CD4+ T cell Regulation of Immune Responses to the Simian Virus 40 Large Tumor Antigen is Complex and Involves Multiple Layers of Coordination Dependent on Immune Conditions

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CD4+ T cell Regulation of Immune Responses to the Simian Virus 40 Large Tumor Antigen is Complex and Involves Multiple Layers of Coordination Dependent on Immune Conditions

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BIOL 498

December 15, 2016
Abstract

Cytotoxic (CD8+) and Helper (CD4+) T lymphocytes play an important role in the immune detection and destruction of tumors. The induction of Cytotoxic T cell responses by multiple CD8 epitopes located within the Simian Virus 40 Large Tumor Antigen (SV40 T ag) oncprotein has been well characterized, and we have begun to characterize the role(s) of CD4+ Helper T cells in controlling cellular immune responses to the SV40 T ag. The goal of this study was to characterize the cytokines produced by activated SV40 T ag-specific Helper T cells in order to identify subset(s) of Helper T cells that may differentially influence regulation of the cellular immune response. Mice were immunized with B6/K-145 cells, which express a SV40 T ag mutant in which the CD8 epitopes I, II/III, IV and V have been inactivated. Frequencies of CD4 epitope 381-, 529- and 581-specific T cells expressing the cytokines IFN-γ, IL-2, or IL-10 were compared using single and double color ELISPOT assays following 24 of 40 hours of in vitro peptide re-stimulation. The data suggests that distinct populations of Helper T cells may secrete IFN-γ, IL-2 or both cytokines. Additionally, the ratio of IL-10 to IFN-γ secreting Helper T cells was reduced in the absence accompanying robust Cytotoxic T cell immune response. Our results begin to support a model in which multiple subsets of Helper T cells, potentially Th1 and Treg, work in parallel to coordinate immune responses to cellular tumor antigens.
Introduction

In the adaptive cellular immune system, CD8+ and CD4+ T cells each have unique strategies for overcoming foreign pathogens. CD8+ T cells recognize epitopes presented on Major Histocompatibility (MHC) class I molecules by host cells infected with viruses or intracellular bacteria, whereas CD4+ T cells recognize epitopes presented on MHC class II molecules by phagocytic antigen presenting cells (APC) that need not be infected. Epitope recognition by CD8+ T lymphocyte triggers destruction (lysis) of host cells harboring pathogens. Since CD4+ T cells recognize epitopes presented on non-infected cells, their main role is to help alert the immune system to infection, and to coordinate and control immune responses. CD4+ T cells accomplish this by secreting cytokines that act as signaling molecules in different immune pathways which promote the clonal expansion and migration of appropriate responders. For example, “help” from CD4+ T cells is thought to promote differentiation of CD8+ T cells into long-lived memory cells, and recruit other immune cells to the site of infection (1).

Both CD8+ and CD4+ T cells are largely activated by two different classes of the molecule known as the Major Histocompatibility Complex (MHC). MHC class I molecules are presented on virus infected host cells and will activate a response from CD8+ T cells whereas MHC class II molecules which are presented on uninfected cells (antigen presenting cells) activate a pathway for cytokine production in CD4+ T cells. CD4+ T cells are able to recognize MHC class II molecules on dendritic cells, B cells, monocytes and macrophages (1). It was observed that MHC class I molecules recognize many more specific tumor antigens than MHC class II molecules are able to recognize, and it is possible that this may limit the ability of CD4+ T cells to respond to immune threats (2).
Additionally, once CD4+ T cells are activated they have the capability to further differentiate into a specific CD4+ T cell subset upon receiving the appropriate cytokine signals (3). Each subset is then characterized by their ability to respond to particular sets of foreign pathogens by the secretion of select “signature” cytokines. The combination of cytokines within this signature mix, provides the immune system with instructions for carrying out a coordinated immune response best suited for the actual type of foreign pathogen. CD4+ T cell subsets and their signature cytokines are outlined in Figure 1.

Cytokines are protein signals that are secreted by select cells in the immune system that regulate innate and adaptive immunity. Release of cytokines is the principal way by which CD4+ T cells alert the immune system to a foreign antigen. This study focuses on IFN-γ, IL-2, IL-4, and IL-10, and IL-17. IFN-γ is required in the induction phase of infection (4). It functions in many key roles which include upregulating expression of MHC class I and II molecules which in turn enhances antigen/epitope processing and presentation on APCs which improves the chances for pathogen-specific lymphocytes to encounter evidence of the antigen. For the purposes of this study IFN-γ is used to indicate a pro-inflammatory response.

IL-17 is a signature cytokine for the T_{H17} CD4+ T cell subset. Additionally the cytokine IL-17 is implicated in pro-inflammatory immune responses specific to bacterial or fungal infections. It functions by recruiting neutrophils to sites of infection which in turn causes inflammation (5). Besides being strongly regulated by the presence of bacterial or fungal pathogens, T_{H17} CD4+ T cells, and subsequently IL-17, is inhibited by the presence of IFN-γ and IL-4 (5). Therefore, when the body decides to elicit a strong T_{H1} or T_{H2} response, T_{H17} responses are diminished, and thus a mixed response is not seen.
On the other hand, IL-4 creates an inhibitory or anti-inflammatory immune response by controlling expression of specific genes in lymphocytes, macrophages, and fibroblasts of the immune system (6). Cytokine IL-4 also induces cell proliferation, and apoptosis (6). Specifically IL-4 is a signature cytokine of the T_{H2} subset of CD4+ T cells, which excel in eradicating helminthes (5). Furthermore, IL-10 is a pivotal cytokine in the inhibitory immune response as well. It opposes many of the pro-inflammatory cytokines (7) by downregulating MHC expression, lowering cytokine release (7), and downregulating T cell activity (8). IL-10 is believed to play a role in the persistence of viral infections, mainly by suppressing the immune response of CD8+ T cells, but it also works to suppress CD4+ T cell response as well. Additionally, many immune system cells besides CD4+ T cells, such as dendritic cells, are able to secrete IL-10 (5).

