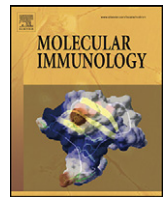




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Human CD4 T cell epitopes selective for Vaccinia versus Variola virus

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ABSTRACT

Due to the high degree of sequence identity between *Orthopoxvirus* species, the specific B and T cell responses raised against these viruses are largely cross-reactive and poorly selective. We therefore searched for CD4 T cell epitopes present in the conserved parts of the Vaccinia genome (VACV) but absent from Variola viruses (VARV), with a view to identifying immunogenic sequences selective for VACV. We identified three long peptide fragments from the B7R, B10R and E7R proteins by *in silico* comparisons of the poxvirus genomes, and evaluated the recognition of these fragments by VACV-specific T cell lines derived from healthy donors. For the 12 CD4 T cell epitopes identified, we assessed their binding to common HLA-DR allotypes and their capacity to induce peptide-specific CD4 T-cell lines. Four peptides from B7R and B10R displayed a broad binding specificity for HLA-DR molecules and induced multiple T cell lines from healthy donors. Besides their absence from VARV, the two B10R peptide sequences were mutated in the Cowpox virus and completely absent from the Monkeypox genome. This work contributes to the development of differential diagnosis of poxvirus infections.

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1. Introduction

Variola virus (VARV), the causal agent of smallpox, was eradicated 30 years ago by extensive worldwide vaccination programs initiated in the 1950s. This success has led to the smallpox vaccine being considered very efficient. It is based on living Vaccinia virus (VACV), which belongs, to the same viral *Orthopoxvirus* genus as VARV. Orthopoxviruses are very large viruses with a double-stranded DNA genome of 130–260 kb, which encodes 180–240 open reading frames. This genus also includes the Monkeypox and Cowpox viruses and certain VACV strains that can be infectious in humans (Essbauer et al., 2010). Attenuated Vaccinia viruses, including Modified Vaccinia Ankara (MVA) (Vollmar et al., 2006) and NYVAC (Belyakov et al., 2003) have also been developed for vaccination purposes, to reduce the risk of adverse effects provoked by Vaccinia injections. Orthopoxviruses are highly conserved and their genome sequences display high levels of identity. As a result, the smallpox vaccine can induce specific immune responses providing protection against various Orthopoxviruses, including VARV and Monkeypox virus (Edghill-Smith et al., 2005; Henderson, 1987). Vaccinia-specific antibodies are cross-reactive with other species

(Edghill-Smith et al., 2005; Sette et al., 2009) and most of the VACV-specific CD8 and CD4 T cell epitopes are highly conserved (Calvo-Calle et al., 2007; Sette et al., 2009; Sirven et al., 2009).

VARV has considerable potential as a biological weapon and is one of the most feared pathogens of the bioterrorism threat. Any Variola outbreak would be very serious, as immunity to poxviruses in populations has waned during the years since smallpox eradication. In 2003, an outbreak of Monkeypox originating from infected prairie dogs affected 71 US citizens (Enserink, 2003). This accidental event highlighted limitations in our ability to detect and respond to a potential bioterrorism threat based on the use of poxviruses. Clinical symptoms are the first line of diagnosis of poxvirus infection, which can subsequently be confirmed by PCR assays and electron microscopy (Breman and Henderson, 2002). Both assays require the active presence of the virus and are therefore not suitable for diagnosis in patients whose infection has spontaneously resolved. This is a limitation to evaluate the extent of viral outbreak. Orthopoxviruses display extensive sequence identity. Thus, serological tests essentially indicate the degree of immunity to orthopoxviruses in general and are of little help for identifying the precise *Orthopoxvirus* species involved in an infection. As a result, half the infected subjects initially went undiagnosed during the US Monkeypox outbreak (Hammarlund et al., 2005). As antiviral immunity persists for many years after smallpox vaccination (Hammarlund et al., 2003), the antibodies induced by smallpox vaccine complicated the diagnosis. It is thus a key challenge to identify the antigenic determinants carried by one virus but not

Abbreviations: VACV, Vaccinia virus; CPXV, Cowpox virus; MPXV, Monkeypox virus; VARV, Variola virus.

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by the others, to facilitate the discrimination of the various species on the basis of their imprints on the repertoire of specific T lymphocytes elicited by the viral infection. Such an approach has been successfully applied to latent tuberculosis, through the detection of ESAT-6- and CFP-10-specific T cells by EliSpot (Arend et al., 2000). These two antigenic proteins are both present in *Mycobacteria tuberculosis* but absent from BCG strains. With a view to applying a similar approach to discriminating between vaccinees and Variola-infected subjects, we searched for CD4 T cell epitopes encoded by the Vaccinia genome but absent from the Variola genome. We describe here the first CD4 T cell epitopes specific for Vaccinia but not for Variola viruses.

