vaccinated with the rHA formulated in alhydrogel, especially after booster vaccination (day 56 timepoint).

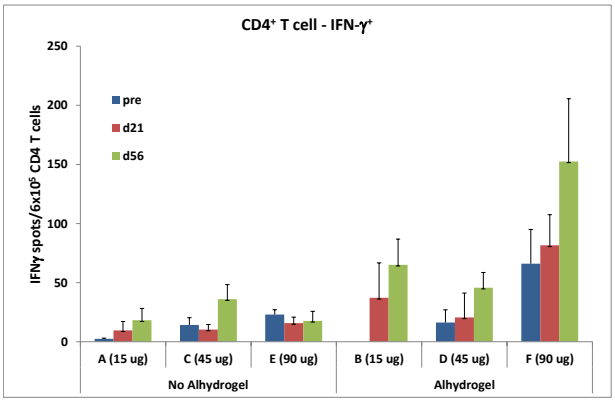


Figure 19. CD4 T cell precursor frequency in groups immunized with recombinant California HA. Purified CD4 T cells were incubated with dendritic cells pulsed with recombinant, plant-derived hemagglutinin and evaluated for expression of IFNg by ELIspot. Average values shown. Error bars represent standard error of measurement

  - Very high $CD4^+$ T cell responses tended to be at the expense of influenza-specific Ab responses
    - Conditions in which the $CD4^+$ T cell responses were enhanced, as observed in 90 µg of HA dose with alhydrogel, generally resulted in substantially lower magnitude of HA-specific antibody responses. This inverse relationship between anti-California HA titer and CD4 T cell

precursor frequency was particularly obvious in group F (alhydrogel+90 μg/ml) and the control vaccine.
        - May explain the decrease in Ab titer for the 90 mg alum-adjuvanted vaccines
- Reactogenicity of the rHA vaccine candidate was evaluated using the MIMIC® PTE module that reflects innate immune responses. The pre-vaccination PBMCs were evaluated under three conditions, no added antigen, addition of the recombinant HA (rHA) or addition of the rHA formulated in alhydrogel (3μg/ml final). The no antigen condition provided a baseline and the alhydrogel formulation (designated as alum in the figures) was included to provide an idea of the effect the adjuvant had on the innate response. All donors were tested under the no antigen (no Ag) and + rHA conditions but only the cells from groups B, D and F (those that were formulated wit alum in the clinical study) were given rHA formulated in alhydrogel (alum+rHA).
    - Phenotyping analysis - The majority of markers show very minor changes between no antigen, +rHA and alum+rHA within a donor.
    - These *phenotyping* results indicate there is little, if any, reactogenicity associated with the rHA antigen.
        - The +rHA does not stimulate the production of any inflammatory (TNFα), TH1 (IFNγ, IL-2) or TH2 (IL-4, IL-5, IL-10) cytokines present in this panel to a level that significantly exceeds that of the no Ag baseline control.
        - There was little difference in expression level for the inflammatory cytokines, GM-CSF, IL-6, IL-8 or MCP-1, between the no Ag treatment and those modules that received rHA or alum+rHA.
    - The cytokine results support the findings reported above for the phenotypic markers, both sets of results indicate there is little, if any reactogenicity associated with the rHA antigen.

2.3. 2012 Summary

One of the issues encountered while conducting the MIMIC® study in parallel with Fraunhofer's clinical trial was the limitation in total cells available for analysis. As a follow up to Task 2 we evaluated MIMIC LTE culture conditions that would allow 'cell-sparing' in situations such as the clinical trial. In a similar manner, elderly blood donors frequently exhibit low cell yield at harvest and small sample volumes. In order to address this issue we performed a study using elderly donors' samples in which we investigated a protocol that differed from our standard LTE setup. This entailed a protocol of "cell sparing where an assay could be set up with the minimum manipulation of the PBMC source and the minimum number of cells that retained the capacity to generate a robust immune response.

- Initial studies focused on modifications that would eliminate the need to prepare both cytokine-derived DCs and purified $CD4^+$ T cells.
    - Purified B cells were mixed with either PBMC or PBMC depleted of $CD8^+$ T cells
    - A 7 donor study demonstrated that the PBMC depleted of $CD8^+$ T cells could efficiently serve as a source for antigen presenting cells and $CD4^+$ T cell help
- A pre/post study was carried out with 22 donors vaccinated with the 2010-11 trivalent influenza vaccine using the modified LTE setup containing CD8+ T cell depleted PBMC and purified B cells. The pre/post study is wherein we collect blood samples both before and after vaccination to compare with in vitro vaccination of the "pre" samples. In this manner, each donor serves as their own control and is considered a good process to further qualify the MIMIC system. Antibodies produced in MIMIC® LTE cultures were evaluated by Antibody Forensics (AF) for magnitude of response and cross-reactivity with other influenza hemagglutinins (HA) and by Surface Assisted-Hemagglutination Inhibition Assay (SA-HAI) for the presence of functional antibodies.
    - Modified MIMIC® LTE cultures were set up with cells isolated from either pre or post vaccination and evaluated for cross-reactivity with HA from other influenza strains found in the previous year's vaccines. A high degree of cross-reactivity was seen for the H1/HA while the elderly donors appeared to have a higher cross-reactive index for H3/HA, indicating the possibility of a more diverse response based on their exposure history.

