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Investigating Cellular Immunity Using the SV40 Large Tumor Antigen

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ABSTRACT

The Simian Virus 40 Large Tumor antigen (SV40 T ag) is a viral oncoprotein that is capable of inducing tumors and serving as the target for anti-tumor T cell responses. Because of this, the SV40 T ag has been studied extensively as a model to better understand immune control of solid tumors. Our laboratory has recently identified three CD4+ epitopes within the T ag and is conducting studies to characterize their role(s) in regulating cellular immunity to the SV40 T ag. Towards this goal, we have generated a cell line (C2a) that expresses a derivative of the T ag in which the three newly discovered CD4+ epitopes have been inactivated by substitution or deletion. The induction of SV40 T ag specific CD8+ memory T cells was investigated using C2a cells which had been grown in serum-free HL-1 media. The role of CD4+ T cells in the negative regulation of the immune response to SV40 T ag was also investigated. A new search was conducted to find additional new CD4+ epitopes that induce the secretion of the cytokine Interleukin-10 (IL-10). The identity of the IL-10-secreting cells was also investigated by ICS. While no new epitopes were uncovered, T cells responding to the known CD4+ epitopes were shown to secrete IL-10, indicating that they may play a role in negative regulation.
INTRODUCTION

The immune system is a complicated network of specialized cells and signaling molecules that work together to coordinate a response to pathogenic challenges. One such challenge is the formation and growth of tumors. Tumors are host cells that have experienced a deregulation of their cell cycle, and as a result proliferate uncontrollably. Several parts of the immune system work to control and destroy tumors if they appear in the body. Since tumor and cancer treatment is a serious concern and area of intense research, understanding more about how the immune system responds to tumors is vital. The use of model tumor producing systems such as SV40 Large Tumor antigen (Tag) is one way to learn more about the way the immune system recognizes and reacts to tumor growth. Understanding the role of signaling molecules such as Interleukin-10 can provide valuable insight into how the immune cells respond to the presence of tumors.

The immune system can be divided into two separate categories, the innate and acquired immune systems. The innate immune system is comprised of immunological defenses that are produced fully functional by inherited host genes. This includes physical barriers between cells, secreted mucus layers, and proteins and molecules produced by cells either constitutively or in response to outside stimulation. The acquired immune system is mediated by B and T lymphocytes which exhibit clonal specificity for certain antigens. T lymphocytes are responsible for identifying and destroying infected cells, based on the presentation of antigens on Major Histocompatibility Molecules (MHC). MHC class I receptors present antigens that have been produced inside the presenting cell, while MHC class II receptors present antigens that have been endocytosed from the environment by the presenting cell (1).
Two major classes of T lymphocytes are CD4+ and CD8+ T cells. CD4+ T cells recognize and bind to MHC II receptors, while CD8+ T cells bind to MHC I receptors. CD4+ T cells primarily regulate the humoral and cellular immune responses, while CD8+ T cells destroy infected host cells (1). CD4+ T cells are also required in order to form memory CD8+ T cells (2). T cells are produced in the thymus, where they develop specificity for a particular antigen sequence. When stimulated by presented antigens, T cells have an increased ability to respond to homeostatic cytokines and resist cell death. This allows T cells that have been stimulated by an antigen to persist and enable the host to develop immunity (3). CD4+ T cells can be further divided into Type 1 (T_{H1}) and Type 2 (T_{H2}) T helper cells. T_{H1} cells produce cytokines, including TNF-α and interferon-γ (IFN-γ), while T_{H2} cells produce cytokines such as Interleukin-4 (IL-4), IL-5, IL-10, and IL-13 (2).