One cytokine however, has both pro-inflammatory and anti-inflammatory properties dependent upon the exact situation. IL-2 is capable of promoting the survival, proliferation, and differentiation of activated CD4+ and CD8+ T cells (5). It is typically secreted by CD4+ T cells soon after their induction by CD4 specific epitopes (5). It is in this same way that IL-2 is capable of promoting the T_{reg} CD4+ T cell subset. T_{reg} T cells however have an anti-inflammatory effect on the body (discussed further) leading IL-2 to also be classified as an anti-inflammatory or inhibitory cytokine.

Normally T cells are introduced to self antigens/epitopes during development in the thymus, and through the process of negative selection, are destroyed if they recognize self antigens too strongly (5). This negative selection works to prevent autoimmunity in the peripheral immune system. T_{reg} CD4+ T cells are T cells that weakly recognize self antigens, but are not deleted through negative selection, and therefore specifically recognize these self
antigens in the peripheral immune system. Once in the periphery, T_{reg} T cells that recognize their antigen induce an inhibitory immune response through three general mechanisms. First, they produce the anti-inflammatory cytokines IL-10 and TGFβ. Second, they reduce T cell stimulation and finally, they consume IL-2 so that other immune cells are deprived of this positive promoting cytokine (5). T_{reg} T cells have been shown to be epitope specific, and therefore may play a role in cellular immunity against foreign pathogens (9-12).

The Simian Virus 40 Large T antigen (SV40 T ag) is a viral oncogene found in the model tumor system SV40 (Figure 2). SV40 T ag contains three H-2D{\textsuperscript{b}}-restricted (I, II/III, and V), two H-2K{\textsuperscript{b}}-restricted (IV, 295) CD8+/CTL epitopes, as well as three I-A{\textsuperscript{b}}-restricted (381, 529, 581) CD4+/helper epitopes (13,14). SV40 T ag is a useful tumor model because of its well-known life cycle, and the ease by which one can manipulate its genetic sequence (15).

Two immunization patterns, primary and secondary, were used to compare cytokine secretion patterns between recently activated, and memory CD4+ T cells respectively. It has been suggested that cytokines secreted by CD4+ T cells are necessary for creating competent memory CD8+ T cells (16). Thus, a possible change in cytokine secretion patterns may affect the development of memory CD8+ T cells, and subsequently the ability of CD8+ T cells to effectively respond to an immune threat.

Moreover, in an effort to better understand the various CD4+ T cell subsets responsible for regulating the immune response to tumors, populations of CD4+ T cells secreting multiple cytokines were explored. Through double color ELISPOT assays, CD4+ T cells were interrogated for their ability to produce IFN-γ/IL-2, IFN-γ/IL-4, or IFN-γ/IL-17. Through
discovering which cytokines are secreted by CD4+ T cells responding to tumor specific epitopes, the CD4+ T cells subset can also be identified due to the distinction of signature cytokines.

Immortal C57BL/6-derived mouse cell lines, B6/K-0 expressing the wild type SV40 T ag or B6/K-145 a mutant T ag which has inactivated CD8 epitopes, were used to immunize C57B/6 male mice (by intraperitoneal injection) in order to induce the activation and differentiation of naïve, SV40-T ag-specific CD4+ T lymphocytes. The results of previous experiments suggested that induction of strong CD8+ response may reduce the CD4+ T helper response to the T ag. Single color and double color ELISPOT assays were used to determine which cytokines such T cells would secrete. It was of interest to determine which cytokines CD4+ T lymphocytes specific for the 381, 529 or 581 epitopes would secrete, and whether expression of any inhibitory cytokines would be enhanced in T cells specific for any of these epitopes.
Methods

**Thawing Frozen Cells and Initial Plating**

Desired cells (B6/K-0 or B6/K-145) were thawed in a 37°C water bath for approximately 1 minute. Cells were then resuspended and placed into a 15mL conical tube containing 7mL of pre-warmed Dulbecco’s +10 media (obtained from PennState Hershey). 0.5mL Fetal Bovine Serum (FBS) (Hyclone) was directly added to the bottom of the tube, and tubes were centrifuged at 4°C for 5 minutes at 1,000 rpm (Jouan CR412 table top refrigerated centrifuge; M4 swinging buck rotor). Supernatent was aspirated. A T75 flask was prepared by adding 20mL of Dulbecco’s +10 media, as well as an appropriate volume of FBS (1mL) in order to achieve 15% v/v FBS concentration. Cell pellet in the conical tube was resuspended using media from the T75 flask, cell suspension was added to the T75 flask, and the flask was placed in a 37°C, 5% CO₂ incubator.

**Passing Cell Line B6/K-145**

When cells appeared to be 85% to 100% confluent (approximately 2-4 days) they were passed into new T75 flasks. Media was aspirated from the older T75 flask, and 4mL of 0.25% Trypsin-EDTA (1x) (Life Technologies) was added to the flask to detach the cell monolayer. Once the monolayer detached from the flask, 8mL of Dulbecco’s media (+5 or +10 depending on desired cell growth) was used to resuspend cells. 18mL of Dulbecco’s media (+5 or +10) was added to a new T75 flask, and the desired amount of cell suspension (appropriate dilution) was also added to the new T75 flask. Flask was placed in a 37°C, 5% CO₂ incubator.