Crossreactivity	H1 Hemagglutinins				H3 Hemagglutinins			
	PRE		POST		PRE		POST	
(CORREL)	California vs		California vs		Perth vs		Perth vs	
	Brisbane	New. Cal.	Brisbane	New. Cal.	Brisbane	Wisc.	Brisbane	Wisc
adult	0.86	0.87	0.98	0.98	0.94	0.57	0.99	0.69
elderly	0.94	0.96	0.83	0.84	0.63	0.92	1.00	0.97

Table 4. Cross-reactivity of antibodies generated in response to the H1 and H3 hemagglutinins present in 2010-11 Fluzone® in MIMIC®. The population was divided into adult (<60) and elderly (>60) for analysis. Individual donor's responses to the various hemagglutinis were compared head to head using the Excel program CORREL to generate the values shown

    - AF results from modified MIMIC® LTE set up with the pre-vaccination PBMC as the cell source were compared to serum samples taken pre and post vaccination. The averaged sera and MIMIC® data showed comparable trends between the *in vivo* and *in vitro* response to all 3 influenza strains present in the vaccine. This was true for both healthy adults and elderly donors; thus, showing the MIMIC system may have applicability for patient segmentation (Figure 20).
    - SA-HAI results from modified MIMIC® LTE set up with the pre-vaccination PBMC as the cell source were compared to serum samples taken pre and post vaccination. Sera and

MIMIC® samples generated similar trends. The adult cohort produced higher levels of functional antibody following vaccination in both systems as compared to the elderly donors (Figure 20).

- The collective data clearly indicate that *in vitro* vaccination in the MIMIC system accurately reflects *in vivo* vaccination of humans.

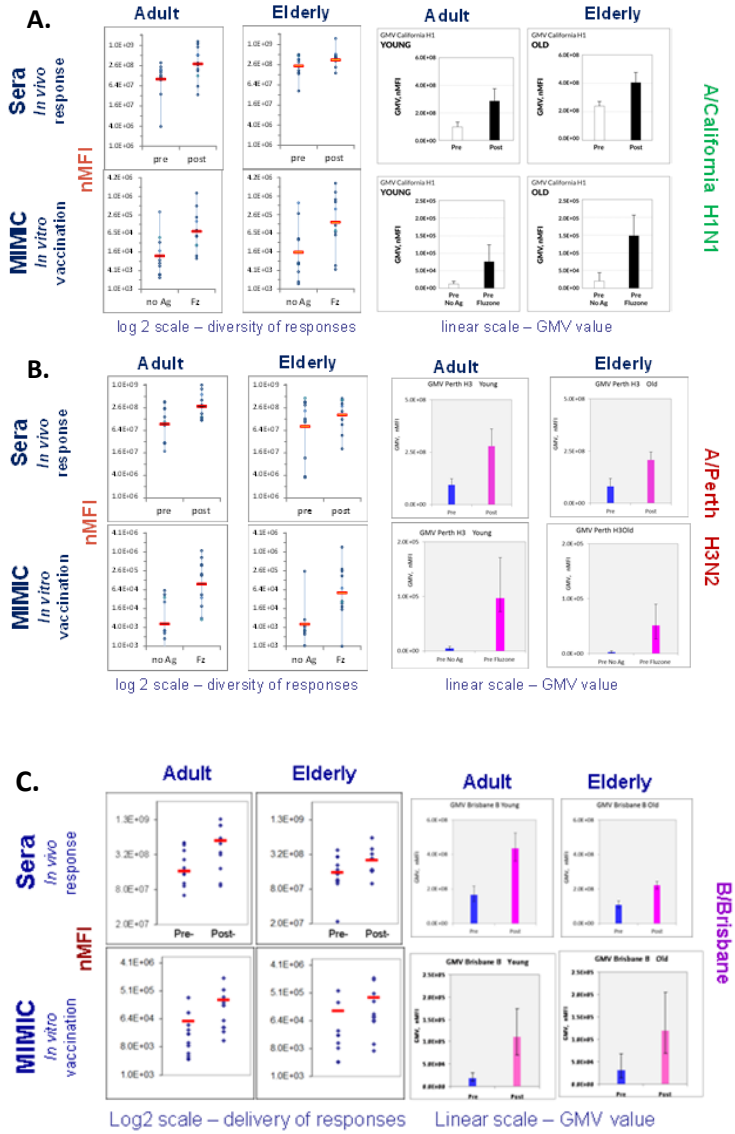


Figure 20. Magnitude of antibody responses to A/California and A/Perth HA in adult and elderly populations. Serum and MIMIC® samples were evaluated for the level of antibody responses to H1 (California, Panel A), H3 (Perth, Panel B) or B (Brisbane, Panel C) hemagglutinins by AF. The left set of panels is arranged to display all of the donor's values and the geometric mean value of the population and uses a log2 scale on the y axis. The right set of panels is in linear scale on the y scale to emphasize the difference in geometric mean value between the cohorts.

To effectively evaluate the influence of vaccine dose in various populations and/or the impact that changes in vaccine formulation might have on potency we considered it necessary to understand the minimum dose at which the standard vaccine could be used at in the MIMIC® LTE system. To determine the ideal dose of Fluzone® for use in the MIMIC® system, we performed a dosing study with both the standard assay using cytokine-derived dendritic cells (DCs) as APCs, purified $CD4^+$ T cells and purified B cells and the modified assay, with CD8-depleted PBMC to serve as a source of $CD4^+$ T cells and antigen presenting cells and purified B cells. We did this to better insure that we had identified, and implemented, the optimal system for use to address questions, such as those posed above, and to distinguish any difference in the two techniques at high dilutions of vaccine.

- An 8 donor study was run with the standard and modified LTE setups described above. Fluzone® vaccine was diluted over a 20 fold range extending from the standard dose, a 1:1000 dilution for B cells to 1:20,000 (Table 5, Figure 21).

