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ABSTRACT

Immune tolerance of dominant tumor-associated antigens presents a particular challenge in developing T cell vaccine-based cancer treatments. T cells specific for weaker T ag epitopes have been shown to escape immune tolerance underscoring their potential in targeted cancer therapies. Because of its ability to induce tumor formation and serve as the target for T cell specific responses, the viral oncoprotein Simian virus 40 large tumor antigen (SV40 T ag) is an ideal model system to study tumor immunity. The SV40 T ag induces a cellular immune response in H-2^b (C57Bl/6) mice which is directed against multiple CD8+ and CD4+ epitopes and exhibits a characteristic hierarchy *in vivo*. Detection of additional weak SV40 T ag epitopes has been confounded by the unknown residual adjuvant effects of fetal bovine serum (FBS) present in media used to culture cells used for immunization. To this end, the immunogenicity of SV40 T ag transformed cells grown in HL-1 serum free or 5% serum media was evaluated by ELISPOT analysis and MHC-tetramer staining. The results of this study suggest that SV40 T-ag expressing tumorigenic cells grown in the absence of FBS induce a robust CD4+ and CD8+ T cell immune response independent of the potential adjuvant effects of FBS. To search for additional epitopes, ELISPOT analysis was conducted using 175 members of a 15mer peptide library corresponding to the complete SV40 T ag amino acid sequence as targets for proinflammatory immune responses. This analysis identified two new candidates corresponding to library peptides beginning at SV40 T ag amino acids 361 and 493. Additional ELISPOT analysis using a synthetic peptide corresponding to SV40 T ag amino acids 361-375 failed to confirm that this region represents a bona fide epitope. MALDI-TOF mass spectrometry analysis will be used to determine whether derivatives of library peptide 295 may have contaminated well(s) supposed to be peptides corresponding to the 361 region of the SV40 T ag.

INTRODUCTION

The adaptive immune system has the unique ability to recognize antigenic peptides distinct to a particular invader and direct an immune response against it with exquisite specificity. A key branch of this adaptive immune response includes the cellular immune system whose functions are primarily carried out by cytotoxic and helper T lymphocytes. Discrete identification by T cells is accomplished by the binding of clonotypic T cell receptors (TCRs) to peptide antigens presented by appropriate Major Histocompatibility Complex (MHC) molecules. The specificity of a TCR for its antigenic peptide is derived from the production of a given TCR from two non-identical peptides whose separate genes form through random recombinational processes (1). In this way, a given T cell is capable of binding an appropriately presented antigen based on specific molecular contacts and ensures that the population of T cells present in an individual represents a diverse repertoire of epitope specificities.

In addition to TCR antigen interactions, the phenomena of MHC restriction also partially defines the molecular interactions of T lymphocytes by ensuring that a single T cell can only recognize peptides displayed by a particular MHC molecule. For example, mature helper T cells bind antigens presented on MHC class II receptors of antigen presenting cells that derive antigen by digestion of extracellular proteins. On the other hand, cytotoxic T cells recognize antigens presented on MHC class I receptors produced from inside the presenting cell (2). The binding interactions between a cell presenting an antigen and a T lymphocyte also require glycoprotein co-receptors present on T cell surfaces. Mature helper T cells display the CD4 glycoprotein on their surface (CD4+) and bind to MHC class II receptors while CTLs display glycoprotein CD8 (CD8+) and bind to MHC class I receptors (1).

An understanding of the functional properties of cytotoxic and helper T cells has resulted from work studying T cell responses to viral infection. Activated CD8+ T cells destroy virus-infected host cells through the recognition of MHC class I receptors bearing viral antigens specific for a given TCR. Following recognition, the cytotoxic activity of CD8+ T cells is induced against the infected cells through a synaptic release of molecules, such as granzymes and perforin, which ultimately result in cellular death (2). The specificity of this cytotoxic activity effectively allows for the elimination of tumor host cells without targeting or destroying healthy cells in the surrounding area.

CD4+ T cells act by different mechanisms to mount an immune response against viral infections. Primarily, they regulate the humoral and cellular immune responses through the secretion of cytokines, the key signaling molecules of the immune system (3). Depending on the type of infection, activated CD4+ T cells can develop into either Type 1 (T_{H1}) or Type 2 (T_{H2}) helper cells which promote defense against intracellular and extracellular parasites respectively. T_{H1} cells produce pro-inflammatory cytokines such as Interleukin-2 (IL-2) and interferon-γ (IFN-γ) to promote cellular immunity. On the other hand, T_{H2} cells produce cytokines such as IL-4, IL-5, IL-10, and IL-13 to evoke antibody-based immunity, eosinophil accumulation, and anti-inflammatory responses (4).

The ability of cytotoxic T cells to mount a defense against viral infections is highly influenced by the regulatory effects of helper T cell secreted cytokines. IL-2 released by helper T cells optimizes CD8+ effector T cell growth and survival, or differentiation into memory cells (5). CD4+ T cells are also important for events during CD8+ T cell priming, the process in which a T cell encounters its specific antigen (6). However, importantly CD4+ T cells are not necessary in many cases in the primary expansion of CD8+ T cells (6,7). Helper T cells also have the

unique ability to recruit immune cells, including CD8+ T cells, to the site of infection. In these ways, it seems that proper functioning of CD8+ to effectively respond to viral infection is, at least in part, dependent on the presence and function of CD4+ T cells and signaling cytokines activated by recognition of specific viral antigens.

In addition to mediating the primary immune response to viral infections, T cells play a critical role defining the immune response to solid tumors. Information gained from studying T cells responses to viral infection has informed tumor immunity research. Particular importance has been placed in studying the responses of CD8+ T cells to solid tumors due their observed cytotoxic effects against intracellular pathogens. This has allowed for the identification of common classes of tumor antigens that may elicit an immune response. These primarily include antigens derived from unique peptide sequences such as overexpressed or mutated self proteins or viral oncoproteins. Activation of tumor specific, pre-cursor cytotoxic T cells is mediated by the presentation of such tumor antigens to cytotoxic T cells in the presence of appropriate co-stimulation. These events generally result in mass proliferation of CD8+ T cell specific for the tumor antigen and generation of tumor-specific effector and memory cells. Successful migration of effector CD8+ T cells to the tumor location, and subsequent recognition of tumor antigen on mutated cells, can result in tumor cell death (1,8). These events closely parallel the cytotoxic immune response to viral infection.

Due to their importance in tumor elimination, emphasis has been placed on understanding memory CD8+ T cells and what allows for their survival. It has been identified that persistence of memory CD8+ T cells requires antigen clearance, costimuatlion, responsiveness to cytokines and CD4+ T cells and is inhibited by chronic exposure to tumor antigen, action of CD4+ T regulatory cells, and the inhibitory vasculature of solid tumors in terms of T cell migration (9).

However, presently much remains undefined about how the tumor microenvironment impacts tumor specific memory CD8+ generation and maintenance.

