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MHC I restricted epitopes conserved among variola and other related orthopoxviruses are recognised by T cells 30 years after vaccination

S. T. Tang^{1,*}, M. Wang^{2,*}, K. Lamberth², M. Harndahl², M. H. Dziegiel³, M. H. Claesson², S. Buus², and O. Lund¹

¹ Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark, Building 208, 2800 Lyngby, Denmark

² Laboratory of Experimental Immunology, Department of International Health, Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen, Blegdamsvej 3, 2200 Copenhagen N, Denmark

³ Blood Bank KI 2034, Copenhagen University Hospital, Blegdamsvej 9, 2100 Copenhagen Ø, Denmark

Abstract

It is many years since the general population has been vaccinated against smallpox virus. Here we report that human leukocyte antigen (HLA) class I restricted T cell epitopes can be recognised more than 30 years after vaccination. Using bioinformatic methods we predicted 177 potential cytotoxic T lymphocyte (CTL) epitopes. Eight epitopes were confirmed to stimulate IFN- γ release by T cells in smallpox vaccinated subjects. The epitopes were restricted by five supertypes (HLA-A1, -A2, -A24 -A26 and -B44). Significant T cell responses were detected against 8 of 45 peptides with an HLA class I affinity of K_D less than or equal to 5 nM, whereas no T cell responses were detected against 60 peptides with an HLA affinity of K_D more than 5nM. All epitopes were fully conserved in seven variola, vaccinia and cowpox strains. Knowledge of the long term response to smallpox vaccination may lead to a better understanding of poxvirus immunity and may aid in the development of new improved vaccines and diagnostic tools.

Keywords

Peptide; CD8+ T cell epitopes; Variola; Vaccinia; Orthopoxviruses; HLA

Introduction

Despite the great success of smallpox vaccination, surprisingly, little knowledge exists of how vaccination induces protection. It has been shown that vaccination with VACV antigens induces antibody responses that contribute to protective immunity against VARV and generate both memory T and B cells that remain > 50 years after vaccination [13,20] despite the absence of smallpox. Neutralizing antibodies appear to be important for protection in mouse model [14] and the neutralizing antibody titres are correlated with anti-vaccinia antibodies [13,36]. In recent years it has become increasingly clear that cellular immunity can also play an

Corresponding author. Tel.: +45 4525 2425; fax: +45 45931585, Email address: lund@cbs.dtu.dk (O. Lund).

*These two authors contributed equally to this work

important role in protection against smallpox. Protection against orthopoxvirus challenge can be obtained after immunisation with single CD8+ T cell epitopes [41]. Most of the CTL responses described are observed shortly after vaccination, but long-term central memory responses associated with CD8+ T cells as well as CD4+ T cells have also been reported [10]. CD8+ T lymphocytes have been shown to be involved in controlling poxvirus infection, and this has prompted a search for potential CD8+ T cell epitopes [16,27,28,31,33,35,41, 45-47]. In recent years over 150 T cell epitopes from human and mouse have been identified (reviewed in Kennedy and Poland 2007)[22]. Orthopoxviruses encode multiple classes of immunomodulatory proteins that interfere with various aspects of the host innate and adaptive immune system [1,5,8,9,11,12,15,21,23,30,34,38,44]. The objective of this study was to find novel CTL epitopes from vaccinia virus strain Copenhagen, which are conserved in six other closely related orthopoxvirus strains, including the highly pathogenic variola virus India-1967 strain.

Materials and Methods

Bioinformatics selection strategy

Orthopoxvirus genomes—All the complete genomes used in this study were retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/GenBank>) and consist of a total of seven orthopoxvirus complete genomes:

1. Variola major virus strain India-1967 (VARV-IND) (Reference Sequence: NC_001611, GenBank accession no: **X69198**), was selected as the reference strain since the first strain is most described and the most cited variola strain [40]
2. Variola major strain Bangladesh-1975 (VARV-BSH, Locus: VARCG, GenBank accession no: **L22579**),
3. Vaccinia virus strain Acambis 3000 Modified virus Ankara (MVA) (VACV-MVA, GenBank accession no: **AY603355**) is well-described and approved in vaccine trial and for vaccine production [4,6,17].
4. Vaccinia virus strain Western Reserve (VACV-WR, GenBank accession no: **AY243312**) is a well-adapted and well-studied laboratory strain.
5. Vaccinia virus strain Copenhagen (VACV, Locus: VACCG, GenBank accession no: **M35027**). The vaccinia strain which has been used for pox vaccine production in Denmark [18,37]
6. Two cowpox virus strains were selected: Brighton Red (CPV-BR, Reference Sequence: NC_003663, GenBank accession no: **AF482758**) and strain GRI-90 (CPV-GRI-90, GenBank accession no: **X94355**) due to the hypothesis that variola and vaccinia virus originated from these [7]. According to Gubser et al., VACV are more closely related to CPV-GRI-90 than CPV-BR thus these authors suggested it to be grouped into a separate species [19].

