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Comparison of ELISA and ELISPOT methods in measuring CD4+ T cell responses to a newly identified MHC class II-restricted epitope within Simian Virus 40 T antigen

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ABSTRACT

ELISA and ELISPOT were used to monitor CD4+ and CD8+ responses to SV40 T ag MHC class I and class II- restricted epitopes. It was found that freezing and thawing IFN-γ samples decreased their ability to be detected by ELISA. Therefore, Femto-HS ELISA reagents were necessary to detect weaker CD4+ responses when using frozen supernatants; responses detected were comparable to those detected by ELISPOT. Later experiments using direct capture ELISA, ELISA of fresh supernatants and ELISPOT showed that the CD4+ responses were stronger when MHC class I-restricted epitopes were absent from the SV40 T ag.

INTRODUCTION

The Simian virus 40 is a DNA tumor virus that was discovered in polio vaccines. It codes for two transforming proteins and three capsid proteins, one of the former being large tumor antigen (Tag) (1). Tag is used as a model system for immunology and cancer research.

In vivo, Tag encodes an oncoprotein that causes tumors in mice (2). C57BL/6 mice express Tag in salivary glands and bones, phenotypically expressing osteoblasts and osteosarcomas (3). Tag is a protein of 94 kDa coded in early regions of SV40 DNA (4). Within the sequence are defined epitopes that are targets for both class I and class II major histocompatibility complexes (MHC) on CD8+ T cells (cytotoxic T-cell lymphocytes, or CTL) and CD4+ T cells (helper T cells) respectively. Four Tag epitopes have been identified that induce a hierarchical CD8+ response. Epitopes I, II/III and IV are immunodominant, such that the CTL response to IV>II/III>I (4). The response to the most dominant, epitope IV, has been studied in detail as the only MHC class I H-2K^{b} restricted response. Known to have been present in the carboxyl terminus of Tag, antigen deletion mutants and synthetic peptides were used to map site IV to amino acids 404-411 of Tag (5). Although amino acids 401-418 are critical to maintain the epitope site, peptides 404-411 is the most efficient target for the MHC class I epitope IV-specific region (5).

A fourth epitope at site V is immunorecessive (6). Therefore, when mutant versions of Tag are introduced to the cell with all other epitopes deleted or inactive, this

is primary the target of CD8+ response (4). However, in cells immunized with wild type SV40, epitope V-specific CTLs were undetectable (6). This hierarchy has been established both in vitro for cell transformation and in vivo for tumor phenotypes (6).

It has been shown that CD8+ T cell immune response potentially decreases immunological effectiveness by developing central tolerance when a transgene is expressed in the thymus; this leads to tumor growth. Peripheral tolerance of CD8+ cells can also lead to various levels of immune system ineffectiveness if Tag epitopes no longer induce immune response (3). Specifically, older mice have been shown to develop tolerance to Tag MHC class I-specific epitopes and fail to prevent tumor growth (3). Studies suggests that tolerance occurs early, before effects of the CTL response to epitopes IV and V is discernable, and when there are also high levels of Tag in the periphery (3).

Recently, studies have investigated the immune system's response to Tag as a combined effort between the CD4+ and CD8+ cells. It was first assumed only CD8+ cells would be significant in the body's response to Tag. In fact, most earlier studies generated an immune response by inducing CTL response with MHC class I-restricted epitope immunizations. Since this procedure directly targets MHC class I targets, it fails to stimulate CD4+ cells and thus leaves the CD8+ cells unsupported. A 1995 study reported that the CTL response of mice lacking CD4+ cells was less efficient, indicating their role in maximizing immune response, but concluding that they are not necessary (4). A 2001 study showed that taking away either the CD4+ or CD8+ T cells from the

immune response resulted in lower tumoricide response, yet not as low as if both helper and killer T cells were deleted together (7).

Therefore, it is shown that both CD8+ and CD4+ T cells are important in generating and maintaining immune response (7). Helper T cells do not directly lyse cells as CD8+ cells do, but they do serve as a support system in the immune system response to SV40. CD4+ cells can help CD8+ cells multiply and develop into effective cytotoxic cells and provide supportive cytokines such as interleukins and gamma interferon (7). However, beyond helper T cells' role in initiating immune response, they also help maintain the immune response. In fact, it has been established that both helper and killer T cells are necessary during the *entire* immune response for tumor rejection (7). Several hypotheses try to explain more specifically why helper T cells are required for immune response. One is that CTL response is too weak or slow to destroy all the tumor cells, and a complementary response with helper T cells might complete the tumor rejection. It is also assumed that helper cells play a supportive role when they produce cytokines such as interferon-γ. Lastly, CD4+ cells might play a role in bringing macrophages and other leukocytes to the area to kill tumor cells (7). In fact, CD4+ cells isolated from Tag-immunized mice secreted greater amounts of cytokines IL-2 and IFN-y only 4 days after Tag-immunizations (7). In this case, T-cells were extracted from the spleen and the tumor area, so the response was both local and systemic (7).

An important research interest now involves further investigating the role that CD4+ helper cells play in immune response so that cancer treatments can better utilize

their effectiveness. Therefore, developing effective methods to quantify both CD4+ and CD8+ response to SV40 T ag is an important step in further understanding immune response to this model system. T cells secrete cytokines, such as IFN-γ, when they recognize and bind to targeted epitopes; therefore, measuring cytokine production in vitro is used to monitor T cell responses to specific epitopes. This paper discusses research that investigateed appropriate techniques for quantifying T cell response to SV40 T ag class I and class II-specific epitopes with Enzyme-linked Immunosorbent Assay (ELISA) and Enzyme-linked Immunosorbent Spot Assay (ELISPOT).

ELISA measures the concentration of cytokine secreted by T cells in wells of 96-well plates. Usually, supernatants from T-cell and peptide solutions are harvested to provide samples with T cell produced IFN-γ. In direct capture ELISA, peptides and splenocytes can be added directly to ELISA wells so that capture antibodies will directly capture the cytokine as it is released. Direct capture is a more sensitive method because no IFN-γ is lost in transfer between wells. In both methods, the concentrations of cytokine in solutions is proportional to the numbers of active T-cells in the wells that recognize the target epitope. The number of T cells present then indicates the strength of the response to earlier immunizations with cells that express specific SV40 T ag epitopes.

The sensitivity of ELISA also depends on reagents used. In this research, regular ELISA reagents detect IFN-γ concentrations in a range from 15 pg/mL-2000 pg/ml. Femto-HS ELISA reagents detect IFN-γ concentrations in a range from 0.7 pg/mL-100

pg/mL. ELISPOT, on the other hand, allows for even greater sensitivity than ELISA, counting the actual numbers of secreting cells in a well.

ELISA and ELISPOT use capture and detection antibodies that sandwich the cytokine of interest, IFN-γ. First, a 96-well plate is coated with capture antibody AN-18 which is specific for IFN-γ. One face of IFN- γ binds to the capture antibody, and added detection antibody R4-6A2 recognizes and binds to another face of IFN- γ to form a sandwich structure. The detection antibody contains biotin, which strongly binds to avidin. Therefore, when an avidin-horseraddish peroxidase (Avidin-HRP) enzyme is added to the assay, the avidin-biotin link ensures that each antibody sandwich structure is now linked to HRP. Next, an HRP-activated color-producing substrate is added to the assay and cleaved by the enzyme. In ELISA, this color solution is released into the media, and the intensity of the color is quantified by finding its absorbance values (optical density). Optical density values correspond to IFN- γ concentrations. In ELISPOT assays, the substrate is also cleaved but forms a precipitates in the location of the antibody sandwich structure, forming a visible dot. These dots are counted and represent the number of actively secreting T cells.

It was shown CD4+ response to the recently identified MHC class II-specific epitope LT529-543 (sequence NEYSVPKTLQARFVK) is weaker than CD8+ response to MHC class I-specific epitopes (epitopes IV, I, II/III and V). ELISA has been shown to detect IFN-γ concentrations that are within the range of a typical CD8+ response to known MHC class I-specific epitopes. It is been predicted that ELISPOT detects T cell

response with greater sensitivity and is necessary to monitor CD4+ response to class II-specific epitopes. However, to compare the two responses it would be helpful to find an assay method that detects CD4+ and CD8+ response within an appropriate range. Since ELISA is faster and easier than ELISPOT, it would be beneficial to develop an ELISA method that monitors CD4+ response with comparable sensitivity as ELISPOT.

The following research investigates which assay methods are best to detect CD8+ and CD4+ within their range of response. Determining the best method to detect T cell response to T ag CD4+ and CD8+ -specific epitopes will aid in further studies that investigate immune response and tolerance to SV40 Tag.

MATERIALS and METHODS

Procedure for Mouse Immunization with Cells Expressing SV40 T ag

2 50 ml conical tubes were prepared with 25 ml Hank's Salt w/o Phenol Red and cells expressing either K-0 or K-1,4,5 SV40 T ag in each. To wash the solutions, they were centrifuged 5 minutes at 1000 rpm. Supernatants were vacuum suctioned and the pellet was resuspended with ~10 ml Hank's. Washes were repeated 3 times. After removing supernatant during final wash, cells were suspended in 3 ml's of Hank's to get a final concentration of 10^8 cells/ml: (7.5 * 10^7 cells)(4 vials)= 3* 10^8 cells total. A third conical tube was prepared with 3 ml Hank's. Contents were transferred from all conical tubes to 12*75 test tubes on ice.