2. Materials and methods

2.1. In silico selection of peptide sequences

Poxvirus sequences were retrieved from the website of the Viral Bioinformatics Resource Center (<http://www.poxvirus.org>). Sublibraries of conserved sequences were created from the genomes of 10 replication-competent Vaccinia strains including Copenhagen, Western Reserve, LC16m8, Lister and Acam2000, and 49 Variola strains on the basis of a complete identity for at least 40 amino acids. Selected sequences from the Vaccinia sublibrary were at least 100 amino acids long and were less than 50% identical to Variola sequences.

2.2. Peptides

We used 15-mer peptides overlapping by 10 amino acids and encompassing the sequences of B10R 40–166 (23 peptides), B7R 1–158 (30 peptides) and E7R 1–115 (21 peptides) from the Vaccinia virus strain Copenhagen (<http://poxvirus.org>). Peptides were synthesized by standard Fmoc chemistry, on an Advanced ChemTech Apex synthesizer (Advanced ChemTech, Louisville), and cleaved from the resin with 95% trifluoroacetic acid. If necessary, peptides were purified by RP-HPLC on a C₁₈ Vydac column (Interchim, Montluçon, France). Their purity was approximately 90%, as indicated by analytical HPLC.

2.3. Vaccinia virus

Vaccinia virus (WR strain, reference: VR-1354, ATCC, Manassas, VA) was amplified by culture in HeLa S3 cells (ATCC) (Earl et al., 2001). After three days, cells were harvested, lysed by repeated freeze–thaw cycles and stored at –80 °C. Vaccinia virus was inactivated by heating at 60 °C for 1 h. The virus was titrated on BSC-1 cells (ATCC) (Earl et al., 2001). Briefly, serial dilutions of the trypsinized virus were used to infect the BSC-1 cell line. After two days, the medium was removed and the cells were stained with crystal violet. Plaques appear as areas of weaker staining due to the retraction, rounding and detachment of infected cells.

2.4. Binding assays specific for HLA-DR molecules

HLA-DR molecules were purified from homozygous EBV cell lines (HPA Culture Collections, Salisbury, UK) by affinity chromatography with the monomorphic mAb L243 (Texier et al., 2000, 2001). Binding to HLA-DR molecules was assessed by competitive ELISA, as previously described (Texier et al., 2000, 2001) on an automated workstation. The peptide concentration that prevented binding of 50% of the labeled peptide (IC₅₀) was evaluated. Data were expressed as relative affinity: ratio of the IC₅₀ of the peptide to the IC₅₀ of the reference peptide, which is a high binder to the HLA II molecule. Unlabeled forms of the biotinylated peptides were used as reference peptides. Their

sequences and IC₅₀ values were the following: HA 306–318 (PKYVKQNTLKLAT) for DRB1*0101 (2 nM), DRB1*0401 (6 nM), DRB1*1101 (8 nM) and DRB5*0101 (3 nM), YKL (AAYAAAKAAALAA) for DRB1*0701 (5 nM), A3 152–166 (EAEQLRAYLDGTGVE) for DRB1*1501 (18 nM), MT 2–16 (AKTIAYDEEARRGLE) for DRB1*0301 (116 nM), B1 21–36 (TERVRLVTRHIYNREE) for DRB1*1301 (29 nM), LOL 191–210 (ESWGAVWRIDTPDKLTGPFT) for DRB3*0101 (33 nM) and E2/E168 (AGDLLAIETDKATI) for DRB4*0101 (43 nM).

2.5. Blood samples and HLA-DR genotyping

Blood cells were collected at the Etablissement Français du Sang (EFS, Rungis, France), as buffy-coat preparations from anonymous healthy donors who gave informed consent, in accordance with EFS guidelines. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation on Ficoll-Hyperpaque gradients (Sigma–Aldrich, St. Quentin Fallavier, France). HLA-DR genotyping was performed with the Gold SSP DRB1 typing kit (Invitrogen, Cergy, France).

