

## **Human Immune Response to Carbon Nanotube Exposure**

David Montelongo, Keith Garrison

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### **Abstract:**

Carbon nanotubes (CNTs) are at the forefront of modern scientific research due to the novel properties that arise from their unique structure. With the many applications of CNTs, we will likely see a dramatic increase in the production of CNTs in the future. However, since they are still a fairly recent discovery, it is necessary to conduct research to evaluate the safety concerns regarding exposure to CNTs before planning on utilizing their promising capabilities. The primary objective of this project was to examine the immune response caused by *in vitro* exposure of human immune cells to carbon nanotubes (CNTs). By using the ELISPOT assay technique to detect human Interferon- $\gamma$  secretion, the pre-existing immune response to CEF (CMV, EBV, and influenza) viral peptides was determined in human Peripheral Blood Mononuclear Cell (PBMC) samples from several different donors. The cells were also incubated with each of two types of multi-walled carbon nanotubes (pristine and -OH functionalized nanotubes), and the extent to which exposure to the CNTs altered the pre-existing response was explored. This experiment has shown that with exposure to pristine MWCNTs, there is a definite trend toward an increased response to CEF peptides in most samples, although no clear trend exists when the samples are exposed to -OH functionalized MWCNTs.

### **Introduction:**

Carbon nanotubes are at the forefront of modern scientific research. Carbon nanotubes, abbreviated hereafter as CNTs, are a recently discovered man-made allotrope of carbon. Their structure is very unique, consisting of a series of benzene rings arranged in a honeycomb lattice, which is rolled on itself and capped on both ends to form a seamless cylinder. This particular structure gives CNTs many novel properties, such as the capability to be chemically modified to impart specific functional groups, peptides, and other macromolecular structures to their surface, which can significantly alter their reactive properties.

The potential applications of CNTs are numerous, as current research has already established that they may be useful in drug carrier systems, gene silencing, and tumor imaging, making their discovery possibly one of the most revolutionary of our age. With the many applications of CNTs, we will likely see a dramatic increase in the production of CNTs in the future. However, since they are a fairly recent discovery, it is necessary to conduct research to evaluate the safety of carbon nanotubes before planning on utilizing their promising capabilities. As of today, most research regarding the subject of CNTs has been focused on their potential applications, with little research going toward exploring their possible detrimental impact on human health. Currently, research has shown that longer CNTs cause frustrated phagocytosis by macrophages (Donaldson and Tran, 2004), and that exposure to CNTs may cause increased susceptibility to bacterial infection (Shvedova et al., 2008), but there is a lack of research in the area of CNTs and their effect on the human immune response. Also, of the few studies that do explore the interactions of CNTs with immune cells, a great majority of them concentrate on intratracheal exposure to CNTs via the inhalation route in mice, and comparatively few studies are conducted *in vitro*.

In a typical human, the immune response to a viral antigen proceeds in a series of steps. When professional antigen presenting cells encounter a viral antigen, the cell takes up the antigen via endocytosis, and proteases break down the viral proteins to produce peptides that are bound by MHC Class II molecules. The MHC Class II molecules are then transported to the cell surface and presented to T-cells, which perceive the antigen through T-cell receptors. The signal received through T-cell receptors, coupled with the co-stimulatory signal provided by interactions between B7 molecules on the antigen presenting cell side and CD28 on the T-cell side, causes activation of the T-cell, leading to an immune response and inflammatory cytokine release by the cell. Without the co-stimulation from the B7/CD28 complex, activation of T-cells does not occur. The hypothesis that this research experiment was designed to test is whether interactions between CNTs and the antigen presenting cells will lead to increased expression of B7 molecules on antigen presenting cells, leading to an increase in co-stimulation of T-cells and an enhanced immune response.