Table 1	Fluzone dose			
	PBMC as APC		DC as APC	
	PBMC	B cells	DC	B cells
Standard Assay	1:500	1:1000	1:500	1:1000
	1:2500	1:5000	1:2500	1:5000
	1:5000	1:10,000	1:5000	1:10,000
	1:7500	1:15,000	1:7500	1:15,000
	1:10,000	1:20,000	1:10,000	1:20,000
control	no Ag	no Ag	no Ag	no Ag

Table 5. Dilutions of Fluzone® used in the study. The B cells and the mixes containing the antigen presenting cells (PBMC or DC) were pulsed with vaccine for 1 day prior and washed prior to mixing for co-culture. B cells received ½ the amount of vaccine given to the APCs. There were a total of 6 conditions tested for each donor.

  - The optimal concentration of Fluzone® for B cell pulsing was 1:15,000 or roughly 3 ng of each of the influenza strains HA and 15 fold lower than the standard dose used previously.

Figure 21. Responses to decreasing doses of Fluzone evaluated using the standard and modified LTE setups. Blue bars represent responses generated in the standard LTE and those in orange represent the modified LTE. Antibody magnitude was measured by AF to beads coupled to HA from A/California H1, A/Perth H3 and B/Brisbane.

  - These results also indicated that under optimal conditions: e.g. adequate cell numbers, viable donor cells etc., the standard LTE assay is superior to the modified assay which uses PBMC as a source for $CD4^+$ T cells and antigen presenting cells. However, seven donors out of the eight studied showed a clearly detectable range of anti-influenza antibody responses suggesting that, in the event that sufficient cell numbers are not available and cell sparing is critical, using the modified assays and substituting PBMCs (depleted of CD8 T cells) as antigen presenting cells is an viable alternative.

In anticipation of upcoming studies with several new formulations of the Fraunhofer A/California H1 HA (as a follow up to Task 1), we screened a number of Fluzone® vaccine formulations to evaluate their use as controls in the anticipated studies. These experiments were performed to observe if there was a discernible difference in Fluzone® formulations from year to year and if there was a difference between different formulations of the same vaccine. 20 donors were evaluated in the standard MIMIC® LTE for their response to different formulations of influenza vaccine; 3 different trivalent inactivated vaccines (TIV) and 4 different formulations of quadrivalent inactivated vaccines (QIV, containing 2 influenza B strains). MIMIC® response to each of the formulations was evaluated using AF and SA-HAI to assess antibody magnitude and functionality.

- AF measurements indicated that all 4 of the QIV formulations tested generated a consistently higher response to the various influenza strains than did the TIV vaccines. The 4 QIV formulations were essentially equivalent in generating response to each of the influenza strains they contained indicating that there was a minimal difference between the preparations. Each of the TIV vaccines also elicited essentially the same level of anti-HA antibody indicating that there was little difference in antigenicity between this year's preparations and last year's.

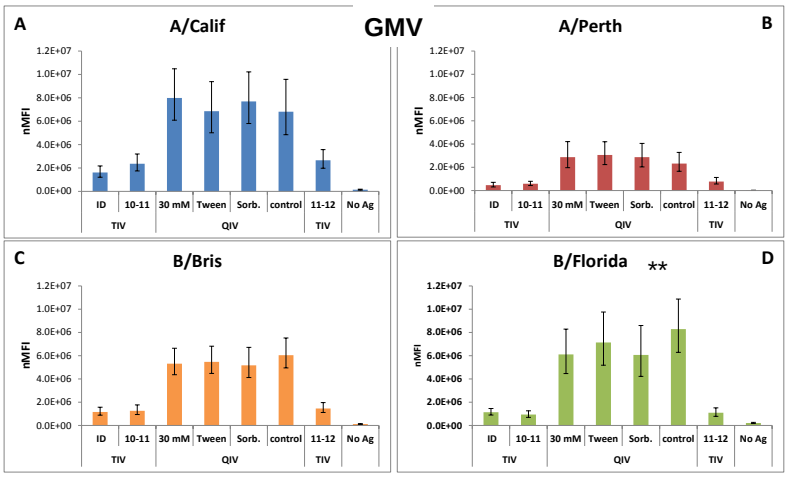


Figure 22. AF values (Geometric mean of all donor's antibody response) for the HA from strains present in the various formulations. Geometric mean values for the population for each HA representing strains in the vaccines are shown. Panel A. A/California H1 HA responses. Panel B. A/Perth H3 HA responses. Panel C. B/Brisbane HA responses. Panel D. B/Florida HA responses (**B/Florida is present only in the QIV vaccine formulations). Error bars represent standard error of measurement.

- SA-HAI measurements closely followed the trends described for AF (Figure 23).

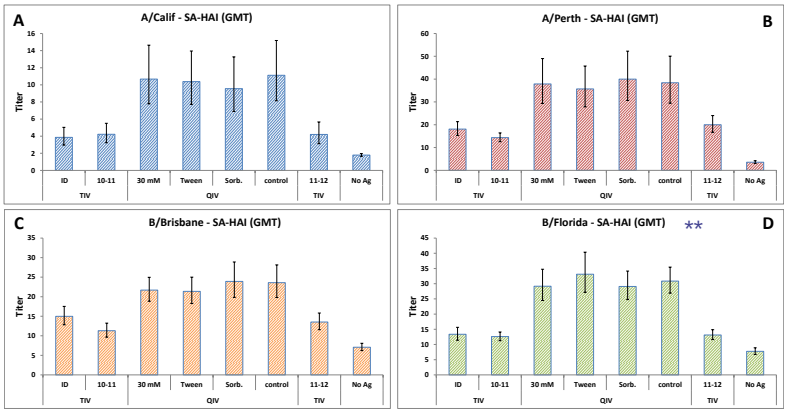


Figure 23. Geometric mean for donor's SA-HAI titers generated by the various Fluzone® vaccine formulations. The geometric mean for all 20 donor's responses was calculated for each of the formulations. Panel A. A/California H1N1, Panel B A/Perth H3N2, Panel C. B/Brisbane and Panel D. B/Florida (**B/Florida is present only in the QIV vaccine formulations). Error bars represent standard error of measurement.