**Freezing Down Cells Remaining After Cell Passage**
Cell suspension not added to new T75 flasks for the next passage of cells was routinely frozen down for future use. After counting cells with hemocytometer, the remaining cell suspension was placed in a 15mL conical tube and centrifuged at 4°C for 5 minutes at 1,000 rpm. Supernatant was aspirated, and the pellet was resuspended in 10% FBS freezing media. Resuspended cells were placed in a microcentrifuge tube specific for frozen cells, and held at -80°C until needed.

**Counting Cells**

100uL of Trypan Blue (obtained from PennState Hershey) was added into 2 microcentrifuge tubes per T75 flask of cells being passed. 100uL of resuspended cells was added to each microcentrifuge tube, and the new cell suspension was counted using a hemocytometer.

**Expanding Cell Lines from T75 to T175**

To pass and expand cells from a T75 to a T175 flask the previous procedure for passing cell lines was used with the following modifications. T175 flask was prepared by adding 36mL of Dulbecco’s media +5 into flask. Normal dilutions for T75 were doubled when expanding into T175 flasks. When passing subsequent cells to a new T175 flask, 6mL of 0.25% Trypsin-EDTA (1x) was used. An appropriate number of T175 flasks were set up in order to have enough cells for immunizing C57BL/6 male mice. Routinely, the number of flasks was doubled from the baseline calculated minimum of T175 flasks needed.

**Harvesting Cells from T175 and Preparing Cells for Immunization**

The following procedure was completed on each T175 flask being harvested. Media was aspirated, and 6mL 0.25% Trypsin-EDTA (1x) was added to trypsinize cells. 8mL of Dulbecco’s
media +5 was added to quench the reaction and cells were resuspended. Cell suspensions from every three flasks were combined in one 50mL conical tube. All tubes containing cells were held on ice moving forward. 50mL conical tubes were centrifuged at 4°C for 5 minutes at 1,000 rpm. Supernatent was aspirated, and cell pellets were resuspended in 10mL of Dulbecco’s +5 media. Cell suspensions from all 50mL conical tubes were combined and cells were counted (routinely at a 1/10 dilution). Cell pellets were centrifuged again at 4°C for 5 minutes at 1,000 rpm, and supernatant was aspirated. Cell pellets were resuspended in 10mL pre-chilled Hank’s Salt w/o Phenol Red (obtained from PennState Hershey). After cells were suspended, the remaining volume in the 50mL conical tube was filled with Hank’s Salt. Tubes were centrifuged at 4°C for 5 minutes at 1,000 rpm and supernatant was aspirated. Cell suspension, washing, and centrifugation with Hank’s salt were repeated 2 times. After the last wash, the supernatant was aspirated and an appropriate amount of Hank’s Salt was added to each cell pellet in order to produce a concentration of 1.0 x 10^8 cells/mL.

**Immunization of C57BL/6 Male Mice**

C57BL/6 male mice (between three and six; Jackson Laboratory) were immunized with B6/K-145 cells. 1mL syringes were attached to 23G1 needles (Becton Dickinson Co). Mice were immunized via intraperitoneal injection, using cell volumes of 0.55 mL for B6/K-145 or 0.50 mL for B6/K-0.

**Harvest and Preparation of C57BL/6 Mice Splenocytes for ELISPOT**

Mice were euthanized by cervical dislocation, and spleens were removed using sterile, aseptic technique and placed into 15mL conical tubes containing 5mL of RPMI + GlutaMAX-I (1x) (RPMI(0))(Life technologies). Spleens being pooled together were collected in the same
15mL conical tube, or spleens from individual mice were placed into individual 15mL conical tubes. Spleens were placed on wire screens previously washed with 5mL RPMI(0). Spleens were punctured with the plunger from a 5mL syringe (Becton Dickinson Co) and mechanically disrupted to release all splenocytes. Cells were suspended using an additional 5mL of RPMI(0) media and placed back in original 15mL conical tubes. All cell suspensions were held on ice. Tubes were centrifuged at 4°C for 7 minutes at 1,000 rpm. Supernatant was aspirated and cells were rapidly resuspended in 7mL prewarmed (37°C) Tris ammonium chloride (obtained from PennState Hershey). Tubes were incubated in a 37°C water bath for 5 minutes, and then 7mL RPMI(0) was added to each tube and mixed by gentle inversion. Tubes were centrifuged at 4°C for 7 minutes at 1,000 rpm, and supernatants were aspirated. Pellets were resuspended in 5.5mL of RPMI(0) media and allowed to stand on ice for 10 minutes. Cell suspension (supernatant) was pipetted into a new 15mL conical tube without disturbing settling cell debris. Cells were counted at an appropriate dilution. Tubes were centrifuged at 4°C for 7 minutes at 1,000 rpm. Supernatant was aspirated, and cell pellets were resuspended in an appropriate volume of HL-1 media (Lonza) to have a cell concentration of 1.0 x 10^7 cells/mL.

Peptide Sequences used in ELISPOT Assays

Peptides were synthesized in the Penn State Hershey Macromolecular Core Facility by Fmoc chemistry using an automated peptide synthesizer (9050 MiliGen Pep Synthesizer; Millipore). Peptides were solubilized in DMSO and diluted to the appropriate concentration with phosphate buffered saline (PBS; for ELISPOT assays) or RP10. Peptides corresponding to MHC class I-restricted determinants from SV40 T ag have been described (Fu et al., 1998; Mylin et al., 2000) and included: site I (LT206-215; SAINNYAQKL), C411L-substituted site IV (LT404-
411L; VVYDFKLKL) and site 295 (AA 295-303; FQYSFEMCL) (Mylin et al., unpublished). The locations and amino acid sequences of peptides corresponding to MHC class II determinants include 381 (AA 381-395; SADIEEWMAGUAWLH), 529 (AA 529-543; NEYSVPKTLQARFUK), 529mut3 (AA 529-543; NEPSVYKTLQPRFUK) and 581 (AA 581-595; WYRPUAEFAQSIQSR). MHC class II- restricted hepatitis B virus core antigen (HBc128-140; TPPAYRPPNAPIL; (Millich et al., 1988; Ressing et al., 1995) was used as the control peptide. Peptide solutions were routinely prepared as 1 mM stocks, stored at -80°C, and freshly diluted prior to use.

