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FAST TRACK

Human T Cell Responses to Endogenously Presented HLA-A*0201 Restricted Peptides of Simian Virus 40 Large T Antigen

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Abstract Presence of the simian virus 40 (SV40) has recently been demonstrated in a relatively high percentage of human mesotheliomas and it is associated with the development of these malignancies in pleural cavities. Therefore, we have initiated a study to identify candidate peptides presented by the human HLA-A*0201 molecule for vaccination approaches against SV40 and monitoring of SV40 directed human immune responses. Initial screening of SV40 large T (Tag) domains required for transformation of cells for HLA-A*0201 binding motifs revealed ten possible binding peptides. Screening of these candidate peptides showed that seven of the ten peptides could bind and stabilize HLA-A*0201 molecules. In an in vitro immunization assay the two peptides with the highest binding affinity for HLA-A*0201, Tag aa 396–405 and aa 577–585, were tested for their ability to induce peptide specific cytotoxic T cells in two healthy donors. One donor developed cytotoxic T cells against Tag aa 396–405 and in T cell cultures of both donors Tag aa 577–585 specific T cells were initiated. The T cells against Tag aa 577–585 not only recognized and killed peptide pulsed cells, but, most importantly, SV40 transformed human mesothelial cells. This is the first demonstration of the induction of SV40 specific human cytotoxic T lymphocytes that recognize endogenously processed peptides from SV40. This peptide identification study opens the possibility to investigate immune responses against SV40 in mesothelioma patients and in individuals exposed to SV40. J. Cell. Biochem. 82: 155–162, 2001.

Key words: SV40; CTL; peptide; HLA-A*0201; mesothelioma; immunotherapy

Simian Virus 40 (SV40) is a member of the Papovaviridae family of DNA tumor viruses that also include human papillomavirus (HPV) and JC and BK viruses. After the initial observation that SV40 could induce mesotheliomas in hamsters [Cicala et al., 1993] in combination with the possible injection of infants between 1955 and 1963 with SV40-contaminated polio vaccines [Carbone et al., 1997a], efforts on the identification of SV40 in human tumors were initiated. Recent studies have demonstrated the presence of SV40 in a variety of tumors including ependymomas, osteosarcomas, choroid plexus tumors, and mesotheliomas [Carbone, 1994; Carbone et al., 1999]. Especially, in mesothelioma upto 80% have been shown to express SV40 sequences including the large T antigen (Tag) [Testa et al., 1998]. Initially, exposure to asbestos was thought to be the only risk factor for the development of mesothelioma, however, <10% of asbestos workers develop mesothelioma [Roggli, 1992]. This suggests that other factors are required for the development of mesothelioma and due to its frequent expression in these tumors, SV40 is a likely candidate.

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Antibody responses against SV40 have been identified in people with possible exposure to SV40 [Butel and Lednicky, 1999]. However, the presence of the virus in tumor specimens indicates that the immune responses induced against the virus during infection are not adequate to eliminate the virus in those patients. Enhancement of immune responses against SV40 by vaccination could induce virus eradication and reduce tumor incidence. A recent study has revealed antibody responses against SV40 in about 5% of individuals born after 1963 [Butel and Lednicky, 1999]. This suggests that SV40 can be present in humans independent of the direct administration of contaminated polio vaccines.

Based on our experience with other DNA tumor viruses including human papillomavirus 16 [Ressing et al., 1995], we have initiated a study to identify the major immunogenic peptides of SV40 for the most widely expressed human MHC allele; i.e., HLA-A*0201 [Imanishi et al., 1992]. These peptides will provide tools to monitor immune responses against SV40 in patients. The same peptides could be used to activate cytotoxic T lymphocytes (CTLs) capable of recognizing and eliminating SV40 infected cells. The size of SV40 Tag restricted the epitope identification. Therefore, the analysis was focused on the regions of SV40 Tag that are required for transformation and thus less likely to be mutated or lost. The screened domains included the p53 and pRb binding sites.