In addition to mediating immunity against viral infections, T lymphocytes play a vital role in the response to tumor cells. Tumor immunotherapy takes advantage of the selectiveness of CD8+ T cells in order to destroy tumor cells. CD8+ T cells are able to selectively kill only infected cells, while leaving healthy host cells relatively unaffected. CD8+ T cells can recognize both tumor antigens presented by antigen presenting cells (APC’s) or antigens presented on the surface of mutated cells. Overexpressed or mutated self-proteins, or the presence of viral oncoproteins are among the kinds of antigens that CD8+ T cells are able to detect. Viral oncoproteins are proteins expressed by viral DNA in host cells that lead to the affected cells becoming deregulated and forming tumors. If CD8+ T cell precursors exist that are specific to an expressed foreign protein, the CD8+ T cells can become activated (4). Once stimulated by an antigen, CD8+ T cells differentiate and proliferate into long-lasting effector and memory cells.
(5). If the T cells are able to expand to an effective level and migrate to the location of the tumor, they can carry out their cytotoxic role and destroy the tumor (4).

Simian Virus 40 (SV40) is a polyomavirus that was discovered in 1960 as a contaminant in polio vaccines. SV40 has a small genome, 5.2 kbp in length, and thus relies heavily on the use of the host cell’s replication machinery in order to propagate (6). One of the most commonly studied features of SV40 is its Large Tumor antigen. Tag is a nuclear oncoprotein that can lead to tumor formation when expressed in vivo under the control of a promoter (7). Tag targets tumor suppressor proteins and inactivates them, which leads to unregulated cell growth and the formation of tumors in the tissues where Tag is expressed (8). Because of this, SV40 Tag has become a popular tool for studying the immunological response to cancer.

CD4+ T cells are vital in the establishment and maintenance of activated CD8+ T cells. CD4+ T cells produce cytokines which are essential for CD8+ expansion and survival, as well as for the activation of dendritic cells. Over time, CD8+ T cell populations become tolerant towards tumor antigens expressed by the host. CD8+ T cells that are specific for tumor proteins can be treated as autoreactive and destroyed. Also, tumor microenvironments frequently inhibit dendritic cell activity, thus hindering the development of an antitumor immune response. Tumor-specific CD4+ T cells are able to activate dendritic cells, which can lead to improved tumor immunity. CD8+ T cells that are stimulated by tumor antigens on APC’s in the presence of activated CD4+ T cells have prolonged cytotoxic effects. However, the mechanism by which this occurs is not fully understood (9).

CD8+ T cells are dependent on CD4+ T cells to maintain their function, without CD4+ T cells the cytotoxic ability of CD8+ T cells is greatly reduced. CD4+ T cells allow CD8+ T cells
to maintain effective levels and cytotoxic capacity, enable them to enter tumor sites, and alter

tumor expression of MHC molecules (10). CD8+ T cells that are activated in the presence of
CD4+ T cells have a prolonged cytotoxic effect in the tumor environment before becoming
tolerized. Repeated additions of new tumor-specific CD4+ T cells have demonstrated the ability
to maintain CTL functions. The CD4+ T cells are able to prevent the tumor environment from
tolerizing the CD8+ T cells (9).

In H$_2^b$ mice, SV40 T ag contains three immunodominant CD8+ epitopes (I, II/III, and IV), and two immunorecessive, or subdominant, CD8+ epitopes (295 and V). Epitopes 295 and V will only induce a CD8+ response when epitopes I, II/III, and IV have been removed (11).

SV40 T ag immunization causes a strong cellular immune response that results the rejection of T ag-induced tumors (12). Mice immunized with T ag display a strong immunological response to epitopes I, II/III, and IV, but not to epitopes 295 and V (11). Presentation of the immunodominant epitopes results in a much greater proliferation of the dominant epitope-responsive CD8+ T cells. The response of CD8+ T cells specific for the dominant epitopes suppresses the response to the subdominant epitopes 295 and V (13). CD4+ T cell specific epitopes in the SV40 T ag have also recently been discovered. To date, three separate epitopes have identified. These epitopes elicit a CD4+ specific response to the SV40 T ag, which is enhanced if the CD8+ T cell epitopes have been removed.