- On the level of individual donors, the AF and SA-HAI values frequently did not correlate. However, when the population was considered as a whole (geometric mean values of AF and geometric mean titers for SA-HAI) the trends predicted by AF were closely mirrored by the SA-HAI values.

- We do not fully understand why the QIV formulations had higher immunogenicity than the TIV influenza vaccines. This is under further study, but highlights the practicality of the MIMIC system to discern differences in formulations and their influence on immunogenicty.

Fraunhofer prepared genetic constructs of their recombinant influenza H1 hemagglutinin antigen that included sequences expected to encode a trimer form or particle-like (VLP). In our first comparative study (as a follow up to task 2) we compared the monomer and trimer forms using 20 donors. Here, we employed a standard MIMIC® LTE culture. The study was performed to evaluate any differences in antigenicity of the different formats and their relative effectiveness in generating functional antibodies. These were compared with a recombinant California-derived baculovirus-expressed H1 hemagglutinin (rHA) from Protein Sciences, seasonal influenza trivalent intramuscular (TIV-IM) or intradermal (TIV-ID) vaccines, and a quadrivalent influenza vaccine (QIV) from Sanofi. We assessed the immunogenicity (Antibody Forensics titer) and functionality (SA-HAI titer) of the antibodies generated by the various vaccines described above.

- Dosing of the various antigens was based on previous experiments. Fraunhofer and Protein Science antigens were used at a concentration of 0.5 µg/ml and the vaccines were diluted to a concentration of 3 ng/ml of each of the hemagglutinins (a total of 9 ng of HA for TIV and 12 ng of HA for QIV). Note the HA concentrations of the Protein Sciences and Fraunhofer antigens are >> than that for TIV or QIV vaccines
- AF results (Figure 24): The antigen constructs generated varying levels of anti-hemagglutinin IgG with Fh monomer generating the highest level and Protein Sciences the lowest. Among the vaccines, the QIV formulation generated the highest level of antibody while both the TIV formulations (TIV-IM & TIV-ID) were roughly equivalent. Fraunhofer monomer and trimer generated anti-HA antibody levels that were roughly equivalent to those generated by the TIV constructs indicating that it is possible to achieve levels of antibody equivalent to that seen with the traditional vaccine, although substantially more recombinant H1 protein (~200 times) was required to do so.

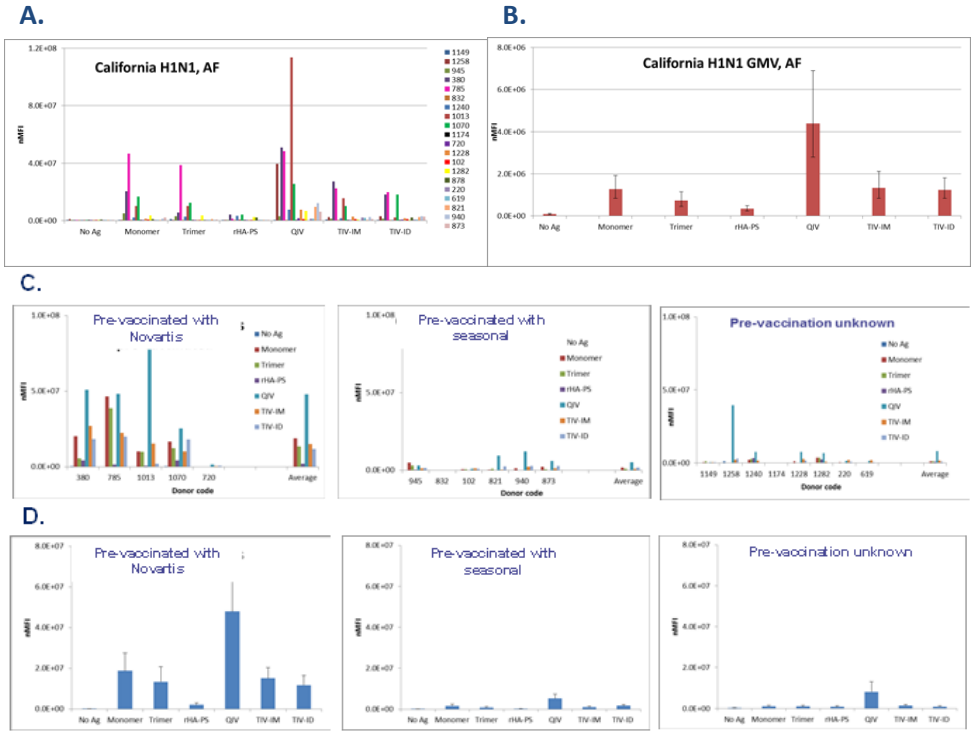


Figure 24. Comparison of anti-California hemagglutinin IgG levels as measured by Antibody Forensics (AF). The hemagglutinins used as antigen in the MIMIC® cultures were in the form of monomer (Fraunhofer), trimer (Fraunhofer), recombinant HA (Protein Sciences) or seasonal trivalent intramuscular or intradermal vaccines (TIV-IM and TIV-ID), quadrivalent vaccine (QIV) or no added antigen (no Ag). Supernatants were evaluated by antibody forensics for reactivity with beads coupled to A/California hemagglutinin from

