VACCINIA VIRUS EXPRESSING ICP47: A NOVEL PLATFORM FOR CANCER VACCINES HIGHLIGHTING TUMOR EPITOPES AND HIDING VIRAL ANTIGENS

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I. AIM

The aim of this project is to design and pre-clinically evaluate a new construct of recombinant vaccinia virus (r.VV) which efficiently presents recombinant antigens and decreases the intrinsic antigenicity of VV as a vector.

Recombinant poxviruses expressing immunomodulatory molecules together with specific antigens might represent powerful vaccines for cancer immunotherapy [1]. A recombinant vaccinia virus encoding tumor associated antigens (TAA) with costimulatory molecules has been used successfully in vitro [2;3] and in phase I/II clinical trials [3;4] to induce specific cytotoxic T lymphocyte (CTL) responses against TAA.

One of the problems encountered by this recombinant viral vector is related to pre-existing immunity to poxviruses and immunodominance of viral vector antigens. Upon re-infection, vaccinia virus specific CD8+ T cells and antibodies are able to rapidly clear out infected cells, therefore limiting the generation of an immune reaction against the antigenic transgenes.

In order to further strengthen the efficiency of those viral vectors, we aim at specifically decreasing the viral MHC class-I restricted immunogenicity without affecting the presentation of the recombinant TAA epitopes. This is essential especially in cancer immunotherapeutic strategies which often require multiple rounds of vaccination to boost specific CD8+ T cells. A r.VV expressing the Herpes simplex virus (HSV) US12 gene coding for infective cell protein 47 (ICP47) was established. ICP47 inhibits TAP dependent presentation of viral and cellular antigens associated with major histocompatability complex class I (MHC class-I) proteins to CD8+ T lymphocytes. This inhibition of viral immunogenicity might improve the effectiveness of viral gene therapy vectors by decreasing epitope competition and cellular anti-viral responses targeting vaccinia virus vectors without affecting responses to transgenic antigens.

II. INTRODUCTION

II. 1. IMMUNE SYSTEM AND CANCER

The role of the immune system in cancer outgrowth and progression has received a great deal of attention. It is widely believed that enhancing immunity against tumors holds much promise for treatment.

II.1.1. Tumor Antigens

To take advantage of the immune system's specificity, one must find antigens that clearly mark the cancer cells as different from untransformed host cells. To achieve this goal immunologists have been interested in defining different types of tumor associated antigens against which the immune system reacts and in investigating how antitumor immunity may be enhanced.

Generally, tumors are poorly immunogenic. There are different types of tumor antigens: specific or associated. Some tumors express unique antigens which are not found in normal cells, called "tumor specific antigens" (TSA). TSA are typically expressed in tumor induced by infectious agents (e.g. EBNA-1 antigen from Burkitt's lymphoma induced by Epstein Barr virus (EBV)) or resulting from mutations, deletions or recombinations (e.g. the 210-kD chimeric protein with abnormal tyrosine kinase activity involved in chronic myelogenous leukemia resulting from the formation of a bcr-abl gene fusion) [5]. However, TSA are rare and not always practical for vaccine design. Most antigens are tumor associated antigens (TAA) which are proteins expressed by normal cells but either produced in significantly higher amounts or with specific expression pattern in tumors [6]. Human TAA can be classified into different groups. One group is represented by the so called differentiation antigens; for example TAA expressed in melanoma and normal melanocytes. This group includes tyrosinase [7], MART-1/Melan-A [8], gp 100 [9] and TRP-2 [10]. Another group of antigens, the so called cancer/testis antigens, are expressed in cancers of

different histological origins and in the testis and it includes the MAGE family [11].

II.1.2. Antigen Processing and Presentation

A major task of the immune system is to discriminate cells that have been infected by a virus, harbor mutations or undergo neoplastic transformation from healthy cells. Potentially antigenic determinants produced inside the cells are typically processed and presented on the cell surface by MHC class-I molecules. The MHC class-I complexes require a peptide to be bound to be stably expressed on cell surfaces [12].