MATERIALS AND METHODS

Cell Lines

Cell lines were cultured in Iscove's modified Dulbecco's medium (Bio Whittaker, MD), supplemented with 10% FCS (Hyclone, UT), 100 μg/ml penicillin (Marsam, NJ), 2 mM glutamine (BioWhittaker, MD), and 5×10^5 M 2-mercaptoethanol (EM Science, NJ) at 37°C in humidified air containing 5% CO_2. MEJT10, a human mesothelial cell line transfected with SV40 early region DNA and the EJras gene [Reddel et al., 1989], was incubated with 100 IU/ml recombinant human IFN-γ (Pharmingen, CA) for 72 h before being used as targets in cytotoxicity assays to increase MHC expression. JY is an HLA-A*0201 EBV-transformed B cell line. T2 (ATCC, CRL-1992) is an HLA-A*0201 antigen-processing defective cell line. The K562 (ATCC, CCL-243) chronic myelogenous leukemia cell line is used to block NK-like activity.

Peripheral Blood Lymphocytes

Peripheral blood lymphocytes (PBL) of healthy HLA-A*0201 positive individuals purified from apheresis blood donations by centrifugation on a Ficoll gradient were routinely stored in liquid nitrogen in 40% FCS + 10% DMSO (Merck, Darmstadt, Germany).

Synthetic Peptides

Peptides were synthesized and purified on HPLC at the macromolecular analysis facility of Loyola University Chicago and The University of Chicago. Peptides were dissolved in 100% DMSO at a concentrations of l00 mg/ml. The peptide solutions were stored at −80°C and diluted upon use in PBS.

Peptide Binding to HLA-A*0201 (T2 Assay)

This assay was performed as described previously [Nijman et al., 1993]. Briefly, T2 cells (2×10^6 cells/ml) were incubated with human β_2-microglobulin (β_2-m: Biodesign, ME) at a concentration of 10 μg/ml. The wells of a 96-well U bottom plate were filled with 80 μl T2 cells and 20 μl peptide at a concentration of 500 μg/ml or with 20 μl PBS and subsequently incubated for 16 h at 37°C in humidified air containing 5% CO_2. The cells were stained for HLA-A*0201 expression with the BB7.2 hybridoma (ATCC, HB-82) culture supernatant for 30 min at 4°C. FITC labeled goat anti-mouse IgG in a dilution of 1:100 was added and incubated for another 30 min at 4°C in the dark. Samples were analyzed by FACS. The fluorescence index (FI) was calculated by the formula: (mean fluorescence experimental sample − mean fluorescence background)/mean fluorescence background.

In Vitro Immunization and Human T Cell Cultures

The in vitro induction of CTL responses was performed as described [Celis et al., 1994]. CD8+ cells from HLA-A*0201 + donors isolated using CD8 Dynal beads (Dynal, Oslo, Norway) were stimulated with dendritic cells (DC) as autologous antigen presenting cells (APC) loaded with peptides. DCs were generated as described by Romani et al. [1996]. Briefly, dendritic cells were loaded with 40 μg/ml peptide in PBS + 1% BSA in the presence of 3 μg/ml β2-m for 4 h at room temperature. The

dendritic cells were irradiated (50 Gy), washed, and distributed into 48-well plates in 100 μl medium at 2.5×10^4 cells/well. Dendritic cells were co-cultured with 5×10^5 CD8 cells in 100 μl containing l0 ng/ml rhIL-7 (Peprotech, NJ). After 24 h of incubation at 37°C rhIL-10 (Peprotech, NJ) was added at a final concentration of 10 ng/ml. At Days 7 and 14 the individual cultures were restimulated with peptide pulsed irradiated adherent PBLs. Human rhIL-10 was added to the cultures on Days 8 and 15 at a final concentration of 10 ng/ml. At Days 9, 11, 16, and 18 human IL-2 was added to the cultures at a final concentration of 50 Cetus U/ml. On Day 21 a cytotoxicity assay was performed for individual wells and 1/3 of the cells from each well was re-stimulated as described.

Cytotoxicity Assay

The presence of specific CTLs was measured in a standard 4 h-chromium release assay as previously described [Rudolf et al., 1999]. Briefly, 1×10^6 target cells were labeled with 50 μCi of ^{51}Cr (Du Pont, MA) for 1 h at 37°C. Effector cells were added to 2,000 targets in 96-well U bottom plates at different effector/target (E/T) ratios. To the labeled target cells, K562 cells were added in an excess of 50 times to block NK activity. Spontaneous and maximal lysis of the target cells were obtained by adding medium or 2% Triton X-100 (Sigma, MO) respectively to the target cells. After 6 h at 37°C, 50 μl of supernatant was collected and added to 125 μl Microscint 40 scintillation fluid (Packard, Meriden, CT). ^{51}Cr release was measured in a scintillation counter. The specific lysis was calculated as follows: $100 \times$ [(cpm experimental release-cpm spontaneous release)/(cpm maximal release-cpm spontaneous release)].