NetCTL epitope prediction—The NetCTL prediction method (Larsen et al., 2005)[24] was used for predicting 9mer pox CTL epitopes for the entire genome of VARV-IND. Prediction was performed for 12 HLA supertypes [25]: A1, A2, A3, A24, A26, B7, B8, B27, B39, B44, B58, and B62. This provides a broad coverage (> 99%) of the HLA alleles worldwide. The 9mer peptides were sorted on the NetCTL score which is a combined score for MHC binding, TAP binding and proteasomal cleavage. A threshold cut-off value of 0.42 in NetMHC corresponding to a predicted binding affinity of better than 500nM was used.

Identification of orthologue proteins—A computer program “Genediff” was developed in order to find the orthologous proteins among the different selected orthopox strains. The Genediff program generated a list of conserved proteins that were shared among all the selected seven orthopox virus strains. The program uses Blast [2] to find the most similar protein to each protein in the reference strain in another strain with the restriction that the Blast E-value had to be lower than 0.05. In this way each of the strains are compared to the reference strain. These results were combined to identify 157 proteins found in all strains.

Selection of epitopes in immunomodulatory proteins—The whole genome of VACV-COP (a total of 262 Open Reading Frames) was scanned in order to find viral immunomodulatory proteins. By using a bioinformatic server, ProtFun (<http://www.cbs.dtu.dk/services/>), finding orthologues to other known vaccinia virus proteins (<http://www.pox.org>) and literature search in PubMed at NCBI 58 potential immunomodulators were found in VACV-COP. 22 of these were found to be among the 157 orthologous proteins conserved among the seven orthopoxvirus strains included in this study. We predicted epitopes in these 22 immunomodulatory proteins and filtered out those 9-mers that were not found in all seven strains, giving 93 selected peptides from 16 different potential immunomodulators.

Selection of Conserved epitopes which were identical in all seven strains of orthopox viruses—Conserved predicted CTL epitopes were selected among the list of remaining orthologues after the immunomodulatory proteins had been picked. These 96 peptides are distributed in 47 different proteins.

Finding “self peptides”—When comparing the entire human genome with VACV-COP using Genediff, 54 orthologues were found. Only one predicted epitope, RYNNNTARY, was found to be identical to a human self peptide, and this was kept in this study.

Selection of known CD8+ T cell epitope—Twelve known pox CTL epitopes were found at the initiation of this study (This number has since increased to more than 150 human epitopes against vaccinia virus [22]. Two of these epitopes; CLTEYLWV (NP_042046.1, gene: D1L) and KVDDTFYYV (NP_042052.1, gene: D8L) were tested on 3 A*0201 positive donors). One known epitope: SLSAYIIRV (NP_042130.1, gene: I3L) was selected to be synthesized.

Collection of blood samples—Buffy coats of 500 ml whole blood from 35 healthy Danish donors (age range: 35–65 years with a sex ratio of 51% female and 49% male; donors had given written informed consent) were obtained from The Blood Bank at Rigshospitalet (Copenhagen, Denmark) and used within 24 h to isolate PBMC. The donors were selected, according to serological typing of their HLA-A and -B haplotype, to maximize coverage of the 12 HLA class I supertypes. A high-resolution sequence-based typing (SBT) of the HLA-A and HLA-B loci was subsequently established (Genome Diagnostics, Utrecht, Netherlands). Data for donors are shown in Table 1. The number of peptides tested in each donor was variable due to different HLA supertypes of the donors. All the HLA-matched donors among the cohort of 35 donors were used for testing a peptide restricted by same HLA supertype as that of donors. On the average 33 peptides (range: 7–47) were tested in each donor.

Isolation of PBMC—PBMC were isolated from buffy coats by density gradient centrifugation using Lymphoprep (Nycomed Pharma AS, Oslo, Norway). The freshly isolated PBMC were cryopreserved for later use at 20×10^6 cells in 1ml RPMI-1640 containing 20% FCS and 10% dimethyl sulfoxide (DMSO) at -140°C .

Peptides—The 9mer peptides were synthesized by standard 9- fluorenylmethyloxycarbonyl (Fmoc) chemistry, purified by reversed-phase high-performance liquid chromatography (at least 80%, usually > 95% purity) and validated by mass spectrometry (Shafer-N, Copenhagen, Denmark). Peptides were distributed at 500 µg/vial and stored lyophilized at -20°C until use. Peptides were dissolved just before use.