A sterile serological pipette with a needle was used to immunize mice with 0.5 ml of one solution (5*10^7 cells/mouse).

Harvesting Mouse Splenocytes and Plating with Peptides for Direct Capture ELISA..

A sterile metal screen was placed into a 100 mm Petri dish and rinsed with at least 5 ml of medium. Two sets of 15 ml conical tubes were labeled, 5 ml of medium was added to one set (to collect spleens), and set on ice. Mice were euthanized by cervical dislocation. Spleens were remove spleen using sterile, alcohol-rinsed scissors and forceps. Excess fatty tissue was trimmed away, and the spleen was placed into a 15 ml tube containing medium (on ice). The spleen and medium were dumped onto a screen (in 100 mm plate). Cells were released from spleen by gently pressing spleen onto/through screen with plunger from 5 ml syringe. A 5 ml serological pipette was used to dissociate, mix cells, and transfer them back into original tube (unless no longer "sterile"). A second 5 ml

pipette to was used to rinse screen and plate with an additional 5 ml of medium, which was combined with original cell suspension. This solution was held on ice until all spleens had been processed. Cells were collected by centrifugation (1,000 rpm, 7 minutes, 4°C). Supernatants were aspirated (sterile 9" Pasteur pipette on vacuum line in hood). A 5 ml pipette was used to separate each cell pellet, rapidly suspending and dispersing the cell pellet by repeated pipetting in 7 ml of pre-warmed (37°C) Tris ammonium chloride solution. Solution was incubated for 5 minutes at 37°C. Each cell suspension was diluted with 7 ml of medium. Mix (inversion). Cells were collected by centrifugation (1,000 rpm, 7 minutes, 4°C). Supernatants were aspirated. Cell pellet were resuspended and dispersed in 5.5 ml medium. They stood on ice for 10 minutes. A fresh 5ml pipette was used in each tube to carefully remove and transfer at least 5 ml of the cell suspension to a fresh, labeled 15 ml tube. A sample was removed for cell counting and diluted into trypan blue. After cell counts had been obtained, cells were collected by centrifugation (1,000 rpm, 7 minutes, 4°C), supernatants were aspirated and resuspended in medium for an appropriate final concentration (routinely 1 x 10^7 cells/ml). Solutions were held on ice until plated.

ELISA (Standard and Femto-HS) Procedure for 1 96-Well Plate

Day 1: Reagents were used from ELISA Ready-Set-Go Kit. 12 ml coating buffer was mixed with 12 µl capture antibody in a 15 ml conical tube. When using Femto-HS ELISA Ready-Set-Go Kit, 12 ml coating buffer was mixed with 48 µl capture antibody in a 15 ml conical tube. Tubes were mixed by inverting and vortexing. 100 µl of mixtures was added to each well of Costar 9018 or NUNC Maxisorp 96-well plates (non-sterile). Plates were covered with plastic covers and incubated in refrigerator overnight at 4°C.

To prepare wash buffer, 2000 ml PBS was mixed with 1 ml Tween-20 (0.05% Tween-20) using a sterile stir-bar. Buffrer was filter sterilized into sterile container and sealed for later use.

Day 2: Plates were removed from 4°C refrigerator and uncovered. Wells in columns 1-6 were aspirated (if using full plate) using NUNC-Immuno Wash vacuum device. 250 μl wash buffer was added to each well using multichannel micropipetter. Aspirating and washing was repeated for wells in columns 7-12. Wash buffer sat in wells for at least one minute. This was repeated for 5 washes. On last wash, all wells were aspirated and plate was inverted and forcefully blotted on diaper to remove residual buffer.

10 ml of 5X unconcentrated Assay Diluent was diluted with 40 ml sterile water and mixed in 50 ml conical tube by inverting and vortexing. 200 μl of diluent was added to each well. Plates were incubated at room temperature for 2 hours on lab bench. Remaining Assay Diluent was stored on ice for later use. Wells were aspirated and washed as described above.

IFN-γ standard serial dilutions were prepared so that IFN-γ concentrations in eight microfuge tubes ranged from 2000 pg/ml to 15.625 pg/ml. 100 μl of each solution was transferred to corresponding wells in ELISA plate. Plate was covered with plastic plate covers and incubated overnight at 4°C. Wells were aspirated and washed as described above. When using ELISA Ready-Set-Go Kit, 12 ml 1X assay diluent was mixed with 12 μl Detection Antibody. If using Femto-HS ELISA Ready-Set-Go Kit, 12 ml 1X assay diluent was mixed with 48 μl Detection Antibody. It was mixed by inverting and vortexing. 100 μl was added to each well and incubated at room temperature for 1 hour on the Orbit Shaker, setting 4. Wells were aspiratd and washed again as described above.

12 ml 1X assay diluent was mixed with 48 μl Avidin-HRP in a 12 ml conical tube by inverting and vortexing. 100 μl was added to each well. Plate was covered and incubated at room temperature for 30 minutes on Orbit Shaker, setting 4. Wells were aspirated and washed again, allowing buffer to sit in wells for 2 minutes. 7 washes were completed.

100 μl substrate solution was added to each well. Plates incubated at room temperature for 15 minutes on Orbit Shaker, setting 4. 50 μl 2N H_2SO_4 (stop solution) was added to each well. All sides of plate were tapped until the solution changed completely from blue to yellow. Optical Density values for each well at 450 nm and 570 nm were found using a BIO-TEK Synergy HT plate reader and KC4 software. Using OD values from known IFN-γ standards, OD values were converted to [IFN- γ] for graphical analysis.

ELISPOT

ELISPOT plates were obtained from a faculty advisory. T cell dots in ELISPOT wells were counted after taking photographs of each well. A photo was viewed on a computer screen protected with a transparency sheet. Dots were then counted and marked with a sharpie pen. The transparency could then be washed with alcohol and kim wipes to count subsequent photos.

RESULTS

Evaluation of ELISA procedure variations to achieve optimal IFN-γ concentration detection.

Before ELISA and ELISPOT results could be compared, it was necessary to develop an ELISA procedure that would allow for detection of IFN-γ concentration with high sensitivity and consistency. First, it was observed if a 2 hour or overnight cytokine incubation produced more sensitive detection of IFN- γ concentration. After coating buffer was washed from four ELISA plates (1,2,3,4) and dilutions of cytokine standards were added to parallel wells in all plates, plates 1 and 2 were incubated overnight at 4°C and plates 3 and 4 were incubated for 2 hours at room temperature. After incubation time, both plates were washed and other reagents were added following a standard ELISA method. Optical Density (OD) values were obtained that corresponded to IFN- γ concentration values in each well. It was found that for wells with known cytokine standard concentrations at or above 250 pg/ml, higher OD was detected in plates 3 and 4, in which cytokine samples incubated 2 hours, than in parallel wells in plates 1 and 2. Conversely, in wells with cytokine standard concentrations less than 250 pg/ml, higher OD was found in plates 1 and 2, which incubated overnight, than in parallel wells in plates 3 and 4 (Figure 1).

It was also investigated if Costar 9018 plates and NUNC ELISA plates detected IFN- γ concentrations with identical specificity. OD values of wells in Costar 9018 and NUNC

plates with solutions of known concentrations of IFN-γ were compared using identical ELISA reagents. Both plates yielded OD values that were not significantly different.

Evaluating the use of frozen IFN-γ solutions in ELISA assays to monitor accurate and precise responses.

Initial trials aliquotted solutions with known IFN-γ concentrations into four sets of microfuge tubes. Three sets were frozen, and one was used in an ELISA assay. Later, the other sets were thawed and two were used in ELISA assays. The last set was frozen again and thawed a second time and used in ELISA assays. Fresh IFN-γ standards samples had highest OD. Previously thawed samples produced suboptimal results, yet still produced a standard curve (Figure 3). It seems possible to use previously thawed samples for experiments, but a standard dilution series should be prepared from sample for comparison.

Therefore, it seemed reasonable to use frozen supernatant samples from plastic plates that were run in parallel to ELISPOT plates in fall 2007 and thawed in summer 2008 to detect IFN-γ concentration with ELISA. IFN-γ concentration detected by ELISA was compared to T cell counts detected by ELISPOT. Overall, ELISA was not sensitive enough to detect the decreased IFN-γ concentration remaining in the solutions.

ELISA results from supernatants frozen on 10-29-07 and 10-31-07 detected IFN-γ concentrations in one well that was to have 275 secreting CD4+ cells detected by

ELISPOT (Figures 7). However, ELISA did not detect any IFN- γ in other wells with more than 275 active CD4+ or CD8+ detected by ELISPOT. Therefore, this data is not sufficiently consistent to suggest a threshold of active T cells detected by ELISPOT which produce IFN-γ concentrations that can be detected by ELISA.

ELISA results from supernatants frozen on 12-6-07 detected IFN-γ in some wells, but IFN-γ concentration values did not correlate with the number of actively secreting T cells measured by ELISPOT (Figures 8).

