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Preventing vaccinia virus class-I epitopes presentation by HSV-ICP47 enhances the immunogenicity of a TAP-independent cancer vaccine epitope

Nermin Raafat[1,2], Charlotte Sadowski-Cron[3], Chantal Mengus[1], Michael Heberer[1], Giulio C. Spagnoli[1] and Paul Zajac[1]

[1] Department of Biomedicine, Oncology group, Institute of Surgical Research and Hospital Management, University of Basel, Switzerland
[2] Department of Biochemistry and Molecular Biology, Faculty of Medicine, Zagazig University, Zagazig, Egypt
[3] Department of Thoracic Surgery, University Hospital of Basel, Switzerland

Herpes simplex virus protein ICP47, encoded by US12 gene, strongly downregulates major histocompatibility complex (MHC) class-I antigen restricted presentation by blocking transporter associated with antigen processing (TAP) protein. To decrease viral vector antigenic immunodominance and MHC class-I driven clearance, we engineered recombinant vaccinia viruses (rVV) expressing ICP47 alone (rVV-US12) or together with endoplasmic reticulum (ER)-targeted Melan-A/MART-1_{27-35} model tumor epitope (rVV-MUS12). In this study, we show that antigen presenting cells (APC), infected with rVV-US12, display a decreased ability to present TAP dependent MHC class-I restricted viral antigens to CD8+ T-cells. While HLA class-I cell surface expression is strongly downregulated, other important immune related molecules such as CD80, CD44 and, most importantly, MHC class-II are unaffected. Characterization of rVV-MUS12 infected cells demonstrates that over-expression of a TAP-independent peptide, partially compensates for ICP47 induced surface MHC class-I downregulation (30% *vs*. 70% respectively). Most importantly, in conditions where clearance of infected APC by virus-specific CTL represents a limiting factor, a significant enhancement of CTL responses to the tumor epitope can be detected in cultures stimulated with rVV-MUS12, as compared to those stimulated by rVV-MART alone. Such reagents could become of high relevance in multiple boost protocols required for cancer immunotherapy, to limit vector-specific responsiveness.

The infected cellular protein 47 (ICP47) also known as immediate early protein 12 (IE12) is encoded by the US12 gene, which is one of the unique short (US) region genes of the HSV genome. ICP47 is an 88 amino acid cytosolic polypeptide which is considered as a key factor in the evasion of HSV-infected cells from cellular immune response. By binding its 3–34 residues[1] to the cytosolic faces of the TAP protein subunits TAP1 and TAP2, ICP47 inhibits the translocation of degradation peptides across the membrane of the endoplasmic reticulum (ER) and prevents their subsequent loading onto major histocompatibility complex (MHC) class-I molecules and recognition by CD8+ T cells.[2] In the absence of a functional TAP, peptide loading onto MHC class-I molecules is inhibited and, as a consequence, empty MHC class-I molecules are retained in the ER.[3]

VV was first used for global smallpox eradication in the early 1980s, but still serves, at present time, as useful recombinant viral vector. Several unique features of VV including safety, wide host range, efficient infection and gene expression, stability, accommodation of large DNA sequence, cytoplasmic replication and ease of administration make it an excellent choice as a vaccine vehicle *in vivo*.[4] In particular, VV represents a delivery vector of choice for cancer immunotherapy. Indeed, a number of cancer vaccines based on VV vectors have shown promising results in preclinical animal models and numerous clinical trials.[5,6]

Despite these advantages, VV used as a gene therapy vector for the delivery of tumor antigens and immunoregulatory molecules has encountered limited success in cancer immunotherapy.[7] Part of this limited efficacy could come from the vector-specific high immunogenicity and long-term memory response to VV which may limit the possibility and efficacy of repeated injections of the virus in prime-boost regimens.[8,9]

Key words: ICP47, vaccinia virus, viral vectors, cancer vaccines
Abbreviations: APC: antigen presenting cells; EBV-BL: Epstein-Barr virus transformed human B-lymphocytes; ER: endoplasmic reticulum; MFI: mean fluorescent intensity; MHC: major histocompatibility complex; NEAA: nonessential amino acids; PBMC: Peripheral blood mononuclear cell; qRT-PCR: quantitative real time PCR; TAA: tumor associated antigens; TEIPP: T-cell epitopes associated with impaired processing
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Correspondence to: Paul Zajac, ICFS, laboratory 404, Hebelstrasse, 20, CH-4031, Basel, Switzerland, Tel.: 0041-0-61-265-2330, Fax: 0041-0-61-265-3990, E-mail: pzajac@uhbs.ch

An additional concern is represented by the immunodominance and immunoprevalence of vaccinia-specific cytotoxic T-lymphocyte (CTL) responses, even in patients who have not previously received smallpox vaccination. Monitoring the magnitude and duration of T-cell responses to vaccinia viral vector, as compared to a foreign recombinant epitope, has shown that the total number of CD8+ cells responding to foreign epitopes is 20- to 30-fold lower than the number of T cells responding to the VV vector.[10,11] On the other hand, the antigenicity of vaccinia virus is of importance in providing, *e.g.*, MHC class-II restricted helper signals. An ideal viral vaccine vector should be safe, efficiently present pathogen or tumor associated antigens (TAA), but it should have a low intrinsic class-I immunogenicity. The latter feature is of peculiar relevance for multiple boost protocols.

Our group has previously generated recombinant vaccinia virus expressing antigenic epitopes derived from three different melanoma differentiation antigens, Melan-A/MART-1_{27-35}, $GP100_{280-288}$ and $tyrosinase_{1-9}$. The antigenic epitopes are encoded together with a polypeptide from adenovirus 19K derived leader sequence (MRYMILGLLALAAVCSA) driving the resulting recombinant products directly into the ER and thereby bypassing antigen processing steps. Genes encoding CD80 and CD86 co-stimulatory molecules, required for T cell activation, have also been added to the vector. After *in vitro* demonstration of its effectiveness,[12,13] this vector (Penta-Mel-rVV) was successfully administered intradermally, followed by boosts with the corresponding specific peptides in solution, in Phase I/II immunotherapy clinical trials for Stage III and IV melanoma patients.[14] A second clinical trial, based on the intranodal administration of the Penta-Mel-rVV was also performed.[15] The results of these two clinical studies show that despite a good immunogenicity of the viral vector, peptide injection is unable to adequately boost the virus induced responses, suggesting that an increased number of viral boosts would be needed to improve the immunization efficiency.