II.1.2.1. MHC class-I Antigen processing pathway

MHC class-I molecules are highly polymorphic, with multiple alleles of several genes giving rise to the protein products. In humans, there are three MHC class-I loci (HLA-A, B and C). In mice the corresponding molecules are H2-K, H2-D and H2-L. MHC class-I dimers consist of a heavy chain and β2-microglobulin. The two chains are associated noncovalently. Only the α-chain is polymorphic. CD8 binds to the α3 transmembrane domain. The α1 and α2 domains fold to make up a groove accomodating peptides, which are 8-10 amino acids in length. The primary purpose of MHC class-I molecules is to present representative peptide fragments produced inside the cell to circulating T cells. For instance, upon infection, viral peptides are presented, allowing the immune system to recognize and kill the infected cell. In the cytosol (fig.1), endogenous proteins are degraded by the proteasome, some of them at the end of their useful lifetime and some of them (about 40%) directly after synthesis.

Most of the peptide fragments generated by the proteasome are further degraded by other cytosolic proteases into single amino acids used for the synthesis of new proteins. Some of the peptides escape degradation and are transported into the endoplasmic reticulum (ER) by the membrane spanning

transporter TAP [13]. There, the peptides can again be degraded by aminopeptidases including ERAP1 [14] or exported back into the cytosol, unless they are able to bind to an empty MHC class-I molecule. The loaded molecules leave the ER via the Golgi apparatus and the *trans*-Golgi network to the cell surface. Several hundred thousand copies of MHC-I molecules each containing a single epitope are presented at any time on the cell surface, where the epitopes are scanned by T cell receptors (TCR) as shown in figure 2 [15].