The primary technique that was utilized in this experiment was the ELISPOT assay. In this technique, antibodies specific for human Interferon- $\gamma$  (IFN- $\gamma$ ) are coated onto microplate wells. The wells are then blocked with non-reactive serum protein, and the cells of interest are plated out into the wells with antigen and are incubated. Inflammatory cytokines, which include IFN- $\gamma$ , are released from the cells and are captured by the antibodies in the region surrounding the cell producing them. The wells are washed free of the cells, and new biotinylated antibodies specific for the target cytokines are added to detect the captured cytokine. These antibodies are then visualized with HRP, which turns a blue color upon addition of a color-changing substrate, and each spot represents an individual cytokine-producing cell. This is used to quantify the amount of inflammatory cytokine produced by the cells.

## Methods and Procedure:

### *Study Participants:*

A total of ten PBMC samples from different donors were obtained from the UCSF Division of Experimental Medicine. The samples were drawn from donors at the Stanford Blood Center at the Stanford University School of Medicine. Samples were stored in a liquid N<sub>2</sub> freezer until needed. Demographic information about the donors can be found below in Table 1.

Stan #	Date processed	Processed by	ABO	CMV	DOB	M/F	Ethnicity
83	5/1/09	Jha	A-	P	3/10/1958	F	Caucasian/european
85	5/15/09	York	A+	P	9/20/1959	F	Caucasian/european
86	5/15/09	York	A+	N	5/20/1959	M	Caucasian/european
87	5/22/09	York	B+	P	1/23/1991	M	Asian/Oriental/Hawaiian/Eskimo
89	6/12/09	Jha	A+	P	9/13/1947	F	Caucasian/european
91	6/19/09	York	B+	N	11/30/1948	M	Asian/Oriental/Hawaiian/Eskimo
92	6/19/09	York	O+	N	7/11/1966	F	Caucasian/european
93	7/10/09	SJH/IEJ	O-	P	5/14/1909	M	Caucasian
94	7/10/09	SJH/IEJ	O+	P	5/19/1909	M	Central/South America
95	7/24/09	SJH	A+	P	9/16/1987	F	Caucasian/european

**Table 1:** Demographic information for the ten study participants.

### *Preparation of Reagents and Solutions:*

**PBS:** All working solutions of PBS consisted of 137mM NaCl, 2.7mM KCl, 4.3mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, and 1.4mM KH<sub>2</sub>PO<sub>4</sub>. The working solution's pH was around 7.3, and the solution was autoclaved prior to use.

**PBST:** PBST consisted of 0.1% (v/v) Tween 20 (TCI America, CAT # T0543) in PBS. The solution was stored at 4°C.

**PBSTB:** PBSTB consisted of 0.2% (m/v) BSA in PBST. The solution was stored at 4°C.

R10: The R10 consisted of 500mL RPMI 1650, 5mL Pen-Strep (10,000U/mL), 5mL 200mM l-glutamine, 5mL 1M hepes buffer solution, and 10% FBS (50ml). The solution was stored at 4°C.

*Suspension of CNTs:*

Tween 80 was added to sterile water to give a final concentration of 20µg/mL Tween 80. MWCNTs (Cheap Tubes Inc., >95wt%, <8nm diameter) were added to this solution to give a final concentration of 40mg/mL. The solution was shaken to suspend the CNTs in solution.

Similarly, -OH functionalized MWCNTs (Cheap Tubes Inc., >95wt%, <8nm diameter) were suspended in sterile water to give a final concentration of 40mg/mL.

*ELISPOT:*

All Steps were done in a sterile hood.

Day 1: Coating

The 96 well plate (Fisher, CAT # MAIPS4510) was pre-washed with PBS (200µL/well), then coated with primary antibody solution. This solution consisted of 12.5µL of MAb 1D1K (Mabtech, Product Code: 3420-3) in 5 mL PBS. 50µL of solution were added per well, and the plate was wrapped in wet paper towels and covered in foil. The plate was then incubated overnight at 4°C.