Protein Sciences. A. Each color bar indicates that individual donor's IgG production under each of the MIMIC® stimulation conditions. Values are represented as normalized mean fluorescence intensity (nMFI) where the Y axis is in linear scale. B. Averaged donor's responses under each condition. Here, geometric mean values are shown. C. The donors were divided in 3 groups based on their vaccination history and each color bar indicates individual donor's IgG production to each of the MIMIC® stimulation conditions. D. Averaged donor's response under each condition in 3 groups of donors. Standard errors are illustrated.

- SA-HAI results (Figure 25): Analysis revealed Fraunhofer's monomer and trimer antigens produced an approximately equivalent level of functional antibodies that was greater than that generated by the rHA from Protein Sciences. When their average response was compared with TIV and QIV vaccines, Fraunhofer's monomer and trimer generated approximately two-fold lower levels of functional antibodies compared to trivalent vaccines (TIV-IM and TIV-ID) and about three to four-fold lower than that of QIV vaccine.

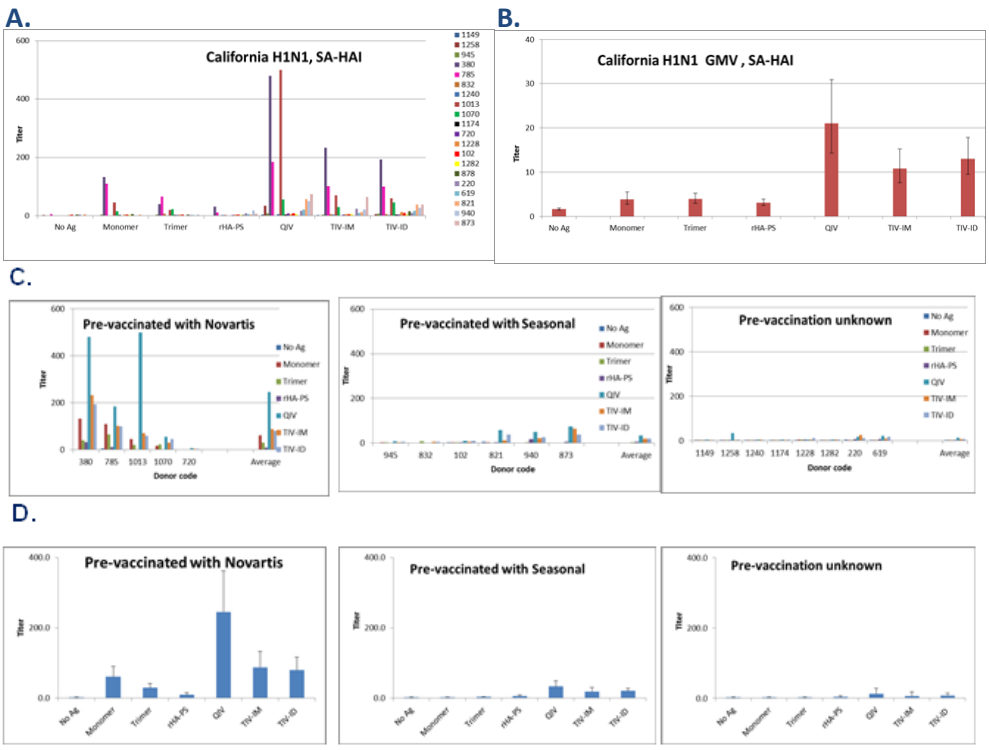


Figure 25. Comparison of anti-California functional antibody levels as measured by surface assisted hemagglutinin inhibiton (SA-HAI). MIMIC® stimulation and sampling conditions are as described in the legend for Figure 1. A. Each colored bar indicates the individual donor's SA-HAI titer generated under each of the stimulation conditions. B. Averaged donor's responses under each condition. C. The donors were divided in 3 groups based on their vaccination history and each color bar indicates individual donor's IgG production to each of the MIMIC® stimulation conditions. D. Averaged donor's response under each condition in 3 groups of donors. Geometric mean titers are shown. Error bars indicate standard error.

In follow-up experiments subsequent to the clinical trial with Fraunhofer's influenza H1 hemagglutinin (Task 2), we evaluated the sera of vaccinated donors for a number of characteristics. Donor sera from volunteers that received Fraunhofer's vaccine or the commercial control vaccine (commercial inactivated monovalent vaccine containing A/California) were evaluated for: the presence of antibodies that cross-react with hemagglutinins from a previous year's seasonal A/H1 strain (Brisbane), the correspondence between the level of antibodies that bound H1 HA (using antibody forensics) and the level of neutralizing antibody (using SA-HAI). In addition we examined the relative level of antibodies capable of binding the stem section of HA and their ability to compete with monoclonal antibodies known to inhibit viral infection.

- Antibodies generated by either the commercial vaccine or Fraunhofer's vaccine showed little cross-reactivity, as measured by their ability to block binding to RBC, to an H1N1 influenza virus present in the previous year's vaccine.
- Although Fraunhofer's vaccine produced a higher level of anti-A/California HA antibodies than the seasonal vaccine the seasonal vaccine generated a higher titer of functional antibodies when analyzed using SA-HAI (Figure 26).