In addition to these gaps of knowledge, much remains unknown about the general role of CD4+ T cells in tumor immunity and their impact on cytotoxic T cells. It was suspected that CD4+ T cell presence and function may be required for the proper functioning of tumor specific CD8+ T cells. Research identified this to be partly true as it was observed that CD4+ T cells have an enhanced ability to migrate through the vasculature of a solid tumor as compared to CD8+ T cells. Upon entry, helper cells secrete IFN-γ and trigger signaling cascades which attract immune cells to a tumor environment and promote infiltration by CD8+ T cells (10, 11). It has also been shown that CD4+ T cells reduce immune suppression in tumor lesions, by an unidentified mechanism, to promote increased sensitivity of the immune response to a solid tumor (12).

Due to the observed roles of cytotoxic and helper T cells to eliminate tumors, many research efforts have tried to use T cells to fight cancer by developing immunotherapies. However, the aforementioned gaps in knowledge make the development of effective T cell-based therapies difficult. The phenomena of immune tolerance presents an additional challenge in developing effective immunotherapies. Immune tolerance by definition is the failure of the immune system to mount a response against an antigen. Within the context of a tumor, this is a developed failure to respond to dominant tumor-associated antigens allowing tumor growth. However, it has been identified that T-cells specific for weaker epitopes escape immune tolerance, suggesting an importance of recessive tumor epitopes in the development of T cell vaccines. Consequently, identifying and understanding the role of weaker tumor antigens in defining immune responses during immune tolerance has potential in overcoming this challenge.

Model tumor producing systems are invaluable tools in studying aspects of T cell based tumor immunity including immune tolerance. Our lab studies T cell responses to the Simian Virus 40 Large T antigen (SV40 T ag). The SV40 5.2 kpb genome codes for at least six viral proteins including: three capsid proteins denoted VP1, VP2, and VP3, two nonstructural polypeptides, an agnoprotein, the small tumor antigen (tag) and the large tumor antigen (T ag) (13). Among these proteins, the T ag has become of particular interest because of its ability to induce neoplasia in a variety of SV40 infected cells in culture and *in vivo* (8, 14).

As an oncoprotein, T ag initiates cell cycle progression and inactivates tumor suppressor proteins retinoblastoma (Rb) and p53 (15). This property makes T ag ideal in the use of a model system because it provides a means to transform cells *in vitro* and develop tumors in nude or immunocompetent mice, or induce tumors in mice containing T ag as a transgene (16). Additionally when immunized in H-2^b mice, SV40 T-ag transformed cells generate a cellular immune response directed against multiple defined MHC class I-restricted and class II-restricted epitopes that ultimately result in rejection of tumors induced by the presence of T ag (16, 17). In H-2^b mice, five SV40 T ag CD8+ epitopes have been identified. These CD8+ determinants include: epitope I (amino acid residues 206-215), epitope II/III (amino acid residues 223-231), epitope IV (amino acid residues 404-411), epitope V (amino acids 489-497) and epitope 295 (amino acid residues 295-303) (16, 17,18). Three H-2^b-restricted CD4+ T cell epitopes have also been discovered and identified within T ag beginning at residues 381, 529, and 581 (17).

The CD8+ epitopes do not induce similar T cell immune responses, but rather form a hierarchy of responses *in vivo.* Epitopes IV, I, and II/III are immunodominant while epitopes V and 295 are immunorecessive. Among the immunodominant epitopes, SV40 T ag-specific T cells preferentially target epitope IV followed by epitopes I, and II/III (19). Accordingly, higher

frequencies of CD8+ T lymphocytes are induced that are specific for the dominant epitopes as compared to the recessive epitopes (V and 295). The recessive determinants will only induce detectable immune responses in the absence of the three dominant epitopes (19). The hierarchy of responses of the CD8+ T cell epitopes observed *in vivo* have been useful in studying immune tolerance. SV11 mice expressing T-ag as transgene result in the deletion of the dominant CD8+ T cell populations, but survival of the known immunorecessive specific cytotoxic T cells. When boosted, these T cell populations significantly expand and allow for effective elimination of choroid plexus tumors. These findings suggest that an effective immunotherapeutic strategy includes targeting and expanding T cells specific for weak tumor antigens.

This study sought to identify other weak SV40 T-ag epitopes that may be important in defining SV40 T-ag tumor immune responses *in vivo*. However, in past stuides SV40 T-ag transformed cells used for immunization have been cultured in media containing fetal bovine serum (FBS), an essential growth supplement for most media formulations. Adjuvant effects of FBS on T cell responses in this model are unknown and have, in turn, limited detection of additional bona fide weak epitopes within SV40 T-ag. To this end, the potential immunogenic effects of FBS within the SV40 T-ag model this study were studied by characterizing the primary CD4+ and CD8+ T cell responses induced by SV40 T-ag tumor cells cultured with or without FBS. Concurrently, this study also sought to identify if FBS limited previous epitope identification studies by searching for new epitopes within SV40 T-ag using cell vaccines produced without FBS-containing medium.

MATERIALS AND METHODS

Mice

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

Synthetic Peptides (Large Scale Synthesis)

Synthetic peptides used in this study were synthesized at the Macromolecular Core Facility of The Pennsylvania State University College of Medicine by 9-fluorenylmethoxycarbonyl chemistry on an automated peptide synthesizer (9050 Milligen PepSynthesizer) or were obtained from Chiron (pin synthesis) and were routinely used without further purification.

Peptide Library

The SV40 T ag peptide library was synthesized by Mimtopes (Clayton Victoria, Australia) and is comprised of 175 15mer peptides which overlap with adjacent members by 11 amino acids residues to cover the entire 708 T-ag amino acid sequence. A3 and B3 Mimtope library sets were stored at -80°C and thawed at 37°C for use. Following this incubation period, 5 µL aliquots of each peptide (3-5mg) stored in DMSO was diluted in sterile polypropylene 96 wells plates at 1:20 in HL-1 (Lonza) media for a total volume of 100 µL. These dilution plates were then used for further dilution transferring 10 µL of the 1:20 dilution to membrane-bottom 96-well plates (Millipore MultiScreen MAIPS4510) containing 90 µL HL-1 media for a total 1:10 dilution. These dilutions are designed to give a final peptide concentration of approximately 10 µM per well. Membrane plates were then incubated at 37°C until the addition of prepared

splenocyte suspensions. Control peptides (SV40 T-ag CD4+ epitopes 381, 529, and 581; SV40 T-ag CD8+ epitopes 295 and I; irrelevant controls mPyt and HBV) were added to each well at a final concentration of 10 μM. Controls were prepared by 1:25 dilution of 1 mM stock peptides to create a 40 μM working stock and transferring 50 μL of working stock to wells containing 50 μL HL-1 and 100 μL splenocytes (final concentration $1x10^6$).

Cell Lines, Cell Culture and Immunizations

The cell line B6/K-0 was generated from the immortalization of C57BL/6 primary cells with wild-type SV40 T-ag DNA. The antigen loss variant B6/K-1,4,5 cell line was derived from *in vitro* coculture of B6/K-0 cells selected for with SV40 T-ag specific CTL clones Y-1, Y-4, and Y-5 and therefore lacks SV40 T-ag CD8+ epitopes I, II/III, IV, and V. The B6/T122B1cell line expresses a T ag derivative in which epitopes I, II/III, IV, and V have been inactivated by alanine mutations in MHC I anchor residues (19).