In this study, we aimed at diminishing the MHC Class I restricted CTL response to the viral vector leading to a prolong survival of rVV infected antigen presenting cells (APC). To achieve this goal, the herpesvirus US12 gene was inserted into recombinant VV vector alone or in combination with a TAP-independent TAA minigene (MART-1/Melan-A_{27-35}). In APC infected with nonreplicating US12-recombinant VV, the presentation of most TAP-dependent epitopes derived from viral proteins should be blocked in the cytosol. Therefore, US12 encoded protein (ICP47) may simultaneously decrease epitope competition and CD8+ anti-vector responses. On the other hand, recombinant ER-targeted vaccine epitopes should not be affected by ICP47 and, profiting from reduced competition and longer survival of infected APC, might induce transgene specific CTL responses with an increased efficiency.

Material and Methods

Cells

CV-1 cells (African green monkey kidney fibroblasts), grown in DMEM 5% FCS, were used for the generation, amplification and titration of the different recombinant vaccinia virus used.

HLA-A0201 positive human Na-8 MEL (melanoma cell line, courtesy of Dr. Jotereau, Nantes, France) and skin-derived fibroblast (generated in the laboratory) were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FCS, 100 μg/ml Kanamycin, 2 mM Glutamine, 1M HEPES, MEM nonessential amino acids (NEAA) and 100 mM Sodium Pyruvate (all from Invitrogen, Carlsbad, CA). HLA-A0201 negative HeLa and Calu-6 cell lines (ATCC, Rockville, MD) were also cultured in DMEM 10% FCS.

Epstein-Barr virus transformed human B-lymphocytes (EBV-BL) were cultured in complete RPMI 1640 medium supplemented with the above mentioned reagents. Peripheral blood mononuclear cells (PBMCs), from HLA-A0201 positive healthy donors, were prepared by gradient centrifugation. $CD14^+$, $CD4^+$ and $CD8^+$ cells were positively selected from total PBMCs by magnetic absorbent cell sorting technique using anti-CD14, CD4 and CD8 antibodies bound microbeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). $CD14^+$, $CD4^+$ and $CD8^+$ cells were cultured in complete RPMI 1640 medium supplemented with 5% human serum (CM).

Gene expression analysis

The expression of specific genes was evaluated by quantitative real time PCR (qRT-PCR), as previously described.[16] Briefly, cells were collected, washed in PBS and total cellular RNA was extracted by using the RNeasy Mini Kit protocol (Qiagen, Basel, Switzerland). Following deoxyribonuclease I (DNase I) (Invitrogen) treatment, cDNA was prepared by reverse transcription using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLT RT, Invitrogen). PCR was performed in an ABI prism 7300 real time PCR system, using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and the following specific primers and probes: IFN-γ, IL-2 gene specific reagents were described in a previous study,[16] β-actin reagents are from a commercial pre-developed assay (Applied Biosystem, Foster City, CA),

US12.

Fwd AAA GGA TCC GCA TGT CGT GGG
Rev AAA GAA TTC TCA ACG GGT TAC CGG ATT ACG
Probe FAM-TCG GTC ACG GTC CCG CCG-TAMRA

US12 (Nest).

Fwd AGG TGC GTG AAC ACC TCT G
Rev GTG GAC CGC TTG CTG CTC

Normalization of US12 or cytokine gene expression was performed by using β-actin as a reference gene and data

were expressed as ratio to a reference sample ($2^{-\Delta\Delta Ct}$ method).

Construction of US12 recombinant vaccinia virus (rVV-US12 and rVV-M_{27-35}-US12)

DNA samples from cerebrospinal fluid from HSV positive patients were kindly provided by Prof. M.E. Lafon (Laboratoire de Virologie, University Hospital of Bordeaux, Bordeaux, France). US12 gene was amplified using US12 specific oligonucleotides by nested PCR technique to reduce contamination in the PCR product. US12 gene was inserted by BamHI and EcoRI restriction into a vaccinia A56R shuttle plasmid under control of a vaccinia-specific early promoter and transcriptional termination sequence.[17] The plasmid containing US12 gene was inserted by homologous sequence recombination, as previously detailed[18] into the wild type (WT, Copenhagen strain) vaccinia virus and the rVV-$MART_{27-35}$ construct. The resulting constructs, rVV-US12 and rVV-$MART_{27-35}$-US12 (named rVV-MUS12), were amplified on CV-1 cells and titered by plaque forming assays. For all viruses used in this study as nonreplicating VV, DNA replication was prevented by psoralen UV inactivation as described in previous work.[16]

In vitro specific T-cell amplification

After magnetic beads sorting, 10^6 CD8+ or CD4+ T-cells were mixed in 2 ml CM with autologous infected CD14+ cells (ratio effector:APC of 1:1). After 3–4 days, culture media were refreshed with CM-IL2 (100U /ml). After 1 week culture, T-cells were restimulated with 10^6 autologous CD14+ either infected or peptide pulsed (20 μg/ml) in CM-IL2 and incubated at 37°C for another week before characterization of the specific responses.

Phenotypic studies

Fluorochrome labelled monoclonal antibodies (mAb) recognizing human HLA-ABC, HLA-A0201, CD14, CD4, CD8, CD80, CD44 and HLA-DR molecules and corresponding control IgG (all from BD Pharmingen, Franklin Lakes, NJ) were used. Soluble MHC-peptide, streptavidin R-PE conjugated pentamers (ProImmune, Oxford, UK) included HLA-A0201 containing L27MART-1/Melan-A_{26-35} or vaccinia virus $H3L_{184-192}$, $B22R_{29-37}$ and $C7L_{74-82}$ peptides.

In particular, regarding MHC class-I, MHC class-II, CD44 and CD80 surface expression, infected cells were stained with specific and control mAb, incubated for 30–45 min at 4°C in the dark, washed twice in cold PBS, fixed 1' in 1% paraformaldehyde, resuspended in 200 μl PBS and then analyzed on a FACSCalibur® cytometer (Becton Dickinson, Franklin Lakes, NJ). Multimer staining was performed according to manufacturer protocol (ProImmune, Oxford, UK) at room temperature in the dark for 10 min followed by an additional CD8 specific staining for 20min at +4°C. For each sample, the measured mean fluorescent intensity (MFI) is represented as a ratio to the MFI of the noninfected control population (100%) according to the formula: Sample value = (MFI sample – MFI IgG control)/(MFI noninfected – MFI IgG).