RESULTS

Selection of Peptides With Probability to Bind to the HLA-A*0201 Molecule

To select SV40 derived peptides with probability to bind to the HLA-A*0201 molecules, the sequence of SV40 Tag regions of interest were analyzed in a peptide binding prediction computer program based on HLA-A*0201 peptide binding motifs identified by Parker et al. [1994]. This program screens a protein or part of a protein for the presence of peptides with specific MHC binding motifs. The program is accessible via Internet at http://bimas.dcrt.-nih.gov/cgi-bin/molbio/ken_parker_comboform. The search was limited to the four regions of SV40 Tag that are considered indispensable for SV40 transformation and therefore, unlikely to be mutated in SV40 induced tumors [Zhu et al., 1992; Sullivan et al., 2000]. These four regions include; the 82 N-terminal amino acids (aa) J domain the pRb binding domain (aa 91–120), the p53 binding domains aa 251–470, and aa 521–640. The results from this computer analysis are shown in Table I, where the peptides that could potentially bind to HLA-A*0201 molecules are arranged according to

TABLE I. Prediction of Peptides Binding to HLA-A*0201 and Prediction of Peptide Generation by Proteasomal Cleavage

Position	Sequence	Score[1]	Proteasome cleavage predictions[2]
285–293	VLLLLGMYL	739	**VLLLLGMYL**
396–404	CLLPKMDSV	290	LH**CLLPKMDSV**
404–413	VVYDFLKCMV	136	S**VVYDFLKCMV**
576–585	LLMLIWYRPV	131	IALLLMLIWYR **YRP**VAEFA
610–619	SVYQKMKFNV	113	**SVYQKMKFNV**AMG
57–66	TLYKKMEDGV	109	NT**LYKKME**D **DGV**KYAHQPD
359–368	QMLTNRFNDL	108	TRE**QMLTNRFND** **LTNRFNDL**
602–611	RLDKEFSLSV	106	EWKE**RLD** **KEFSLSV**Y
396–405	CLLPKMDSVV	105	LHCL**LPKMDSV**V
577–585	LMLIWYRPV	95	IALL**LMLIWYR** **YRPV**AEFA

[1]Score according to Parker represents the estimate of disassociation half-time of a molecule containing this sequence.
[2]Prediction of the proteasomal cleavage according to the Holzhütter algorithm. The HLA-A*0201 peptide sequence is printed in bold.

their predictive HLA-A*0201 binding score. Except for peptide aa 57–66 from the J domain, all potentially binding peptides were identified in the p53 binding domains of SV40.

Binding of HLA-A*0201 by Selected Peptides

The peptides predicted by the Parker analysis to bind HLA-A*0201 were synthesized and tested for stabilization of the HLA-A*0201 on TAP deficient T2 cells. These cells cannot transport peptides into the endoplasmic reticulum to load HLA molecules and only exogenous addition of binding peptides will stabilize the HLA expression on the cell surface. Therefore, the HLA expression levels of these T2 cell after peptide addition directly correlate with peptide binding levels. As shown in Figure 1, most but not all predicted peptides from Table I bound to the HLA-A*0201 on the T2 cells. Peptides were regarded as binding when the fluorescence index >1. The HBV aa 18–27 peptide served as a reference positive control peptide in these assays as binding of this peptide to HLA-A*0201 has been established previously [Bertoletti et al., 1993]. Some peptides showed relatively high binding levels and two of the best binding peptides aa 396–405 and aa 577–585 were selected for testing of their ability to induce human peptide specific cytotoxic T lymphocytes (CTL).