SV40 T-ag cells lines B6/K-0, B6/K-1,4,5, and B6/T122b1 were adapted to grow in serum-free HL-1 media (Lonza) or Dulbecco's Minimal Eagle Medium supplemented to 5% fetal calf serum. For cells grown in serum-free HL-1 media, cell passage required rinsing cell layers at confluence with Mg^{2+}/Ca^{2+} free phosphate buffered saline (PBS) followed by the addition of TrypLE™ Express Enzyme (1X) with no phenol red and allowed to incubate at 37°C and 5% CO_2 for five minutes. Following this, TrypLE™ Express Enzyme was inactivated upon dilution with Mg^{2+}/Ca^{2+} free PBS and cells pelleted upon centrifugation at <1000 rpm (M4 rotor in Jouan CR412 centrifuge), 4°C and the final cell pellet resuspended in HL-1 media. Tumor cells cultured in Dulbecco's 5%media were passaged by the common method utilizing trypsin.

For immunizations, cell were harvested at confluence using the aforementioned passaging techniques, washed three times with ice cold Hank's balanced salt solution (HBSS), suspended to 10^8 cell/mL in HBSS, and injected intraperitoneally in 0.5 mL. All experiments were carried out ten days after immunization of mice.

Isolation of Splenic Lymphocytes

Mice were euthanized by cervical dislocation and spleens were surgically removed and placed into ice cold serum-free RPMI-1640 plus Glutamax medium supplemented with antibiotics referred to here as RPMI(0) and stored on ice. The collected spleens were then homogenized in RPMI(0) and centrifuged at 1000 rpm, 4°C for seven minutes. The cell pellet was resuspended in prewarmed Tris Ammonium Chloride and incubated for five minutes at 37°C to deplete the suspension of red blood cells via osmotic lysis. Following centrifugation, the tan-white pellet was resuspended in RPMI(0) and cellular debris allowed to settle for only 10 minutes. The liquid suspension was collected and, after samples were taken for cell counting, centrifuged. Cell pellets were then resuspended in enough HL-1 medium to produce a final living cell concentration of $1x10^7$ cell/mL.

ELISPOT Assays

ELISPOT Ready-Set-Go kits to detect IFN- γ or IL-10 were purchased from eBiosciences and the respective protocols carried out. Specifically, capture antibody at a 1/250 dilution in ELISPOT Coating Buffer (included in kit reagent set) was prepared and 100 µL of this mixture added to membrane-bottom 96-well plates (Millipore MultiScreen MAIPS4510). Following an overnight incubation period at 4°C, the buffer was aspirated, and the plates rinsed twice with Coating Buffer. The plates were then blocked with 1% (w/v) Bovine Serum Albumin (BSA) in

PBS for one hour at room temperature. Added blocking solution was aspirated and the plates rinsed twice with Coating Buffer. Peptides (SV40 T ag CD4+ epitopes 381, 529, and 581; SV40 T ag CD8+ epitopes 295 and I; SV40 T ag peptide 361; irrelevant controls mPyt and HBV) were prepared by 1:25 dilution of 1 mM stock peptides to create a 40 µM working stock and transferring 50 µL of working stock to wells containing 50 µL HL-1 and 100 µL splenocytes (final concentration $1x10^6$). SV40 T-ag peptide library screen were prepared as described above. After a 24 hour incubation period at 37°C and 5% CO_2, cells and media were aspirated and the wells washed six times with 200 µL ELISPOT Wash Buffer (0.05% Tween 20 in PBS). Following this, biotinylated detection antibodies diluted 1/250 in Assay Diluent were added to the wells at 100 µL and incubated for 2 hours at room temperature. The plates were then washed six times with Wash Buffer and incubated for 45 minutes with 100 µL of Avidin-conjugated horseradish peroxidase (A-HRP) reagent diluted in Assay Diluent. After this incubation period, the plates were washed four times with Wash Buffer and three times with PBS lacking Tween 20. To detect the captured cytokines, 100 µL of filtered AEC substrate solution (0.1 M sodium acetate pH 5.0, 333 µg/mL 3-amino-9-ethyl carbazole, 0.015% H_2O_2) was added to each well and spots allowed to develop for 10 minutes at room temperature. Lastly, a five minute wash period with deionized water was conducted and the plate cover removed from the membrane to allow for drying overnight. The resultant spots were then counted using an automated C.T.L. ImmunoSpot S5 Core Analyzer with ImmunoSpot 5.0.3 analysis software.

MHC Tetramer Staining

To conduct MHC Tetramer staining, 200 µL of a $1x10^7$ cell/mL RBC-depleted splenocyte cell suspension was added to a 96 well round bottom plate for a total cellular concentration of $2x10^6$ cells per well. Following centrifugation for three minutes at 2 000 rpm, the supernatant

was removed and the cell pellet washed twice in 200 µL ice cold FACS buffer [PBS, 2% FCS (v/v), 0.1% NaN_3 (w/v)]. The cell pellet was then resuspended in 100 µL of FACS buffer with 1:100 diluted Fc block and incubated at room temperature for 10 minutes. Wells were then diluted with 100 µL of FACS buffer and centrifuged as before and wells washed once more with FACS buffer. The cell pellets were then resuspended in 50 µL FACS buffer containing 1:200 diluted Tet I-PE tetramers or Tet IV-PE tetramers and 1:100 diluted αCD8-APC. Following at 15 minute incubation period at room temperature, the wells were diluted with 150 µL FACS buffer and centrifuged. Two additional washes with FACS buffer were conducted. Finally, the cell pellet was resuspended in 300 µL of 2% paraformaldehyde, transferred to FACS tubes, and stored in foil at 4°C until analysis. Single color controls were also prepared by 1:100 dilution of αCD8-APC and αCD8-PE in FACS buffer as described for sample above. Analysis was conducted using the BD LSR Fortessa flow cytometer at the Penn State Hershey Core Research Facility.

RESULTS

In the absence of FBS, B6/K-1,4,5 and B6/K-0 SV40 transformed cells generate robust primary CD4+ T cell responses.