2.6. Generation and specificity of antigen-specific T-cell lines

Immature and mature dendritic cells (DCs) were generated from plastic-adherent PBMCs by culture for five days in AIM-V medium supplemented with 1000 U/ml of rh-GmCSF (Miltenyi Biotec, Paris) and 1000 U/ml of rh-IL-4 (R&D Systems). LPS (Sigma) (1 µg/ml) was used as a maturation agent. CD4⁺ T lymphocytes were isolated by positive selection on –CD4 antibody-coated immunomagnetic beads (Miltenyi Biotec, Paris, France). They were diluted in 1 ml of complete IMDM medium (Invitrogen) supplemented with 0.24 mM glutamine, 0.55 mM asparagine, 1.5 mM arginine (all amino acids from Sigma), 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen), and 10% human serum. Immature DCs were incubated overnight with inactivated VACV and LPS, at a MOI of 1. Mature DCs were incubated with a pool of peptides, each at a concentration of 10 µg/ml. Pulsed DCs (10⁴) were washed and added to 10⁵ autologous CD4⁺ lymphocytes in 200 µl of complete IMDM supplemented with 1000 U/ml IL-6 (R&D Systems, Abingdon, UK) and 10 ng/ml IL-12 (R&D Systems) per well of a round-bottomed microwell plate, to generate either VACV-specific T-cell lines with DCs loaded with VACV or peptide-specific T-cell lines with DCs loaded with the peptide pool. The CD4⁺ T lymphocytes were restimulated on days 7, 14 and 21 with autologous DCs loaded with the peptide pool and were grown in complete IMDM medium supplemented with 10 U/ml IL-2 and 5 ng/ml IL-7 (R&D Systems). Peptide-specificity of the CD4⁺ T cells was investigated by IFN-γ enzyme-linked immunosorbent (EliSpot) assays carried out between days 28 and 32, as previously described (Castelli et al., 2007). VACV specificity was assessed with immature DCs loaded with inactivated virus by incubation at a MOI of 100 for 4 h. T cell lines were considered specific if the number of spots produced in the presence of antigens was at least twice that produced in the absence of antigens, with a minimum of 20 spots.

3. Results

3.1. VACV-specific T cell lines are stimulated by overlapping peptides derived from B10R, B7R and E7R

By comparing the conserved sequences of VARV and VACV, we identified three long conserved sequences that were present in multiple VACV genomes but absent from all the VARV genomes considered (Table 1). These sequences were encoded by the B10R, E7R and B7R genes of VACV and varied in length between 115 and 158 amino acids. We investigated whether these sequences could participate to a CD4 response specific for VACV, by deriving VACV-specific T cell lines and assessing their stimulation by pools

Table 1
Selection of VACV sequences absent from VARV.

Name	Length	Sequence
B10R 40–166	126 AA	IVVNNMEKLDIYKDKQWSIHEMPMARVYHGIDSTFGMLYFAGGLSVTEQYGNLEKNNEISCYNPRTNKWFDISYTIYKISSLCKLNNVYFVFSKDIGYVEKYDGA-WKLVHDLRPAIKALSTSPY
B7R 1–158	158 AA	MYKKLITFLFVIGALASYSNNEYTPFNKLSVKLYIDGVNIENSYTDDNNELVLFNKFEYTSIITESCDVGFSDSIDIDVINDYKIIMDYIDSSTIQRRGHTCRISTKLSCHYDKYPYIHKYDGDERRQYSITAEKGYKGIKYEISMINDDTLLRKHT
E7R 16115	115 AA	MGTAATIQTPTKMLMNKENAEMILEKIVDHIVMYISDESSDENNPEYIDFRNRYEDYRSLIISDHEFVKLCKNHAEKSSPETQQMIKHIEYQLIPVSEVLLKPIMS-MGDIIT

Vaccinia sequences were retrieved from the Poxvirus Bioinformatics Resource Center (<http://www.poxvirus.org/>) and selected on the basis of their conservation in VACV strains and their absence from VARV, as described in Section 2.

of peptides encompassing the B10R 40–166, B7R 1–158 and E7R 1–115 fragments. Twelve peptide pools were constituted, each containing 10–11 overlapping 15-mer peptides. VACV-specific T cell lines were obtained by weekly rounds of stimulation with autologous DCs loaded with inactivated VACV. Six healthy donors with different HLA-DRB1 typing were included in this study (Fig. 1A). Between four and eight polyclonal T cell lines per donor were found to be specific for VACV. Some T cell lines also responded to the peptide pools. B10R peptide pools were found to be more likely to stimulate T cells than E7R and B7R pools (Fig. 1A). In a second EliSpot experiment, we assessed the ability of specific T cells to recognize individual peptides from the active peptide pools (Fig. 1B). For example, T cell line 563.4 was specific for VACV and for the B10R 130–164 peptide pool. In the second EliSpot, this T cell line was found to be specific for the B10R 145–159 peptide. The VACV-specific T-cell line 570.19 was stimulated with the B7R 1–40 peptide pool (Fig. 1C). Two peptides, B7R 11–25 and B7R 21–35, were identified as the most active, whereas peptide B7R 1–15 yielded the lowest level of T-cell stimulation. By this approach, we identified 12 CD4 T cell epitopes in the B7R and B10R fragments: B10R 75–89, 110–124, 145–159, 150–164 and B7R 1–15, 11–25, 21–35, 46–60, 51–65, 56–70, 61–75, 71–85. However, no individual peptide was identified in the E7R fragment, although multiple T cell lines reacted with E7R peptide pools.