Immunogenicity of SV40 Derived HLA-A*0201 Restricted Peptides

The induction of peptide specific T cells was tested in vitro by co-cultivation of HLA-A*0201 positive PBL from healthy donors with autologous peptide loaded dendritic cells (DC). Individual cultures of two PBL donors were initiated for peptide Tag aa 396–405, Tag 577–585, and HBV aa 18–27 in 48 cultures/peptide. After three weeks the individual cultures were tested in a cytotoxicity assay for recognition of HLA-A*0201 expressing JY cells pulsed with the relevant peptides. Cultures were scored positive when the percentage of specific lysis of peptide loaded JY cells minus percentage lysis of unloaded JY cells was >10. Donor A had 3/48, 33/48, and 13/48 positive cultures for peptides Tag aa 396–405, Tag 577–585, and HBV aa 18–27, respectively. Donor B had 21/48, 33/48, and 41/48 positive cultures for peptides Tag aa 396–405, Tag 577–585, and HBV aa 18–27, respectively (data not shown). To confirm the induction of CTLs from these donors against the SV40 derived peptides, positive individual cultures were pooled, restimulated with peptide pulsed targets and tested in a cytotoxicity assay at different E/T ratios against peptide loaded JY cells. The results presented in Figure 2 indicate that in contrast to donor A, donor B did induce CTLs against SV40 Tag peptide aa 396–405 and that both donors induced a response against SV40 Tag peptide aa 577–585.

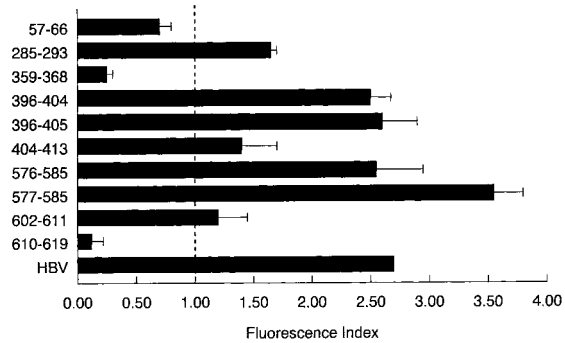

Fig. 1. The fluorescence index (FI) of different peptides for binding to HLA-A*0201 on T2 cells. The mean fluorescence index±SEM of four individual binding assays is displayed on the X-axis. The amino acids numbers of the tested peptides is indicated on the Y-axis. A fluorescence index >1 (indicated by the dotted line), is an indicator for binding. The peptide binding is normalized for HBV peptide binding, which is used as a positive control.

Recognition of SV40 Infected Cells

To achieve eradication of SV40 infected cells by T cells against a SV40 derived peptide, it is necessary that the SV40 infected cells endogenously process the peptide and present it in the HLA molecules on the cell surface. To test this, MEJ-T10 mesothelial cells transformed with SV40 and EJ-ras were tested in vitro for recognition by the SV40 Tag aa 577–585 and aa 396–405 specific T cells from both donors A and B (tested for peptide specificity, see Fig. 2). T cells against Sv40 Tag aa 577–585 specifically killed mesothelial cells expressing SV40 Tag. The T cells against SV40 Tag aa 396–405, however, were unable to kill the mesothelial cells (Fig. 3).

DISCUSSION

The recent association of the oncogenic SV40 virus with several different human tumors [Carbone et al., 1999] has initiated the interest in vaccine development against this virus