Day 2: Cell Thawing and Plating

The plate was washed with PBS four consecutive times, then blocked with R10 media (80µL/well) and incubated at 37°C for 1 hour. While incubating, the PBMC samples were removed from the liquid N<sub>2</sub> freezer, and kept on dry ice prior to thawing. The cryovials were

then transferred to 37°C water until a small bit of ice remained inside the cryovial. The PBMC samples were then transferred to pre-chilled 15mL tubes, and 5mL of cold R10 were promptly but slowly added dropwise to the tubes over a 1-2 minute period. R10 was then added slowly to all samples to a total volume of 12-13mL, and centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatant was decanted, and the samples were resuspended in 5mL R10 for cell counting. 10µL of sample were mixed with 10µL of 0.4% Trypan Blue, and 10µL of mixture were injected into a hemacytometer. Cells were counted and volumes adjusted to bring the concentration of cells to about  $10^6$  cells/mL. 20µL of CEF peptide solution (diluted to 0.8µg/mL, Mabtech, Product Code: 3615-1) were added to the appropriate wells, 20µL R10 were added to control wells, 5µL of MWCNT or fMWCNT solutions were added to the appropriate wells, and 100µL of cell suspension solution were added to all wells. The plate was then incubated for 16-18 hours at 37°C.

### Day 3: Development

Wells were washed twice with PBS, twice with PBST, and 50µL of secondary antibody solution were added to each well. This solution consisted of 5µL of IFN-γ biotinylated MAb 7B6-1 (Mabtech, Product Code: 3420-6) diluted in 5mL PBSTB. The plate was left to equilibrate at room temperature for 30 minutes. After two PBST washes, 100µL of Streptavidin-Alkaline Phosphatase conjugate solution were added to each well. This solution consisted of 10µL of SA-ALP (VWR, CAT# 100181-230) diluted in 10mL PBSTB. The plate was incubated for another hour at room temperature. After a PBST wash, the plastic backing of the plate was removed, and the plate was soaked in PBST for 30 minutes. While soaking, the Vector Blue substrate solution was prepared using the Vector Blue kit (VWR, CAT# 101098-454). Four drops of solutions 1, 2, and 3 were added to 10mL of 100mM Tris HCl, pH 8.0. This solution

was protected from light at all times. 100 $\mu$ L of substrate solution were added to each well after the soak, and spots were done developing after about 15 minutes. The plate was washed in tap water and distilled water, then left to dry in a closed drawer overnight.

*Spot Counting:*

Spots were counted by hand using a dissecting microscope, and the spot counts were confirmed using an automated ELISPOT plate counter.

*Statistical Analysis of Data:*

For each set of wells that corresponded to a particular PBMC sample, the average spot count, standard deviation, SFU per million cells, percentage of CEF baseline response, and percentage of “medium alone” baseline response values were calculated. Results can be found in the appendix. When comparing two different sets of test conditions, raw spot counts for each well were also subjected to the Student’s T-Test to determine if the results were statistically significant (Level of significance:  $<0.05$ ). Results of the statistical tests can be found in the appendix.

**Results:**

Three main comparisons were drawn from the compiled data. These three comparisons were: wells containing cells + medium alone vs. wells containing cells + CNTs, wells containing cells + medium alone vs. wells containing cells + CEF peptide antigen, and wells containing cells + CEF peptide antigen vs. wells containing cells + both CEF peptide antigen and CNTs. Since the extent to which the cell samples secreted IFN- $\gamma$  varied, the number of spots varied

between PBMC samples. To standardize the values, enhancement was expressed as a percentage. Results of these comparisons can be found below. It should be noted that three of the samples (Stan 86, Stan 89, and Stan 93) had extremely low spot counts relative to the others, so data pertaining to these samples were excluded from the tables below.