Biochemical peptide–HLA class I binding assay—The biochemical assay for peptide–MHC-I binding was performed as previously described [42,43]. Briefly, denatured and purified recombinant HLA heavy chains were diluted into a renaturation buffer containing HLA heavy chain, β_2 -microglobulin, graded concentrations of the test peptide, and incubated at 18°C for 48 h allowing equilibrium to be reached. We have previously demonstrated that denatured HLA molecules can de novo fold efficiently, but only in the presence of appropriate peptide [32]. The concentration of peptide–HLA complexes generated was measured in a quantitative enzyme-linked immunosorbent assay and plotted against the concentration of peptide offered [42]. Because the effective concentration of HLA (3–5 nM) used in these assays is below the equilibrium dissociation constant (K_D) of most high-affinity peptide–HLA interactions, the peptide concentration leading to half-saturation of the HLA is a reasonable approximation of the affinity of the interaction. An initial screening procedure was employed whereby a single high concentration (20,000 nM) of peptide was tested. If no complex formation was found, the peptide was assigned as a non-binder to the HLA molecule in question, conversely, if complex formation was found in the initial screening, a full titration of the peptide was performed to determine the affinity of binding.

IFN- γ ELISPOT assay—PBMC were thawed, washed and resuspended to a concentration of $6-8 \times 10^6$ cells per ml in RPMI-1640 supplemented with 5% heat-inactivated AB serum (Valley Biomedical, Winchester, USA), 2mM l-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were cultured in 24-well plates (Nunc, Roskilde, Denmark). Individual peptides were added to a final concentration of 20 µg/ml per well and incubated for 10 days at 37°C, 5% CO₂ in humidified air. RhIL-2 (Proleukin; Chiron, The Netherlands) 20 U/ml was added on day 1. Cells were harvested on day 10, washed twice in RPMI-1640 and resuspended in complete medium to a final concentration of 1×10^6 cells/ml. The IFN- γ ELISPOT assay was performed as described previously [49] to quantify peptide-specific CD8 + T cells after *in vitro* expansion. Briefly, 96-well ELISPOT plates (Multiscreen, MAHAS4510; Millipore, Molsheim, France) were coated overnight at 4°C with 7.5 µg/ml anti-human IFN- γ (M-700A, Endogen; Pierce Biotechnology, Rockford, IL, USA). The plates were washed six times with sterile phosphate buffered saline (PBS) to remove unbound coating antibody. Cell suspensions were prepared and six replicate wells were seeded with 10^5 cells per well. Peptides were added to a final concentration of 10 µg/ml. As positive controls, cells were stimulated with 10 µg/ml phytohaemagglutinin (PHA) (Sigma-Aldrich, UK). Cells were incubated with complete medium alone as negative control. After incubation for 18–20 h at 37°C and 5% CO₂, plates were washed with PBS containing 0.05% Tween-20. Biotinylated antihuman IFN- γ (M-701B, Endogen; Pierce Biotechnology, Rockford, IL, USA) was added at 0.75 µg/ml and plates were incubated for 2 h at room temperature. After incubation, plates were washed and incubated further for 1 h with streptavidin-conjugated peroxidase (Dako, Copenhagen, Denmark) diluted 1:500 in PBS/1% BSA. Next, the plates were washed as before and 200 µl of substrate [30 mg 4-chloro-1-naphthol (057h8927, Sigma) dissolved in 10 ml methanol, diluted in 50ml triethanol-buffered H₂O (T1377, Sigma) at pH 7.5 and 25 µl H₂O₂ 30% (H1009, Sigma)] were added to the wells. Finally, plates were washed under running tap water and dried at room temperature. IFN- γ spot-forming cells (SFC) were enumerated using an ELISPOT reader (KS ELISpot, Zeiss, Munich, Germany). In order to block MHC-I restricted responses, anti MHC-I antibody W6/32 ascites (ATCC, Rockville, MD, USA) was added in a dilution of 1/40 for 30 min before

adding peptides in ELISPOT assays. Results are expressed as mean number of spot-forming cells (SFC) per 10^5 PBMC, which is a measure for T cell frequency.

Statistics—Wilcoxon rank-sum test was used to analyze the quantitative differences between the experimental wells and control in ELISPOT assays. A P-value below 0.05 was considered significant.