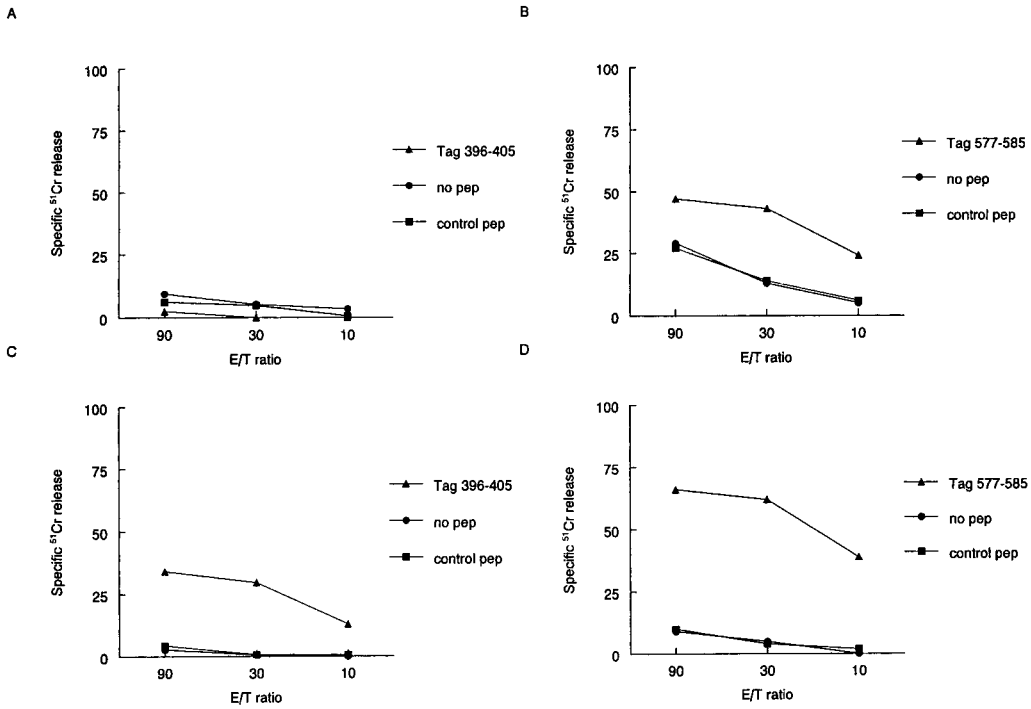

Fig. 2. Cytotoxic activity of human CD8+ T cell lines against peptide loaded JY cells was determined at different E/T ratios as indicated for different donors. Specific ^{51}Cr release by CD8+ T cells from donor A (A + B) or donor B (C + D) initiated by in vitro immunization against DCs loaded with peptide 396–405 (A + C) or peptide 577–585 (B + D) is depicted on the Y axis. The targets for these assays were JY cells loaded with SV40 Tag peptides 396–405 or 577–585 or JY cells without external peptide loading (no pep). Tag 396–405 served as a negative control peptide for T cells induced against Tag peptide 577–585 (B + D) and Tag 577–585 served as a negative control peptide for T cells induced against Tag peptide 396–405 (A + C). This figure is a representative display of three individual assays.

[Macedo et al., 1998]. This report describes the identification of several peptides for the HLA-A*0201 haplotype that could be used in the monitoring and development of such SV40 directed vaccines.

Transformation of cells by SV40 is a well studied phenomenon and is dependent on the expression of the SV40 Tag, reviewed by Fanning and Knippers, [1992] and Testa and Giordano, [2001]. Binding of several tumor suppressor antigens (p53, pRb, p107, and p130) by Tag interferes with the inactivation of the cell cycle to arrest and promotes cell division. Tag is directly mutagenic and favors malignant transformation. Inactivation of p53 through binding to Tag, prevents apoptosis of

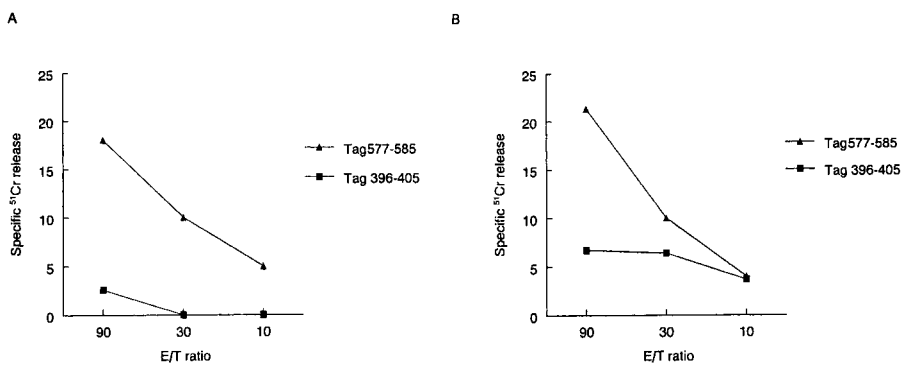

Fig. 3. Specific cytotoxicity of peptide induced human CD8 + T cells from donor A **(A)** and donor B **(B)** against a human mesothelial cell line transformed with SV40 and EJ-ras (MEJT10) at different E/T ratios. Both Tag aa 577–585 and Tag aa 396–405 specific T cells from both donors were tested twice in the same cytotoxicity assay against MEJT10. The specificity of the T cells was confirmed against peptide loaded JY cells (Fig. 2).