**Table 2: Medium Alone vs. CNTs**

<b><u>Sample</u></b>	<b><u>% Enhancement by MWCNTs</u></b>	<b><u>% Enhancement by fMWCNTs</u></b>
Stan 83	1.5%	7.3%
Stan 85	34.7%	-2.0%
Stan 87	76.7%	36.7%
Stan 91	43.1%	103.1%
Stan 92	N/A	N/A
Stan 94	-60.0%	-60.0%
Stan 95	588.2%	182.4%

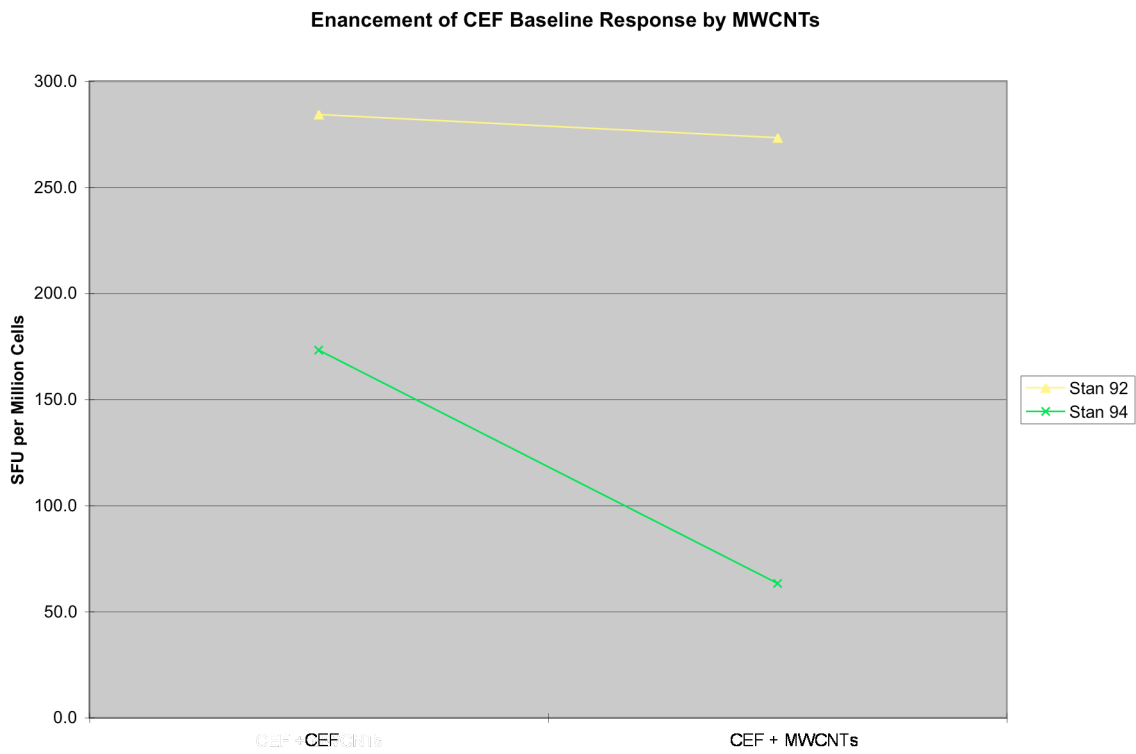
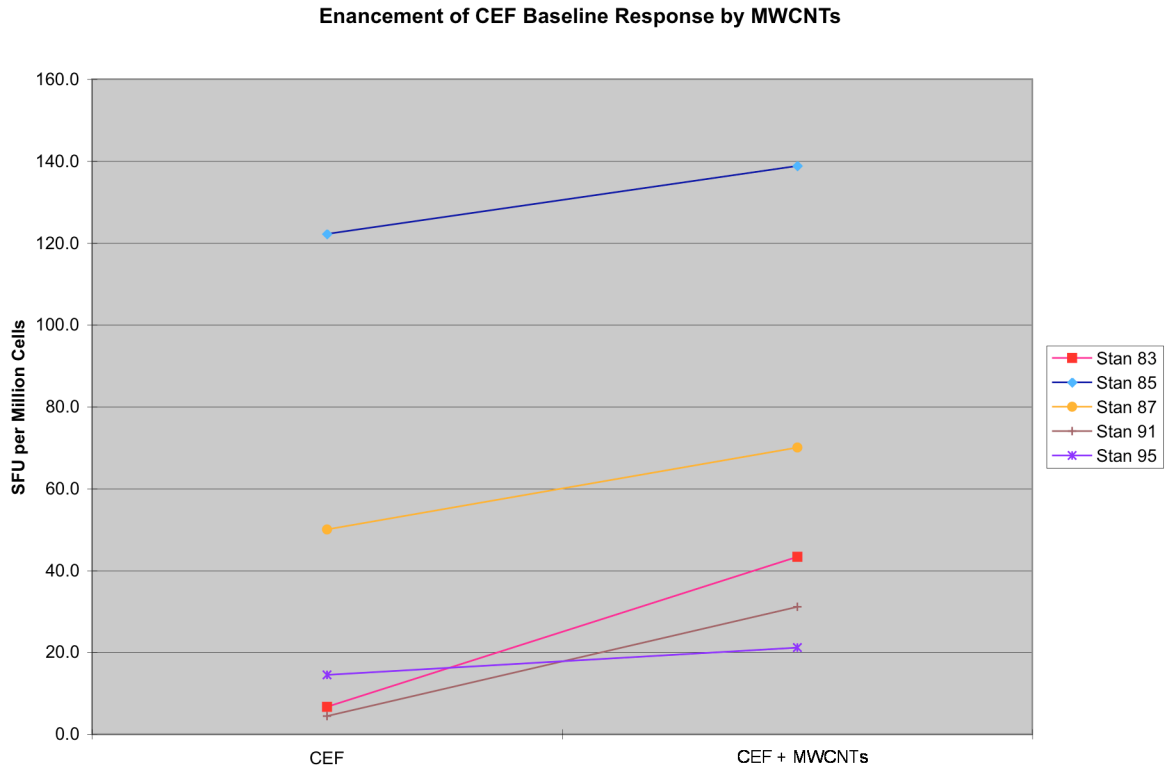
**Table 3: Medium Alone vs. CEF**