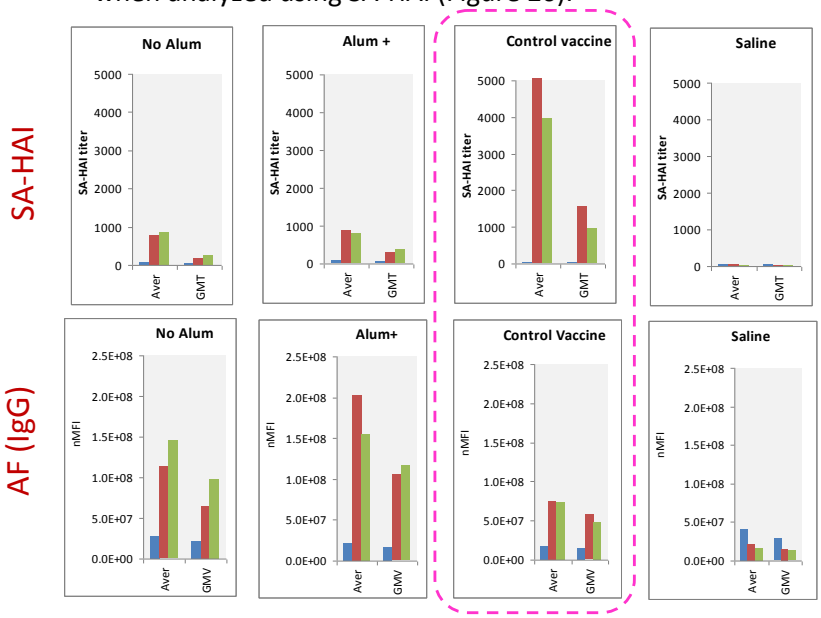


Figure 26. Relative levels of HAI titers and and IgG for sera immunized with FhCBM and control vaccines. GMT: Geometric Mean Titer, GMV: Geometric Mean Value

- The level of antibodies produced following vaccination that were capable of binding the stem portion of the HA and competing with stem-specific monoclonal antibodies known to neutralize influenza infection were analyzed using a novel technique designated epitope forensics (Figure 27). In these experiments the control vaccine and Fraunhofer's vaccine generated a similar level of these stem-binding antibodies. This shows that the monomer form of HA does not have more stem vs head related Abs as compared to the inactivated commercial vaccine (Table 6).

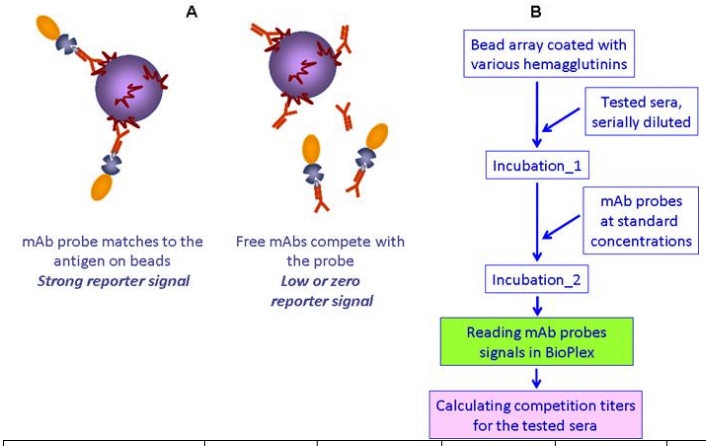


Figure 27. Flow Scheme for Epitope Forensic experiments. A: Diagramatic representation of competition of unlabeled antibodies from sera or MIMIC® with the mAb probe (e.g., a biotinylated monoclonal antibody coupled with SA-PE conjugate). B. Flow scheme of the EF experiment.

Assay	SA-HAI	AF	EF, M145 Stem	EF, F10 Stem	EF, M3 Head
FhCBM, GMT	1105	2.21E+08	668	375	296
Control vaccine, GMT	3729	7.83E+07	288	115	92
Control vaccine / Fh	3.37	0.35	0.43	0.31	0.31

Table 6. Comparison of SA-HAI, AF and EF titers for the sera immunized with Control monovalent H1N1 vaccine and with FhCMB vaccine candidate, group D. GMT stands for Geometrical Mean Titer

$CD4^+$ T cells are essential players in developing an anti-influenza response. They provide the necessary T cell help in driving B cell activation and antibody production specific for both new (naïve) and previously encountered (recall) antigens. We employed the MIMIC® $CD4^+$ T cell analysis to evaluate the difference in T cell response in vaccinated adult and elderly donors to seasonal trivalent influenza vaccine (Fluzone®). Our primary interest was to compare the CD4+ T cell response in adult and elderly donors vaccinated with 2010-11 seasonal influenza vaccine to determine if a higher dose of vaccine is required in elderly subjects to stimulate a level of response similar to that observed in adults. Intracellular cytokine staining (ICCS) assays were performed to examine the quantity and the quality of the CD4+ T cell responses.

- The study was relatively small. Three adults and 4 elderly donors were assessed following vaccination with the 2010-2011 seasonal trivalent influenza vaccine; Fluzone® (A/California/07/2009; A/Victoria/210/2009 (an A/Perth/16/2009 – like virus) and B/Brisbane/60/2008).
- The elderly donors showed a reduction in putative functional T cell responses to influenza vaccine. Furthermore, elderly populations required higher antigen doses for their T cells to respond. Overall, the adult donors responded more vigorously to flu antigens than the elderly by producing a higher proportion/magnitude of cells that expressed all 4 cytokines monitored.