ELISA with Femto-HS reagents was able to detect IFN-γ concentrations produced by CD4+ cells in frozen supernatants. Except for one column of wells (from mouse 7), Femto-HS ELISA detected IFN-γ concentrations in supernatants that were imprecisely proportional to the active number of T cells detected by ELISPOT in each well that had more than 40 active T cells. Therefore, this data supports that ELISA with Femto-HS reagents can detect IFN-γ concentrations in frozen supernatants that contained more than 40 active T cells. However, this threshold value is approximate because Femto-HS ELISA did detect IFN-γ in frozen supernatants with which ELISPOT detected less than 40 active T cells per well (Figure 8).

Using ELISA to monitor CD4+ T cell response to SV40 T ag LT529-543 epitope.

To get fresh splenocyte samples, mice were immunized to activate CD4+ T cells that specifically recognize SV40 T ag. Three mice were primed and boosted with cells expressing a wild type SV40 T ag (K-0), and another three mice were primed and boosted

with cells expressing a mutant form of SV40 T ag (K-1,4,5) in which immunodominant and immunorecessive CD8+ -specific epitopes were eliminated or mutated. Two other mice served as controls and were immunized only with buffer solution. Ten days after buffering, spleen cells were harvested from mice and used to monitor immune response in the animal.

In the first experiment, splenocytes and synthetic peptide solutions were added directly to ELISA wells, so that peptides would be presented on antigen presenting splenocytes and recognized by activated T cells in the solution. The T cells that recognized the peptides in vitro then released IFN-γ into solution. The IFN-γ released was captured and detected by ELISA reagents. It was shown that direct capture ELISA detected response to class I-specific epitope V; however, response to the class II-specific epitope was no higher than the naïve mouse response (Figure 5). Response detected by supernatants collected in parallel plates also only detected epitope V response and with less sensitivity (Figure 6). The direct assay epitope V OD value for prime and boosted mouse 173 was ~0.5, while the supernatant assay epitope V OD value for mouse 173 was ~0.2. This suggests that direct capture and supernatant ELISAs are sensitive enough to detect CD8+ response to epitope V but not to CD4+ epitope 529-543 response.
The mice immunization was repeated, and Femto-HS ELISA reagents were used to detect IFN-γ concentration in wells with harvested splenocytes and peptides. It was found that using a direct capture ELISA procedure with Femto-HS reagents will successfully detect CD8+ response to epitopes IV and I from mice immunized with K-0 and CD4+ response from mice immunized with K-1,4,5 to the MHC class II-restricted epitope LT529-543 but

with OD values that are too high for the plate reader (Figure 9). However, ELISA detected CD8+ response from mice immunized with K-0 to epitopes IV and I within range for the plate reader when regular ELISA reagents were used to detect IFN-γ concentration present in supernatants. However, regular ELISA reagents are not sensitive enough to detect CD4+ response to LT529-543 (Figure 10). A parallel ELISPOT plate showed that the number of secreting CD8+ cells from mice immunized with K-0 that recognized epitope IV were too numerous to count. However, ELISPOT detected numbers of CD8+ cells from mice immunized with K-0 to epitope I and numbers of CD4+ from mice immunized with K-1,4,5 to LT529-543 within a countable range (Figure 11).

The experiment was repeated using regular ELISA reagents to detect IFN-γ concentrations in a direct capture assay. Only CD8+ response from mice immunized with K-0 to epitope IV was detected, between 37-179 pg IFN-γ/ml (Figure 12). The second plate used FEMTO-HS ELISA reagents to detect IFN-γ concentrations in supernatants. This ELISA assay detected IFN-γ produced by CD8+ cells from mice immunized with K-0 in response to epitope IV and I mostly in range (Figure 13). CD4+ cells from mice 5 and 6 showed weak but detectable response to LT529-543 using IFN-γ concentrations in supernatants using FEMTO-HS ELISA reagents. However, CD4+ cells from mouse 4 showed undetectable response. Therefore, Femto-HS ELISA detected IFN-γ produced by both CD8+ and CD4+ cells in an appropriate range.
For this trial, an ELISPOT plate was used in parallel to direct capture and supernatant ELISA. ELISPOT detected a high number of active CD8+ cells recognizing epitopes IV

and I (1500+) (Figure 14). However, the number of CD8+ cells responding to epitope IV were too numerous to count. CD4+ response to LT529-543 and LT525-543 (a larger version of the class II-restricted T ag epitope) in mice 5 and 6 was weak but detectable. CD4+ response to mouse 4 was not significantly above background. Results from ELISPOT support results from Femto-HS supernatant ELISA. Both assays show CD8+ and CD4+ responses that roughly correlate and can be compared.

A stronger CD8+ response from mice immunized with K-0 was seen than CD4+ response from mice immunized with K-0 and K-1,4,5. However, IFN-γ concentrations produced by CD4+ cells exposed to K-1,4,5 was higher than IFN-γ concentrations produced by CD4+ cells exposed to K-0.

DISCUSSION

It was seen that slight inconsistencies in OD values for IFN-γ standard dilutions require that fresh dilution sets be prepared for each ELISA plate if they are to be used to find the relationship between OD values and IFN-γ concentrations in wells. While variation among OD values was low, it is recommended that fresh IFN-γ standard dilutions are prepared for each ELISA plate to determine unknown IFN-γ concentrations in each well.

It was seen that the best ELISA results were achieved using fresh IFN-γ samples. It is predicted that IFN-γ may be lost by adhering to the sides of the plastic storage tubes. Therefore, smaller tubes with a greater area of contact between the sample and the tube would have lower IFN-γ concentrations when taken from the tube. This would account for the lower OD values of IFN-γ frozen and thawed once that transferred from one tube to another because some cytokine may had adhered to the sides of the tube and was lost in transfer, and was stored in a tube with greater sample/tube contact area. It is also possible that the freezing and thawing process cause some irreversible denaturing of IFN-γ. Therefore, IFN-γ concentrations detected in frozen supernatants will be lower than the IFN-γ originally in the media produced by CD4+ or CD8+ cells. IFN-γ concentrations produced by CD8+ cells specific for immunorecessive epitope V were detected just over background using standard ELISA reagents. Therefore, Femto-HS ELISA reagents are necessary to detect CD4+-produced IFN-γ in frozen supernatants.

Running direct capture ELISA, ELISA using supernatant samples, and ELISPOT plates in parallel showed that each detect CD4+ and CD8+ responses to MHC class II and class I-restricted epitopes in different ranges. ELISPOT was found to be an appropriate tool to quantify the number of CD4+ cells from K-1,4,5 immunized mice secreting IFN-γ in response to LT529-543. However, since ELISPOT takes more time to complete than ELISA, it is helpful to know that Femto-HS ELISA can be used to detect IFN-γ concentration in supernatants produced by CD4+ response to LT529-543 when a CD8+ response is lacking. On the other hand, CD8+ cell response to wild type T ag can be detected in a an appropriate range using standard reagent ELISA, either using a direct capture method or harvested supernatants.

It was also confirmed that CD4+ response to LT529-543 is stronger when a T ag-specific CD8+ response was lacking. This could be explained by the role of CD4+ and CD8+ cells in vivo. CD8+ cells kill cells expressing T ag MHC class I-restricted epitopes. Therefore, CD8+ activity shortens the duration that cells expressing SV40 T ag are living in the body. This shortens the time in which CD4+ cells have an opportunity to recognize MHC class II-restricted epitopes in infected cells and proliferate. Therefore, less proliferation of CD4+ cells that recognize LT529-543occur in the animals. However, when CD8+ response is absent, cells expressing SV40 T ag are not killed quickly and CD4+ cells have a longer duration to mount response and proliferate. The data therefore suggests that a regulatory interplay exists between strong CD8+ and CD4+ T cells. Specifically, strong CD8+ responses can inhibit CD4+ responses to the same antigen.

Further studies could test to see if CD8+ response is inhibited or enhanced by CD4+ response by using cells that express a SV40 T ag mutant lacking LT529-543. ELISA with standard reagents would be an appropriate tool to detect CD8+-produced IFN-γ in response to MHC class I-restricted epitopes. If SV40 T ag mutants are available with LT529-543 mutated, these could also be used to screen for additional CD4+-specific epitopes. Femto- HS ELISA measuring IFN-γ concentration in supernatants harvested from wells with splenocyte and peptide solutions would be an appropriate assay to monitor CD4+ response to other SV40 T ag mutants or K-1,4,5.

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FIGURE LEGENDS

Figure 1. 2 hour versus overnight incubations. Average Optical Density (OD) values are compared between plates with 2 hour and overnight incubations. OD values from wells with 2000, 125, 31.25 and 15.125 pg/ml are higher with a 2 hour incubation, and OD values from wells with 1000, 500, 250 and 62.5 pg/ml are higher with an overnight incubation.

Figure 2. Optical density of IFN-γ standards. The Optical Density (OD) values for wells with identically prepared and diluted IFN-γ standards follow a similar curve even though OD values are not precisely identical.

Figure 3. IFN-γ detection of fresh, freeze-thawed and twice freeze-thawed samples with overnight incubations, 6-17-08. The fresh IFN-γ samples showed the highest OD values. Previously thawed samples produce suboptimal results, yet still produce a standard curve. The samples that were frozen and thawed twice had the lowest detectible concentrations of IFN-γ.