<b><u>Sample</u></b>	<b><u>% Enhancement by CEF</u></b>
Stan 83	1.0%
Stan 85	124.5%
Stan 87	50.0%
Stan 91	6.2%
Stan 92	691.9%
Stan 94	520.0%
Stan 95	76.5%

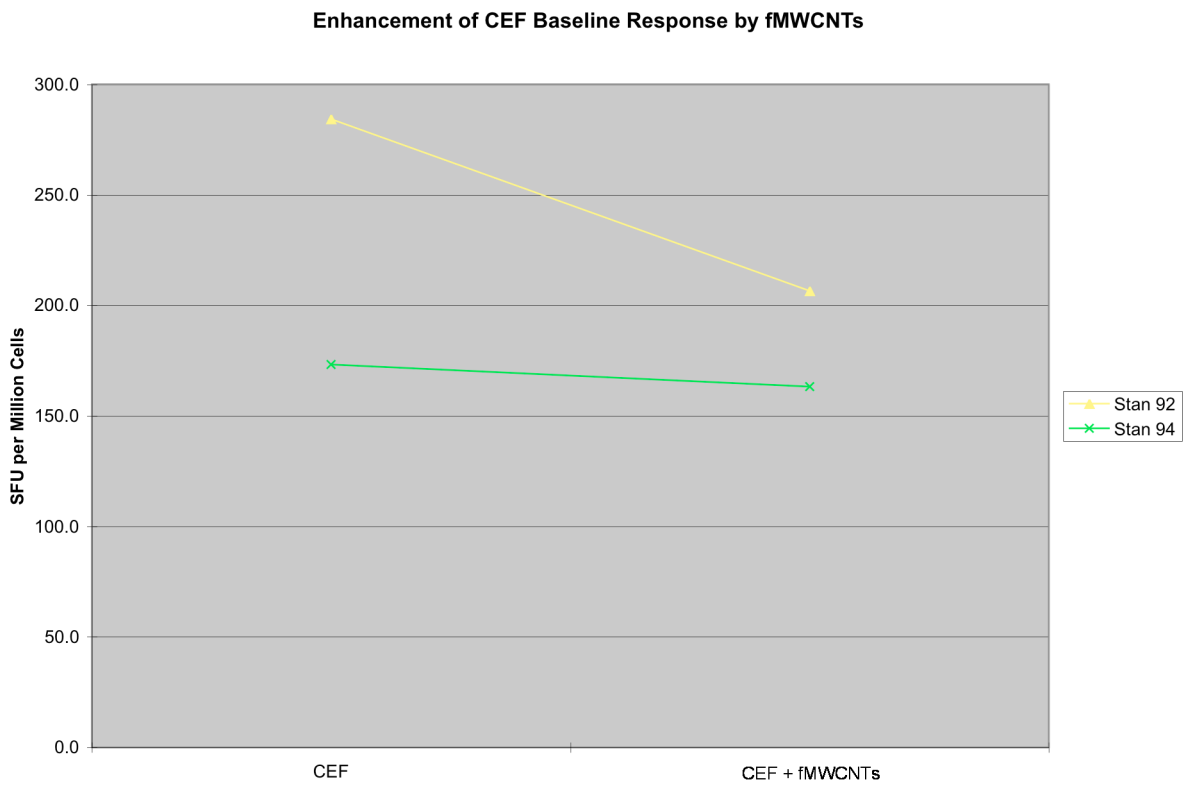
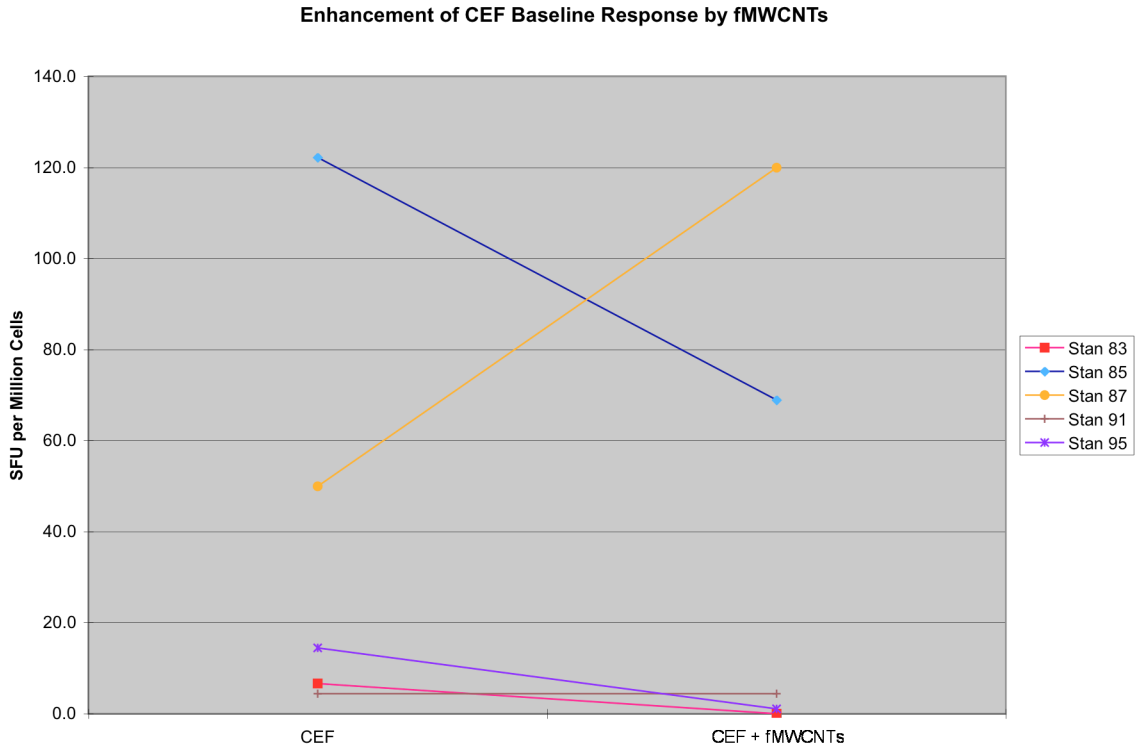
**Table 4: CEF vs. CEF + CNTs**