3.2. B10R and B7R T cell epitopes display broad specificity for HLA-DR molecules

A response to the identified B7R and B10R T cell epitopes was observed in donors with different HLA-DR haplotypes (see legend Fig. 1). We therefore investigated the binding of these epitopes to various common HLA-DR molecules (Table 2). HLA-DR are the HLA class II molecules most frequently involved in the antigen-specific response. We selected HLA-DR molecules with a high worldwide frequency for these experiments, including seven HLA-DR molecules encoded by HLA-DRB1 genes and the three corresponding 2nd HLA-DR molecules (DRB3, DRB4 and DRB5). All these alleles are present at a frequency of over 5% in the Caucasian population (Table 2) and are present in 85% of individuals. They are also very common in other populations. The four T cell epitopes present in the B10R fragment bound five to eight HLA-DR molecules. The B10R 145–159 peptide had a very low binding ratio and was therefore identified as a very good promiscuous binder. Three of the peptides derived from B7R fragments (1–15, 21–35 and 51–65) bound to six to seven HLA-DR molecules, whereas the other five peptides bound to one to three HLA-DR molecules only. Four B10R peptides and three B7R peptides were therefore expected to elicit a T cell response in a large proportion of individuals.

3.3. Four B10R and E7R T cell epitopes had high levels of immunoprevalence

We then evaluated the capacity of the B10R and B7R peptides to stimulate T cells from multiple donors. CD4⁺ T cells were seeded

in 96-well plates and were stimulated weekly with mature DCs loaded with either B7R or B10R T cell epitopes. Peptide-specific CD4 T lymphocytes were amplified by three weekly stimulations and characterized by IFN-γ EliSpot. Nine healthy donors were included

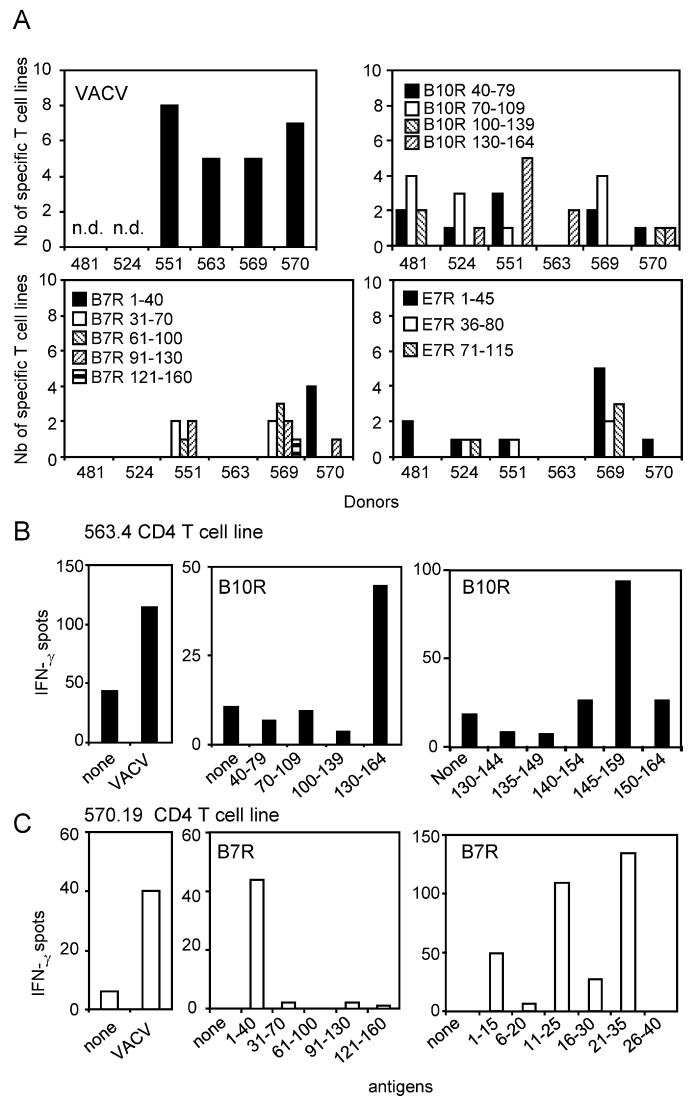


Fig. 1. Peptide specificity of VACV-specific T cell lines. VACV-specific T cell lines were derived from blood samples collected from six different healthy donors, by weekly *in vitro* stimulation with VACV-loaded dendritic cells. HLA-DRB1 typing was performed for four donors: #551, 0404, 1501; #563, 0102, 0301; #569, 0301, 1101; #570, 0401, 1101. T cell line specificity was assessed by IFN-γ EliSpot with immature dendritic cells loaded with inactivated VACV and PBMCs loaded with peptide pools or individual peptides. (A) Number of T cell lines specific for VACV and the B10R, B7R and E7R peptide pools. The specificity of the T cell lines 563.4 (B) and 570.19 (C) was evaluated in two successive EliSpot experiments. VACV- and peptide pool-specificity was evaluated in the first EliSpot assay (first and second panel), whereas individual peptide specificity was investigated in a second EliSpot assay (third panel).