Intracellular cytokine staining

Cultured cells were resuspended in 1 ml CM and Brefeldin was added to a final concentration of 10 μg/ml. Cells were then incubated for 5 h at 37°C. Subsequently, they were washed twice in PBS 2 mM EDTA supplemented with 2% FCS. The pellet was resuspended in 2 ml paraformaldehyde 1% for 5 min at room temperature and washed. Cells were finally resuspended in 500 μl FACS™ permeabilizing solution (BD, Pharmingen, Franklin Lake, NJ) diluted 1/10 in H_2O. They were gently vortexed and incubated for 10 min at room temperature. Fifteen microliters of IFNγ specific antibody (BD, Pharmingen, Franklin Lake, NJ) were added to the pellet. After 30–45 min incubation at 4°C in the dark, cells were washed twice in cold PBS, fixed 1 min in paraformaldehyde 1%, resuspended in 200 μl PBS and analysed by flow cytometry.

Cell proliferation analysis

T lymphocytes stimulated with rVV infected autologous CD14+ cells were cultured in flat bottomed 96-well plates, in a final volume of 200 μl per well for 6 days. 3H-thymidine (1 μCi) was then added to each well and cultures were incubated for 18 h at 37°C. The cells were harvested by a Micro96TM Cell Harvester (Skatron, Sunnyvale, CA). Liquid scintillation cocktail was added (OPTI-FLUOR®, PerkinElmer, Waltham, MA) and light emission was measured in a scintillation counter (TopCount, PerkinElmer, Waltham, MA).

Results

Characterization of ICP47 expression on MHC class-I and nontargeted surface molecules on infected cells

The capacity of the newly engineered and UV-inactivated[19] rVV-US12 and rVV-MUS12 to express US12 gene was first confirmed by qRT-PCR. As expected, levels of US12 mRNA were similarly detected for both constructs and were consistent, with multiplicity of infection values (Supporting Information Figure). Preliminary kinetics experiments in infected Na-8 cells demonstrated that ICP47 induced downregulation of MHC-I surface expression becomes visible after 12 h and reaches its maximal extent 36–48 h after infection (data not shown). The latter time was chosen for the following experiments.

Effects on MHC class-I surface expression of the recombinant ICP47 protein and HLA-A0201 TAP-independent peptide were evaluated by phenotypic characterization in infected cells. As depicted in Figure 1*a*, HLA-A0201 negative Calu-6 cells, infected with inactivated rVV-US12 or rVV-MUS12, showed a significant ($p < 0.05$) and similar decrease of total HLA-ABC expression as compared to cells infected with inactivated WT or rVV-MART control viruses which did not modify surface MHC-I expression as compared to