Results

Computational prediction of peptides binding to MHC-class I molecules from the vaccinia virus genome which are 100% conserved in related orthopox viruses

In this study artificial neural networks (ANNs) were used to predict vaccinia virus CTL epitopes. We have developed an algorithm program, Genediff, in order to find all the orthologous proteins that were shared among seven strains of related orthopoxviruses. Vaccinia virus strain Copenhagen with 262 ORFs, was chosen to be our reference strain due to the fact that this strain was used as the vaccine strain for the Danish donor cohort included in this study. The orthologous proteins were found in the following orthopox viruses: variola virus strain India-1967 (VARV-IND), variola virus strain Bangladesh (VARV-BSH), vaccinia virus strain Acambis 3000 Modified Virus Ankara (VACV-MVA), vaccinia virus strain Western Reserve (VACV-WR), cowpox virus strain Brighton Red (CPV-BR) and cowpox virus strain GRI-90 (CPV-GRI-90). 157 orthologues were found to be shared among the seven selected strains; these were divided into two selections. The first selection consists of 22 potential immunomodulators that were present in all seven strains, while the second selection contains the remaining conserved orthologous proteins. Since pox viruses in general do have many immunomodulatory proteins we have here studied if the human T cells were able to target peptides within these proteins.

The web server NetCTL was used for *in silico* CTL epitope prediction [24]. We synthesized up to 20 peptides for each supertype with the highest NetCTL score that had a NetMHC score of at least 0.42, and were found to be 100% conserved in all seven fully sequenced orthopoxvirus strains. Due to difficulties with the synthesis some of the predicted peptides were discarded. Thus, at the end, we tested 86 out of 93 selected predicted peptides originating from 16 of the immunomodulators and 91 out of 96 predicted peptides from 46 of the conserved proteins.

Validation of MHC-I binding of predicted epitopes

A total of 177 peptides were synthesized and tested in a biochemical *in vitro* binding assay [42,43] to determine whether the peptides bind to the relevant MHC class I molecules. The numbers of peptides predicted to bind to each of the eleven different superotypes were: HLA-A1 (15), A2 (15), A3 (19), A24 (20), A26 (18), B7 (17), B15 (15), B27 (11), B39 (17), B44 (15), B58 (15) and HLA-B08 (0). The reason none was selected for B08 was that all predictions were below the NetMHC threshold of 0.42, because the predictor used was in an early stage of development, giving low output values.

The measured binding affinities (K_D) of the 9-mer predicted peptides can be divided into five different groups: very high binders ($n = 45$; $K_D \leq 5\text{nM}$), high binders ($n = 52$; $5\text{nM} < K_D \leq 50\text{nM}$), intermediate binders ($n = 42$; $50\text{nM} < K_D \leq 500\text{nM}$), low binders ($n = 28$; $500\text{nM} < K_D \leq 5000\text{nM}$) and finally peptides with low or no binding affinity for MHC class I molecules ($n = 10$, $K_D > 5000\text{nM}$) (see Table 2). In this study, 139 out of 177 measured peptides (79%) bound to their respective HLA molecules with a $K_D \leq 500\text{ nM}$.

Eight VACV-COP epitopes elicited IFN- γ release in ELISPOT assays

The peptides were evaluated for their ability to stimulate IFN- γ production in an ELISPOT assay by peripheral blood mononuclear cells (PBMC) from individual donor vaccinated more than 30 years ago with VACV. In order to increase the low frequency of peptide-specific T cells, PBMC were exposed for 10 days to peptides prior to performing the ELISPOT assays. A total of 105 HLA class I binding peptides were assayed for IFN- γ ELISPOT formation. Immunogenicity was observed for eight out of 45 peptides that bound with a $K_D \leq 5$ nM. None of 60 tested peptides with an affinity > 5 nM induced a response. This correlation of affinity and immunogenicity is highly significant ($p < 0.001$, two-tailed, Fischer exact test).

Eight positive peptides belonging to five different supertypes (A1, A2, A24, A26 and B44) were found to elicit a significant ($p < 0.05$) immune response in at least one donor in two independent experiments. These responses ranged from 8-208 IFN- γ spots per 10^5 PBMC. All the significant ELISPOT data are listed in Table 3 and a donor-specific overview is given in Table 1. In order to verify that these T cell responses were restricted by MHC class I molecules, anti-MHC class I antibody W6/32 was added in IFN- γ ELISPOT assays for the identified epitopes. The anti-MHC class I antibody was found to block peptide-induced IFN- γ release (data not shown), which together with the peptide-MHC binding data support that the epitope-induced responses are MHC class I restricted. Table 4 shows the reactivity of each of the donors against each of the peptides. Each peptide was only tested in donors that had at least one allele within the supertype that the peptide was predicted to be restricted by. Individual donor immunity was only directed against a few of the peptides shown in the table, and not against several peptides as would have been the case if all reactivity was generated during the *in vitro* culture. These data thus suggest that the responses were generated from pre-existing immunity in the donors. None of the eight peptides have been found in non pox viruses suggesting that this immunity must have been elicited by the pox vaccinations. Six epitopes were recognised by more than one donor (Table 3). One peptide (N1L₇₀_RMIAISAKV (A2) was recognised by four different HLA-A2+ donors (donors 13, 19, 30 & 31) and five peptides were recognized by two different donors with matched HLA supertype. The last two epitopes were only recognised by one donor.