Interferon-$\gamma$ (IFN-$\gamma$) plays a critical role in the immune response. Secreted by numerous
different immune cells, IFN-$\gamma$ stimulates macrophages to enhance their antimicrobial and
antitumor effects, as well as antigen processing and presentation. IFN-$\gamma$ increases the
presentation of antigens by both MHC I and II molecules, the effect of which is an increase in
cell-mediated immunity. IFN-$\gamma$ causes the formation of special proteasomes that specifically
degrade antigens for presentation on MHC I molecules. Increased presentation on MHC I molecules increases the likelihood of CD8+ T cells detecting viral or oncogenic antigens. IFN-γ also increases presentation on MHC II molecules, inducing a greater reaction from CD4+ T cells (4).

IL-10 is a major growth factor for B cell activation (14). It is also an important immunoregulatory cytokine that protects the host from autoimmunity. IL-10 can be produced by a variety of immune cells, and IL-10 deficient mice display a more aggressive and prolonged response to antigens. IL-10 is associated with the reduction of IL-4 and IL-3 during allergic responses (15). IL-10 has been found to reduce proinflammatory immune reactions by suppressing the release of inflammatory cytokines. It also delays APC maturation, increasing the time they have to take up antigens while delaying their movement to the lymph nodes, where they can interact with T cells. It is suggested that IL-10 plays a role in reducing the inflammatory response once the immune stimulus has been removed (10). IL-10 also appears to inhibit cross-presentation by DC’s, which could limit T cell responses. This is thought to possibly lead to anergy in T cells (16). IL-10 has been found to increase the activity of natural killer (NK) cells. IL-10 also demonstrates anti-tumor properties, and it is suggested that this occurs through the activation of NK cells or CD8+ T cells (10). The addition of exogenous IL-10 has been found to inhibit tumor growth and lead to tumor regression. Debate still exists over whether IL-10 is an immunostimulatory or immunosuppressive cytokine (16).

In this paper, the role of the SV40 T ag CD4+ epitopes in the generation of memory CD8+ T cells was investigated. The IL-10 response to the CD4+ epitopes was also examined, and the SV40 T ag was screened for new, IL-10-specific epitopes. It was determined that the presence of the CD4+ epitopes in secondary immunizations decreases the CD8+ response, and
the CD4+ epitopes are capable of inducing IL-10 secretion. While no new IL-10 specific epitope were found, a portion of the T ag that induces non-memory secretion of IL-10 was identified. The cells responsible for this secretion will be a topic of further study, as well as the nature of the IL-10 response to the CD4+ epitopes.
MATERIALS AND METHODS

Mice

Male C57BL/6 (H-2^b) mice (B6) were purchased from The Jackson Laboratory. All experiments were performed in accordance with active protocols approved by the Messiah College Institutional Animal Care and Use Committee.

Peptide Library

A 175-peptide library corresponding to the amino acid sequence of the SV40 T ag was synthesized by Mimotopes (Clayton Victoria, Australia). The library peptides contained 11 residue overlaps and were provided in a 96 well format.

Cell Lines, Cell Cultures and Immunizations

The B6/WT-19 cell line (WT-19) was generated by immortalization of C57BL/6-derived primary cells with the wild type SV40 DNA (17). The antigen loss variant B6/K-1,4,5 cell line (K145) was selected by sequential in vitro of B6/K-0 cells with the SV40 T ag-specific CTL clones Y-1, Y-4 and Y-5 (18). The C2a cell line was produced by site-directed mutagenesis inactivation of the three known CD4+ epitopes (381, 529, 581).

T ag-expressing cells used in immunizations, unless otherwise specified, were cultured in Dulbecco’s Minimal Eagle Medium supplemented to 5% Fetal Calf Serum. For immunizations, cells were harvested at confluence using trypsin, washed three times with ice cold Hank’s balanced salt solution (HBSS), suspended to 10^8 cells/ml in HBSS, and injected intraperitoneally in 0.5 ml.
All experiments on primary immunized mice were carried out ten days after immunization unless otherwise noted.