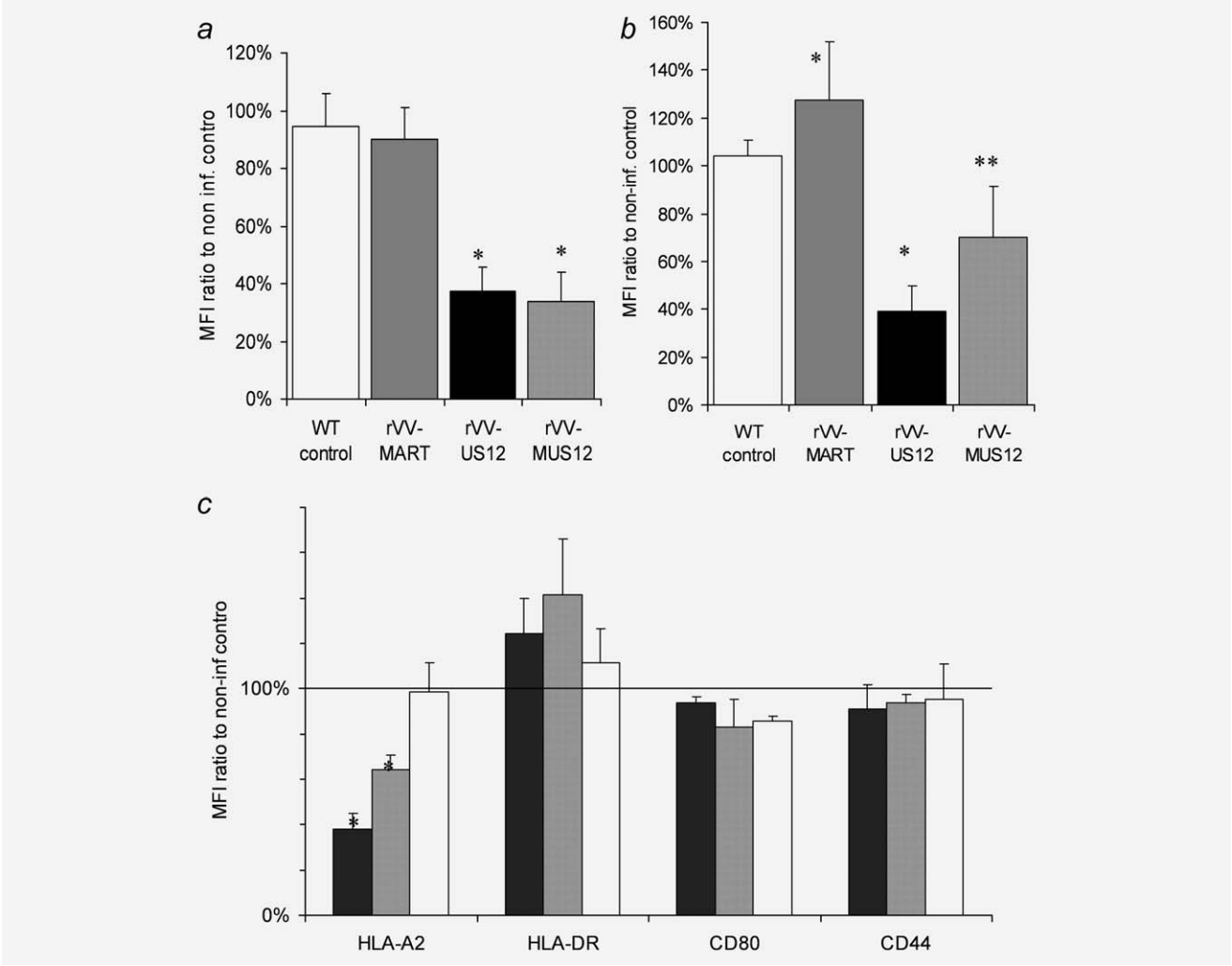

Figure 1. Effect of rVV-US12 infection on ICP47 targeted and nontargeted surface molecules. (*a*) Total MHC-I staining on HLA-A2 negative Calu-6 cells infected with nonreplicating WT-VV, rVV-MART, rVV-US12 or rVV-MUS12 at m.o.i. of 10. Cell surface expression of MHC class I was characterized after at least 36 h infection by staining with a FITC-labelled monoclonal antibody specific for a monomorphic epitope of the HLA-A, -B and -C determinants or with an isotype control antibody. Samples mean fluorescence intensities are normalized following a calculation described in "Material and Methods." (*) indicates a significant ($p < 0.05$) difference from WT control infection. (*b*) Cells surface HLA-A0201 staining of similarly infected HLA-A0201 positive human skin fibroblasts. (**) indicates significant increase as compared to ICP47 expressing counterpart. (*c*) 10^6 EBV-BL were infected with nonreplicating rVV-US12 (black bars), rVV-MART-US12 (stripped bars) or rVV-WT (white bars) at 10 m.o.i. After 48 h, cells were stained by labeled mAbs specific for HLA-A0201, HLA-DR, CD80 or CD44 and analyzed by flow cytometry. Data refer to three independent experiments. Neither HLA-DR, CD80 nor CD44 display significant variation in the different conditions.

noninfected population. These data also confirm that the two viral constructs expressing the TAP-independent HLA-A0201 epitope MART-1/MelanA_{27-35} (rVV-MART and rVV-MUS12), behave similarly as their MART negative counterpart (WT and rVV-US12) in HLA-A0201 negative cells.

Analysis of ICP47 related HLA-A0201 decreased surface expression

HLA-A0201 allele is frequently expressed in different populations and it is known to restrict T cell responses to several TAA epitopes, including Melan-A/MART-1_{27-35}. Therefore, its differential regulation potentially due to ICP47 and ER targeted recombinant MART-1/MelanA_{27-35} epitope expression was also characterized in detail.

Figure 1*b* shows the result of experiments ($n = 16$) performed on different HLA-A0201 positive infected cells with rVV-US12, rVV-MUS12 and control viruses WT or rVV-MART. Similar to total MHC-I showed in Figure 1*a*, HLA-A0201 surface expression in rVV-US12 infected cells was found to be significantly ($p < 0.05$) decreased as compared to all the other conditions. In presence of TAP-independant (ER-targeted) HLA-A0201 MART-1/Melan-A epitope, expressed

by rVV-MART, in agreement with previous reports,[20–22] a significant ($p < 0.05$), increase of HLA-A0201 expression was observed. Most importantly, rVV-MUS12 infection induced a significantly higher surface expression of HLA-A0201 as compared to rVV-US12 infected cells. In this condition, MFI values were 50% higher than in ICP47 containing cells.

The measurable compensation of ICP47 induced downregulation indicates that HLA-A0201 molecules were efficiently loaded with the recombinant TAP independent MART-1/Melan-$A_{27–35}$ peptide. Furthermore, it also suggests that concurrent to the strong inhibition of the surface expression of TAP dependent endogenous peptides, the recombinant epitope might represent a significant fraction of those presented by HLA-A0201 molecules expressed on the cell surfaces.

HLA-DR, CD80 and CD44 in ICP47 expressing cells

Activation of $CD4^+$ T cells is important not only to help the generation and expansion of $CD8^+$ T cell responses but also to mediate anti-tumor effector functions.[23] In this context, presence of cell surface co-stimulatory or adhesion molecules plays decisive roles in the generation of responses to antigenic challenges, steering it toward the induction of effector cells instead of tolerance.[24,25] Based on this background, we verified whether, following r.VV infection, ICP47 over-expression and interference with class-I presentation also affected the expression of other immunoregulatory surface molecules. The phenotypic analysis (Fig. 1*c*) of HLA-A2, DR, CD80 (B7.1) and CD44 on the surface of infected HLA-A0201 positive EBV-BL cell lines confirmed that ICP47 specifically decreases HLA class-I expression (compensated by TAP-independent MART-1/Melan-A peptide in rVV-MUS12), without affecting the expression of any other molecule tested.

Characterization of VV antigen specific CD8+ T cell responses

Next, we investigated the effects of ICP47 expression on the capacity of APC to induce VV specific responsiveness. Healthy donors CD8+ or CD4+ T cells were co-cultured with autologous CD14+ cells infected with rVV-US12 or rVV-MUS12. WT-VV or rVV-MART were alternatively used as controls of vaccinia virus cellular immunogenic profile. All cultures were re-stimulated on Day 8 with WT-VV infected autologous CD14+ cells. On Day 15 after the priming, cells were stained with a pool of MHC-multimers specific for common VV derived HLA-A0201 restricted epitopes, including $H3L_{184–192}$, $B22R_{29–37}$ and $C7L_{74–82}$.[26,27] As shown in Figure 2*a*, similarly to the condition "primed" with noninfected APCs (0.8%), CD8+ T-cells cultures stimulated with rVV-US12 infected APCs only displayed 0.6% positive cells. Sample primed with rVV-MUS12 generated 1.3% MHC-multimer(+) cells while the virus control priming, here rVV-MART, led to the expansion of 4.2% VV antigen specific CD8+ T cells.

Functional characterization of T-cell activation induced, in the same conditions, by VV, was then addressed by IFN-γ intracellular staining. Data reported in Figure 2*b* confirm that following WT VV restimulation, CD8+ T-cell cultures first stimulated with rVV-US12 yield results comparable to those obtained in the "noninfected" condition (0.7% and 0.6%, respectively). Importantly, though rVV-MUS12 appears in this experiment to induce a slightly increased fraction of IFN+ cells (1.1%), this response remains far below the one observed in cultures primed by a non-ICP47 expressing VV control (here rVV-MART).

Quantification of IFN-γ and IL-2 cytokine gene expression was also performed by qRT-PCR. Data from three experiments, reported in Figure 2*c*, confirmed the previous result whereas, upon restimulation, CD8+ T-cell cultures initially primed with rVV-US12 or rVV-MUS12 infected cells expressed about 5- to 10-fold less IFN-γ than those primed by WT-VV. Similar to IFN-γ expression profile, IL-2 gene expression in response to VV re-stimulation in CD8+ primed with rVV-US12 or rVV-MUS12 infected APC was four or two times (27% ± 2% and 48% ± 12%), lower, respectively, than that detectable in cultures initially primed with WT-VV.

These results demonstrate that expression of ICP47 protein strongly inhibits the induction of MHC class-I restricted VV antigen specific CD8+ T cell responses.