One donor (donor 19) exhibited a broad response against half of the identified epitopes (4 out of 8), three of these were HLA-A2 restricted (E1L₂₁₂_FLIDLAFLLI, A42R₇₃_LMDENTYAM & N1L₇₀_RMIAISAKV) and one was B44 restricted (H1L₄₇_SEVKFKYVL). In total 11 donors responded to at least one peptide (Table 1).

Nature of the antigens recognised in VACV-COP

The eight epitopes were derived from eight different VACV-COP proteins (A10L, A18R, A20R, E1L, A38L, A42R, H1L and N1L). Table 5 lists each of the antigens with their name as defined in the Poxvirus Bioinformatics Resource Center (<http://www.poxvirus.org>). The functional categories of the recognised VACV-COP antigens are broadly distributed into three classes: virion structure, virus regulation of translation and transcription and virulence factors. Epitopes in four different potential immunomodulatory proteins (A38L, A42R, H1L and N1L) were found for VACV-COP. In order to examine whether the eight positive epitopes have been described previously, a search was performed in the Immune Epitope Database and Analysis Resource (IEDB, <http://www.immuneepitope.org/>). Seven of the epitopes (A10L₆₃₅_DTRGIFSAY, A18R₂₅₄_LSDLKKTIIY, A20R₁₄₃_KYQSPVNIF, E1L₂₁₂_FLIDLAFLLI, A42R₇₃_LMDENTYAM, H1L₄₇_SEVKFKYVL, N1L₇₀_RMIAISAKV, with the following IEDB ID numbers: 1045360, 1082606, 1084526, 1083691, 2444, 1087630, 1083932, respectively) were found in the IEDB.

Discussion

In this article we have identified eight VACV-COP MHC I restricted T cell epitopes. The epitopes were selected so they were 100% conserved among VARV-IND, VARV-BSH, VACV-MVA, VACV-WR and two other orthopoxviruses, the cowpox virus strains: CPV-BR and CPV-GRI-90. The identified epitopes are restricted by HLA-A1, -A2, -A24, -A26 and -B44. To our knowledge the epitope: A38L₂₅₆-VSEHFSLLF has not been studied before. Seven of the epitopes identified in the present study were reported as non-immunogenic in previous studies (<http://www.immuneepitope.org/>). Such differences can be expected since each subject responds to different sets of peptides and testing a peptide in more subjects increases the chance that it is recognised as an epitope in at least one subject.

The subjects in our study cohort were healthy Danish blood bank donors with an age range from 35-62 years. Most Danes were vaccinated > 30 years ago with the vaccine strain; vaccinia virus strain Copenhagen. Only one study has examined epitope recognition in long term vaccinia virus vaccinees without re-vaccination [33]. Ostrout and colleagues tested 46 HLA-A2 restricted peptides and found a positive IFN- γ response towards 6 of these [33]. In the present study we have screened peptides from 11 different HLA supertypes which have been calculated to have an average population coverage of more than 99% [39]. The experimental setup of Ostrout differ from ours in that they did not expand the donor PBMC *in vitro* before performing their ELISPOT assay, but instead they use a longer incubation time for the ELISPOT assay (42h instead of 18-20h in the present study). Ostrout et al. included both non-A2 vaccinees and a group of HLA-A2 donors, who were never vaccinated. The age-range of the latter group was not provided but we believe that these donors must be below 27-30 years of age. We have decided not to include such a control group since these donors are younger than the group that have been exposed to poxvirus and one could discuss whether it is good as a control group. The present study is the first study describing epitope T cell responses restricted to HLA-A1, -A24, -A26 and -B44 supertypes in long term vaccinia virus vaccinees without revaccination. Most of the other studies involving pox epitope recognition have used T cells from donors re-vaccinated with vaccinia vaccine [31], established VACV-specific CTL lines [46], or by using inbred mice expressing mouse alleles (K^b or D^b) [28,48] or transgenic mice expressing human HLA molecules [35]. The T cell responses detected in the present study can be expected to be long term T cell memory responses. The data in Table 4 strongly support that spots are formed by *in vivo* peptide-specific preexisting cells and not by peptide-specific cells generated in the *in vitro* culture. The argument goes as such: The five HLA-A1- or A2-binding peptides in the table are immunogenic, but reactivity is only present in 9 of 28 different HLA-1 or A-2 matched donors. Thus, if immune reactivity was only generated *in vitro*, all five immunogenic peptides should have induced formation of spots in the 9 immune HLA-A1 or -A2 donors, respectively. Apparently this is not the case. Thus immune reactivity against pox peptides must already have been existing *in vivo*.