Single Color ELISPOT

Three 96-well filtration plates (Multiscreen-IP sterile 0.45um hydrophobic) (Merck Millipore) were used in parallel for cytokines IFN-γ, IL-4, and IL-10 respectively. Capture antibodies were prepared for IFN-γ (eBioscience), IL-4 (eBioscience), and IL-10 (BD Biosciences) with dilutions in ELISPOT coating buffer (powder from eBioscience, 1L dH2O) at 1/250, 1/250, and 1/200 respectively. Appropriate capture antibody was added to each plate with 100uL of capture antibody per well. Plates were incubated at 4°C overnight. The following day (routinely 16-20 hours following addition of capture antibody) coating antibody was aspirated from each well, and each well was washed twice with 200uL of ELISPOT coating buffer. Each plate was blocked using 1% (w/v) BSA (eBioscience) in PBS (1x eBioscience) for one hour at room temperature. Blocking solution was aspirated from wells and each well was washed twice with 200uL of ELISPOT coating buffer. Depending on the controls used Class I peptides gB, SV40 T ag IV, or SV40 T ag 295 in any combination with Class II peptides SV40 381, 529, 581, 529 mut 3, or HBV core were used (obtained from PennState Hershey). Class I peptides were routinely diluted in HL-1 media at a concentration of 2uM, and Class II peptides were routinely
diluted in HL-1 media at a concentration of 10uM. ELISPOT coating buffer was aspirated from wells and 50uL HL-1 media, 50uL HL-1 media peptide mix, and 100uL cell suspension was added each well for a total well volume of 200uL. Plates were incubated at 37°C, 5% CO₂ for 24 hours (IFN-γ) or 40 hours (IL-4, IL-10). After appropriate incubation times, plate(s) were removed from the incubator. Assay diluent was routinely prepared with sterile water at a 1/5 dilution using 5x ELISA/ELISPOT Diluent (eBioscience) for a total volume of 25mL per 96-well plate. Detection antibodies were prepared for IFN-γ (eBioscience), IL-4 (eBioscience), and IL-10 (BD Biosciences) with dilutions in 11mL Assay Diluent at 1/250. Cells and medium were aspirated from 96-well plate, and washed six times with ELISPOT Wash Buffer (200uL/well) (1x PBS with 0.05% Tween-20). 100uL of appropriate detection antibody was added to each well, and incubated at room temperature for two hours on a plate rocker. Avidin-HRP for IFN-γ, IL-4 (eBioscience) and IL-10 (BD Biosciences) was diluted in 11mL Assay Diluent at a dilution of 1/250. Following incubation on plate rocker, plate(s) were aspirated and washed six times with ELISPOT wash buffer (200 uL/well) allowing wells to soak for 1 minute between washes. 100uL of the appropriate Avidin-HRP was added to each well and the plate(s) were allowed to incubate at room temperature for 45 minutes on plate rocker. AEC substrate solution was prepared during this incubation by adding 400uL of AEC stock solution (100mg AEC in 10mL DMF) to 12mL acetate solution (0.1M, pH 5) and filtered through a 0.45um syringe-mounted filter (Millex-HA). AEC substrate solution was held on ice. After incubation on the plate rocker Avidin-HRP was aspirated from wells and wells were washed four times with ELISPOT wash buffer (200uL/well) and three times with PBS lacking Tween 20 (200ul/well). Immediately before AEC substrate solution was added to wells, 6uL of H₂O₂ was added to the AEC substrate solution. 100uL of AEC substrate solution was added to each well, and plate(s) were covered
with aluminum foil to protect from light. Spots were allowed to develop (protected from light) on a plate rocker (routinely between 25-60 minutes). After it was determined that the reaction had run to completion, the reaction was terminated by rinsing with distilled water (routinely >20 times). Plates were allowed to air dry 24+ hours protected from light.

**Double Color ELISPOT**

Three 96-well filtration plates (High-protein-binding PVDF Filter Plates) (ImmunoSpot) were used in parallel for cytokines IFN-γ/IL-2, IFN-γ/IL-4, and IFN-γ/IL-17 respectively. Capture antibodies were prepared for IFN-γ (Immunospot), IL-2 (Immunospot), IL-4 (Immunospot) and IL-17 (Immunospot) in Diluent A (Immunospot) separately with IFN-γ capture antibody being prepared for each plate. Each plate required 10 mL of Diluent A per set of capture antibodies, with volumes of capture antibody 60 µL (IFN-γ), 80 µL (IL-2), 80 µL (IL-4), and 120 µL (IL-17) being added.