**Isolation of Splenic Lymphocytes**

Mice were euthanized by cervical dislocation and the spleens were removed and placed in ice cold RPMI-1640 plus Glutamax medium (Invitrogen) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 50 μM 2-mercaptoethanol, and 25μg/ml pyruvic acid (referred to as RPMI(0)). The spleens were then homogenized in RPMI(0), centrifuged, resuspended in prewarmed Tris Ammonium Chloride, and incubated at 37°C for 5 minutes. The cell suspension was then diluted with RPMI(0) and centrifuged. The cells were resuspended in RPMI-0, and allowed to settle for 10 minutes, at which point the liquid suspension was collected. The suspension was centrifuged and resuspended in RPMI(0) supplemented to 10% with Fetal Calf Serum (RPMI(10)).

**Intracellular Cytokine Staining**

Harvested splenocytes were resuspended to 2x10^7 cells/ml in RPMI(10). Cells were attended to 96 well plates at 2x10^6 cells/well (100 μl). 100 μl of RPMI(10) containing 2 μM peptide and 2 μg/ml Brefeldin-A were added to each well. The cells were incubated for 6 hours at 37°C in 5% CO₂. After incubation, cells were washed twice with 200μl of FACS buffer (2% Fetal Calf Serum, 0.125% sodium azide in PBS) and incubated for 10 minutes in 100 μl 1:100 FC block: FACS. Between all washes, the plates were centrifuged for 3 minutes at 2000 rpm in a Jouan CR412 refrigerated centrifuge. The cells were washed again with FACS buffer and incubated for 15 minutes in 50 μl of 1:100 αCD8+-APC (Tonbo Biosciences):FACS. The cells were washed twice with FACS, and incubated for 20 minutes in 150 μl Cytofix/Cytoperm (BD
The cells were washed twice with Perm/Wash (1:10 10x Perm/Wash: H₂O) (BD Bioscience), and incubated for 15 minutes in 50 μl 1:50 αIFN-γ-PE (Tonbo Biosciences):Perm/Wash. The cells were washed twice with Perm/Wash, and suspended in 300 μl of 2% paraformaldehyde. Samples were analyzed with an LSR Fortessa Flow Cytometer.

**ELISPOT Assays**

ELISPOT assays were performed using IFN-γ or IL-10 ELISPOT kits purchased from eBioscience or BD Biosciences, respectively. Membrane-bottom 96-well plates were prepared by the addition of the capture antibody in 100 μl Coating Buffer according to the manufacturer’s specifications. The plates were then incubated overnight. The buffer was then aspirated, and the plates were washed twice with Coating Buffer. The plates were then blocked with 1% Bovine Serum Albumin in PBS for 1 hour. The blocking solution was removed, and the plates were washed twice with Coating Buffer. 1x10⁶ splenocytes were added in 100 μl to each well in serum-free HL-1 media, as well as the 10 μl of 1mM peptide solution. The plates were then incubated at 37°C for 24 or 48 hours for IFN-γ or IL-10 respectively. After incubation, the plates were washed 6 times with 200 μl ELISPOT Wash Buffer (0.05% Tween 20 in PBS). The plates were then incubated for 2 hours with 100 μl of Detection antibody in Assay Diluent. The plates were then washed 6 times with Wash Buffer and incubated for 45 minutes with 100 μl of Avidin-HRP reagent in Assay Diluent. The plates were then washed 4 times with Wash Buffer, then 3 times with PBS lacking Tween 20. 100 μl of AEC Substrate Solution were added to each well, and spots were allowed to develop. The plates were then washed for 5 minutes with deionized water, and were allowed to dry overnight. Spots were enumerated using a C.T.L ImmunoSpot S5 Core Analyzer with ImmunoSpot 5.0.3 analysis software.
RESULTS

Role of CD4+ Epitopes in CD8+ Memory Response

The goal of this experiment was to determine the effect of the CD4+ epitopes on the generation of memory CD8+ T cells. In order to examine this, mice were immunized with $1 \times 10^7$ of either WT-19 or C2a cells. Two months later, the mice received a boosting immunization with $1 \times 10^7$ of either WT-19 or C2a cells. Seven days later, the splenocytes were harvested and analyzed for responsiveness to the CD8+ epitopes I and IV by ICS (Figure 1). As shown in the figure, substantial number of SV40 T ag epitope I and epitope IV responsive CD8+ T cells were detected in each of the four groups. The highest level of responsive CD8+ T cells was generated in the group of mice that received the WT-19 primary immunization and the C2a boosting immunization. In each group, the frequencies of epitope IV-specific CD8+ T cells exceeded the number of epitope I-specific cells.