31 percent of the donors responded (11 out of 35 donors) to one or more of the eight identified epitopes. The responders were on the average older than the non-responders but this difference was not significant ($p = 0.18$, paired, two-tailed Student t-test). In our study we showed that long term memory T cells directed towards vaccinia and variola epitopes persists in human in the complete absence of original antigens. A previous study indicated that vaccination gave a 90% protection against lethal smallpox infection for more than 20 years [26]. CD4 T cells have been shown to be the principal component of long-term cellular memory to VACV [3,20]. Hammerlund et al. (2003) studied the duration of antiviral immunity after smallpox vaccination [20]. They found that detectable antiviral CD8+ T cell memory was maintained in only ~50% of the vaccinees 20 years after last vaccination, regardless of how many booster vaccinations these individuals had received. These authors question whether CD8+ T cell memory is required for protection and conclude that CD4+ T cell memory or neutralizing antibody

responses (or both) may constitute the main components of long-term immunity against smallpox after CD8⁺ T cell memory has faded. The study by Combadiere et al., 2004 showed that *in vitro* stimulation increased the frequency of effector memory cells producing IFN- γ , and their study indicated that both CD4⁺ and CD8⁺ T cells are involved in VACV-specific memory responses. According to Combadiere et al., T cell effector memory vanished, but T cell proliferative memory persisted or stabilized. The latter remained stable independently of the time since priming with > 70% responses. Anti-vaccinia proliferative T cell responses were shown to be maintained many years after antigen exposure, while T cell effector memory/central memory was shown to vanish 45 years after first vaccination [10].

Many studies focus on genes that are expressed at the early stages of infection as, due to their relatively abundant expression, the proteins may be more available for the host immune system [28,29,31,46]. Here, we identified three epitopes in three late gene products (A10L, E1L and A42R), two were early expressed (A20R and A38L) and finally three were found to be expressed both early and late (A18R, H1L and N1L). From these data we cannot observe any bias towards early-expressed gene products being preferentially targeted by T cells. The 21 CD8⁺ restricted T cell epitopes found by Pasquetto et al. were also primarily from late expressed genes [35].

It can be expected that the number of epitopes hosted by a protein should be proportional to the length of the protein. Oseroff and colleagues [31] studied the protein size differences in proteins with and without epitopes. They studied approximately 6000 peptides and observed that all the identified 48 positive epitopes were contained in relatively large proteins (> 100 residues), whereas no epitopes were found in small proteins (< 100 residues). All our positive epitopes were from proteins with > 100 residues and appear to support Oseroff and colleagues findings. In this study, we found four epitopes in each of the two selections of proteins: the group of conserved and the group of immunomodulatory proteins. Thus, in our study, the function of a protein does not appear to be of importance for immunogenicity. Many epitopes have been discovered in the mean time by other groups and these are found to be distributed over all functional classes of vaccinia virus proteins [22]. Previously, we have shown that most influenza CTL epitopes found among peptides that bind to a HLA molecule, bind with $K_D \leq 500\text{nM}$ [49]. This is in agreement with a previous review [50] stating that 500nM is the HLA binding affinity threshold for immunogenicity. Here we show that pox CTL epitopes more than 30 years after immunization are exclusively found among peptides binding with an affinity of $\leq 5\text{nM}$ suggesting that long term memory is associated with high peptide-HLA binding affinities.

In conclusion we have identified a total of eight T cell epitopes. This investigation confirmed that bioinformatic analysis can facilitate the discovery of epitopes with a broad conservation in a fast and efficient way. This may be used in the design of a future improved vaccine or of a diagnostic tool for smallpox.