Plates were prepared by removing the underdrain, pipetting 15 µL of 70% ethanol into wells and allowed to incubate at room temperature for less than one minute. Plates were then washed with 150 µL of PBS (PennState Hershey) three times. The underdrain was replaced and appropriate capture antibodies were added to each plate with 80 µL of capture antibody per well. Plates were sealed with parafilm and incubated at 4°C overnight. The following day (routinely 16-20 hours following addition of capture antibody) coating antibody was aspirated from each well, and each well was washed once with 150uL of PBS. Depending on the controls used Class I peptides gB, SV40 Tag IV, or SV40 Tag 295 in any combination with Class II peptides SV40 381, 529, 581, 529 mut 3, or HBV core were used (obtained from PennState Hershey). Class I peptides were routinely diluted in HL-1 media at a concentration of 2uM, and Class II peptides
were routinely diluted in HL-1 media at a concentration of 10uM. 50uL HL-1 media and 50uL HL-1 media peptide mix were placed in wells and allowed to incubate at 37° C for 10-20 minutes before plating cells. Following this, 100uL cell suspension (routinely 5 x 10^7 cells) was added to each well for a total well volume of 200uL. Plates were incubated at 37°C, 5% CO₂ for 24 hours.

After incubation, plates were removed from the incubator. Detection antibody sets were prepared for IFN-γ/IL-2, IFN-γ/IL-4 and IFN-γ/IL-17 in 10 mL Assay Diluent at a volume of 10 µL per detection antibody per set. Cells and medium were aspirated from 96-well plate, and washed twice with PBS (200 µL/well) and twice with ELISPOT Wash Buffer (200uL/well) (1x PBS with 0.05% Tween-20). 80 µL of appropriate detection antibody set was added to each well, and incubated at room temperature for two hours on a plate rocker. 10 µL of FITC-HRP and 10 µL of Strep-AP were added to 10 mL of Diluent C (Immunospot). Following incubation on plate rocker, plates were washed three times with ELISPOT wash buffer (200 uL/well). 80 µL of the FITC-HRP and Strep-AP solution was added to each well and the plates were allowed to incubate at room temperature for one hour on plate rocker.

Blue developer solution was prepared during this incubation by adding 160 µL of S1 (Immunospot), 160 µL S2 (Immunospot) and 92 µL of S3 (Immunospot) solutions to 10 mL of Diluent Blue (Immunospot) vortexing between each addition. Blue developer solution was held on ice protected from light. After incubation on the plate rocker the FITC-HRP and Strep-AP was removed from wells and wells were washed two times with ELISPOT wash buffer (200uL/well) and two times with filter sterilized water (200 µL/well). 80 µL of blue developer solution was added to each well, with filter sterilized water used in the red color control wells. Plates were incubated at room temperature for 15 minutes on plate rocker protected from light.
During the incubation, the red developer solution was prepared by adding 180 µL of R1 (Immunospot) and 160 µL of R2 (Immunospot) solutions to 10 mL of Diluent Red (Immunospot) vortexing between each addition. Following incubation with the blue developer solution, the reaction was stopped by gently washing with deionized water at least three times. Plate was washed once with filter sterilized water (200 µL/well). 80 µL of red developer solution was added to each well, with filter sterilized water used in the blue color control wells. Plates were incubated at room temperature for 30 minutes on plate rocker protected from light. After it was determined that the reaction had run to completion, the reaction was terminated by rinsing with deionized water (routinely >10 times), and the underdrain was removed. Plates were allowed to air dry 24+ hours protected from light.

*Counting ELISPOT Spots*

Spots formed on ELISPOT membranes were counted at Penn State Hershey’s laboratory of Dr. Todd Schell using the C.T.L. ELISPOT Plate Scanner. Pictures of all single color ELISPOT plates were taken using ImmunoCapture 6.3.5, and counted using ImmunoSpot 5.0 Academic Basic Count or Smart Count. Double color ELISPOT plates were sent to ImmunoSpot (Shaker Heights, OH) for scanning and spot counting.
Results

This research begins laying the groundwork for the characterization of CD4+ T cell responses to the Simian Virus 40 Large Tumor antigen (SV40 T ag). While CD8+ T cells have been actively researched in this model system, there has been little advancement in our understanding of CD4+ T cells. In particular, understanding how inhibitory cytokines are used during a CD4+ response is of great interest since there is relatively little that is known about the role of inhibitory cytokines compared to pro-inflammatory cytokines. Additionally, determining the specific CD4+ T cell subsets that control and regulate the immune response to tumors in crucial for understanding how the overall immune system responds to tumors. Continuation of this research may lead to a better understanding of how CD4+ T cells help to induce or work to hinder a strong CD8+ T cell response.

C57BL/6 mice were immunized with either the B6/K-0 (wild type SV40 T ag) cell line or the B6/K-145 (CD8 epitopes inactivated) cell line. B6/K-0 cell line was used to induce a strong CD8+ response, as well as a strong CD4+ response. The B6/K-145 cell line was used to determine if there was a difference in cytokine expression when only a CD4+ response was induced.

In our first experiment, the splenocytes from B6/K-145 primary immunized mice were collected and used in three parallel double color ELISPOT assays specific for cytokines IFN-γ, IL-2, and/or IL-17. IFN-γ was used as a control since previous studies have shown that T cells induced by SV40 T ag CD4 epitopes reliably produce IFN-γ. IFN-γ is also a pro-inflammatory cytokine which served as a basis for comparison of the differences between pro-inflammatory and inhibitory cytokines during a CD4+ response. Several different peptides were chosen (listed...
in Methods) based on desired control peptides. The ELISPOT assays focused on the cytokines produced by T cells induced by CD4 epitopes 381, 529, and 581. The irrelevant peptide HBV core was used as a control peptide.

The double color ELISPOT assay allowed for the visualization of overlapping or distinct populations of CD4+ T cells secreting various sets of cytokines (Figure 3). Each plate was prepared to indicate the presence of cytokine pairs; IFN-γ/IL-2, IFN-γ/IL-4, or IFN-γ/IL-17. Spots on ELISPOT membranes correlate to the presence of one cytokine secreting T cell. Red spots indicated the presence of an IFN-γ secreting T cell, blue spots represented either IL-2, IL-4, or IL-17 secreting T cells, and purple spots signal a T cell that secretes both cytokines in the specific pairing.