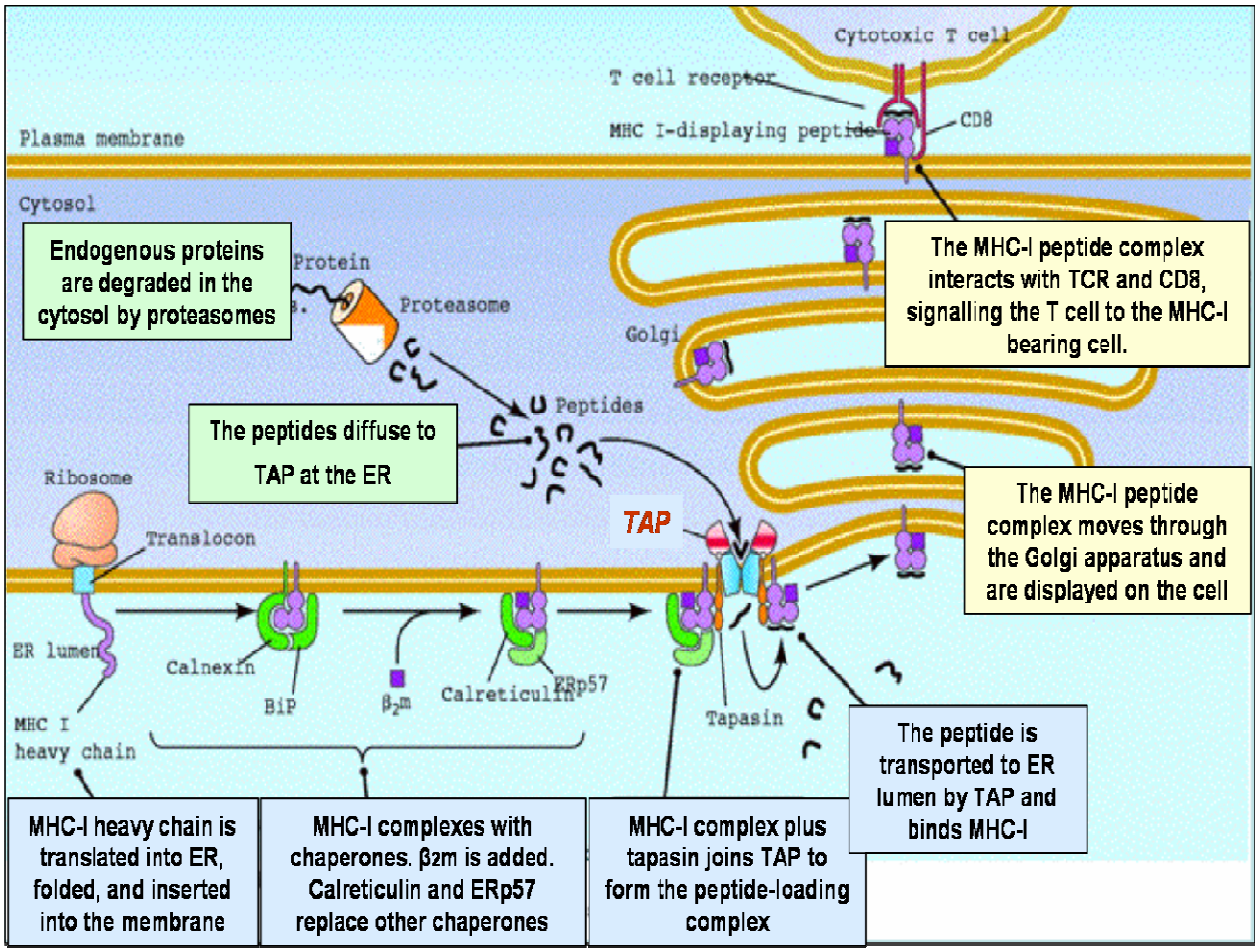


Figure (1): Overview of MHC I Antigen-processing pathway. TAP, transporter associated with antigen processing; ER, endoplasmic reticulum; MHC I, major histocompatability complex I; BiP, immunoglobulin binding protein; ß$_2$m, ß$_2$-microglobulin (modified from Abele et al., 2004) [16].

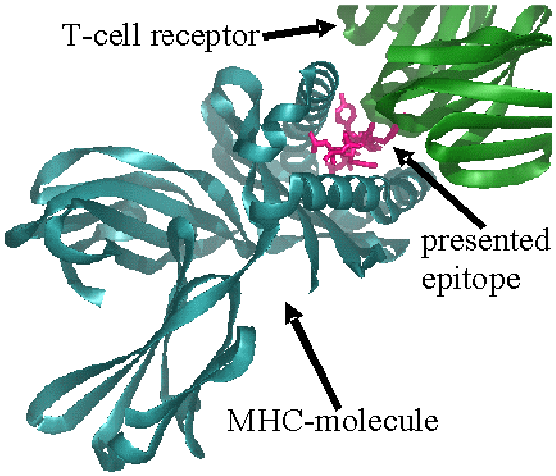


Figure (2): MHC-I bound epitope is scanned by T-cell receptor [15].

The MHC I-peptide complex interacts with the TCR and CD8 molecules of CD8+ T cells, allowing eventual activation of T-cells and resulting in specific immune responses. In normal, noninfected cells, the MHC class-I molecules bind to self protein derived peptides, but specific circulating T cells are tolerant to these epitopes and will not get activated. However, when the cell is infected by a virus, the MHC class-I presents pathogen-derived peptides at the cell surface [16].

II.1.2.2. Transporter Associated with Antigen Processing (TAP)

TAP is an ATP-binding cassette (ABC) transporter, which belongs to the largest and most diverse family of membrane-spanning transport proteins. ABC transporters transport diverse molecules, from large proteins to ions, across plasma membranes, and are found in the animal and plant kingdom, bacteria, and archea [17].

TAP consists of two subunits, TAP1 and TAP2, both of which contain a C-terminal hydrophilic domain that binds ATP and a more hydrophobic N-terminal transmembrane domain which may span the membrane six to eight times (fig.3). Both subunits must be present for TAP to function, and both subunits hydrolyze ATP to power peptide transport. Although the ability of a

peptide to bind to TAP does not require ATP, peptide transport requires ATP hydrolysis [18].

The genes encoding human TAP1 and TAP2 are located in the MHC class-II locus of chromosome 6, between the DQB1 and DPA1 loci, and they are 8-12 kb in size. Deletion of either or both of the TAP genes results in greatly reduced surface expression of MHC class-I molecules and failure to present cytosolic antigens to cytotoxic T cells [17]. Promoter region of TAP contains interferons responsive elements which induce TAP1 gene expression and participates to the increase of MHC class-I presentation on the surface of infected cell [19].