In order to study the potential adjuvant effects of FBS on the CD4+ T cell response induced by SV40 T-ag tumor cells, B6/K-1,4,5 and B6/K-0 SV40 cells lines were growth adapted to HL-1 serum-free medium or Dulbecco's Minimal Eagle Medium supplemented to 5% FBS. Adapted cells were then used for immunization of H-2^b mice and ten days later, the splenocytes were harvested and analyzed for responsiveness to SV40 T-ag CD4+ T cell epitopes by ELISPOT analysis to detect IFN-γ-expressing cells (Figure 1). Values for an irrelevant control peptide (HBV-core) were subtracted from the enumerated spots values presented. As shown in Figure 1A, primary immunization with B6/K-1,4,5 tumor cells lacking most SV40 T-ag CD8+ T cell epitopes (excluding epitope 295) generated a considerable number of SV40 T-ag epitope specific, IFN-γ-expressing cells. There was no statistically significant difference between the responses induced by serum-free or 5% FBS cultured tumor cells. The greatest response for both serum-free or 5% FBS cultured B6/K-1,4,5 tumor cells was observed in the presence of the CD8+ T cell epitope 295. There was no statistically significant difference between the responses to the CD4+ T cell epitopes 381, 529 and 581. In a separate experiment, it was observed that B6/K-0 tumor cells containing a wildtype version of SV40 T-ag also produced robust IFN-γ expression from SV40 T-ag specific T cells (Figure 1B). As observed with the response induced by the B6/K-1,4,5 cell line, serum-free or 5% FBS cultured B6/K-0 cells did not generate immune responses with statistically significant differences. In comparison to the immune response generated by B6/K-1,4,5 tumor cells, B6/K-0 cells produced a less impressive response to SV40 T-ag CD8+ T cell epitope 295.

In the absence of FBS, B6/K-0 SV40 transformed cells induce a comparable percentage of SV40 T-ag epitope specific CD8+ T cells

This experiment sought to monitor the SV40 T-ag CD8+ T cell response in the absence of FBS to evaluate the potential adjuvant effects of this growth supplement. To this end, the percentage of CD8+ T cells that were SV40 T-ag epitope specific among the total CD8+ T cell population was determined upon immunization of H-2^b mice with B6-K-0 cells cultured in either serum free or 5% FBS media. SV40 T-ag epitope I and IV specific T cell receptors were stained through binding of tetrameric MHC class I molecules joined to a PE-flurochrome bound streptavidin molecule by a biotinylated domain and detected via subsequent flow cytometry analysis. For both epitope I and IV, B6-K-0 cells cultured in either serum-free or 5% FBS media induced a statistically comparable percentage of SV40 T-ag epitope specific CD8+ T cells among the total CD8+ T cell population (Figure 2). Specifically, the SV40 T-ag CD8+ specific cells generated by immunization with serum-free cells represented an average of 5.4% (± 1.6) and 12.9% (± 3.0) of the total CD8+ T cell population for epitopes I and IV respectively. Similarly, percentages in response to immunization with cell cultured in 5% FBS media for epitopes I and IV were 4.21% (± 3.4) and 12.57% (± 2.8). Statistically these values did not differ based on the results of a student's T test ($p > 0.1$). Generally, epitope IV specific T cells represented a greater percentage of the total population as compared to T cells specific for epitope I.

Two potentially new SV40 T cell epitopes were identified with a 175 member SV40 T-ag peptide library and SV40 T-ag tumor cell vaccines cultured without FBS

To evaluate if the potential adjuvant effects of FBS previously masked the clear identification of additional SV40 T-ag epitopes, a SV40 T-ag peptide library screen was

conducted to detect the immune response against B6/K-1,4,5 tumor cells cultured in serum-free media. Specifically, the SV40 T-ag peptide library was comprised of 175 15mer peptides which overlap with adjacent members by 11 amino acids residues to cover the entire 708 T-ag amino acid sequence. To this end, H-2^b mice were immunized with serum-free growth adapted B6/K-1,4,5 and splenocytes harvested 10 day later. The primary immune response of IFN-γ-secreting cells to the library peptides was then detected via ELISPOT analysis (Figure 3). Minimal background responses were observed. Wells containing the SV40 T-ag peptide 15 mers corresponding to the previously identified SV40 T-ag epitopes 295, 381, 529, and 581 produced observable immune responses. Importantly, two responding regions corresponding to library peptides beginning at SV40 T-ag amino acids 361 (361-379) and 493(493-515) were identified. Surprisingly, the region beginning at SV40 T-ag amino acid 361 produced a larger response than the known CD4+ T cell epitope regions. In addition to these regions identified, other potentially important responses were observed with peptides correlating to SV40 T-ag amino acid sequences beginning at 269 (269-287) and 309 (309-323). However, these responses were weaker or produced in response to short (1, 15 mer peptide) stretches of amino acids than the other unidentified responding regions.

ELISPOT analysis failed to confirm if SV40 T ag region 361-375 contains an SV40 T ag epitope

To test the immunogenicity of the 361 responding region, the SV40 T ag transformed cell lines B6/ T122B1 and B6/ K-1,4,5 were growth adapted to HL-1 serum-free medium or Dulbecco's Minimal Eagle Medium supplemented to 5% FBS. Adapted cells were then used for immunization of H-2^b mice and ten days later, the splenocytes were harvested and analyzed for responsiveness to a newly synthesized 361 peptide (corresponding to SV40 T ag amino acids 361-375) or known SV40 T-ag CD4+ (529) or CD8+ (295) T cell epitopes by ELISPOT

analysis to detect IFN-γ-expressing cells (Figure 4A) or IL-10 expressing cells (Figure 4B). Values for an irrelevant control peptide (HBV-core) were subtracted from the enumerated spot values presented. As shown in Figure 4A, no significant proinflammatory (IFN-γ) immune response was generated against the 361 peptide by immunization with any of the cell lines. Similarly, peptide 361 did not induce detectable numbers of anti-inflammatory (IL-10) secreting T cells following immunization with any of three cell lines (Figure 4B). Significantly, the B6/T122B1 cells did generate a strong cellular immune response when cultured in the absence of FBS confirming the immunogenicity of these cells independent of culturing conditions. Similar to previous experiments, there was no statistically significant difference between the pro-inflammatory or anti-inflammatory immune responses induced by B6/K-1,4,5 cells grown in either HL-1 serum-free medium or 5% FBS containing medium.

DISCUSSION

In the absence of FBS, B6/K-1,4,5 and B6/K-0 SV40 transformed cells generate robust primary CD4+ and CD8+ T cell responses

In order to evaluate the potential adjuvant effects of FBS, the CD4+ and CD8+ primary immune response of H-2^b mice when immunized with SV40 T-ag transformed tumor cells cultured in either serum-free of 5% FBS containing media was evaluated. Importantly, SV40 T-ag tumor cells grown in the absence of FBS generated robust primary CD4+ and CD8+ T cell responses. These results suggest that SV40 T-ag tumor cells have CD4+ and CD8+ T cell specific immunogenicity independent of the potential adjuvant effects of FBS. Although not statistically significant, general trends suggested that FBS may actually repress primary CD4+ T cell responses against B6/K-1,4,5 tumor cells lacking most (except epitope 295) CD8+ T cell epitopes (Figure 1). Further analysis with larger experimental populations would be required to further investigate these observations.

It is also relevant to note the observed decrease in the immune response to epitope 295 when comparing the ELISPOT results for experiments that immunized with B6/K-1,4,5 and B6/K-0 cell lines. The B6/K-0 cell line contains all of the CD8+ epitopes while the B6/K-1,4,5 cell line contains only the CD8+ epitope 295. Accordingly, due to the aforementioned immunorecessive nature of epitope 295, this result can be expected as the immune response was greatly decreased in the presence (B6/K-0) of the immunodomiant CD8+ epitopes as compared to the response observed in their absence (B6/K-1,4,5).