Results for each pair of cytokines are mixed and generally unsubstantial due to low cell counts and high background from the HBV core peptide. High HBV core peptide background for IFN-γ/IL-4 and IFN-γ/IL-17 double color ELISPOT plates prevented the detection of any IL-4, IL-17 or double color spots (data not shown). Spot counts for the IFN-γ/IL-2 pairing however was comparatively higher, and thus the following results and subsequent discussion of CD4+ T cell populations revolves around these specific results.

381-, 529-, and 581- T ag specific T cells are shown to have the ability to secrete IFN-γ and IL-2 in distinct populations as well as a unified overlapping population (Figure 4). Numbers of T cells that secreted only IFN-γ or IL-2 were relatively similar in 381- T ag specific T cells, with slightly greater numbers of IFN-γ only secretors over IL-2 only secretors seen in 529- and 581- T ag specific T cells. The population of T cells that was able to secrete both cytokines was relatively small overall (less than 10%) and was only seen in 381- and 529- T ag specific T cells.
Cytokine production by SV40 T ag-specific CD4+ T cells was further investigated by using parallel single color ELISPOT assays. The first condition under which it was investigated is a comparison of the two cell lines; B6/K-0 and B6/K-145 during a primary immunization. The second condition is a comparison of the two cell lines between a primary and a secondary immunization. This second condition was carried out twice for the B6/K-145 cell line, but has only been done once for the B6/K-0 cell line. Results have been analyzed using the averages from both experiments where applicable.

Overall results from primary ELISPOTs (data not shown) show that CD4 epitopes 381, 529, and 581 in B6/K-0 and B6/K-145 immunized mice induced T cells to produce both pro-inflammatory (IFN-γ) and inhibitory (IL-4, IL-10) cytokines. CD4 epitope 529 seemed to induce a higher frequency of T cells capable of secreting the three cytokines, and appeared to be dominant over CD4 epitopes 381, and 581. In general, the number of T cells secreting cytokines was greater in mice originally immunized with B6/K-0 cells compared to B6/K-145 cells.

Ratios of the frequency of induced cytokine producing T cells were calculated by taking the average spot counts for each inhibitory cytokine and dividing them by the average spot counts for IFN-γ (Figure 5). Ratios between inhibitory cytokines (IL-4, IL-10) in relation to pro-inflammatory cytokine (IFN-γ) for a primary immunization showed increased numbers of IL-10 secreting T cells compared to IFN-γ secreting T cells (particularly in mice with B6/K-0 primary immunizations), and decreased numbers of IL-4 secreting T cells compared to IFN-γ secreting T cells (Figure 5). B6/K-0 immunized mice generally had overall higher ratios than the B6/K-145 immunized mice. Of particular interest is the IL-10 ratio for CD4 epitopes for the B6/K-0 cell line during a primary immunization which showed significant increase in the numbers of IL-10
secreting T cells. Additionally, the B6/K-0 IL-4 ratio for epitope 581 appeared to be higher or at least comparable to the same IFN-\(\gamma\) ratio.

In the secondary immunization experiments, C57BL/6 mice were immunized with the B6/K-0 or B6/K-145 cell line 46 days and again 7 days before harvesting splenocytes. Once again, the splenocytes were collected and used in three parallel ELISPOT assays specific for cytokines IFN-\(\gamma\), IL-4, or IL-10.

Results from secondary ELISPOTs (data not shown) indicate that CD4 epitopes 381, 529, and 581 in B6/K-0 and B6/K-145 secondarily immunized mice induced T cells to produce both pro-inflammatory (IFN-\(\gamma\)) and inhibitory (IL-4, IL-10) cytokines. Overall, secondary immunized mice had greater pro-inflammatory (IFN-\(\gamma\)) and inhibitory (IL-4, IL-10) cytokine production compared to T cells from mice with primary immunization (data not shown). There also appeared to be more IL-4 secreting T cells for B6/K-0 immunized mice than for mice immunized with B6/K-145 (results not shown). Furthermore, of the three CD4 epitopes, the 529 epitope is dominant over 381 and 581 both in the primary and secondary immunizations.

Ratios between inhibitory cytokines (IL-4, IL-10) in relation to pro-inflammatory cytokine (IFN-\(\gamma\)) between a primary and secondary immunization pattern showed increased IL-4 and IL-10 ratios for B6/K-0 immunized mice compared to the B6/K-145 immunized mice (Figure 5). An overall trend for all CD4 epitopes for the B6/K-0 cell line was a decrease in IL-10 ratio from a primary immunization to a secondary immunization. This trend was not readily observed for the B6/K-145 cell line, as the ratios between primary and secondary immunized mice remained consistent. Additionally, CD4 epitope 581 did appear to induce more IL-4
producers than IFN-γ producers in mice immunized with B6/K-0. CD4 epitope 581 also seemed to have comparatively higher ratios than epitopes 381 or 529.

Additionally, single color ELISPOT assays specific for IFN-γ or IL-10 after 40 hours of in vitro restimulation were carried out following a primary immunization of B6/K-145 cells. The ratio of the number of IL-10 secreting T cells to the number of IFN-γ secreting T cells was significantly less than 1 (Figure 6). This data is consistent with the findings detailed previously regarding the ratio between IL-10 and IFN-γ in a B6/K-145 primary immunization with an in vitro restimulation period of 40 hours (IL-10) and 24 hours (IFN-γ) respectively.
Discussion

Experiments were carried out to observe CD4+ T cell cytokine production in two different environments - differing cell lines (B6/K-0 vs B6/K-145), and differing immunization patterns (primary vs secondary) – as well as to learn if various populations of CD4+ T cells secrete multiple cytokines and to determine the subsets of CD4+ T cells that regulate immune responses to tumors.