Since both the B cell and T cell responses are thought to be of importance in the response to influenza, we investigated the possibility of utilizing the same MIMIC® setup to generate both humoral and cellular responses. This would also allow us to maximize the usage of the cells in cases in which we received samples containing low cell numbers. For this purpose the standard MIMIC® B cell assay was setup and supernatants were collected as described for antibody analyses. From the same setup, the cell pellets containing CD4 T cells from the assay were harvested, re-stimulated with antigen-pulsed target DCs and intracellular cytokine production was determined by flow cytometry in a manner equivalent to the standard T cell assay.

- We compared the responses of the $CD4^+$ T cells from 4 elderly donors that were stimulated with Fluzone under standard CD4 ICCS assay conditions or the standard B cell conditions (the 'combined' ICCS assay).
- In the standard ICCS assay, two of the four donors showed strong (5-15%) cytokine responses. In the combined assay, all donors showed 'significant' T_H-1 responses (~5%). Thus there were more Fluzone-reactive $CD4^+$ T cells present after 14 days in the standard B cell assay than in the standard ICCS assay. In addition, these reactive T cells were present in cultures that were set-up with much lower antigen concentrations than standard $CD4^+$ ICCS. It is possible that the B cells present in the culture served as antigen presenting cells that were both longer-lived than the DCs present in the standard $CD4^+$ ICCS assay and were capable of concentrating and efficiently presenting antigen present at low concentrations.

2.4. Follow-up assessment of Fraunhofer's VLP construct (data not included in previous reports)

During the past decade, increased research in VLP (virus-like particle) based technology has revealed that VLP based vaccines have strong immune stimulating properties as demonstrated by high antibody titer production and T cell activation. This study was therefore intended to evaluate Fraunhofer's plant derived A/California influenza hemagglutinin antigen formulated as a VLP. Previously a MIMIC study of FhCMB's monovalent California derived hemagglutinin showed the ability to accurately predict vaccine responses in a Phase I influenza clinical trial. Here, we compared three different forms of plant derived H1 hemagglutinin, monomer, trimer and VLP and measured their response against Fluzone trivalent inactivated influenza vaccine (TIV).

MIMIC© culture setup

As an initial study to determine the optimal dosing for use with the VLP construct, LTE cultures were stimulated with recombinant California hemagglutinin presented as a monomer, trimer or envelope VLP. To identify the optimal quantity of Fraunhofer pHA VLP we stimulated MIMIC cultures at doses ranging from 2ng/ml-500ng/ml. Cultures also received 2011-2012 Fluzone TIV, consisting of A/California/07/2009 X-179A (H1N1), A/Victoria/210/2009 X-187 (H3N2), and B/Brisbane/60/2008. After 14 days incubation, supernatants were harvested and assessed for antibodies capable of binding immobilzed California HA protein (Antibody Forensics). Five adult donors randomly selected from our donor pool were used for this study.

Results

Cell Viability: The dosing range was chosen based on previous experiments with influenza antigens in the MIMIC©. After 14 days in culture, cells were harvested and cell viabilty determined by trypan blue exclusion (Figure 28). Cultures stimulated with pHA monomer or trimer constructs had recoveries of ~30%, very similar to unstimulated cultures (Figure 28). Cell recovery is 2.5X lower for cultures that received Fraunhofer's HA VLP.

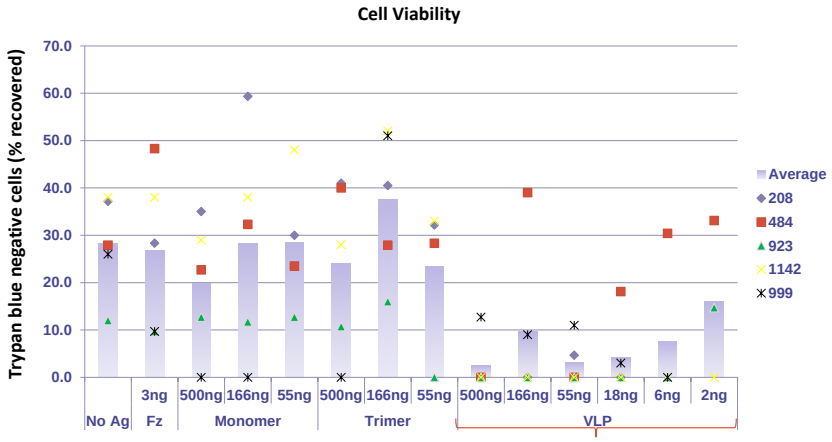
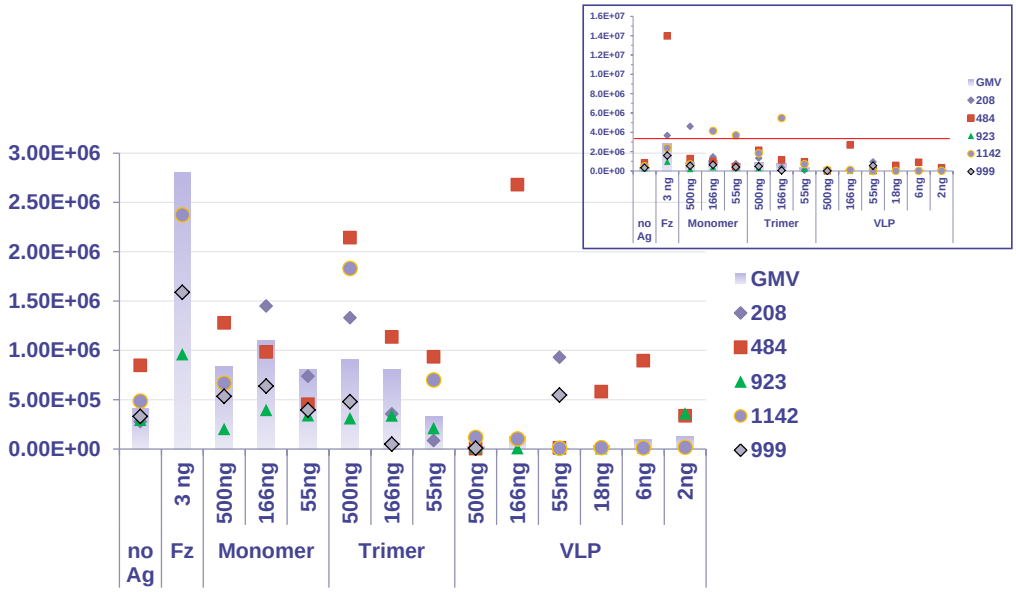