In order to test the validity of these findings, this experiment was repeated under the same conditions. Splenocytes were again analyzed for responsiveness to the CD8+ epitopes I and IV by ICS (Figure 2). The second set of data indicated that the strongest response was generated in response to homologous primary and secondary immunizations (WT19/WT19 and C2a/C2a).

Induction of T ag-specific CD8+ Memory Cells with T ag CD4+ Epitope-deficient Cells Grown in Serum-free Medium

To determine whether SV40 T ag-specific memory CD8+ T cells can be generated in the absence of the T ag CD4+ epitopes and fetal calf serum, mice were immunized with C2a cells that had been grown in HL-1 serum-free media. After four months, these mice received a boosting immunization of C2a cells grown in DMEM supplemented with 5% fetal calf serum,
and the splenocytes were harvested seven days later. The splenocytes of these mice were compared to those of mice that received seven- or ten-day primary immunizations with C2a cells grown in DMEM supplemented with 5% FCS. The analysis was carried out by ICS for CD8+ cells producing IFN-γ in response to the CD8+ epitopes I and IV (Figure 3).

**IL-10 Response to CD4+ Epitopes**

In order to determine whether the CD4+ epitopes were capable of inducing IL-10 secretion, mice were immunized with $5 \times 10^7$ K145 cells, which lack the CD8+ epitopes. Ten days later, the splenocytes were harvested and analyzed for IFN-γ and IL-10 secretion in response to the CD4+ epitopes by ELISPOT (Figure 4). The IL-10 ELISPOT indicated above baseline levels of IL-10 secretion in response to the CD4+ epitopes.

**IL-10 Library Screen**

To determine if any new IL-10-specific epitopes existed in the SV40 T ag, IFN-γ and IL-10 secretion were measured by ELISPOT with a 175-peptide library with splenocytes from K145 primary immunized mice ($5 \times 10^7$ cells/mouse). The IFN-γ ELISPOT showed the expected results, with responses to the three CD4+ epitopes and the recessive CD8+ epitope. The IL-10 ELISPOT showed small responses to the CD4+ epitopes, and a strong response to two new peptide residues (peptides 19 and 117). An IFN-γ response was also seen to peptide 124, with a small IL-10 response observed as well (Figure 5).

**IL-10 Response to Selected Library Peptides**

To determine whether peptides 19 and 117 induced genuine, memory-associated IL-10 secretion and to further examine peptide 124, a second experiment was performed using K145
immunized mice (5x10⁷ cells/mouse). Splenocytes from both immunized and naïve mice were analyzed for IFN-γ and IL-10 responses to the new peptides by ELISPOT (Figure 6).

Analysis of IFN-γ, IL-10 and IL-4 Production by ICS

In order to determine whether cells responding to the T ag CD4+ epitopes were secreting a single cytokine or several in combination, mice were given a ten-day K145 primary immunization with 5x10⁷ cells per mouse. The splenocytes were harvested and analyzed by ICS for production of IFN-γ, IL-10 and IL-4 in response to the three CD4+ epitopes. This analysis was unsuccessful, as none of the cells gave any form of cytokine response (data not shown).
DISCUSSION

Role of CD4+ Epitopes in CD8+ Memory Response

In order to elucidate the role of the CD4+ epitopes in generating memory CD8+ T cells, a memory experiment was performed using wild type WT-19 and CD4+ epitope deficient C2a cells. The response to the CD8+ epitopes I and IV was then examined by measuring the number of IFN-γ secretion by ICS. In the initial results, the highest number of responding T cells was generated against epitope IV in response to the WT-19 primary immunization followed by secondary immunization with the helper epitope deficient C2a cells. This suggests the possibility that the one or more of the three CD4+ epitopes may exert negative regulation during secondary/recall responses. While the CD4+ epitopes are necessary in primary immunizations for the generation of memory CD8+ T cells, this suggests that the presence of the CD4+ epitopes in subsequent immunizations can have a negative effect on the CD8+ population size (2).