The results have indicated that cell lines B6/K-0 and B6/K-145 are capable of inducing T cell production of pro-inflammatory (IFN-γ) and inhibitory (IL-4, IL-10) cytokines in response to all three SV40 T-ag CD4 epitopes (381, 529, and 581). In general, there were more B6/K-0 responding CD4+ T cells, than B6/K-145 responders. This suggests that a strong CD4+ T cell response is correlated to a strong CD8+ T cell response.

It was also seen that inhibitory cytokine IL-4 seemed to have fewer producers induced by all three CD4 epitopes than IFN-γ or IL-10. One possible explanation for these findings is that IL-4 is a signature cytokine for Th2 helper cells, which excel in destroying helminthic parasites, and are not as effective against tumors (5). IL-4 is also known to induce cell apoptosis (7) which is a more extreme form of inhibition of the immune system than lowered MHC, T cell, or cytokine activity which is induced by IL-10. Thus, IL-4 may act as a final resort for the immune system if IL-10 or other inhibitory cytokines are not able to suppress the immune system sufficiently, which may account for the decreased IL-4 producers found in this study.

Numbers of IL-10 secreting CD4+ T cells however seemed to be increased compared to IFN-γ secreting CD4+ T cells in B6/K-0 primary immunizations. This was confirmed by an IL-
10/IFN-γ ratio that is greater in 381, 529, and 581 specific CD4+ T cells. As stated before, the IL-10 ratio was most enhanced following a primary immunization with B6/K-0 cells relative to either a secondary B6/K-0 immunization, or a primary or secondary B6/K-145 immunization. It is therefore possible that a strong CD8+ T cell response promotes a strong IL-10 inhibitory cytokine response. Also, newly experienced CD4+ T cells, as seen in a primary immunization, may elicit a higher IL-10 inhibitory response compared to memory CD4+ T cells seen in a secondary immunization.

Another general trend observed was CD4 epitope 529 inducing higher T cell cytokine secretion of both pro-inflammatory and inhibitory cytokines. This finding suggested that CD4 epitopes are able to induce different immune system responses, and work in different capacities. In particular CD4 epitope 529 induces greater cytokine production, and is dominant over CD4 epitopes 381 and 581.

The next experiment used mice administered a secondary immunization pattern with either B6/K-0 or B6/K-145 cells. Overall results indicated that T cells that had been re-stimulated in vivo by CD4 epitopes, had higher productions of each of the three cytokines – IFN-γ, IL-4, or IL-10. These results are expected since it is well known that immunity is increased with an increasing number of immunizations, due to the creation of memory T cells. Thus, the secondary immunization allows for the cytokine profiling of memory CD4+ T cells.

Once again, B6/K-0 immunized mice appeared to have an increased number of T cell responders compared to B6/K-145 immunized mice, and we propose that a strong CD8+ T cell response is needed in order to see a strong CD4+ T cell response.
It was noted that IL-4 producers seem to be lower in frequency than IFN-γ and IL-10 producers. This is particularly true for B6/K-145 mice immunized with CD4 epitope 381, where there was no noticeable indication of CD4+ activity. This may be due to the fact that CD4 epitope 529 is dominant over epitopes 381 and 581.

The next portion of this study was concerned with determining if there was a single population or multiple populations of T ag specific CD4+ T cells that secreted the cytokines previously observed. Double color ELISPOT assays allowed us the opportunity to observe if T cells were capable of secreting multiple cytokines at once. Plates with one of the cytokine pairings (IFN-γ/IL-2, IFN-γ/IL-4, or IFN-γ/IL-17) were done in parallel. Unfortunately, numbers for the IFN-γ/IL-4 and IFN-γ/IL-17 plates were too low to draw any accurate conclusions. This is surprising for the IFN-γ/IL-4 plate in particular since IL-4 secreting T cells have previously been observed. This may be attributed to error with the learning curve associated with learning a new technique. If this is true, then it is possible that there may in fact be T ag specific T cells capable of secreting IL-17, and they simply went unnoticed in this assay. We suggest repeated experiments to assess the validity of this claim.

The IFN-γ/IL-2 plate however proffered spot counts high enough to offer new information. With it, we see evidence for three possible populations of CD4+ T cells involved with IFN-γ and IL-2 secretion. The first population secretes IFN-γ only, the second solely secretes IL-2, and the third population secretes both cytokines. This suggests that multiple populations, and potentially multiple subsets, of CD4+ T cells regulate the immune response to tumors – a much more complex model than previously surmised.
Variability between individual mice is a factor that needs to be considered when interpreting these results. In general, the sample size of C57BL/6 male mice was small, and each CD4 epitope was only used to induce between two and four wells for ELISPOT. As the sample size increases and more data is obtained, the average spot counts and ratios should become more consistent, and the standard deviation should decrease.