Table 2
Affinity of binding to HLA-DR molecules of B10R and B7R epitopes.

Proteins	Peptides	Relative binding affinity										Bound HLA II
		DR1 18%	DR3 21%	DR4 11%	DR7 26%	DR11 18%	DR13 12%	DR15 15%	DRB3 18%	DRB4 48%	DRB5 15%	
B10R	75–89	1	17	655	6	600	>3464	2	157	19	124	5
	110–124	12	1	23	1	9	>3464	3	9	8	104	8
	145–159	55	1	2	2	1	12	231	0.4	>2321	45	8
	150–164	8	>861	11	126	183	1477	68	242	2	43	5
B7R	1–15	8	1	289	12	29	>3464	11	10	2	13	7
	11–25	2000	426	645	107	>1217	214	1	>1598	>2321	>28787	1
	21–35	25	44	1080	10	0.1	90	59	>1598	>2321	18	7
	46–60	>4472	>861	>1543	>1826	516	>3464	15	>1598	>2321	>28787	1
	51–65	1600	1	104	8	911	21	0.4	57	3	1151	6
	56–70	122	>861	8	115	72	>3464	432	>1598	>2321	>28787	2
	61–75	33	467	873	316	2000	2182	467	>1598	0.1	>28787	2
	71–85	>4472	>861	>1543	58	236	1279	3	19	>2321	>28787	3

The 12 T cell epitopes identified in the B10R and B7R fragments were subjected to competitive ELISA. Data are expressed as relative activity (ratio of the IC₅₀ of the peptide to the IC₅₀ of the reference peptide, which binds strongly to the HLA II molecule). Relative activities less than 100 are shown in bold and indicate a binding. Means were calculated from at least two independent experiments. Percentages are the phenotypic frequencies of the HLA-DR alleles in the Caucasian population.

Table 3
CD4⁺ T cell response induced *in vitro* by B10R peptides.

Donor	HLA-DR typing		Number of T cell lines			
	DRB1	2nd DR	75–89	110–124	145–159	150–164
#577	0101, 0701	DRB4				
#580	0701, 1101	DRB3, B4			1	1
#600	0101, 0301	DRB3, B4			1	
#604	0101, 1601	DRB5		1	3	2
#613	1104, 1301	DRB3				
#631	0404, 1104	DRB3, B4		1		1
#632	0301, 1501	DRB3, B5				
#633	0101, 1101	DRB3			3	
#634	0801, 0901	DRB4				2
		Total	0	2	8	6
		Responder frequency	0/9	2/9	4/9	4/9
			0%	22%	44%	44%

CD4⁺ T cell lines from nine healthy donors were obtained by weekly stimulation with autologous mature dendritic cells loaded with the pool of B10R peptides. Their peptide specificity was assessed by IFN- γ ELISpot assays, with autologous PBMCs used as the antigen-presenting cells.

in these experiments. These donors were selected on the basis of HLA-DRB1 typing, to ensure that all the common HLA-DRB1 allotypes were present (Table 3). Each peptide-specific T cell line was essentially specific for a single peptide (Fig. 2). T cell lines 604.18 and 600.3 were specific for the same peptide, B10R 145–159, whereas T cell line 604.22 was specific for the B10R 110–124 peptide. Two peptides (B10R 145–159 and B10R 150–164) specifically stimulated T cell line 604.16, but were overlapping. Several T cell lines (604.60, 604.87 and 604.89) recognized a common peptide

derived from the B7R protein (B7R 21–35). Other B7R peptides induced specific T cell lines, as illustrated by T cell line 604.43, which was stimulated by the peptide B7R 51–65. Only one peptide derived from the B10R protein was completely inactive (Table 3). The B10R 145–159 and 150–164 peptides induced eight and six T cell lines, respectively, and these two peptides were each immunogenic with cells from four different donors. Two T cell epitopes also emerged as the most T cell stimulating from the B7R peptides, namely 21–35 and 61–75 (Table 4). All other peptides induced

Table 4
CD4⁺ T cell response induced *in vitro* by B7R peptides.

Donor	Number of T cell lines							
	1–15	11–25	21–35	46–60	51–65	56–70	61–75	71–85
#577							1	1
#580			1					
#600			1					
#604		1	13	1	1	4	2	2
#613			1	1	1	1	1	
#631								
#632		1			3		1	
#633			3					
#634							1	
Total	0	2	19	2	5	5	6	3
Responder frequency	0/9	2/9	5/9	2/9	3/9	2/9	5/9	2/9
	0%	22%	56%	22%	33%	22%	56%	22%

Same legend as for Table 3.