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Table 1

Overview of the donors used in this study. Highlighted in grey are donors which have responded to one or more epitopes

Donor #	Sex	Age (y)	Serological typing HLA-A	HLA-B	Sequence based typing HLA-A	HLA-B	Immune reactivity (# number of epitopes)
1	M	36	A2, A3	B35, B44	*0201	*3501	*4402
2	M	47	A1, A3	B49, B60	*0101	*4901	*4001
5	F	59	A1, A24	B37, B39	*0101	*2402	*3906
6	M	41	A1, A3	B8, B35	*0101	*0301	*3501
7	M	40	A1	B8, B52	*0101	*0801	*5201
8	F	43	A11	B22, B35	*1101	*5501	*3501
9	F	43	A3, A32	B7, B44	*0301	*0702	*4402
10	F	38	A3, A25	B7, B18	*0301	*0702	*1801
11	M	47	A2, A3	B7, B45	*0201	*0702	*4501
12	F	37	A1, A24	B8, B35	*0101	*0801	*3503
13	M	60	A2	B39, B44	*0201	*3901	*4402
14	M	58	A1, A2	B8, B37	*0101	*0801	*3701
15	M	44	A2, A26	B7, B64	*0201	*0702	*1401
16	M	50	A2, A29	B5, B45	*0201	*5101	*4501
17	F	35	A2, A29	B44, B62	*0201	*4403	*1501
19	F	46	A1, A2	B8, B40	*0101	*0801	*4001
20	F	43	A11, A31	B22, B38	*1101	*5501	*3801
21	M	54	A1, A24	B7, B39	*0101	*0702	*3901
22	M	57	A11, A32	B13, B15	*1101	*1302	*1517
23	F	58	A1, A24	B7, B8	*0101	*0702	*0801
24	F	36	A2, A26	B38, B58	*0206	*3801	*5801
25	F	62	A24, A26	B7, B38	*2402	*2601	*3801
26	M	58	A1, A3	B8, B27	*0101	*0801	*2705
27	F	43	A2, A28	B17, B44	*0201	*5701	*4402
28	F	62	A3, A26	B7, B14	*0301	*0702	*1401
29	M	52	A3, A11	B13	*0301	*1302	*1302
30	F	49	A2, A11	B52	*0201	*5201	*5201
31	F	63	A2, A3	B7, B22	*0201	*0701	*5601
32	M	55	A2, A32	B5, B15	*0201	*5201	*5101
33	M	60	A1	B8	*0101	*0801	*0801
34	M	50	A1, A25	B8, B18	*0101	*0801	*1801
35	F	43	A29, A33	B17, B27	*2902	*3303	*2705
36	F	50	A2, A24	B17, B35	*0201	*2402	*3508
37	M	46	A3, A28	B14, B28	*0301	*1402	*1402
38	F	47	A28, A29	B44, B45	*6801	*2902	*4501

Table 2

In vitro measured binding affinity (K_D) values for pox peptides.

Supertype	Very high binders $K_D \leq 5nM$	High binders $5nM < K_D \leq 50nM$	Intermediate binders $50nM < K_D \leq 500nM$	Low binders $500nM < K_D \leq 5000nM$	Low or -non-binders $K_D > 5000nM$	Total
A1	8(2 [*])	4	0	1	2	15
A2	10(3 [*])	2	3	0		15
A3	1	6	6	5	1	19
A24	8(1 [*])	2	5	5		20
A26	7(1 [*])	4	5	2		18
B7	2	9	4	2		17
B15	0	5	8	1	1	15
B27	3	4	0	4		11
B39	0	4	5	3	5	17
B44	5(1 [*])	4	4	2		15
B58	1	8	2	3	1	15
Total	45(45)	52(37)	42(23)	28(0)	10(0)	177(105)

* number of positive epitope(s) within this supertype. In bold bracket, actual peptide number tested for each group of binders in IFN- γ ELISPOT assay

IFN- γ ELISPOT analysis of peptide-specific donor responses.

Peptide	Sequence	HLA Restriction	No. of donor screened	Responding donors	Elispot assay 1		Elispot assay 2	
					+ peptide ^a	- peptide ^b	+ peptide ^a	- peptide ^b
A10L ₆₃₅	DTRGIFSAY	A26	4	1 (Donor 28)	43 $\pm$ 0	0 $\pm$ 0	54 $\pm$ 26	1 $\pm$ 2
A18R ₂₅₄	LSDLKTTY	A01	16	2 (Donor 7,9)	14 $\pm$ 3	5 $\pm$ 3	20 $\pm$ 2	4 $\pm$ 3
A20R ₁₄₃	KYQSPVNI	A24	6	2 (Donor 21,23)	26 $\pm$ 6	3 $\pm$ 2	36 $\pm$ 8	1 $\pm$ 1
E1L ₂₁₂	FLJDLAFLI	A02	15	1 (Donor 19)	22 $\pm$ 5	0 $\pm$ 1	50 $\pm$ 6	5 $\pm$ 1
A38L ₂₅₆	VSEHFSLLF	A01	16	2 (Donor 14,23)	8 $\pm$ 5	1 $\pm$ 1	10 $\pm$ 2	0 $\pm$ 1
A42R ₇₃	LMDENTYAM	A02	15	2 (Donor 19, 24)	44 $\pm$ 6	1 $\pm$ 1	89 $\pm$ 7	6 $\pm$ 2
H1L ₄₇	SEVKFYVL	B44	10	2 (Donor 13, 19)	35 $\pm$ 9	8 $\pm$ 3	15 $\pm$ 3	4 $\pm$ 3
N1L ₇₀	RMIAISAKV	A02	15	4 (Donor 13,19,30,31)	191 $\pm$ 16	1 $\pm$ 1	208 $\pm$ 19	19 $\pm$ 13