CD4+ T cells were also stimulated with autologous CD14+ cells infected with rVV-US12, rVV-MUS12 or WT-VV. Figure 2*d* reports IFN-γ gene expression data from three independent experiments indicating that despite the strong blockade of MHC-class I observed in rVV-US12 and rVV-MUS12 infected APCs (see above), specific CD4+ T cells were activated, *via* MHC-class II presentation, at least as efficiently as by the WT-VV control virus (143% ± 66% and 146% ± 42%, respectively).

Effect of ICP47 on ER-targeted recombinant epitope

In rVV-MUS12 and rVV-MART constructs, the recombinant epitope is formulated in a TAP independent ER-targeted form (E3/19K-MART-1/Melan-$A_{27–35}$). We and others have previously demonstrated that this antigenic formulation for specific HLA restriction can bypass a number of antigen processing steps, promoting surface expression, presentation and immunogenicity of recombinant epitopes.[28]

To address the effect of rVV-MUS12 on MART specific CD8+ T cell stimulation, CTL priming experiments were performed by using PBMC from healthy donors. CD8+ T cell cultures were initially stimulated with autologous CD14+ cells infected with rVV-MART as positive control, rVV-MUS12, or rVV-US12 as negative control. 8 days after priming, cultures were restimulated with peptide pulsed autologous CD14+ cells. On Day 15, resulting $MART_{27–35}$ specific CD8+ T cells were characterized with MHC-pentamers. As shown in Figure 3*a*, our experiments demonstrated that rVV-MUS12 stimulated cells resulted in a significant expansion of MART-1/Melan-$A_{27–35}$ specific CTL, similar to the level induced by the positive control rVV-MART (3.6%).

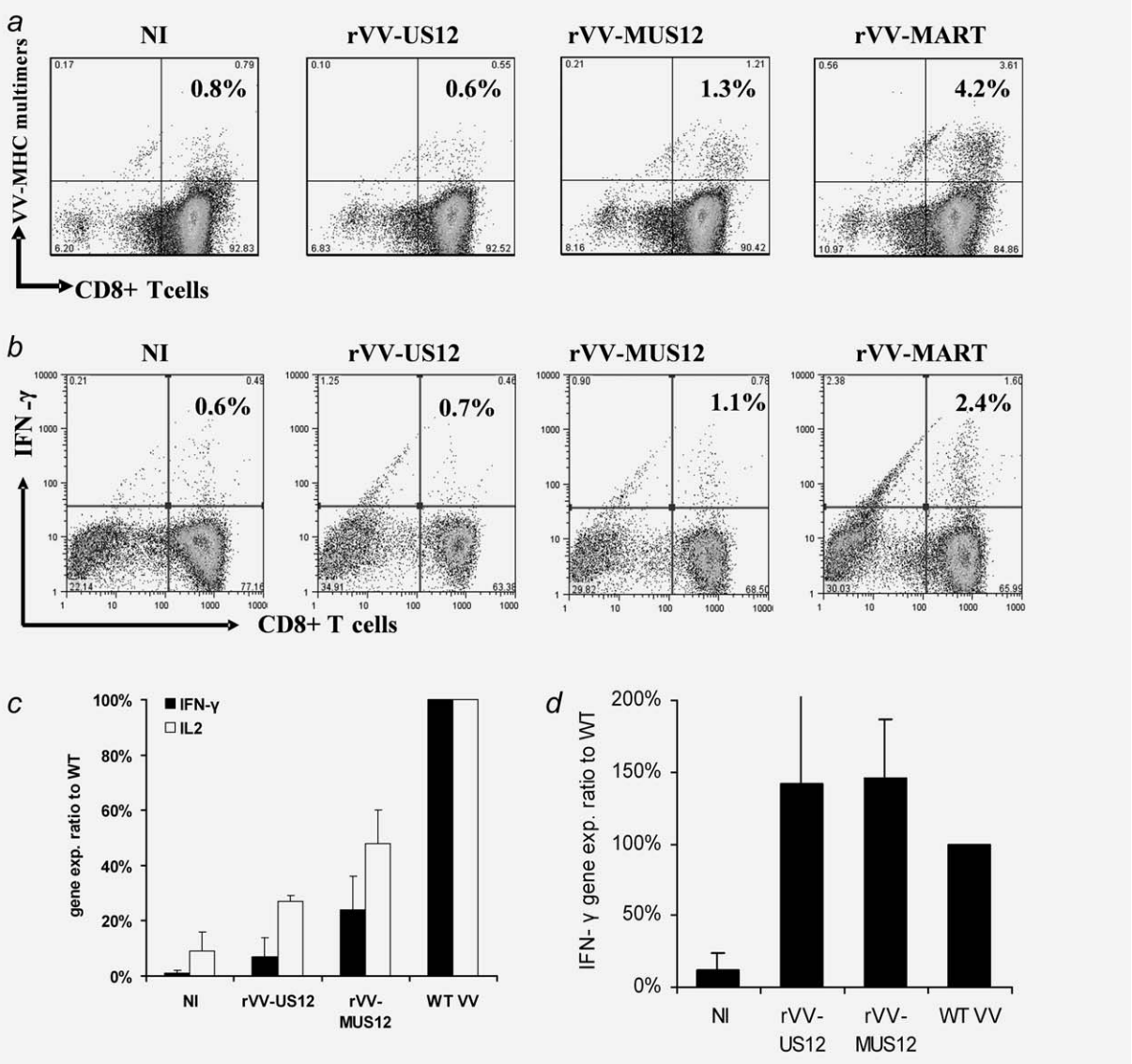

Figure 2. Characterization of VV antigen specific CD8+ T cell response. CD14+ cells (1×10^6) from HLA-A0201 positive healthy donors were infected with replication incompetent rVV-US12, rVV-MUS12 or control VV (WT or MART) at 10 m.o.i. and, 48 h after infection, co-cultured with 10^6 autologous CD8+ or CD4+ T cells. Noninfected (NI) CD14+ cells were used as control. On Day 8, cultures were boosted with 5×10^5 autologous CD14+ cells, infected with WT-VV. On Day 15, half of the CD8+ cultures were analyzed by flow cytometry (*a*) on staining with APC-labeled anti-CD8 mAb and PE-labeled specific $H3L_{184-192}$, $B22R_{29-37}$, $C7L_{74-82}$ HLA-A0201 multimers. To characterize the functional responses of VV specific T-cells, the remaining cultures were mixed with nonreplicating WT VV infected autologous CD14+ cells for 16 h. IFN-γ intracellular staining (*b*) was then performed with PE-labeled anti-IFN-γ mAb and APC labeled-anti-CD8 mAb. IFN-γ and IL-2 gene expression levels in CD8+ cells cultures (*c*) and IFN-γ gene expression level in CD4+ cultures (*d*) were also evaluated by qRT-PCR. Data in graphs (*c*) and (*d*) represent percentages of cytokine gene expression as compared to the level detectable in cultures "primed" with positive control WT-VV (used as 100% in each experiment) and are reported as average (± SD) of three separate experiments.