Figure 4. 2 hour versus overnight dilutions in assay buffer and HL-1 media. It is shown again that ELISA that uses 2 hour incubations detect greater IFN-γ concentrations inconsistently than identical procedures using overnight incubations. It is also shown that HL-1 media will produce OD values on a standard curve proportional to but less than assay buffer. Data occurs in pairs between plates that had the same preparation except for incubation time.

Figure 5. Optical density (OD) values from direct capture ELISA. A) T cells from a naïve mouse detected equal response to all epitopes (~0.1), with a slightly higher response to the LT529-543 epitope (~0.2); B) T cell response from a mouse twice immunized shows significant increase in CD8+ response to epitope V (~0.5) but not a significant increase in response to the other epitopes; C) and D) Mice with unknown immunization histories show T cell responses similar to the naïve mouse.

Figure 6. Optical density (OD) values from supernatant ELISA. OD values that correspond to IFN-γ concentration in supernatants show that A) T cells from a naïve mouse detected equal response to all epitopes (~0.05), with a slightly higher response to the LT529-543 epitope (~0.1); B) T cell response from a mouse twice immunized shows significant increase in CD8+ response to epitope V (~0.2) but not a significant increase in response to the other epitopes; C) and D) Mice with unknown immunization histories show T cell responses similar to the naïve mouse. Enzyme contaminants in the wells with the Flu peptides and cells from mouse 170 show higher OD values, but should be ignored as outliers. The OD values from supernatants are approximately half the OD values from produced in the same experiment that used direct capture ELISA (FIGURE 7).

Figure 7. Comparing ELISA and ELISPOT as tools to detected IFN-γ concentration in frozen solutions- trial 1. ELISA (A) detected IFN-γ concentrations in supernatants produced by cells all mice, but they do not correlate to the active number of T cells detected by ELISPOT.

Figure 8. Comparing ELISA and ELISPOT as tools to detected IFN-γ concentration in frozen solutions-trial 2. Except in mouse 7, Femto-HS ELISA detected IFN-γ concentrations in supernatants that were somewhat proportional to the active number of T cells detected by ELISPOT in each well that had more than 40 active T cells.

Figure 9. T cell response to SV40 T antigen MHC class I and class II-restricted epitopes measured by IFN-γ Femto HS- ELISA. Femto-HS ELISA detects IFN-γ concentration produced by CD4+ response and CD8+ response to MHC class II and class I epitopes with OD values that are out of range for the plate reader.

Figure 10. T cell response to SV40 T antigen MHC class I and class II-restricted epitopes measured by IFN-γ ELISA of 48 hour supernatants. ELISA with standard reagents detected IFN-γ in fresh supernatants produced by CD8+ cells immunized with K-0 in

response to eptiope IV and I. However, it was not sensitive enough to detect IFN-γ concentration in supernatants produced by CD4+ cells.

Figure 11. T cell response to SV40 T antigen MHC class I and class II-restricted epitopes measured by IFN-γ ELISPOT. ELISPOT detected numbers of CD8+ cells from mice immunized with K-0 to epitope I and numbers of CD4+ from mice immunized with K-1,4,5 to LT529-543 within a countable range

Figure 12. T-cell response to SV40 T ag class I and class II-specific epitopes; IFN-y concentration measured with ELISA. Direct Capture ELISA with standard reagents detected IFN-γ produced by CD8+ cells from mice immunized with K-0.

Figure 13. T cell response to SV40 T ag class I and class II-restricted epitopes, IFN-γ concentration in supernatants measure by Femto-HS ELISA. Femto-HS ELISA detected IFN-γ concentrations in supernatants produced by CD4+ and CD8+ cells in range (except for mouse 3/epitope IV) in fresh supernatants.

Figure 14. T cell response to MHC class I and class II- restricted epitopes measured by IFN-γ ELISPOT. ELISPOT enumerates number of IFN-γ secreting CD4+ and CD8+ cells in response to MHC class II and class I-restricted epitopes.