Data represent mean SFC/105 $\pm$ SD. Two independent experiments for each epitope were included.

^a positive responses were defined by mean SFC of experimental wells (+peptide) > 2 times background (-peptide) and p< 0.05 (Wilcoxon rank-sum test).

^b the background level with no peptide (-peptide): 0-19 SFC/1 $\times$ 105 PBMC.

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Structural and phylogenetic characteristics of vaccinia virus strain Copenhagen antigens. Shown are the protein names for each of the antigen as defined by the Poxvirus Bioinformatics Resource Center (<http://www.poxvirus.org>), name in the VACV-COP strain, corresponding names and percentage homology to the six other orthopoxviruses (variola major virus strain India-1967 (VARV-IND), variola major virus strain Bangladesh-1975 (VARV-BSH), vaccinia virus strain Acambis 3000 Modified virus Ankara (VACV-MVA), vaccinia virus strain Western Reserve (VACV-WR), cowpox virus strains; Brighton Red (CPV-BR) and GRI-90 (CPV-GRI-90)), functional category, predicted protein size, the start residue of the epitope within the protein and protein expression kinetics: early versus late (either known or predicted – <http://www.poxvirus.org/>), NetCTL predicted combined score for binding affinity, proteasomal cleavage and TAP binding and the measured binding affinity. The prediction server is available at <http://www.cbs.dtu.dk/services/NetCTL/>. K_D , measured affinity in nM.

Table 5

VACV-COP	Protein	Protein size	Start residue of epitope	Protein Description	Functional Group	Epitope	%Similarity with other related orthopox viruses	VARV-BSH	VV-MVA	VV-WR	CPV-BR	CPV-GRI-90	Combi score	K _d (nM) measured						
CONSERVED PROTEINS:																				
A10L	early/late	891	635	precursor p4s of core protein 4s, complexes with A4L	virion structure	DTRIGFSAY	A10L	97	A11L	100	ANVA123	97	VACVIR129	97	CPV142	96	A11L	98	2.23	1
A18R	early	483	254	DNA helicase, effects elongation and termination of postreplicative viral transcription	virus regulation	LSDLKITY	A18R	97	A19R	100	ANVA132	97	VACVIR138	97	CPV151	95	A19R	98	5.18	4
A20R	early	428	143	stoichiometric component of the DNA polymerase processivity factor	virus regulation	KYQSPYNF	A20R	98	A21R	100	ANVA135	98	VACVIR141	98	CPV154	98	A22R	98	1.65	2
A22R	late	428	212	poly-A polymerase catalytic subunit, VP55	virus regulation	FLDGLRLI	E1L	96	E1L	100	ANVA650	99	VACVIR257	95	CPV1067	96	F1L	98	1.69	3
IMMUNOMODULATORY PROTEINS:																				
A38L	early	277	256	CD47-like putative membrane protein	virulence factor	VSEHFSLF	A41L	95	A41L	100	ANVA151	94	VACVIR162	95	CPV175	95	A40L	96	3.90	5
A42R	late	133	73	profilin-like or profilin homolog	virulence factor	LMDEITYAM	A41R	97	A45R	100	ANVA156	94	VACVIR167	97	CPV179	97	A44R	99	1.42	1
H1L	late/early	171	47	tyrosine protein phosphatase, blocks IFN-γ signal transduction	virus regulation	SEVKPKYWL	H1L	96	H1L	100	ANVA683	97	VACVIR269	96	CPV110	96	J1L	96	1.47	4
R1L	early/late	117	70	virulence, host defense modulator	virulence factor	RIMASATV	P1L	93	P1L	99	ANVA622	92	VACVIR283	93	CPV037	91	G1L	95	1.45	3

* Protein immunogenic in human (reviewed in Kennedy and Poland, 2007)

** Protein immunogenic in mouse (reviewed in Kennedy and Poland, 2007)

In bold, peptide tested negative in other studies