The functional capacity of the expanded specific T cells was also characterized by IFN-γ intracellular staining, after antigenic stimulation. Data reported in Figure 3*b* confirm that CD8+ T cells priming with rVV-MUS12 leads to the expansion of as many IFN-γ positive cells as detectable upon rVV-MART stimulation (2.6% and 2.8%, respectively). The quantitative analysis of IFN-γ and IL-2 gene expression in these cultures (Fig. 3*c*) showed that T cells, induced by the rVV-MUS12, express IFN-γ and IL-2 close to levels comparable to those induced by rVV-MART stimulation.

These experiments clearly indicate that the capacity to initiate a CD8+ immune response against a TAP independent recombinant epitope is not affected in vectors encoding ICP47.

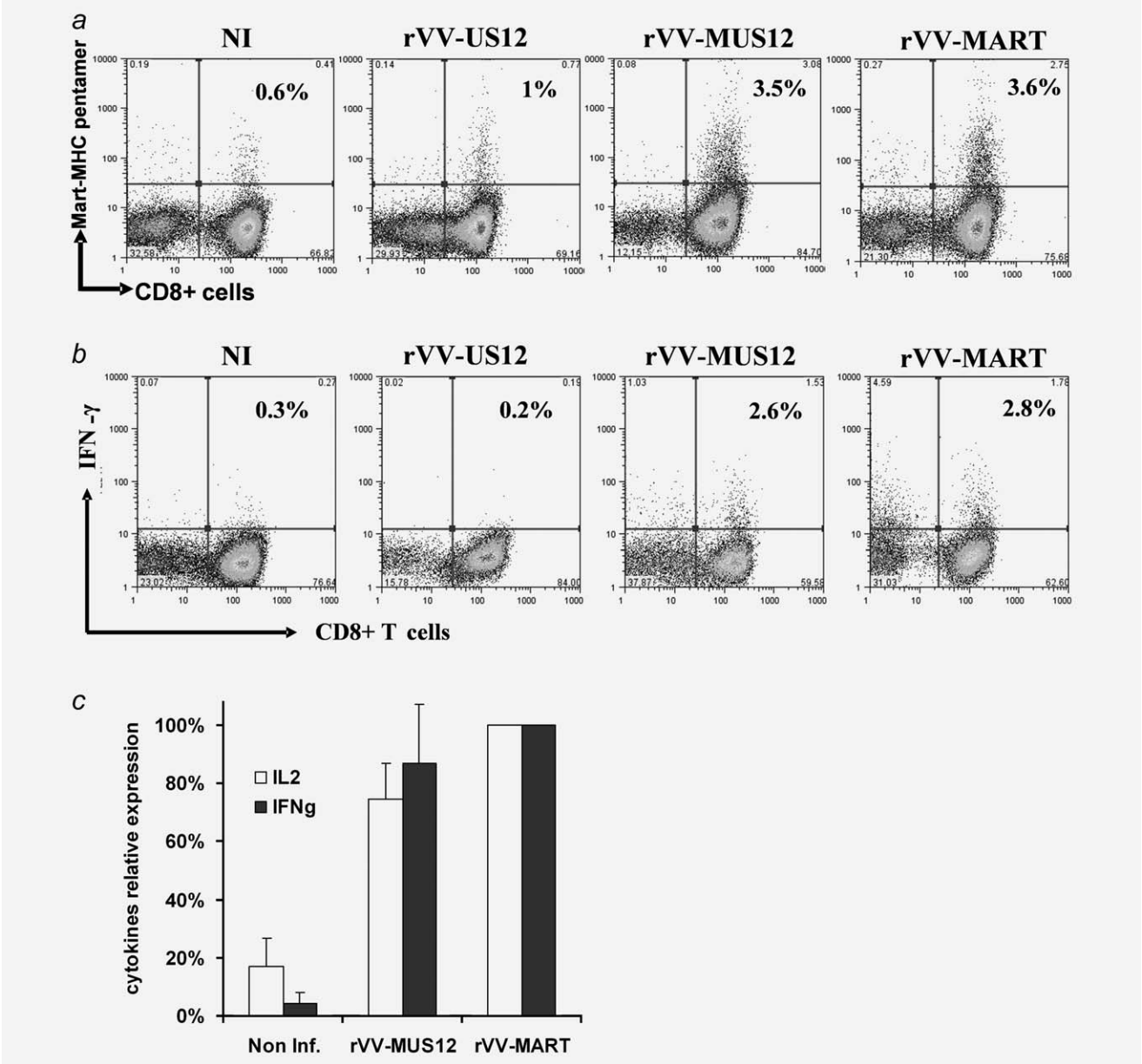

Figure 3. Induction of MART-1/Melan-A_{27-35} specific CD8+ T cell response. CD14+ cells from healthy donors (1×10^6) were infected with replication inactivated rVV-US12, rVV-MART-US12 or rVV-MART at 10 m.o.i., and co-cultured with 1×10^6 autologous CD8+ T cells. Noninfected CD14+ cells were used as control. On day 8, cultures were boosted with 1×10^5 autologous CD14+ cells pulsed with L27MART-1/Melan-A_{26-35} peptide (20 μg/ml). One week later (Day 15), flow cytometry analysis (*a*) of specific response was performed with APC-labeled anti-CD8 mAbs and PE-labeled specific L27MART-1/Melan-A_{26-35}-HLA-A2 pentamers. For functional assays based on cytokine expression, the remaining cells were re-stimulated with peptide pulsed autologous CD14+ cells and analyzed either by intracellular staining (*b*) or qRT-PCR (*c*). Data on IFN-γ and IL-2 mRNA levels are reported as percentages of the level detected in cultures stimulated with the rVV-MART (used as positive control; 100%) and represent the average of two independent experiments.