The results upon repeating this experiment seem to contradict these initial findings, instead indicating the strongest response is generated when the mice are immunized with the same cell line in the primary and secondary immunizations.

Induction of Tag-specific CD8+ Memory Cells with Tag CD4+ Epitope-deficient Cells Grown in Serum-free Medium

In order to determine if the three known SV40 Tag CD4+ epitopes are required to generate CD8+ memory T cells, mice were immunized with C2a cells, which lack the CD4+ epitopes, which had been grown in HL-1 serum-free media. The reason for using HL-1 media is that the normal DMEM media contains 5% fetal calf serum, which could act as an adjuvant and replace the role of the CD4+ helper epitopes. After four months, the mice received a boosting
immunization with DMEM seven days prior to splenocytes harvest. These splenocytes were compared to those from mice that had received either a seven- or ten-day primary immunization with C2a cells grown in DMEM. The comparison was done using ICS for responding CD8+ cells after stimulation with the CD8+ epitopes I and IV.

In comparison with the mouse that received the seven-day primary immunization, the HL-1-grown, C2a-immunized mice generated very strong CD8+ responses against the target epitopes. This indicates that the HL-1 immunization did generate memory CD8+ T cells, despite the absence of the CD4+ epitopes and the absence of the fetal calf serum. Previous research has suggested that CD4+ epitopes are required for generating CD8+ memory, but this research involved a viral model, not cancerous cells (19).

Several possible explanations exist for these findings. One is that CD4+ helper epitopes are not required for the generation of long-lasting CD8+ memory cells. Another is that the HL-1 media contains something that is capable of acting as an adjuvant to replace the CD4+ help. A third explanation is that additional CD4+ epitopes exist within the SV40 T ag that have not yet been characterized.

*IL-10 Response to CD4+ Epitopes*

To determine whether the CD4+ epitopes were capable of inducing IL-10 secretion, mice were immunized with K145 cells. The use of K145 cells allowed for better detection of responses to the CD4+ epitopes, as the presence of the CD8+ epitopes masks the response to the CD4+ epitopes. The immunization generated an IL-10 response to the CD4+ epitopes above background response to an irrelevant peptide and above the level of the naïve mice. This
indicates that the IL-10 response is memory-specific and is directed against the CD4+ epitopes. A normal IFN-γ response to the CD4+ epitopes was observed.

**IL-10 Library Screen**

To determine if any IL-10-specific epitopes existed in the SV40 T ag, ELISPOTs for IFN-γ and IL-10 were performed using a 175-peptide library. The IFN-γ ELISPOT generated responses corresponding to the locations of the three CD4+ epitopes, as well as a response to the location of the immunorecessive CD8+ epitope 295, which is present in the K145 cells. The IL-10 ELISPOT generated minimal responses to the locations of the CD4+ epitopes, most likely due to the peptides being overlapping segments of the T ag, and not the actual CD4+ epitopes. Two unique peptides stimulated IL-10 secretion above background. These peptides did not correspond to any known epitopes in the T ag, and both generated IL-10 specific responses.

**IL-10 Response to Selected Library Peptides**

To determine if the IL-10 response to peptides 19 and 117 was repeatable and memory-associated, the responses to the peptides from immunized and naïve mice were tested by ELISPOT. The inclusion of naïve mice allowed the response to be characterized as either memory related or non-memory related. Both peptides 19 and 117 did not induce significant IFN-γ secretion, echoing what had been seen in the initial library screen. In the IL-10 ELISPOT, peptide 19 (residues 73-87) failed to stimulate above-background IL-10 secretion, indicating that it is not capable of inducing IL-10 secretion. The response seen in the IL-10 library ELISPOT may have been caused by bacterial contamination, which may have induced IL-10 secretion by monocytes present in the splenocytes. Peptide 117 (residues 465-479) did generate above background IL-10 secretion; however, this IL-10 response was seen in both the immunized and
naïve mice. This indicates that while peptide 117 is capable of inducing IL-10 secretion, this response is not memory related. The responding cells were not correlated with SV40 T ag immunization and may have been monocytes responding to the peptide. The possibility also exists that the cells responding to peptide 117 are regulatory T cells, but further work will be needed in order to determine this.