Throughout the experiments, splenocytes were collected from C57BL/6 male mice that had not been immunized (naïve) to be used as a background comparison of all cytokine ELISPOTS produced. When all CD4 epitope ELISPOT counts had been averaged, the peptide HBV core averages were subtracted to account for background from irrelevant peptides. Expected results would have no ELISPOT counts for naïve mice, but this result was not seen as CD4 epitopes 381, 529 and 581 were occasionally able to induce T cells to secrete cytokines. These results would suggest that naïve mice are already experienced to these CD4 epitopes even without immunization with wild type or mutated SV40 T ag. Further analysis will be needed in order to see if there is a basis for these current results.
Conclusions

In conclusion, tumor cell immunizations delivering the wild type SV40 T ag (B6/K-0) or a mutated SV40 T ag (B6/K-145) both induce pro-inflammatory (IFN-γ) and inhibitory (IL-4, IL-10) cytokine-expressing CD4+ T lymphocytes. Ratios of IFN-γ and IL-10 secreting T cells are comparable (especially in B6/K-0 primary immunizations), while the IL-4 ratio for secreting T cells appears to be low and inconsistent. It was also noted that a strong CD8+ T cell response may be needed in order to induce a robust CD4+ T cell immune response. This is gathered from data that shows that the B6/K-0 cell line induces an increased number of T cells compared to the B6/K-145 cell line. Regardless of the cell line, CD4 epitope 529 is dominant compared to the other CD4 epitopes 381 and 581. Additionally, a secondary immunization pattern induces more cytokine producers than a primary immunization pattern. Furthermore, we show that both distinct and overlapping populations of CD4+ T cells are capable of secreting IFN-γ and IL-2.

From this, we suggest a model that contains the possibility of two CD4+ subsets, T_H1 and T_reg, involved in coordinating immune responses to tumors but that are induced by different stimuli (Figure 7). In this model, the T_H1 subset is activated in response to immunization with SV40 T ag no matter whether a robust CD8+ T cell response is present. It is unclear at this time, which signals help to differentiate naïve T cells into this subset. Then, we see the plausibility of a T_reg subset that secretes IL-10, being activated only in the presence of a strong CD8+ T cell response. We hypothesize that IL-10 is then capable of initiating a negative feedback loop that could potentially down-regulate IL-2, IFN-γ, and/or CD8+ T cells themselves. Taken collectively, these results begin to support a model in which CD4+ T cells play a much more complex role in regulating the immune response to tumors than previously thought.
Acknowledgements

I would like to thank Dr. Lawrence Mylin and Shaun Egolf for helping to immunize mice, prepare splenocytes, and perform ELISPOT. Thank you also to the laboratory of Dr. Todd Schell (Penn State Hershey, Microbiology and Immunology) for providing the use of the C.T.L. ELISPOT Plate Scanner, and to Messiah College for funding this project.
References


Figure Legend

Figure 1. Differentiation of Naïve T lymphocytes into one of the CD4+ subsets. Naïve T cells undergo differentiation into specific CD4+ subsets after receiving the appropriate cytokine signal(s)(3). Once differentiated, CD4+ T cells will secrete the signature cytokines associated with their particular subset.

Figure 2. Locations of currently known H-2Kb- and H-2Db-restricted CD8/CTL and I-Ab-restricted CD4/helper epitopes within the SV40 T ag8 amino acid sequence.

Figure 3. Representative ELISPOT wells for a double color assay designed to detect individual cells that secrete two cytokines. Red spots indicate an IFN-γ secreting [Helper] T cell, blue spots signify an IL-2 secreting [Helper] T cell and purple spots represent a [Helper] T cell that secretes both IFN-γ and IL-2.

Figure 4. IL-2 is secreted by different populations of Helper T cells: those that do or do not also secrete IFN-γ. C57Bl/6 mice were immunized with syngeneic cells expressing a mutant SV40 T ag missing four CD8 epitopes (B6/K-145). Spleens were harvested ten days later. Results of an ELISPOT enumerating epitope peptide specific (381,529,581,295) production of IFN-γ and IL-2 in 5x10^5 immune or naïve splenic lymphocytes. Percentages are calculated from the total number of cytokine secreting cells specific for each epitope: cells that secreted only IL-2, only IFN-γ or both IL-2 and IFN-γ (double color). The total number of cytokine-secreting cells represented by each set of bars is indicated above.

Figure 5. Frequencies of ELISPOT counts representing immune T cells specific for each of three CD4 epitopes (381, 529, 581) are presented as a ratio between responders secreting inhibitory
cytokines (IL-4, IL-10) in relation to the pro-inflammatory cytokine IFN-γ following a secondary immunization with the cell lines B6/K-0 or B6/K-145.

**Figure 6.** Ratios of the frequencies of ELISPOT counts representing B6/K-145-immune T cells specific for the CD4 epitope (529) or the CD8 epitope (295) secreting inhibitory cytokine (IL-10) in relation to the pro-inflammatory cytokine IFN-γ following a 40 hours of *in vitro* peptide stimulation time point with the cell line.

**Figure 7. Potential Mechanisms of Helper T cells Responding to SV40 T ag.** Based on current and past results, three different populations of Helper T cells may exist. The first are T_{H1} which secrete the pro-inflammatory cytokine IFN-γ. The second are Helper T cells that secrete IL-2 and possibly lead to the induction and/or survival of the third population; T_{reg} cells which secrete IL-10.
Figure 2

CD8 Epitopes
- I: 206-215
- II/III: 223-231
- 295: 295-303
- IV: 404-411
- V: 489-497

CD4 Epitopes
- 381: 381-395
- 529: 529-543
- 581: 581-595
Figure 5

SV40 Tag Epitopes

B6/K-0

B6/K-145
Figure 6

The bar chart shows the ratio of SV40 Tag Epitopes for CD4 (529) and CD8 (295) cells. The chart compares the levels of IFN-γ (dark blue) and IL-10 (light blue). The ratio values are as follows:

- For CD4 (529): IFN-γ is at a ratio of 1.2, IL-10 at 0.4.
- For CD8 (295): IFN-γ is at a ratio of 1.0, IL-10 at 0.2.

This indicates a higher ratio of IFN-γ over IL-10 for both CD4 and CD8 cells, with CD4 cells showing a slightly higher ratio for IFN-γ compared to CD8 cells.