Recombinant VV immunogenicity in presence of pre-existing VV specific CTL response

To highlight, *in vitro,* the advantageous capacity of ICP47 expressing VV to promote the induction of transgenic epitope specific responses despite the presence of strong vector-specific immune responses, experiments of MART-1/Melan-A specific CTL induction were performed by using, as responders, VV presensitized PBMCs. This "*in vitro*" strategy might reflect more closely *in vivo* conditions of pre-immunization or multi-boosting protocols with a recombinant virus.

Peripheral blood CD8+ and CD4+ T cells were isolated from three smallpox-vaccinated healthy donors, and anti-viral responses were stimulated with autologous WT-VV infected

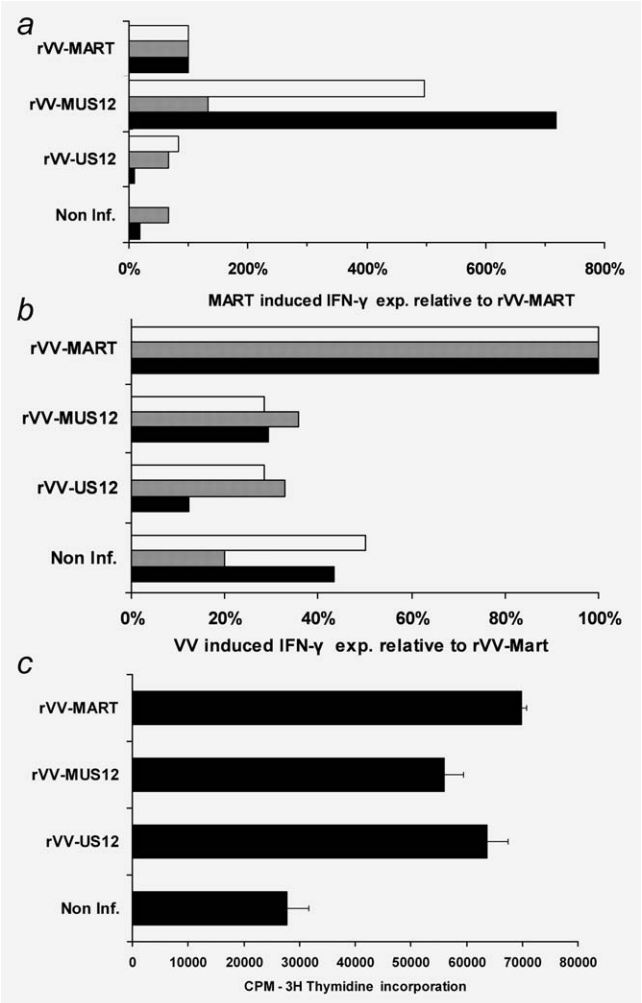

Figure 4. CD8+ T-cell activation in presence of pre-existing VV specific CTL response. 8×10^6 CD14+ cells from three healthy donors were infected with replication inactivated WT-VV at 10 m.o.i. and mixed at 1:1 ratio with autologous CD4+ or CD8+ T cells. After 8 days of culture, CD8+ cell cultures were subdivided in four groups and mixed with 5×10^5 monocytes either infected for 48 h at 10 m.o.i with replication inactivated rVV-US12, rVV-MUS12 or rVV-MART or left uninfected. Each of these four cultures was again subdivided in two subcultures to separately amplify and evaluate responses for the transgenic epitope and the viral vector. On days 15 and 21, culture were boosted with autologous CD14+ cells either pulsed with L27MART-1/Melan-A_{26-35} peptide (20 μg/ml) or infected with WT-VV. On Day 27, peptide (*a*) or vaccinia virus (*b*) specific IFN-γ gene expression was quantified by qRT-PCR following 6 h of specific antigenic stimulation. For each donor, data are expressed as ratio to values detected in the rVV-MART primed cultures. (*c*) For the third donor, 4×10^6 CD4+ T cells were also isolated and stimulated similarly to CD8+ cells. Proliferation of CD4+ T cells in presence of infected autologous CD14+ cells was evaluated after one boost only (day 15) by thymidine ^{3}H incorporation. Data are represented as average of triplicate measurements.

CD14+ cells. After 8 days, these cultures were primed for MART-1/Melan-A_{27-35} epitope specific responses with either rVV-MART or rVV-MUS12 infected CD14+ cells.

In this setting, the critical differential event for MART-1/Melan-A_{27-35} specific T-cells activation should be related to the degree of infected-APC clearing by pre-existing VV specific CTL. Noninfected and rVV-US12 infected CD14+ were used as control stimuli.

After re-stimulations with MART-1/Melan-A_{27-35} peptide or WT-VV, cultures were tested for peptide or virus specific induction of IFN-γ gene expression.

Interestingly, results reported from three donors in Figure 4*a* show a 5-, 1.5- and 7-fold increased level of MART-1/Melan-A specific IFN-γ gene expression in CD8+ T cell primed with rVV-MUS12 as compared to rVV-MART stimulated cultures. These data suggest that the decreased generation of antigenic VV peptides, resulting from ICP47 blockade, did play an "enhancing" role for MART-1/Melan-A_{27-35} immunogenicity in the presence of reactive VV specific CD8+ T cell response.

Concomitantly, as observed in the previous experiments (see above), VV specific IFN-γ gene expression in CD8+ T cell stimulated with an ICP47 expressing virus, *e.g.*, rVV-US12 or rVV-MUS12, is more than two-fold decreased as compared to cultures stimulated by control rVV-MART (Fig. 4*b*). These data confirm that ICP47 is able to diminish vaccinia viral antigens presentation leading to diminished vector-specific CD8+ T cell responses which may be responsible for a rapid clearance of the infected APC.

Characterization of T-helper responsiveness was also performed with CD4+ T cells cultures similarly "presensitized" with WT-VV virus and differentially restimulated. The effect of ICP47 encoding viruses on CD4+ T cell activation was evaluated by measuring their proliferative responses to viral antigens. In line with the previous results from nonpresensitized cultures, CD4+ T cell proliferation data (Fig. 4*c*), confirmed that ICP47 expressed by rVV-US12 or rVV-MUS12 does not affect the capacity of these vectors to stimulate vaccinia-specific MHC class-II restricted responses to an extent similar to those induced by the rVV-MART as virus positive control.