Two potentially new SV40 T cell epitopes were identified with a 175 member SV40 T-ag peptide library and SV40 T-ag tumor cell vaccines cultured without FBS

The SV40 T-ag peptide library screen had been previously used to identify the SV40 T-ag CD4+ T cell epitopes 381, 529, and 581 and helped to identify the immunorecessive CD8+ T cell epitope 295. However, to determine if additional epitopes may exist within SV40 T-ag that had previously been masked or difficult to identify because of the unknown adjuvant effects of FBS, this study utilized the peptide library screen with B6/K-1,4,5 cells grown in the absence of FBS. A previously unidentified responding region was observed beginning at the SV40 T-ag amino acid sequence 361. Responding wells correlated to the entire responding region covering the amino acid residues 361-379. Importantly this stretch of amino acids (LTNR**F**ND**LL**DRMDIM) contains potentially key amino acids that could act as anchor residues for MHC molecules. Specifically, it contains the amino acid phenylalanine three amino acids away from the hydrophobic amino acid leucine as is observed in other antigens with H-2K^b anchor residues (Mylin, personal contact). However, none of the canonical I/A^b MHC anchor residues were observed. The unidentified responding region beginning at amino acid sequence 493 (493-515) had been previously considered as a responding region by Lauver *et al* in SV40 T-ag library screens identifying IFN- γ or IL-10 secreting cells. Importantly, this stretch of amino acids partially overlaps with the CD8+ T cell epitope inactivated in B6/K-1,4,5 tumor cells lines. Additional analysis will be required to identify which amino acids may act as key anchor sequences in this responding region.

ELISPOT analysis failed to confirm if SV40 T ag region 361-375 contains an SV40 T ag epitope

Additional ELISPOT analysis to test the immunogenicity of the 361 responding region failed to confirm if an SV40 T ag epitope is contained within the SV40 T ag 361-375 amino acid region. The response observed from the 361-375 SV40 T ag amino acid region using the SV40 T ag peptide library may be the result of contamination of the 361-corresponding well from nearby wells containing CD8+ epitope 295. MALDI-TOF mass spectrometry analysis will be used to determine whether derivatives of library peptide 295 may have contaminated well(s) supposed to be peptides corresponding to the 361 region of the SV40 T ag. If however, no contamination is found, it may also be the case that the C-terminus of synthetic peptide 361 contained enough extra amino acids as to interfere with MHC binding and therefore efficacy as an epitope. Creating an additional synthetic peptide with a shorter C-terminus, but containing the potential H-2K^{b} anchor residues may act to confirm if this region does, in fact, contain a weak SV40 T ag epitope. An additional scope of this experiment was to test the immunogenicity of the newly adapted cell line B6/T122B1. Importantly, these cells were able to generate a strong cellular immune response when compared to other FBS grown SV40 T ag cell lines (Figure 1, 4). In addition to this finding, it was also identified that there was no statistically significant difference between the pro-inflammatory or anti-inflammatory immune responses induced by B6/K-1,4,5 cells grown in either HL-1 serum-free medium or 5% FBS containing medium. This confirms the previous results of those experiments corresponding to Figure 1, 2 in which a robust CD4+ and CD8+ immune response is observed independent of culturing conditions.

CONCLUSION

In the absence of FBS, immunization of tumor cells bearing a wild type SV40 (B6/K-0) or mutated CD8+ deficient variants (B6/K-1,4,5, B6/T122B1) generated a robust SV40 T-ag CD4+ T cell response. These results suggest that these SV40 T-ag tumor cells have CD4+ T cell specific immunogenicity independent of the potential adjuvant effects of FBS. These effects were also evaluated for the CD8+ T cell response and a comparable immunogenicity was observed between cells cultured in the absence or presence of FBS. This study also aimed to search for new SV40 T-ag epitopes using a peptide library screen and SV40 T-ag tumor cells grown without the confounding adjuvant effects of FBS. Two potentially new SV40 T-ag epitopes beginning at amino acid residues 361 and 493 were detected. However, additional ELISPOT analysis using a synthetic peptide corresponding to SV40 T ag amino acids 361-375 failed to confirm that this region represents a bona fide SV40 T ag epitope. Follow up analyses failed to detect significant numbers of proinflammatory or anti-inflammatory 361-375-specific T cells. To test for potential contamination of the SV40 T ag peptide library, MALDI-TOF mass spectrometry analysis will be used specifically to identify whether derivatives of library peptide 295 contaminated those well intended to contain peptides corresponding to the 361 region of the SV40 T ag.

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FIGURE LEGENDS

Fig. 1. In the absence of FBS, B6/K-1,4,5 and B6/K-0 SV40 transformed cells generate robust primary CD4+ T cell responses. (A) C57BL/6 mice were immunized with B6-K-1,4,5 cells expressing a mutant T-ag missing CD8+ T cell epitopes (except 295) or (B) B6-K-0 cells containing a wild-type of SV40 T-ag cultured in either 5% FBS or 0% FBS. The spleens of immune or naïve mice were harvested 10 days later and epitope specific (381, 529, 581), proinflammatory (IFN-γ) immune responses detected by ELISPOT. Values for an irrelevant control peptide (HBV-core) were subtracted from the spot counts presented.

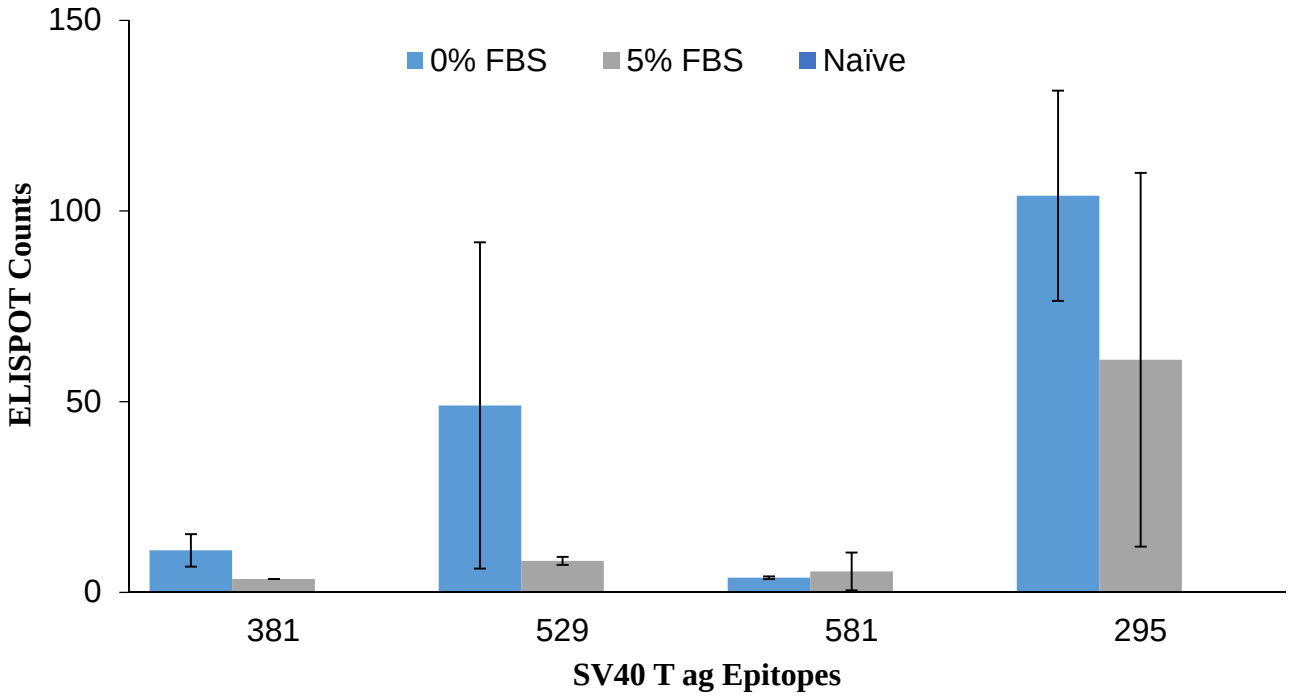
Fig. 2. In the absence of FBS, B6/K-0 SV40 transformed cells induce a comparable percentage of SV40 T-ag epitope specific CD8+ T cells. C57BL/6 mice were immunized with B6-K-0 cells cultured in either 5% FBS or 0% FBS. The spleens of immune or naïve mice were harvested 10 days later and splenotcytes stained with either Tet-I-PE or Tet-IV-PE and αCD8-APC and visualized using the BD LSR Fortessa flow cytometer at the Penn State Hershey Core Research Facility