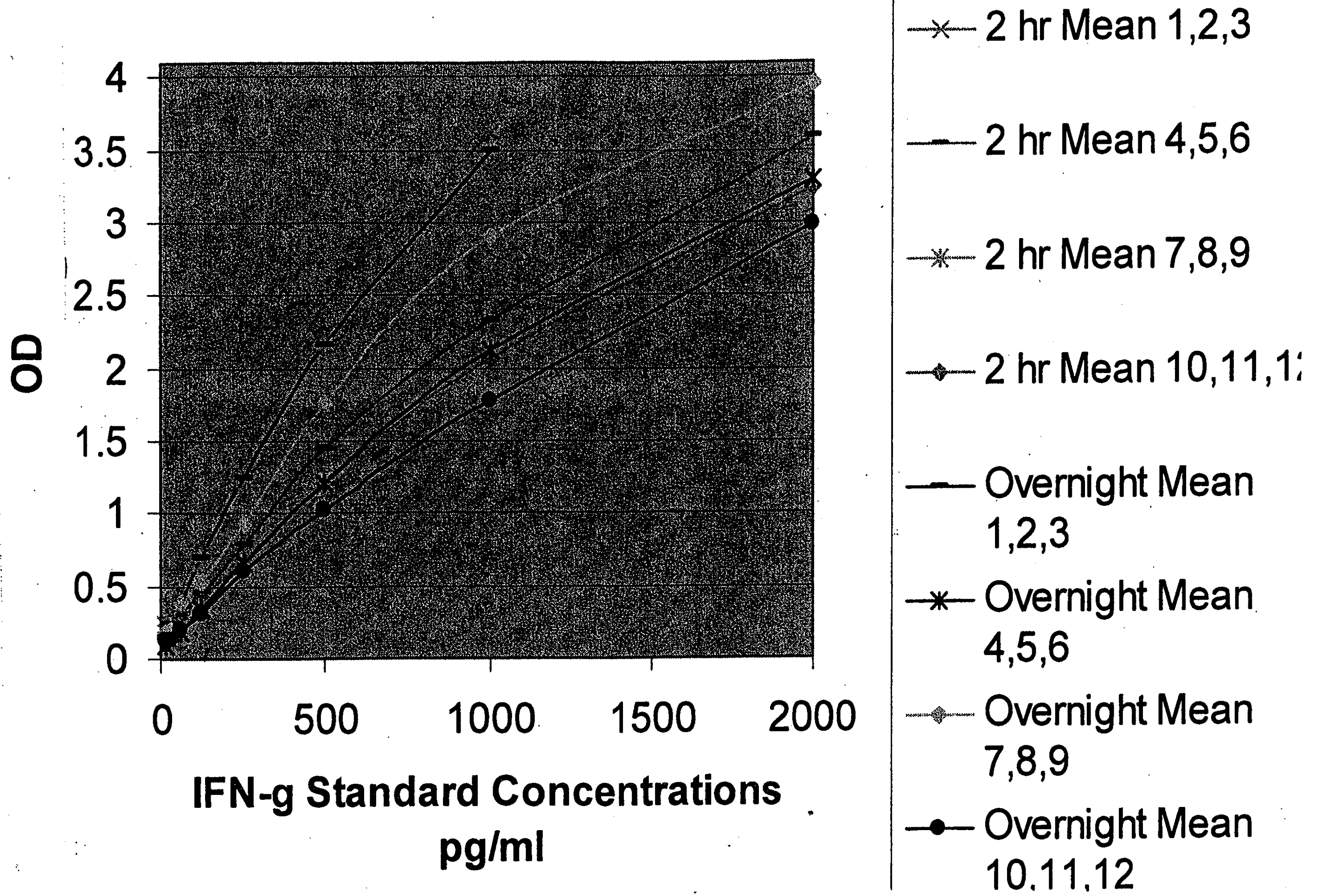


Fig 1

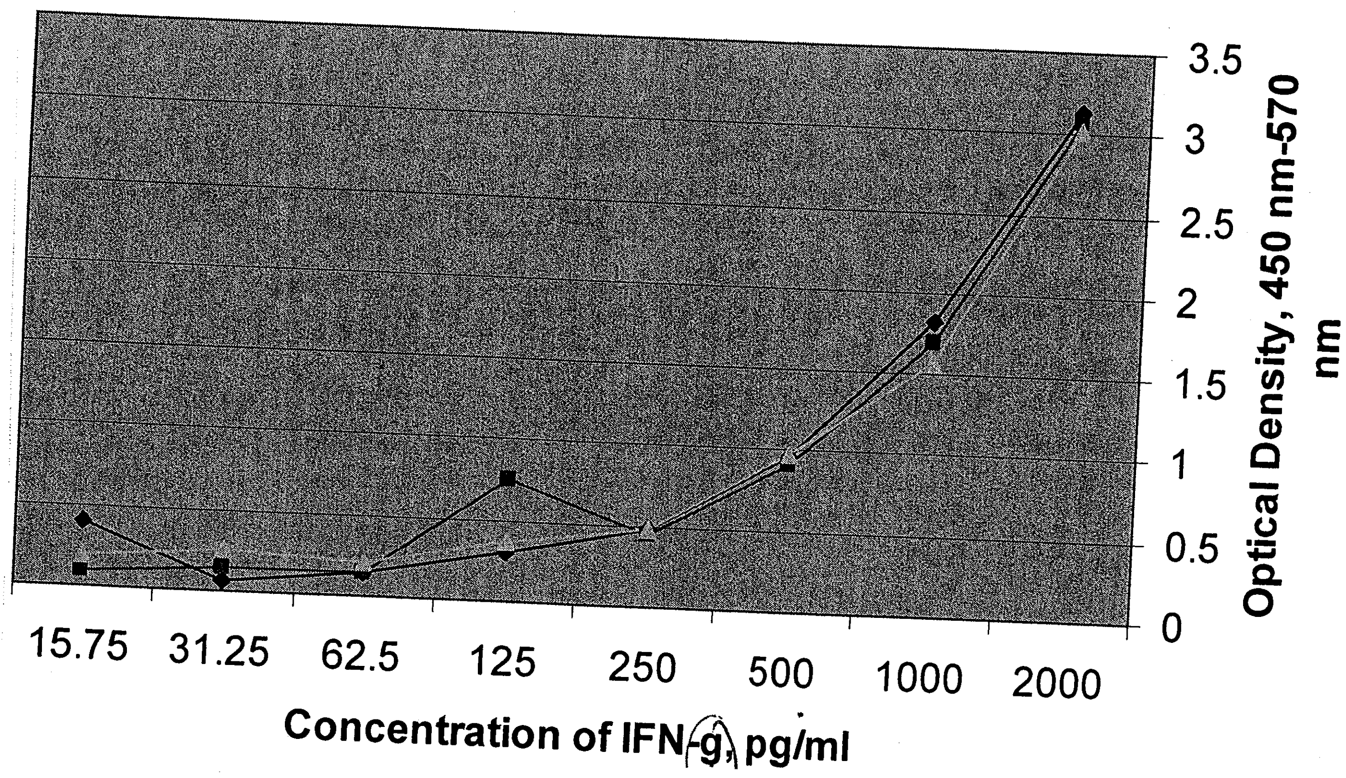


Fig 2

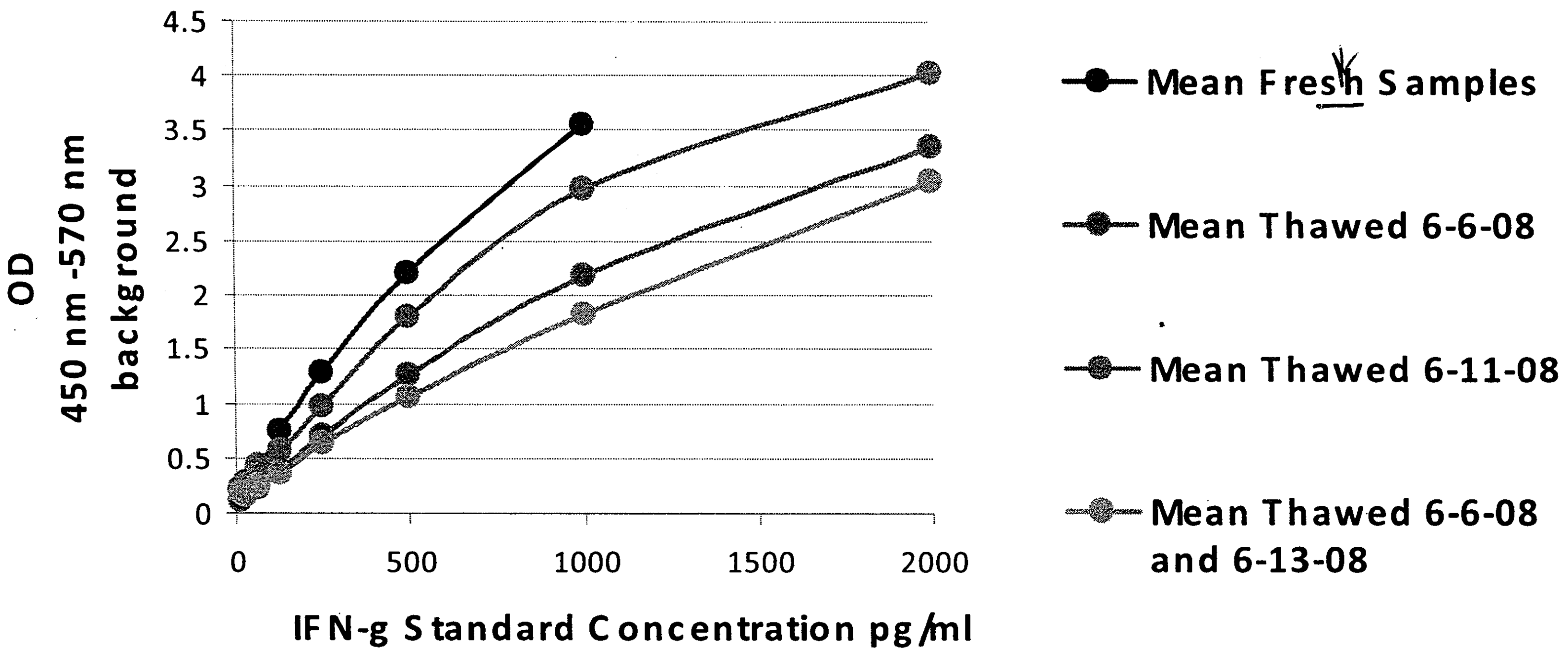


Fig 3

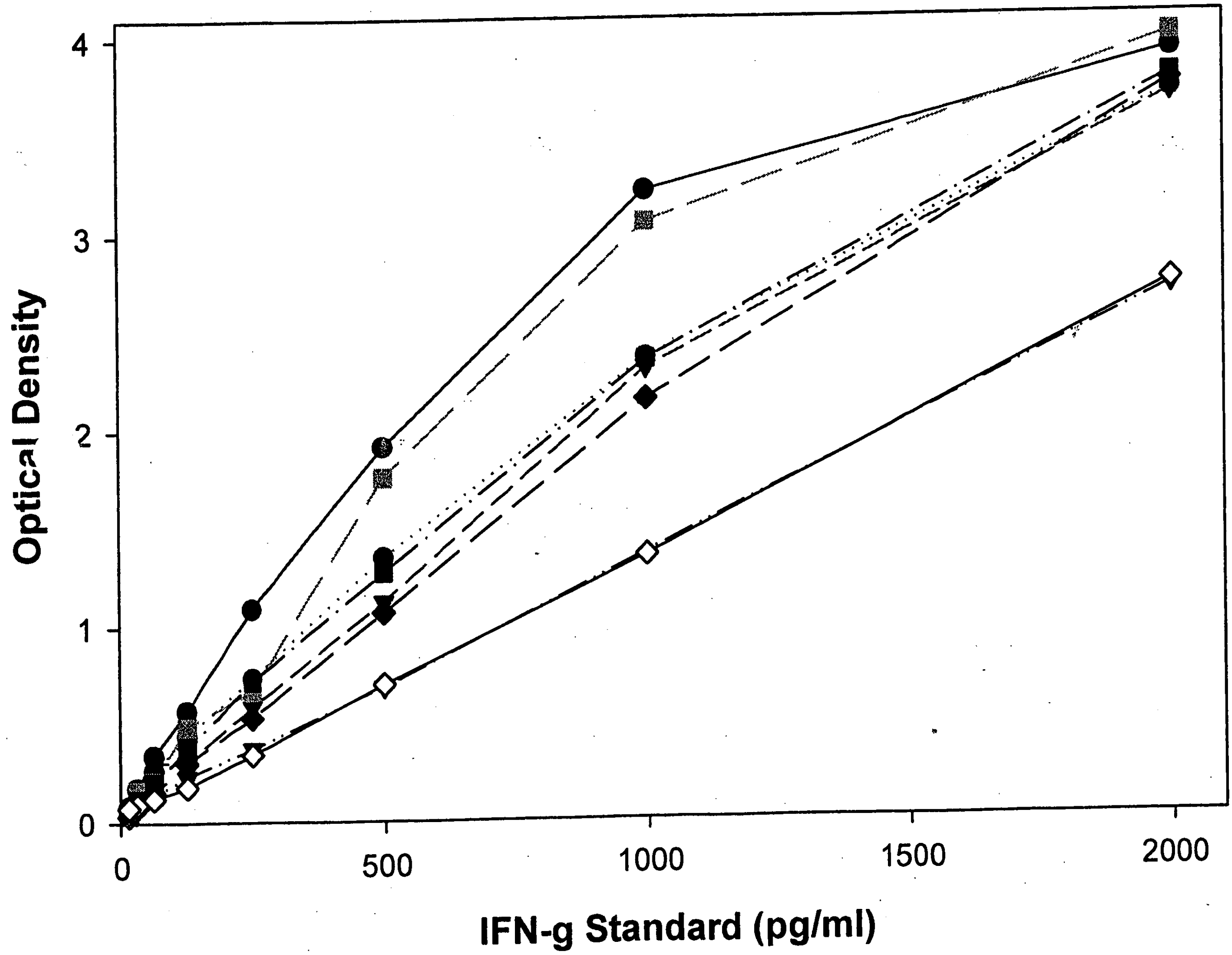
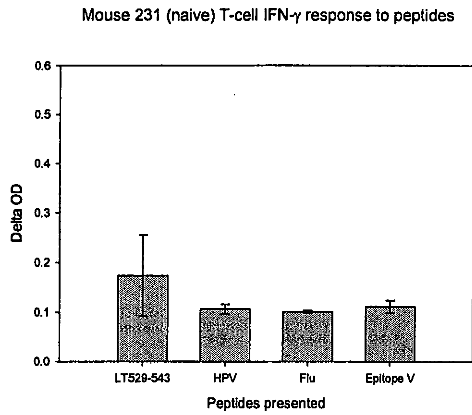