Figure 28. Cell viability 14 days post stimulation. California derived hemagglutinin monomer, trimer and VLP were added to LTE cultures at doses ranging from 2ng-500ng of HA. At day 14 cells were harvested and counted by trypan blue exclusion. Bars represent the average viability of 5 donors at a given hemagglutinin concentration.

Antibody Response: Fraunhofer monomer and trimer constructs generated comparable levels of anti-HA antibodies specific for California hemagglutinin (Figure 29). Cultures that were stimulated with at least 166ng/ml HA produced 2X more antibody compared to unstimulated control (no Ag). These results are consistent with avprevious study with the trimer form . However, when cells from the same donor were incubated with Fraunhofer VLP, essentially no antibody was produced in culture (Figure 29). This result

was thought likely to be due to contamination of VLP lots which led to failure of the cells to survive during co-culture as well (see below).

Figure 29. Antibody Forensics. MIMIC culture supernatants were tested for the presence California HA specific antibodies after being challenged with varying doses of Fraunhofer plant derived HA constructs. Fluzone 2011-2012 seasonal TIV was used as a positive control at the predetermined optimal concentration of 3ng/ml of HA antigen. Magnitude of response reported as normalized mean fluorescence (nMFI= dilution x [sample fluorescence-background fluorescence]). Light blue bars represent the geometric mean value, where n= 5. **donor 484 yielded 1.4E7 nMFI when stimulated with 3ng/ml Fluzone vaccine (data point shown in inset). Inset shows all values. Red line indicates cutoff point of y axis used for larger graph so GMVs could be more easily compared.*



To confirm that Fraunhofer pHA VLP lots were contaminated, we performed sterility and endotoxin testing on all materials received from FhCMB (Figure VLP3). Each rHA protein, monomer, trimer and VLP were added to XVIVO-15 media without antibiotics and phenol red at concentrations equivalent to that used in MIMIC cultures. After 4 days of incubation at 37°, bacteria growth was seen in wells that contained the VLP HA, even at the lowest dose of 2ng/ml (Figure VLP3 B). Fraunhofer monomer and trimer cultures maintained were sterile. Endotxin test results show that pHA VLP constructs have significantly higher levels contaminating endoxtoxins (1,700x). Due to the limitations of time and finances no further testing of Fraunhofer's VLP construct is planned.

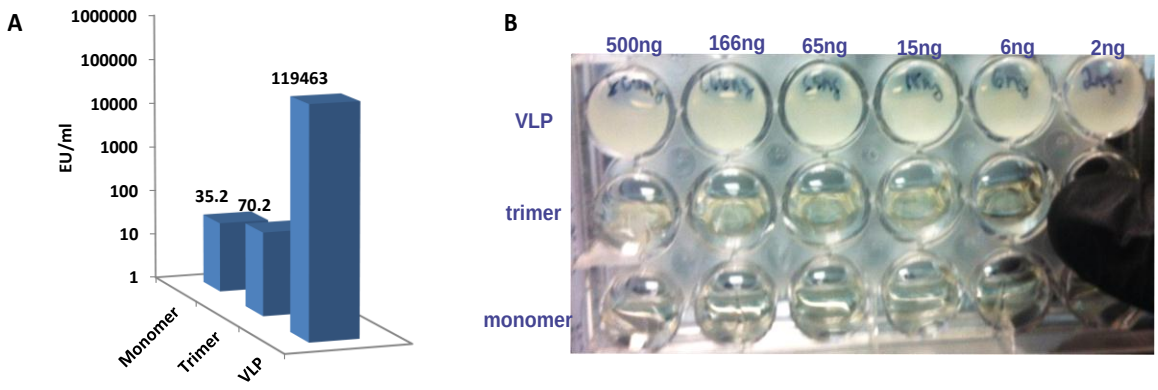


Figure 30. Endotoxin level and bacterial contamination in VLP lots. (A) Fraunhofer HA monomer, trimer and VLP were tested for presence of endotoxin using the Endosafe PTS system. (B) Each rHA formulation was serially diluted in XVIVO-15 medium without phenol red and antibiotics. After 4 days, cultures were observed for bacterial growth.

Summary

Antibody levels induced by stimualtion with Fraunhofer California HA monomer and trimer constructs were consistent with previous studies, yielding optimal levels when challenged with ~500ng/ml of HA. Due to bacterial contamination in VLP lots, we were unable to assess the antigenicity of California HA expressed as VLP.

3. Reference

1. B. C. Schanen *et al.*, Coupling sensitive in vitro and in silico techniques to assess cross-reactive CD4(+) T cells against the swine-origin H1N1 influenza virus. *Vaccine* **29**, 3299 (Apr 12, 2011).