Fig. 3. Two potentially new SV40 T cell epitopes were identified with a 175 member SV40 T-ag peptide library. C57BL/6 mice were immunized with B6-K-1,4,5 cells cultured in 0% FBS media. Harvested splenocytes were used in a SV40 T-ag peptide library comprised of 175, 15 mer peptides which overlap adjacent members by 11 residues to cover the entire 708 T ag amino acid sequence**.** Proinflammatory T-ag specific responses (IFN-γ) were detected by ELISPOT and two potentially new epitopes were identified beginning at SV40 T-ag amino acids 361 and 493 denoted (*)

Fig. 4. ***ELISPOT analysis failed to confirm if SV40 T ag region 361-375 contain an SV40 T ag epitope*** C57BL/6 mice were immunized with B6-K-1,4,5 cells which express a mutant T ag missing CD8+ T cell epitopes (except 295) or 122B1 cells which express a mutant T ag derivative in which epitopes I, II/III, IV, and V had been inactivated by alanine substitutions of MHC I anchor residues and were cultured in media formulated with either 5% or 0% FBS. The spleens of immune or naïve mice were harvested 10 days later and epitope specific (361, 529, 295), (A) proinflammatory (IFN-γ) or (B) anti-inflammatory (IL-10) immune responses detected by ELISPOT. Spot counts from wells supplemented with an irrelevant control peptide (HBV-core) were subtracted from the spot counts presented.

Figure 1

A


150
100
50
0
0% FBS
5% FBS
Naïve
ELISPOT Counts
381
529
581
295
SV40 T ag Epitopes

B

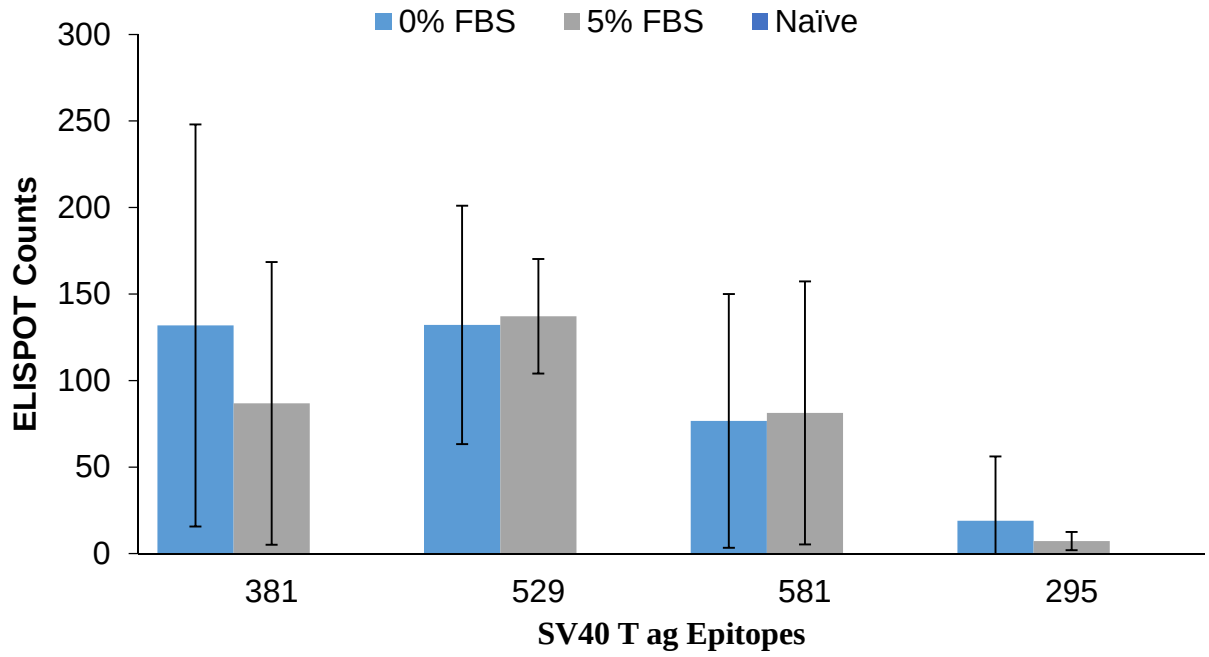

300
250
200
150
100
50
0
0% FBS
5% FBS
Naïve
ELISPOT Counts
381
529
581
295
SV40 T ag Epitopes