Fig 4

A



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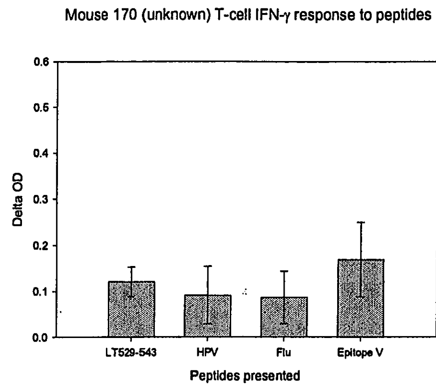

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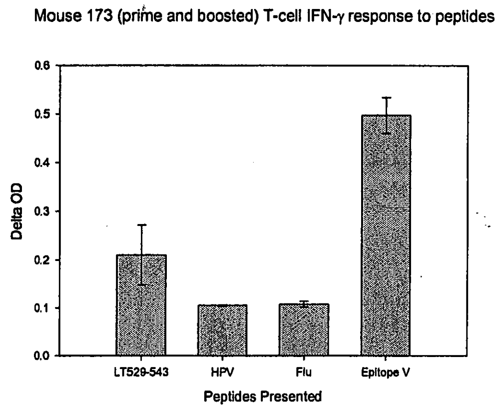

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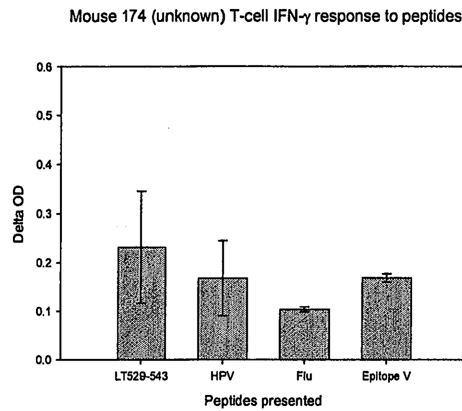
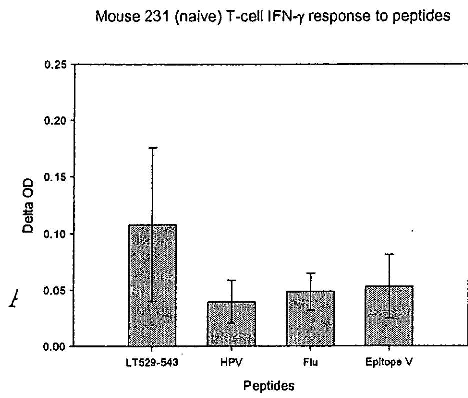
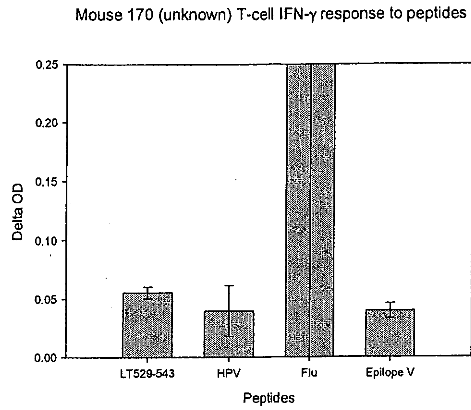


Fig 5

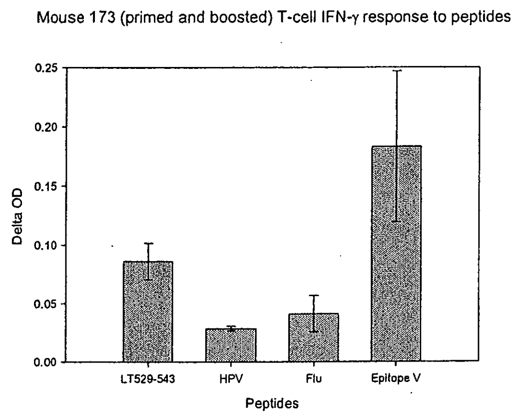
Mouse 231 (naive) T-cell IFN-γ response to peptides



Mouse 170 (unknown) T-cell IFN-γ response to peptides



Mouse 173 (primed and boosted) T-cell IFN-γ response to peptides



Mouse 174 (unknown) T-cell IFN-γ response to peptides

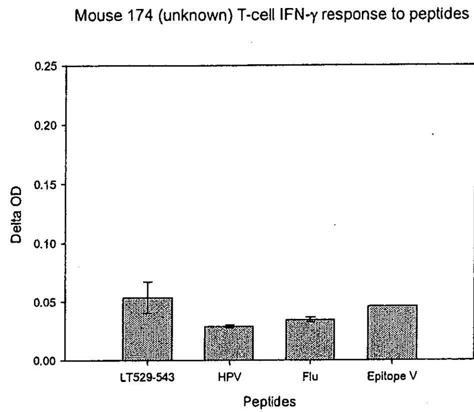
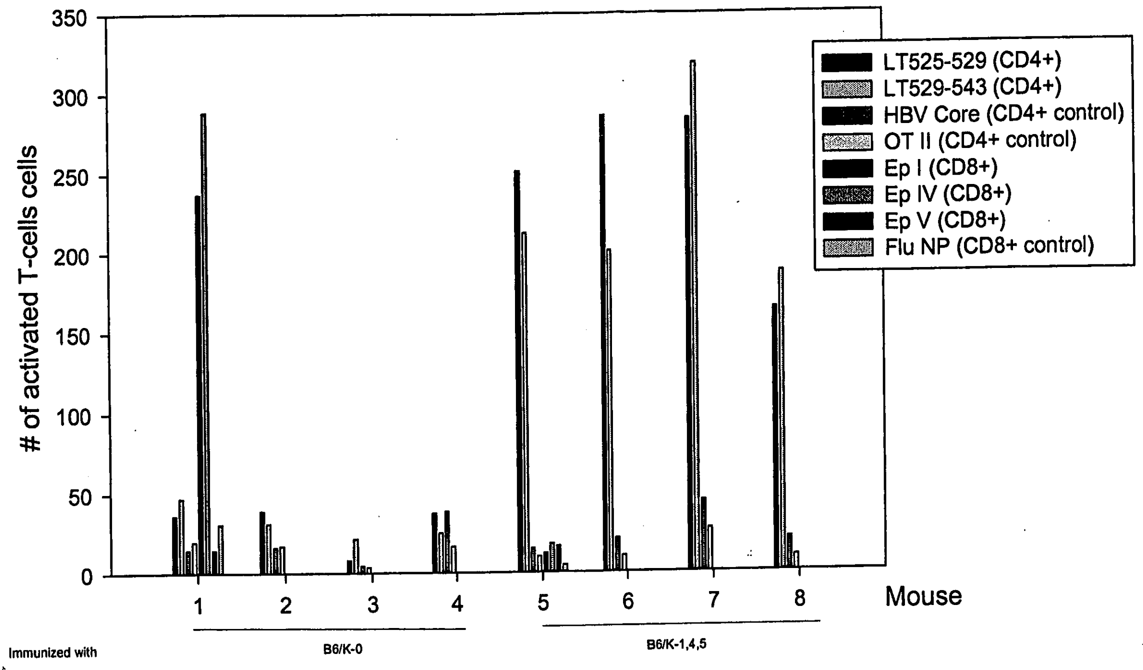