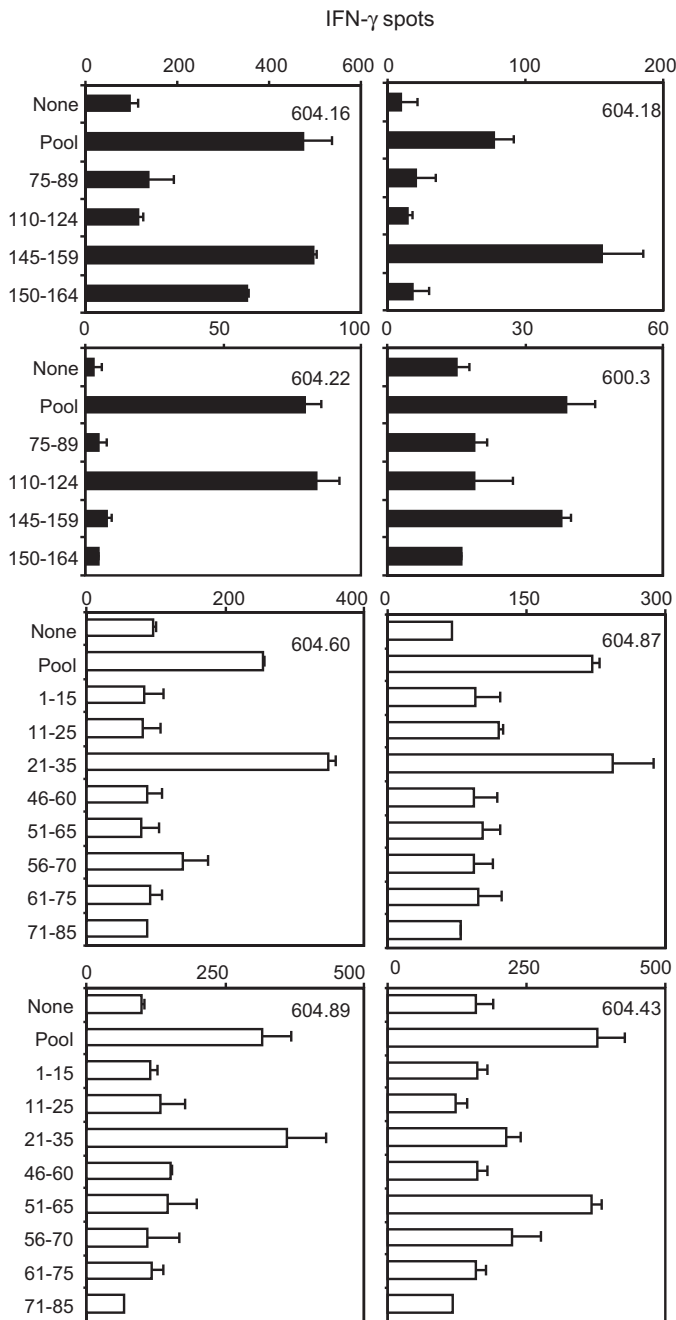


Fig. 2. Peptide specificity of T cell lines induced with the B10R and B7R T cell epitopes. The T cell lines presented in this figure were from two donors (600 and 604). They were produced by weekly *in vitro* stimulation with mature dendritic cells loaded with a pool of either B10R (black) or B7R (white) peptides identified as T cell epitopes with VACV-specific T cell lines. The specificity of the T cell lines was assessed by IFN- γ EliSpot with PBMCs loaded with peptide pools or individual peptides.

a reduced number of T cell lines and in a few donors only. We therefore found that among the B7R and B10R T cell epitopes, four peptides (B10R 145–159, B10R 150–164, B7R 21–35 and B7R 61–75) stimulated T cells for approximately half of the donors. We characterized the HLA-DR molecules supporting the presentation of these epitopes to T cells, using L cells stably transfected with HLA-DR-encoding plasmids corresponding to the HLA typing of the donors, as antigen-presenting cells. B7R 21–35 was presented by HLA-DR1 to T cell line 633.33 and by HLA-DR11 to T cell lines 613.53 and 633.37 (Fig. 3). The B7R 61–75-specific T cell line 577.44