Peptide 124 (residues 493-507) did induce above background IFN-γ secretion in three of the mice, as well as IL-10 secretion in two of the mice. This response does appear to be memory associated and correlates with SV40 T ag immunization. Peptide 124 overlaps a section of the CD8+ epitope V, but this epitope is inactivated in the K145 cell line due to a point mutation. This mutation occurs at residue 491, and thus does not occur in the segment of the T ag that corresponds to peptide 124. The positive responses for both IFN-γ and IL-10 in towards peptide 124 indicate that this peptide may represent a new CD4+ epitope in the T ag. Further studies will be required to determine if that is the case.

*Analysis of IFN-γ, IL-10 and IL-4 Production by ICS*

The attempt to determine the cytokine profile of cells responding to the CD4+ epitopes was unsuccessful, as none of the cells stained for any of the cytokines investigated. The reason for this is unknown. Some non-T cell populations of cells did stain with the cytokine stains, indicating that the stains were present. It is possible that the peptides or the Brefeldin-A were added to the splenocytes incorrectly, or that splenocytes were incorrectly blocked or stained. It is also possible that the IL-10 and IL-4 stains interfered with the IFN-γ staining. Regardless of the cause, this experiment will need to be repeated in order to determine the cytokines expressed by individual CD4+ cells responding to the CD4+ epitopes.
CONCLUSION

While initially it appeared that secondary immunizations with cells expressing the wild-type T ag decreased the levels of T ag specific CD8+ T cells in comparison with secondary immunizations lacking the CD4+ epitopes, repeating the experiment failed to confirm this. Therefore further study is required to determine whether the presence of the CD4+ epitopes do induce negative regulation that decreases the CD8+ response. Immunizations with cells lacking the three known CD4+ epitopes that where grown in HL-1 serum-free media were still capable of generating long-term memory CD8+ T cells. This indicates that either the CD4+ epitopes are not required for memory CD8+ T cell generation, the HL-1 media can act as an adjuvant, or additional CD4+ epitopes exist in the SV40 T ag. Immunizations with cells expressing an SV40 T ag derivative lacking the immunodominant CD8+ epitopes demonstrate that the CD4+ epitopes do induce memory-related IL-10 secretion. From the screen of the SV40 T ag library, cells responded to the peptides 19 and 117 by secreting IL-10, but not IFN-γ. However, the numbers of cells appeared to secrete IL-10 following stimulation by the library peptide 117 in the ELISPOT assay was similar in immune and naïve mice, suggesting that the responders were not T cells. Peptide 19 failed to trigger IL-10 secretion above background when compared with an irrelevant control. The library peptide 124 was found to induce memory-associated IFN-γ and IL-10, suggesting that it may be a previously uncharacterized CD4+ epitope. Further studies will be necessary to determine the identity of the cells responding to peptide 117 and to determine if peptide 124 corresponds to a new CD4+ epitope. Further study will also be required to determine if individual CD4+ T cells secrete IFN-γ and IL-10 in response to the CD4+ epitopes.
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REFERENCES


FIGURE LEGENDS

Figure 1. **CD8+ memory response to presence/absence of CD4+ epitopes.** Mice received a primary immunization with cells expressing either the wild type T ag (WT-19) or a T ag derivative in which the CD4+ epitopes had been removed (C2a). After two months, the mice received a boosting immunization of either WT-19 or C2a cells. The splenocytes were harvested 7 days later and were analyzed for IFN-γ production in response stimulation with peptides corresponding to the CD8+ epitopes I and IV by Intracellular Cytokine Staining.