damaged cells and may also increase the sensitivity of cells to other carcinogens such as asbestos [Bocchetta et al., 2000]. This study focused on the highly conserved Tag protein domains that are required for SV40 mediated transformation and included the pRb and p53 binding domains. The focus on these regions comprised the risk of missing immunogenic peptides in other regions that could be expressed in SV40 transformed cells. However, identification of peptides in other protein domains of SV40 may result in peptide candidates that are mutated or not expressed in transformed cells. Our analysis has yielded ten potential peptide candidates for the most abundantly expressed HLA haplotype HLA-A*0201. Nine of the ten candidate peptides were derived from the Tag regions involved in p53 binding and no peptide candidates were identified in the pRb binding domain. This finding is most likely a result of the size of the respective binding domains and the selection of the HLA haplotype and does not reflect the contributions of these domains in the transformation of human cells. Seven of ten peptides predicted by the computer analysis to bind to HLA-A*0201 molecules, were determined as binding peptides in a T2 binding assay. The two best binding peptides induced SV40 Tag specific human CTL responses indicating that the human T cell repertoire harbors T cell receptors against SV40 derived peptides. Furthermore, we found that the Tag peptide aa 577–585 is endogenously processed by SV40 transformed cells and that these cells can be lysed by CTL specific for this peptide. Based on the Parker prediction this peptide was not the most likely candidate for a CTL epitope since nine other possible binding peptides had a higher Parker score. However, the prediction program is limited and may not always predict potential binding peptides in the right order. Therefore, final selection of peptides for in vitro immunization assays was based on the actual peptide binding to HLA-A*0201 molecules in a T2 assay.

Peptides are generated from proteins that are cleaved by the proteasome after transportation into the endoplasmic reticulum (reviewed in Rock and Goldberg, 1999). After cleavage by the proteasome the amino terminus of a peptide can still be trimmed by amino peptidases in the endoplasmic reticulum, however, the C-terminus of the peptide requires the correct cleavage to fit the HLA allele (reviewed in Yewdell and Bennink, 2001). Holzhütter et al. [1999,2000] have developed a proteasomal cleavage prediction algorithm. Computer analysis of the potential SV40 HLA-A*0201 peptides (Table I) revealed that the peptide that was identified as a naturally processed epitope (Tag 577–585) was not predicted to be generated by the proteasome. Treatment of cells with interferon-γ reverts the constitutive proteasome into an immunoproteasome [Rock and Goldberg, 1999]. Protein cleavage by constitutive proteasomes is different from immunoproteasomes as not all predicted cleavage sites would be used by both forms. This can result in presentation of different peptides from the same protein and affect the immune recognition [Morel et al., 2000]. The computer algorithm is based on cleavage patterns of the immunoproteasome. In this particular case, the results seem to prove the prediction wrong. Thus our results may contribute to refine the proteasomal cleavage prediction program.

Vaccination studies in mice have indicated that an immune response induced against SV40 Tag can effectively prevent formation of an SV40 expressing tumor [Bright et al., 1998; Mylin et al., 2000]. This supports the development of vaccination strategies against SV40 in humans [Xie et al., 1999]. However, there is a controversy concerning the requirement for SV40 expression in the maintenance of the oncogenic phenotype in SV40 related tumors. Most of the tumor cell lines isolated from mesothelioma patients rapidly become PCR-negative for SV40 during in vitro culture [Pilatte et al., 2000] and this is also our own experience (data not shown). In agreement with this observation is the heterogeneous expression of SV40 in mesothelioma detected by immunohistochemistry [Carbone et al., 1997b] and loss of SV40 after tumor induction in other tumors [Tzeng et al., 1998; Salewski et al., 1999]. These data indicate that there are individual cells within SV40 positive mesotheliomas that do not require SV40 for the maintenance of the tumorigenic phenotype. At first glance, this seemed in contrast with the observation that anti-sense SV40 Tag induced some apoptosis and partly decreased proliferation of mesothelioma cells in vitro [Waheed et al., 1999]. However, the latter study did not discriminate between tumor cells that were positive vs. negative for SV40 Tag, and cells that had previously lost SV40 were obviously not

affected. Considering these data, treatment of tumors with SV40 directed vaccines might not be useful, as SV40 negative tumor cells will evade the treatment and promote the outgrowth of SV40 negative tumors. Instead vaccinations against SV40 should be directed towards people who have been exposed to SV40, but have not yet developed SV40 related malignancies. These include all individuals that have received their polio vaccine between 1955–1963 and have been exposed to other carcinogens such as asbestos.

In conclusion, this study provide knowledge of HLA-A*0201 binding SV40 Tag peptides recognized by the immune system, required for the evaluation of T cell responses after SV40 directed vaccinations. At least two of these are immunogenic in humans and at least one is endogenously processed by SV40 transformed cells.

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