Discussion

This study show that a replication incompetent recombinant vaccinia virus encoding for the HSV1-US12 gene is capable of decreasing cell surface expression of HLA class-I on infected cells and the induction of vector-specific CTL. These data are in agreement with published reports[29,30] showing that ICP47 binds to TAP and blocks peptide transport from the cytosol to ER thereby preventing HLA class I loading and translocation to the cell surface.[3,31] The maximum downregulating effect, related to ICP47 expression and HLA turnover is detectable after 36 h of infection with a nonreplicating virus. The extent of HLA "downregulation" could reach 80% of expression levels observed in noninfected cells. Most importantly, the downregulation of HLA class I due to ICP47 was partially "compensated" in cells infected with rVV-MART-US12. The HLA-A0201 increasing surface

density is also detected on cells infected with a recombinant virus over-expressing the ER-targeted MART-1/Melan-A peptide. Thus, the compensation of TAP-blockade related HLA downregulation, in rVV-MUS12 infected HLA-A0201 cells, can "bona fide" be attributed to the same phenomenon of TAP-independent MART-1/Melan-$A_{27–35}$ HLA restricted epitope, stabilizing the HLA0201-peptide complex onto cell surfaces.

Full T cell receptor mediated activation requires not only antigenic peptide recognition signal but also triggering of co-stimulatory molecules, such as CD80 as a second signal. This effect is also reinforced by other signals mediated by adhesion molecules, such as CD44, during APC-T cell interactions[25] and by soluble factors, such as T helper I cytokines produced by activated CD4+ T-cell. The characterization of the cell surface expression of these molecules in rVV-US12 infected APC confirms that the downregulating effect of ICP47 is specific for HLA class-I and that it does not affect the expression of other surface molecules.

The results of T cell stimulation with rVV-US12 and rVV-MART-US12 infected monocytes are consistent with a decreased MHC class-I restricted responsiveness towards VV protein derived peptides, as indicated by the characterization of CD8+ lymphocytes specific for HLA-A0201 restricted immunodominant viral epitopes $H3L_{184–192}$, $B22R_{29–37}$ and $C7L_{74–82}$ peptides. These data, limited to a single HLA determinant and three epitopes, are also confirmed by the decreased levels IFN-γ and IL-2 gene expression detected in CD8+ T cells in response to HLA class I restricted epitopes presented by WT-VV infected monocytes.

Using MART-1/Melan-$A_{27–35}$ as a model epitope of transgenic TAP-independent HLA class I restricted presentation, we have demonstrated that the co-expression of ICP47 does not affect the generation of MART-1/Melan-$A_{27–35}$ specific CTL response. Thus, in TAP-blocked conditions, ER-targeted epitopes remain highly immunogenic and are able to induce specific HLA class I restricted responsiveness. The immunogenic advantage related to co-expression of ICP47 with the recombinant epitope for CD8+ activation, can be attributed to different simultaneously acting mechanisms. First, the strong inhibition of the expression of HLA complexes containing TAP-dependent peptides from VV proteins decreases the induction of VV vector-specific CD8+ T cell response. Second, the removal of immunoprevalent and immunodominant competing determinants may favor an increased expansion of CD8+ T cells specific for subdominant epitopes deriving from TAA[32–34] or from commonly expressed protein which epitopes are not presented in normal cells. Indeed, Van Hall and colleagues have demonstrated that in many TAP impaired tumors, these T-cell epitopes associated with impaired processing (TEIPP) could readily represent a relevant repertoire for tumor targeting.[35–37] Finally, and most importantly, the decreased presentation of VV specific class-I restricted epitopes may also decrease the VV related cytolytic clearance of infected APC. The resulting prolongation of the survival of APC may then favor the stimulation of immune responses against recombinant antigens.

During the initial CD8+ T cell prime-boost experiments with the minigene rVV encoded epitope, we did not observe an increased immunogenicity toward the ER-targeted peptide encoded within the ICP47 co-expressing vector as compared to a control virus. Indeed, in view of the "hyper" saturation of a large number of HLA-A0201 molecules with the over-expressed TAP-independent epitope, enhanced priming could have been expected. Yet, the CTL response generated in these conditions was not significantly different from that induced by the control ER-MART-1/Melan-$A_{27–35}$ expressing rVV. We speculated that in these *in vitro* conditions, the saturating amount of HLA-peptide on APC surface might have been already reached by the control rVV-MART despite the presence of vector-specific TAP-dependent competing epitopes. This is in agreement with previously published data where we showed that APC infected with rVV-MART were able to reach stimulation plateau even at very low doses of vector.[28]

Therefore, to more effectively highlight the advantage provided by ICP47 mediated block of the expression of vector-derived epitopes, we investigated the differential priming for MART epitope in conditions where VV-specific CTL driven APC clearance could become a limiting factor, *e.g.*, following stimulation with WT VV vector. In these particular conditions, we were able to observe that in the presence of a strong pre-existing VV-CTL response, stimulation by a recombinant virus co-expressing ICP47 resulted in an improved response to the ER transgenic epitope as compared to the conventional viral vector.

Several studies suggest that cytokines, such as IFN-γ, secreted by type I CD4+ T cells might be involved in antitumor and anti-angiogenic activities, leading to inhibition of tumor growth.[38,39] Furthermore, the role of CD4+ T cells is especially relevant in the immunogenic adjuvant effect of viral vectors and CD4 helper responses represent a key feature of vaccine efficacy. In this study, we have demonstrated that the ability of VV antigen specific CD4+ T cells to proliferate and produce IFN-γ in response to VV stimulation is fully preserved in cultures stimulated with rVV-US12 or rVV-MART-US12 infected APC, as compared to cultures stimulated with control virus. Our data clearly indicate that rVV-US12 and rVV-MART-US12 provide efficient activation of VV antigen specific CD4+ T cells thereby maintaining their capacity to promote CD8+ T cells activation and expansion.

A possible limitation of this TAP-blocking strategy relies in the innate NK response against low MHC-I bearing cells. However, as demonstrated here, a single TAP-independent epitope co-expressed is leading to a partial compensation of the MHC-I downregulation. Therefore, such combinatory design could eventually prevent the NK surveillance. Moreover, as recently described,[40] poxviruses have intrinsic complex NK regulatory mechanisms which can lead to a decrease

NK lytic activity induced by cell surface HLA decreased expression.

Altogether, the results obtained with rVV-MUS12 indicate that this reagent is able to increase the immunogenicity of the recombinant epitope. In clinical applications, this type of vaccine may simplify prime-boost protocols, as these would no longer require complex heterologous vaccine formulation. Moreover, it could also increase the possible numbers of effective boosts by limiting vector-specific responsiveness. Such ICP47-vaccine strategy should favor the induction of the strong and long lasting tumor specific immune responses required to eliminate tumor burden and to prevent recurrences.

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