<b><u>Sample</u></b>	<b><u>% Enhancement by MWCNTs</u></b>	<b><u>% Enhancement by fMWCNTs</u></b>
Stan 83	550.0%	-9.7%
Stan 85	13.6%	-43.6%
Stan 87	40.0%	140.0%
Stan 91	600.0%	0.0%
Stan 92	-3.9%	-27.3%
Stan 94	-63.5%	-5.8%
Stan 95	46.2%	-96.3%





Figures 1 & 2: Enhancement of CEF Response by MWCNTs. Two charts were necessary because two different scales on the Y-axis were needed.



Figures 3 & 4: Enhancement of CEF Response by MWCNTs. Two charts were necessary because two different scales on the Y-axis were needed.

In addition to the above comparisons, the extent to which the CNTs enhanced the SFU count in the “medium alone” wells and the “CEF baseline” wells were also compared. This was done in order to determine whether the CNTs had the effect of truly enhancing the immune response when used in conjunction with the CEF peptides, or whether any increases in SFU count could simply be accounted for by some kind of detergent or chemical reaction caused by the CNTs or CNT suspension solution.

**Table 5: % Enhancement Comparisons (MWCNTs)**

<b><u>Sample</u></b>	<b><u>% Enhancement of Medium Alone</u></b>	<b><u>% Enhancement of CEF</u></b>	<b><u>Increased % Enhancement?</u></b>
Stan 83	1.5%	550.0%	Yes
Stan 85	34.7%	13.6%	No
Stan 87	76.7%	40.0%	No
Stan 91	43.1%	600.0%	Yes
Stan 92	N/A	-3.9%	N/A
Stan 94	-60.0%	-63.5%	No
Stan 95	588.2%	46.2%	No

**Table 6: % Enhancement Comparisons (fMWCNTs)**

<b><u>Sample</u></b>	<b><u>% Enhancement of Medium Alone</u></b>	<b><u>% Enhancement of CEF</u></b>	<b><u>Increased % Enhancement?</u></b>
Stan 83	7.3%	-9.7%	No
Stan 85	-2.0%	-43.6%	No
Stan 87	36.7%	140.0%	Yes
Stan 91	103.1%	0.0%	No
Stan 92	N/A	-27.3%	N/A
Stan 94	-60.0%	-5.8%	Yes
Stan 95	182.4%	-96.3%	No

### **Discussion:**

From the comparisons drawn in the above tables, some trends become immediately apparent. In the first comparison (see Table 2) of wells containing cells + medium alone with wells containing cells + CNTs, it is clear that most individuals showed some changes in SFU count when treated with either type of CNT. However, the extent to which the SFU count was

altered greatly varied depending on the individual, and none of the changes in SFU count were great enough to make the results statistically significant according to the student's T-test. However, in spite of this, this experiment suggests that there is a possibility that CNTs may trigger the secretion of inflammatory cytokines on their own in some individuals.

In the second comparison (see Table 3), it is clear that exposure of the cells to CEF peptides was very successful in catalyzing the secretion of IFN- $\gamma$ . Most of the samples had a dramatic increase in SFU count when compared to the "medium alone" baseline SFU count.

In the third comparison (wells with cells exposed to CEF antigen vs. wells with cells exposed to both CEF antigen and CNTs), many interesting points can be observed. From the table, it is clear that most of the cell samples, when exposed to pristine MWCNTs along with CEF antigen, had an increased immune response when compared to the CEF baseline SFU count. However, this trend did not hold true for the -OH functionalized MWCNTs. From Table 4 and Figures 3 and 4 above, one can easily observe that some samples experienced almost no change in SFU count from the CEF baseline SFU count, some samples experienced a decrease in SFU count, and one of the samples showed an increased SFU count. This highly variable data shows that no clear conclusions can be drawn with regard to the effect of fMWCNTs on the CEF baseline response, and also suggests that this is an area that should be investigated in future studies.