Figure 2

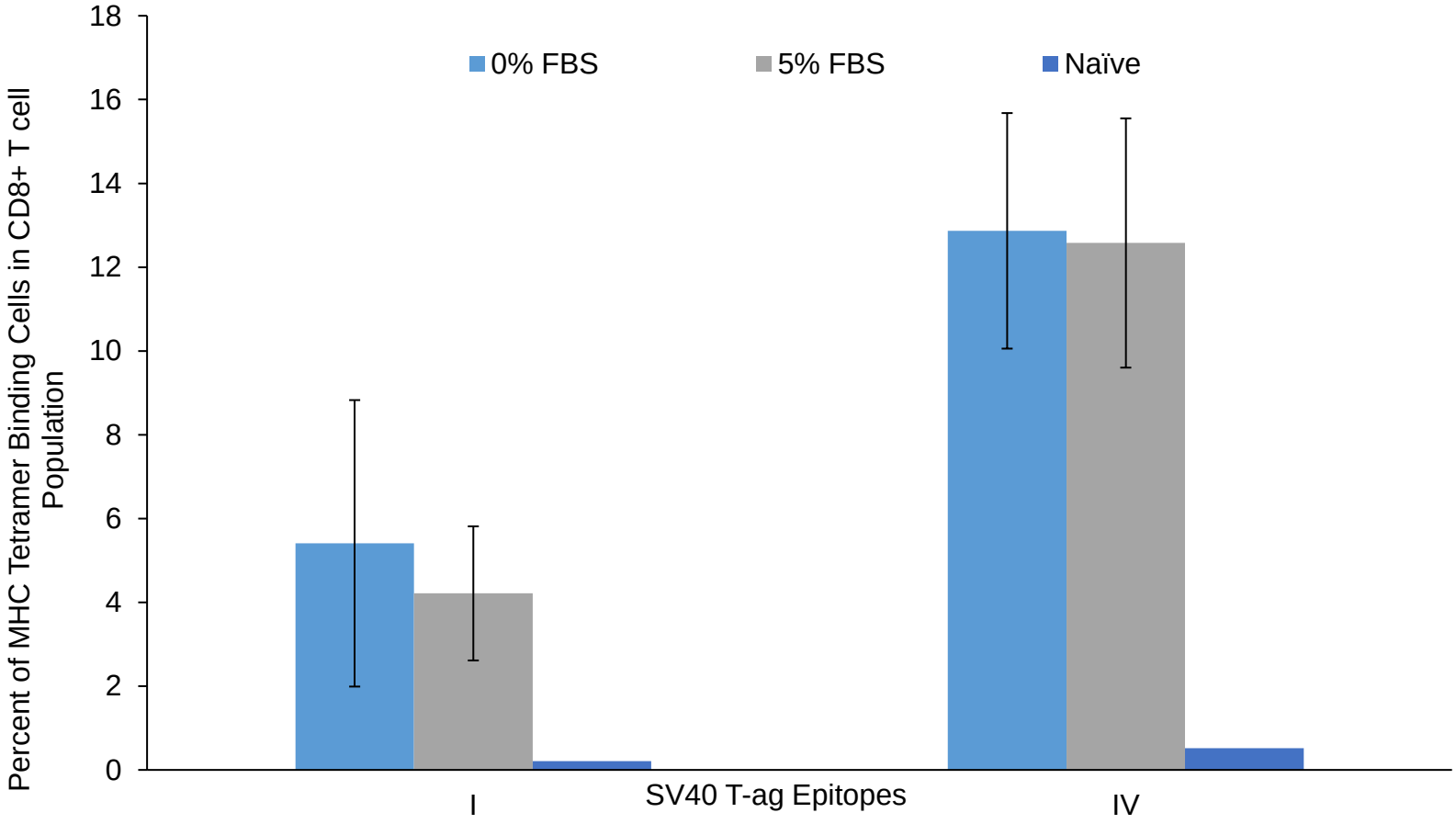

18
16
14
12
10
8
6
4
2
0
0% FBS
5% FBS
Naïve
Percent of MHC Tetramer Binding Cells in CD8+ T cell Population
I
SV40 T-ag Epitopes
IV