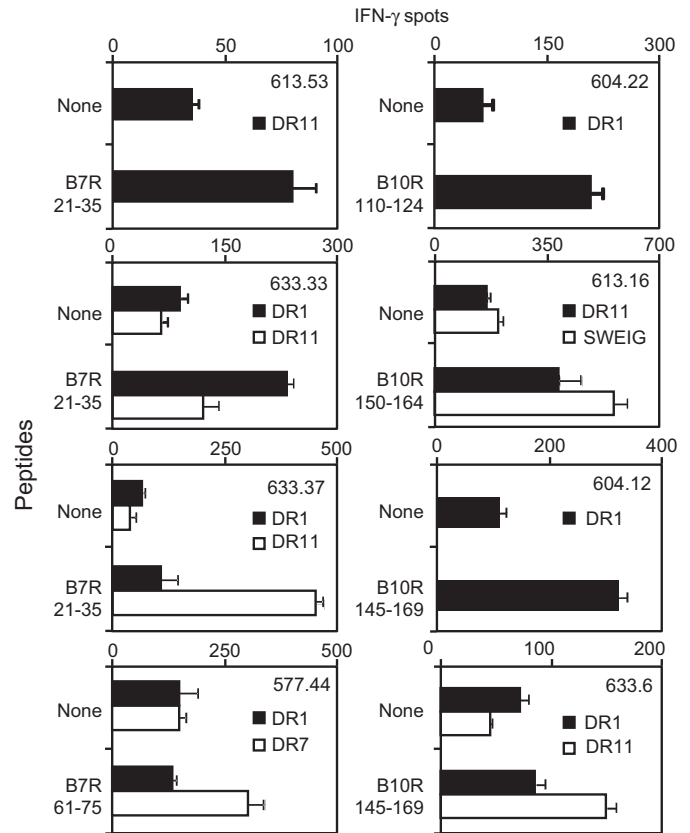


Fig. 3. HLA-DR restriction of B10R and B7R T cell epitopes. Peptide-specific T cell lines were incubated with HLA-DR transfected L cells or homozygous EBV cell line in the presence or in the absence of the appropriate peptide. Their activation was investigated by IFN- γ EliSpot.

was restricted to HLA-DR7, while B10R 110–124 was restricted to HLA-DR1. T cell line 613.16 recognized the B10R 150–164 peptide presented by HLA-DR11-transfected L cells and the homozygous HLA-DR11 EBV cell line SWEIG. Finally, the B10R 145–169 peptide was presented by HLA-DR1 and HLA-DR11 to T cell lines 604.12 and 633.6, respectively. Consistent with their broad specificity for HLA-DR molecules, the four peptides thus appear to be restricted to different HLA-DR molecules.

4. Discussion

We have mainly identified four CD4 T cell epitopes present in VACV but absent from VARV. These epitopes can be combined in three fragments: B10R 145–164, B7R 21–35 and B7R 61–75. The four CD4 T cell epitopes are recognized by VACV-specific T cell lines, bind to different HLA-DR molecules and induce specific T cell lines from multiple donors after *in vitro* stimulation. There is currently no unambiguous immunological approach to discriminate infections by *Orthopoxvirus* species. The detection of a CD4 T cell response specific for these peptides is therefore potentially useful in this respect.

The four VACV-specific CD4 T cell epitopes were identified by comparing the conserved parts of the genomes of Vaccinia and Variola viruses and the subsequent screening of overlapping peptides for their ability to stimulate VACV-specific T cells. These T cell epitopes were derived from the B7R and B10R proteins (Goebel et al., 1990). E7R was also found to contain VACV-specific CD4 T cell epitopes, but we were unable to identify these epitopes, due to the small number of E7R-specific T cells available for further studies. The B7R protein has been reported to be produced late

Table 5
Sequence homology of the B10R 145–164, B7R 21–35 and B7R 61–75 T cell epitopes.

Species	Strain	B10R 145-164	
VACV	Western Reserve	GAWKLVHDLRLEPAIKALSTSPY	
VACV	Copenhagen	GAWKLVHDLRLEPAIKALSTSPY	
VACV	Lister	GAWKLVHDLRLEPAIKALSTSPY	
VACV	Acambis3000 MVA	G-----LEPAIKALSTSPY	
CPXV	Brighton Red	GAWKLVHDLRLEPAIKAFSTSPY	
MPXV	USA_2003	-----	
VARV	Bangladesh 1975	-----	
Species	Strain	B7R 21-35	B7R 61-75
VACV	Western Reserve	NEYTPFNKLSVKLYI	ISIIITESCDVGFDSI
VACV	Copenhagen	NEYTPFNKLSVKLYI	ISIIITESCDVGFDSI
VACV	Acambis3000 MVA	NEYTPFNKLSVKLYI	ISIIITESCDVGFDSI
CPXV	Brighton Red	NEYTPFNKLSVKLYI	VSIIITESCDIGFDSI
MPXV	USA_2003	NEYTPFNKLSVKLYI	ISIIITESCDVGFDSI
VARV	Bangladesh 1975	-----	-----

VACV, Vaccinia virus; CPXV, Cowpox virus; MPXV, Monkeypox virus; VARV, Variola virus. “-”: absence of amino acid.