Fig. 6



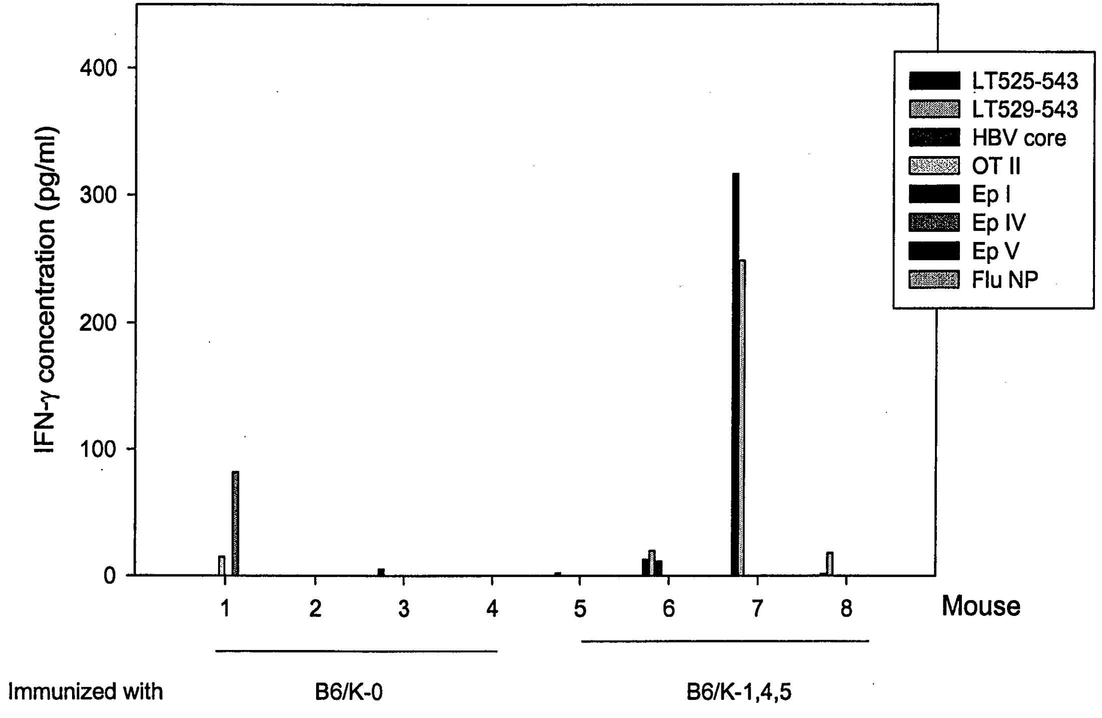
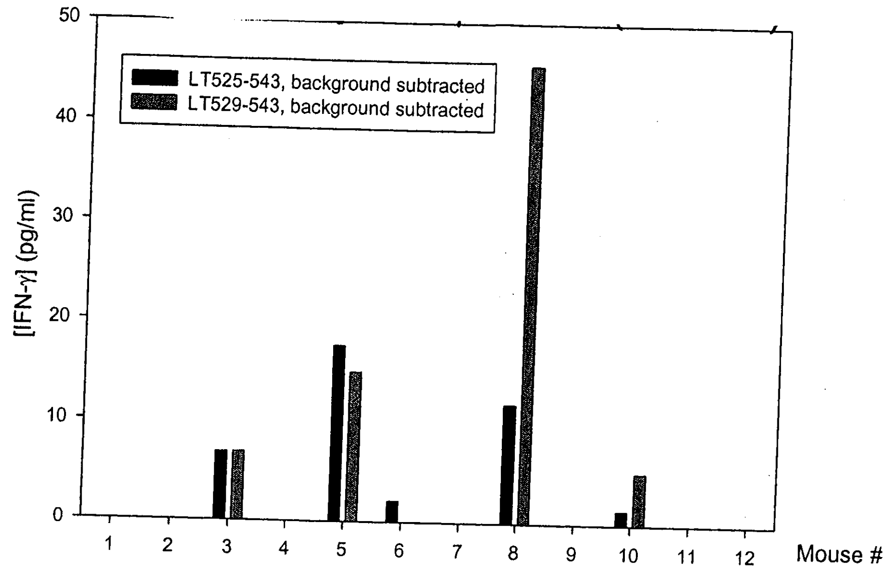