Figure 3

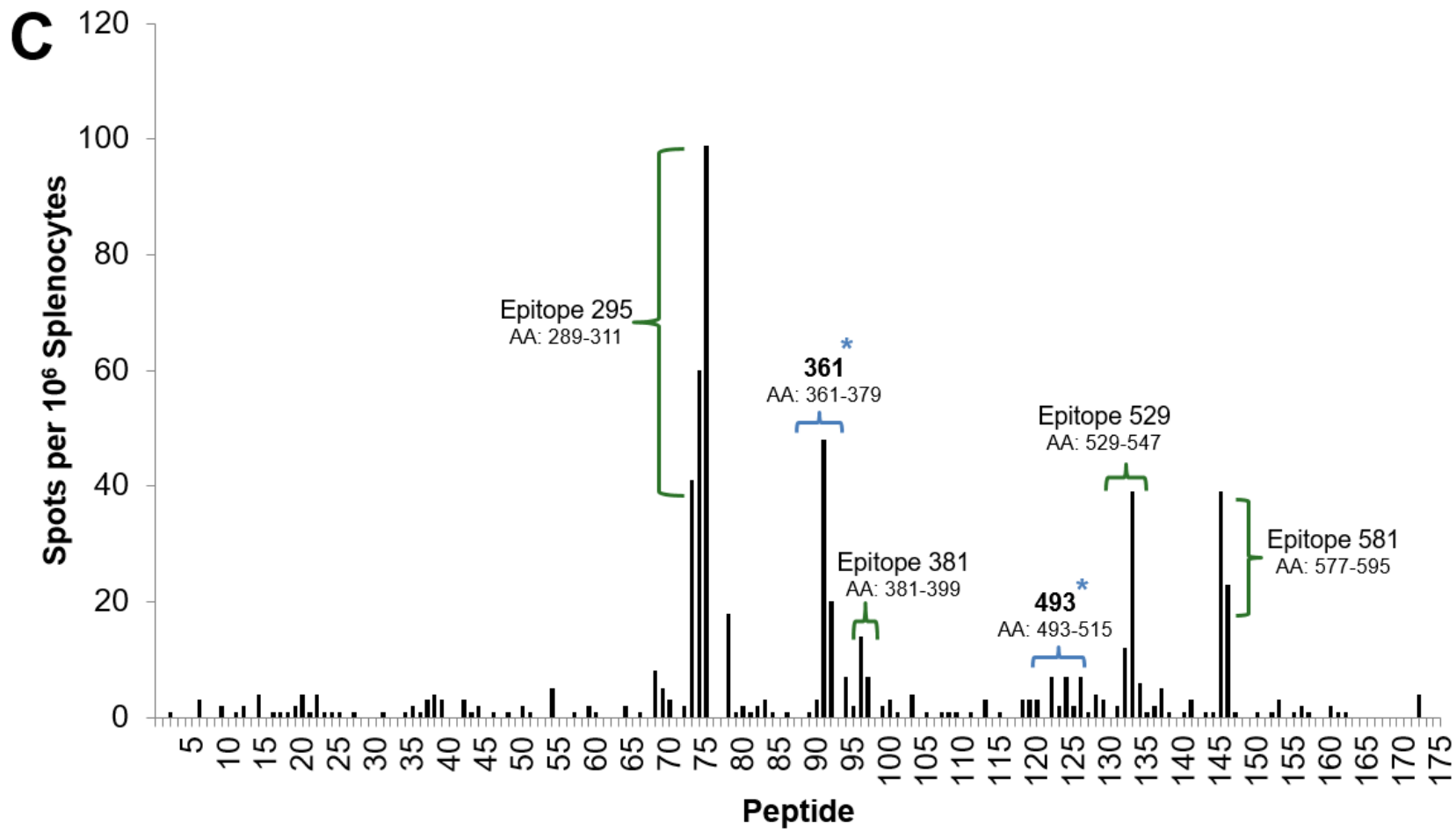
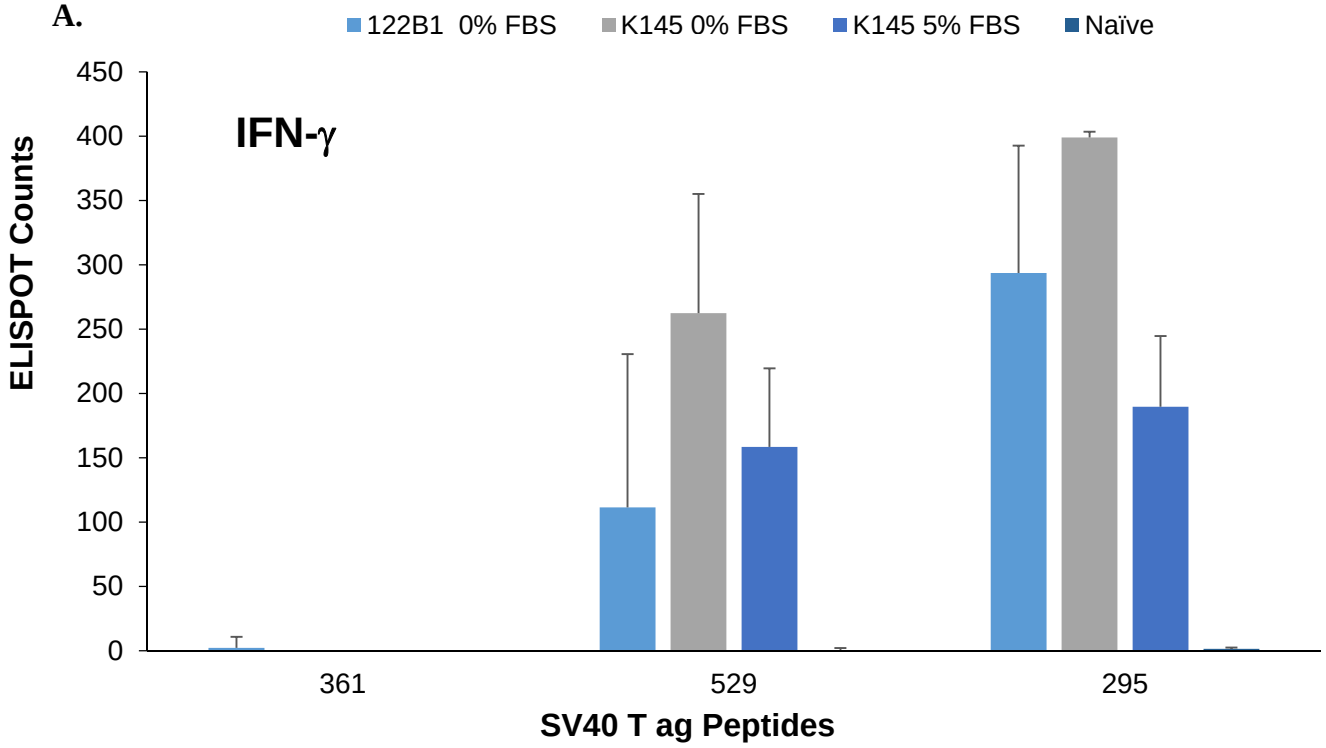
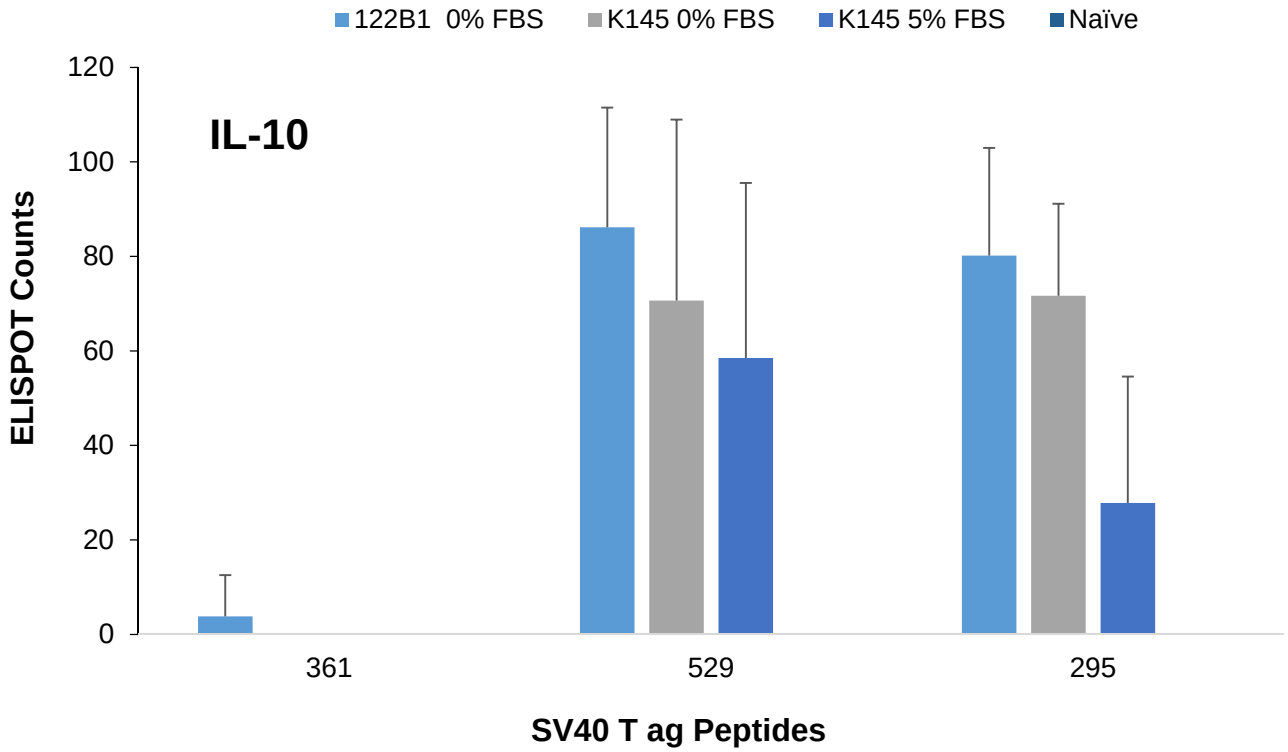

C
Spots per 10⁶ Splenocytes
120
100
80
60
40
20
0
Epitope 295
AA: 289-311
361 *
AA: 361-379
Epitope 381
AA: 381-399
493 *
AA: 493-515
Epitope 529
AA: 529-547
Epitope 581
AA: 577-595
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140 145 150 155 160 165 170 175
Peptide

Figure 4

A.


122B1 0% FBS
K145 0% FBS
K145 5% FBS
Naïve
IFN-γ
ELISPOT Counts
450
400
350
300
250
200
150
100
50
0
361
529
295
SV40 T ag Peptides

B.


122B1 0% FBS
K145 0% FBS
K145 5% FBS
Naïve
IL-10
ELISPOT Counts
120
100
80
60
40
20
0
361
529
295
SV40 T ag Peptides