In addition to the three primary comparisons, Tables 5 and 6 compare the extent to which the CNTs enhanced the SFU count in the "medium alone" wells and the "CEF baseline" wells. This was done to test the experimental hypothesis that CNTs would cause a true enhancement of the immune response due to an interaction between the CNTs and the cells, which causes the cells to become stimulated by CEF antigen to a greater extent than normal. If this were the case,

one would expect that when the CNTs were exposed to the cells along with the CEF antigen, there would be a large increase in the percent enhancement value when compared to the percent enhancement of the “medium alone” baseline value. This would verify that the CNTs could in fact be causing the antigen presenting cells to increase expression of B7 molecules, which would increase the amount of co-stimulatory signals transmitted to the T-cells, and would increase the production of IFN- $\gamma$ . From Tables 5 and 6, one can see that Stan 83 and Stan 91 experienced far greater percent enhancements when the pristine MWCNTs were coupled with the CEF antigen peptides. Similarly, Stan 87 and Stan 94 also experienced greater percent enhancements when the functionalized MWCNTs were coupled with CEF. Thus, there is some supporting evidence that in some of the samples that were tested, the CNTs may be interacting with the cells in such a way that they are causing an amplification of the immune response to CEF peptides.

It should be noted that this experiment does not examine the effect of the CNT suspension solution on the cells. In a study published by Wick et al., it was determined that Tween 80 in low concentrations is biocompatible, and should not have any cytotoxic effects on cells *in vitro* (Wick et al., 2007). The same concentration of Tween 80 was used in this study as in the biocompatibility study, which would suggest that the suspension solution should have had no effect on cytokine secretion by the cells; however, since the effect of the CNT vehicle solution was not tested in the ELISPOT, it cannot be stated with absolute certainty that the solution had no effect on the results. Also, some of the samples had fairly high SFU counts in the “medium alone” wells, which means that the amount of background “noise” in this study was fairly high. This was accounted for by determining the average SFU count for the “medium alone” wells for each sample and subtracting this value out of the total SFU counts. In doing this, the amount of background “noise” should have been minimized.

There are also specific advantages and drawbacks associated with the ELISPOT technique itself. The ELISPOT technique creates a very basic model of the human immune response that is easy to replicate and quantify within the lab. The various components incorporated within the technique are used to mimic and measure a natural immune response to an antigen, though as with any other biological model, a few shortcomings are present. The primary way in which this model differs from the actual human immune response is that the viral peptide antigens from the CEF pool are artificially loaded onto the MHC Class I or Class II molecules already present on the cell surfaces, rather than having whole viral proteins being processed within the cell and presented on the MHC molecules. This also means that the extent to which any one individual will respond to an antigen is largely dependent on whether or not that study participant had encountered that antigen in the past. The ELISPOT assay cannot generate a *de novo* immune response, which could explain why some of the samples did not generate very high SFU values, and why the SFU values were so variable between individual samples. However, this assay cannot differentiate between the individual variation that occurs naturally between any two samples and the unintentional experimental variation that could occur with inconsistencies in the contents of any two individual wells.

Also, only two of the many different types of CNTs that exist were used in this experiment. Because of this, it is still uncertain whether the trends observed in this experiment would be observed in samples exposed to any of the other types of CNTs that exist.

**Conclusion:**

This experiment has shown that with exposure to pristine MWCNTs, there is a definite trend toward an increased response to CEF peptides in most samples, although no clear trend

exists when the samples are exposed to –OH functionalized MWCNTs. Furthermore, several immediate and practical implications have resulted from this experiment. This project has provided some insight into the safety aspects of nanotechnology. Since nanotechnology is a rapidly growing field of study with seemingly limitless potential, it is important to elucidate the effect of nanoparticle exposure on the human body. The data from this experiment seems to suggest that safety precautions should be taken when handling CNTs to limit the amount of exposure to the CNTs that a person receives. This project also suggests that it could be worthwhile to further explore the potential of carbon nanotubes to be used pharmaceutically as an adjuvant to deliberately enhance the immune response in humans.

**References:**

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