Fig 7



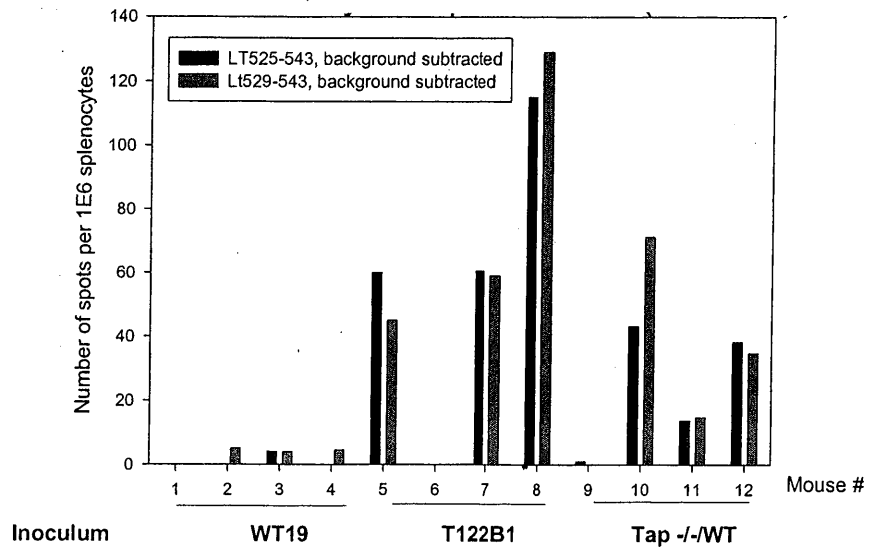


Fig 8

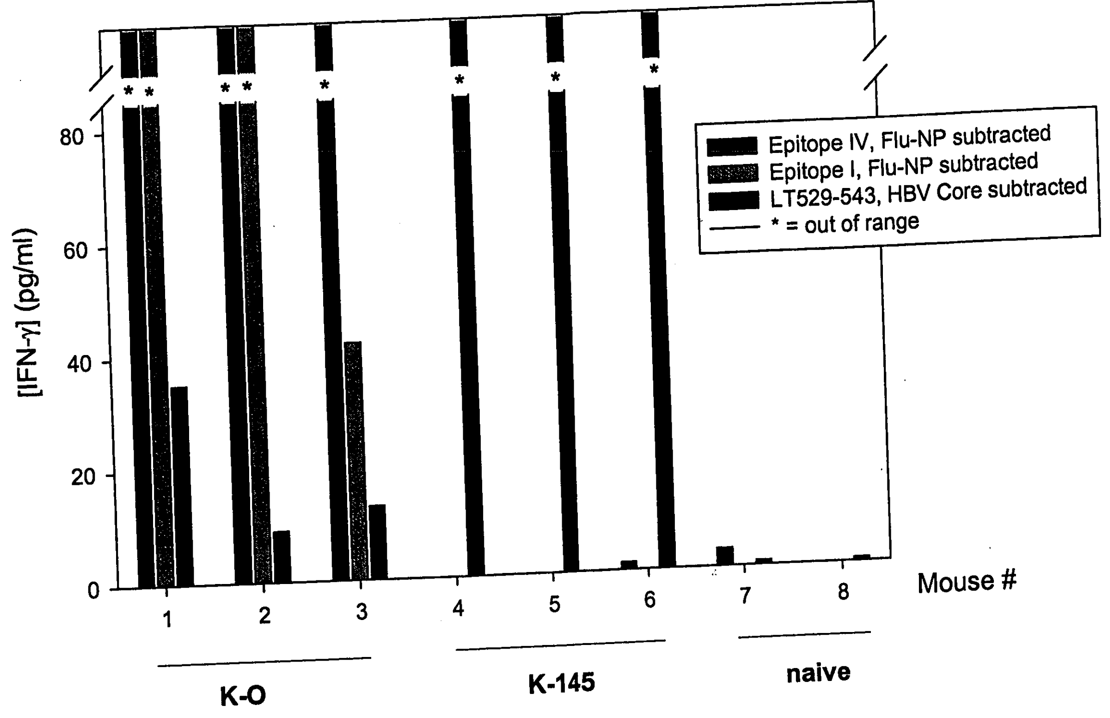


Fig 9

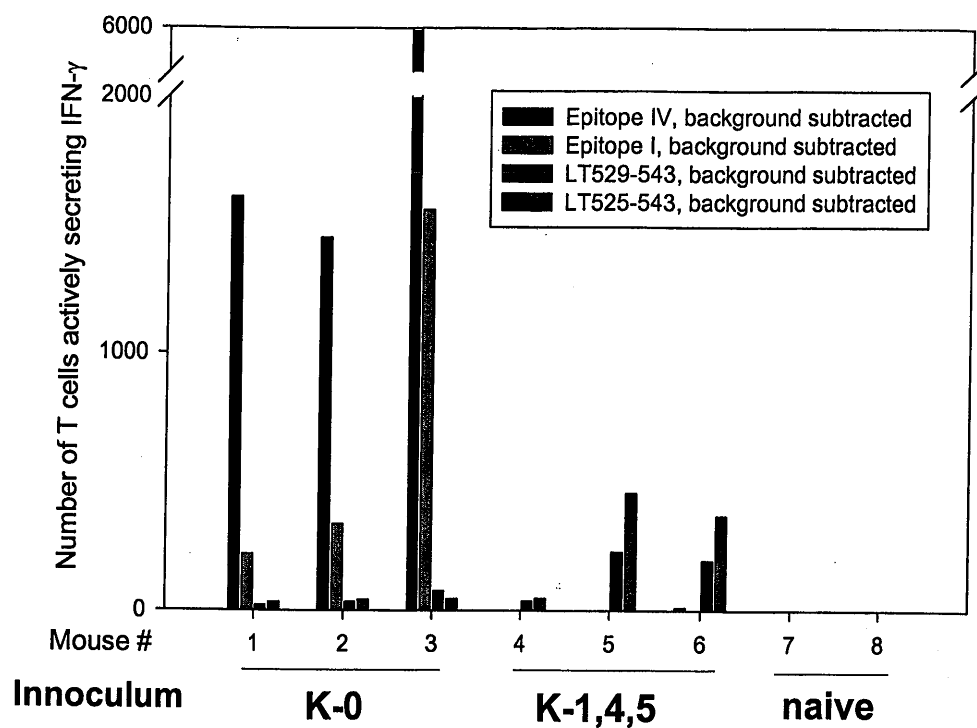


Fig 10

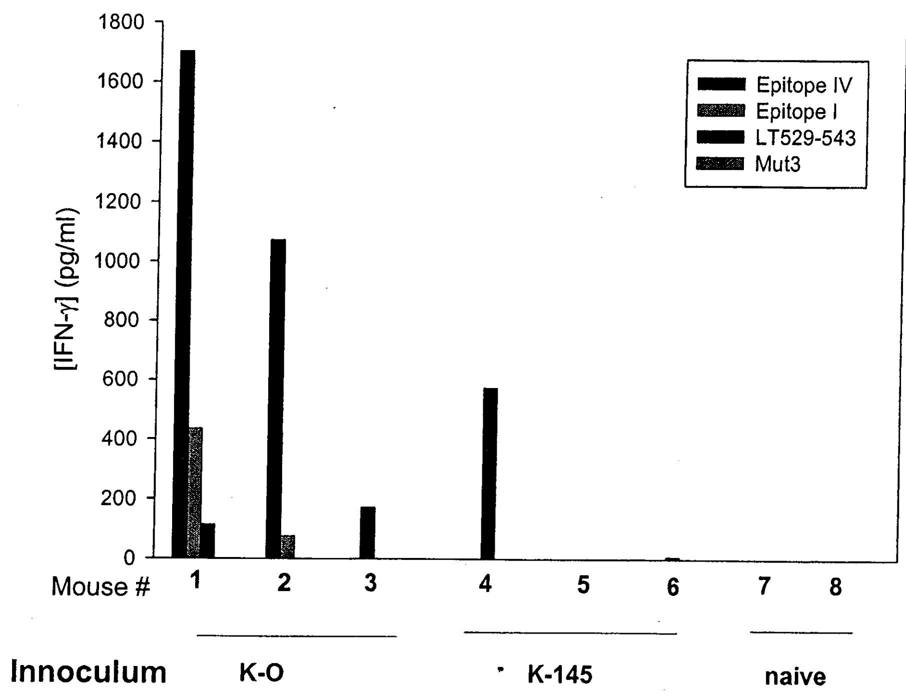


Fig 11

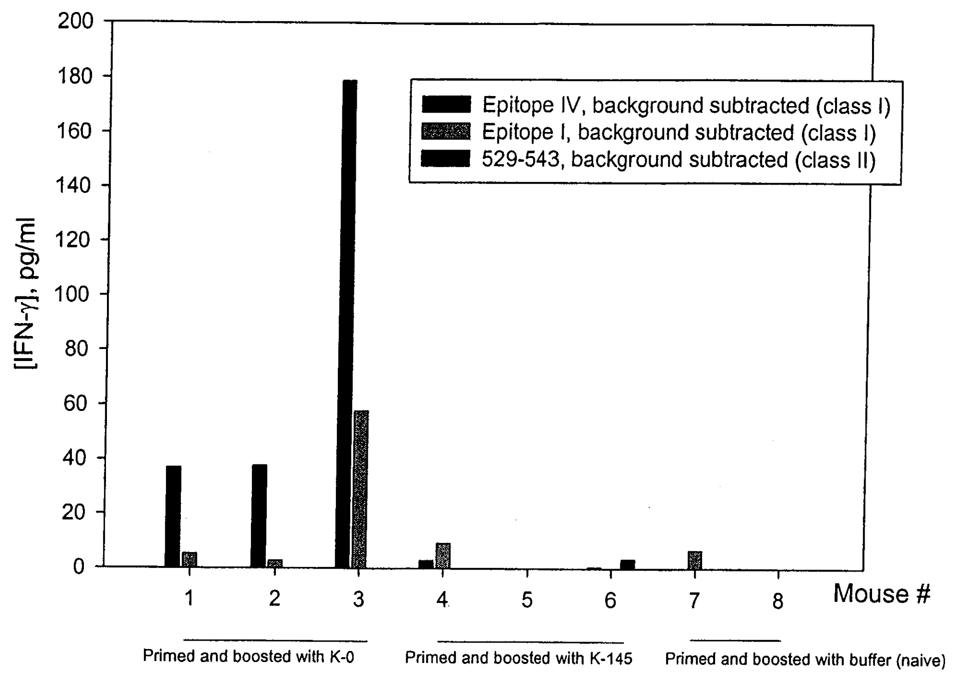


Fig. 12

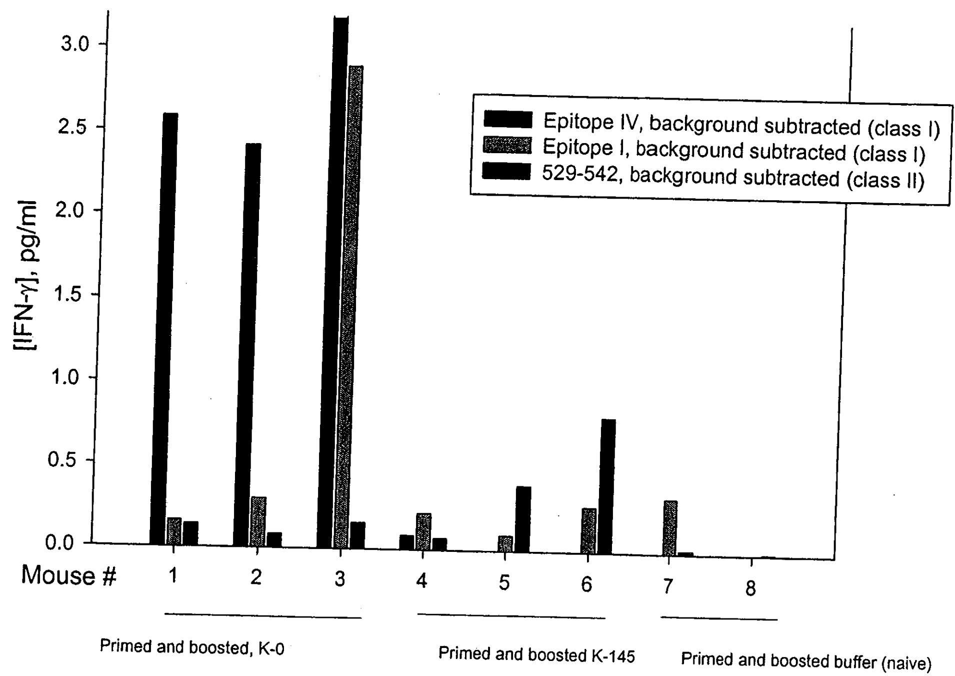


Fig 13

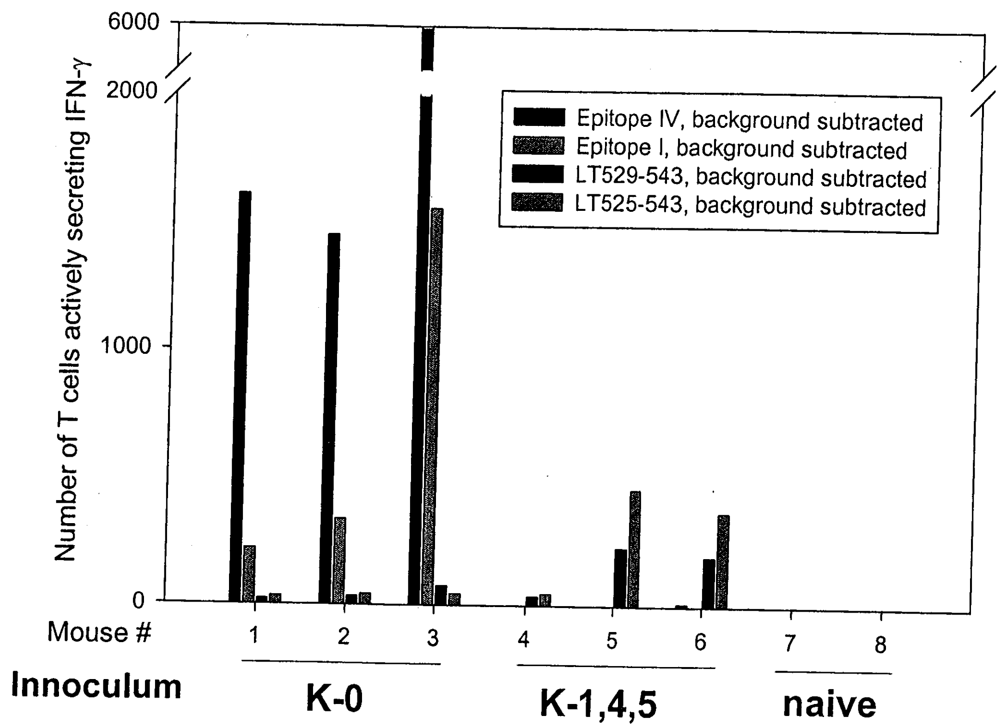


Fig 14