in the viral cycle and to contribute to virus virulence (Price et al., 2000). Oseroff et al. identified this protein as the target of CD8 T cells restricted to HLA-A3 and A11 molecules (Vita et al., 2010), but to our knowledge no CD4 T cell epitopes have been reported for this protein. Both the B7R 21–35 and B7R 61–75 peptides are highly conserved among the *Orthopoxvirus* species, including MVA and the Cowpox and Monkeypox viruses (Table 5). The detection of CD4 T cell responses specific for these peptides would not discriminate VACV vaccination from infection with Monkeypox or Cowpox but would exclude VARV infection. B10R has been described as a Kelch-like protein, but its function remains unknown (Goebel et al., 1990). B10R-specific CD8 T cell epitopes have been described in humans (Vita et al., 2010) and mice (Oseroff et al., 2008), but no CD4 T cell epitopes specific for B10R have been characterized. The B10R 145–164 sequence is profoundly altered in MVA strains, because the initial *in silico* screening of peptide sequences included replication-competent viruses, only (Table 5). The central part of the corresponding sequence is also mutated in Cowpox. As a result, the detection of a CD4 T cell response specific for this peptide appears to be highly selective for Vaccinia and rules out the possibility of infection with the Variola and Monkeypox viruses. However, it cannot identify cases of vaccination with MVA, a current vaccine strain. Nevertheless, it may be useful for detecting cases of vaccination with other VACV strains and of zoonotic diseases triggered by VACV infections, as observed in Brazil (Essbauer et al., 2010).

The four CD4 T cell epitopes described here were identified on the basis of their ability to stimulate the production of VACV-specific T cell lines. They are thus involved in the VACV T cell response and hence correspond to so-called immunodominant epitopes (Oseroff et al., 2008). As illustrated by the responder frequency in T cell stimulation assays (Tables 2 and 3), they also correspond to immunoprevalent CD4 T cell epitopes (Castelli et al., 2008; Oseroff et al., 2008). Five of nine donors responded to peptides B7R 21–35 and B7R 61–75, whereas the B10R 145–159 and 150–164 peptides together induced a T cell response in six of nine donors. These donors exhibit multiple HLA typing, including very common allotypes. Consistent with their capacity to elicit a response from multiple donors, the four peptides identified displayed broad binding specificity for very common HLA class II molecules. All the HLA-DR molecules introduced in the assays are present at a frequency of over 5% in the Caucasian population and are present in 85% of individuals. Moreover, it seems that at B10R 150–164 and B7R 61–75 can be restricted to other HLA-DR molecules, as they induce specific T cells in donors #631 and #634. Both donors exhibited HLA-DR molecules we did not introduce in the binding assays. It remains unclear which population of naïve or memory T cells is activated by these peptides. Their T cell reactivity has been revealed after several rounds of *in vitro* stimulation and, thus, in culture conditions compatible with the priming of naïve T cells (Castelli et al., 2007, 2008). VACV-specific antibodies were

detected for several donors (not shown), suggesting that the residual memory VACV-specific T cells may also have been amplified by the rounds of stimulation.

The CD4 T cell epitopes identified here are of potential diagnostic interest, due to their sequence selectivity. Current approaches to the diagnosis of poxvirus infection are based on the clinical symptoms and the detection of the virus by PCR or electron microscopy (Bremant and Henderson, 2002). Both assays require the presence of active virus and therefore exclude patients whose infection has resolved spontaneously. This could be a limitation to evaluate the extent of an outbreak. Half the subjects infected with the Monkeypox virus initially remained undiagnosed in the US outbreak (Hammarlund et al., 2005). These subjects would be expected to react with Monkeypox-selective peptides, whereas a T cell response specific for VACV would be revealed by reactivity to the B10R 145–164 peptide, except for the MVA strain. However, the difficulty of demonstrating the potential diagnostic value of the B7R and B10R peptides lies in recruiting appropriate donors with a well-defined immune status for poxvirus infection, including subjects recently vaccinated with VACV. This would make it possible to evaluate peptide reactivity by *ex vivo* ELISpot without the need for several rounds of stimulation. It would be also interesting to evaluate the polyfunctionality of these T cells. Moreover, selective peptides from other Orthopoxviruses are also needed and we are currently investigating whether VARV-selective sequences are the target of the CD4 T cell response. Finally, although not characterized yet, selective CD8 T cell epitopes might also exist and could be advantageously used to cover a larger part of the population, several HLA-A and -B alleles being very common.

In conclusion, we have identified four CD4 T cell epitopes selective for VACV and in particular absent from VARV genome. This is of particular importance because most of the VACV-derived CD4 T cell epitopes identified to date are highly conserved among the Orthopoxviruses (Sette et al., 2009). These previously identified peptides are of potential use for vaccination, but our more selective peptides have potential value for use in diagnosis. Well controlled vaccination trials are now required to validate this possibility.

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