Figure 2. **Repeated CD8+ memory response to presence/absence of CD4+ epitopes.** Mice received a primary immunization with cells expressing either the wild type T ag (WT-19) or a T ag derivative in which the CD4+ epitopes had been removed (C2a). After two months, the mice received a boosting immunization of either WT-19 or C2a cells. The splenocytes were harvested 7 days later and were analyzed for IFN-γ production in response stimulation with peptides corresponding to the CD8+ epitopes I and IV by Intracellular Cytokine Staining.

Figure 3. **CD8+ Memory Cells generated in the absence of CD4+ epitopes and fetal calf serum.** Mice 1 and 2 received an immunization of $2.5 \times 10^7$ C2a cells grown in serum-free HL-1 media. After 4 months, mice 1 and 2 received a boosting immunization of $2.5 \times 10^7$ C2a cells grown in DMEM with 5% FCS. Mouse 3 received a primary immunization of $2.5 \times 10^7$ C2a cells grown in DMEM with 5% FCS at the same time, and the splenocytes were harvested seven days later. Mouse 4 received a primary immunization of $2.5 \times 10^7$ C2a cells grown in DMEM with 5% FCS ten days prior to the splenocytes harvest. The
spleenocytes were analyzed for IFN-γ production in response stimulation with peptides corresponding to the CD8+ epitopes I and IV by Intracellular Cytokine Staining.

**Figure 4. IL-10 response to CD4+ epitopes.** Mice received a primary immunization with K145 cells. Splenocytes were tested by ELISPOT for either IFN-γ (A) or IL-10 (B) secretion in response to *in vitro* stimulation with peptides corresponding to the three known CD4+ epitopes. Results shown were first reduced by the number of cells that responded to an irrelevant CD4+ epitope peptide (HBV core).

**Figure 5. SV40 T ag library screen.** Mice were immunized with K145 cells. A 175 member peptide library representing overlapping 15mer segments of the entire SV40 T ag was used to search for either IFN-γ (A) or IL-10 (B) secreting cells by ELISPOT.

**Figure 6. Selected epitope screen.** ELISPOT analysis of the IL-10 responses to three 15mer library synthetic peptides corresponding SV40 T ag sequences. Mice 1-4 were immunized with K145 cells, while mice 5 and 6 were unimmunized (naïve). The splenocytes from the mice were tested by ELISPOT for either INF-γ (A) or IL-10 (B) secretion. Results shown were first reduced by the number of cells that also responded to an irrelevant CD4+ epitope peptide (HBV core).
Figure 1

- Epitope I
- Epitope IV

Percent Responding CD8+ Cells

- WT-19 1°/WT-19 2°
- WT-19 1°/C2a 2°
- C2a 1°/WT-19 2°
- C2a 1°/C2a 2°
- Naive
Figure 2

Bar chart showing the percent responding CD8+ cells for different groups: WT19 1°/WT19 2°, WT19 1°/C2a 2°, C2a 1°/WT19 2°, C2a 1°/C2a 2°, and Naïve. The chart compares Epitope I (white bars) and Epitope IV (black bars) for each group.
Figure 3

![Graph showing percentage of CD8+ cells responding to Epitope I and Epitope IV in different mice. Bars represent mean ± standard error. The y-axis is labeled "Percent Responding CD8+" and the x-axis is labeled "Mouse" with categories 1, 2, 3, and 4, and "Naïve." The legend indicates that Epitope I is represented by filled bars and Epitope IV by open bars.]
Figure 4

A

![Bar graph showing IFN-γ production in naive splenocytes from different mouse strains.]

B

![Bar graph showing IL-10 production in naive splenocytes from different mouse strains.]

- Responding cells/10^6 splenocytes
- Mouse
- IFN-γ
- IL-10
- 381
- 529
- 581
Figure 5

A

B
Figure 6

A

**IFN-γ**

Responding cells/10^6 splenocytes

Mice

B

**IL-10**

Responding cells/10^6 splenocytes

Mice