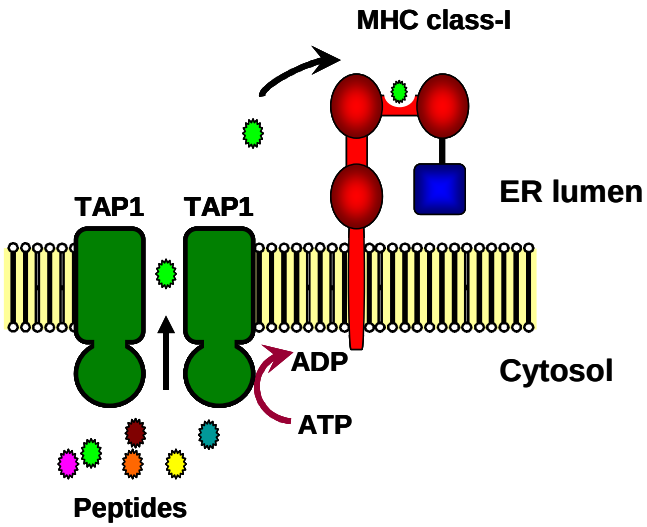


Figure (3): structure of TAP. Both subunits, TAP1 and TAP2 (for **T**ransporter **A**ssociated with antigen **P**rocessing), are required for normal presentation of intracellular antigens to T cells. These polypeptides form a heteromeric "peptide pump". The TAP1 (also known as RING4 or PSF1) and TAP2 (also known as RING11 or PSF2) possess an ATP binding region and 6 to 8 transmembrane helical segments. They are responsible for peptides selection in the cytosol and movement across the ER membrane to the binding site of MHC class-I molecules.

II.1.2.2.1. Role of TAP in the function of the immune system

After proteasome degradation of endogenous proteins into 3-22 residue peptides in the cytoplasm, a small fraction of the peptides diffuse to TAP at the ER. Meanwhile, MHC class-I heavy chains assemble and bind to calnexin (fig.2), a chaperone protein, in the ER membrane. Then β_2-microglobulin binds

to the MHC class-I heavy chain and the dimers separate from calnexin and bind to calreticulin and Erp57 to form the MHC class-I complex. Next, tapasin binds the complex to form the peptide-loading complexes and joins the complex with TAP. Then the degraded cytosolic peptides are transported by TAP into the lumen of the ER and loaded onto an MHC class-I molecule. It is uncertain whether the TAP-tapasin complex directly loads peptides onto MHC class-I molecules or whether the MHC class-I molecule scans the peptides once they are transported into the ER [16].

The TAP-MHC class-I interaction in the peptide complex may help stabilize MHCclass-I:β_2-Microglobulin [20]. In addition, the nucleotide binding site of TAP may transmit a signal for the peptide-loaded MHC class-I to dissociate from the complex.

II.1.2.2.2. TAP related disorders

A. Viruses affecting TAP

Several viruses, especially persistent viruses, interfere with TAP to decrease the presentation of MHC class-I, and thus, avoid the immune response. Herpes simplex virus type 1(HSV-1) encodes the protein ICP47, which binds the peptide binding site of TAP, blocking the first step in the translocation pathway. The critical amino acids of ICP47 required for TAP inhibition are located in the NH2-terminal region from residue 3 to 34. Moreover, This interaction between TAP and ICP47 is species specific [21].

Human cytomegalovirus (HCMV) encodes an ER-resident protein called gpUS6 that inhibits TAP mediated peptide transport. The mechanism is probably due to binding of US6 to ER luminal part of TAP inhibiting peptide translocation but not affecting peptide binding [22]

Adenoviruses (AdV) encode a protein, E3/19K that is well established to bind MHC class-I molecules, trapping them in the ER. It was also demonstrated

that E3/19K binds to TAP and inhibits the tapasin action, thereby preventing MHC class-I/TAP association [23].

Human papilloma virus (HPV) may evade immune recognition by inducing downregulation of TAP1 protein expression. More significantly, the amount of TAP1 protein expression correlates inversely with the frequency of disease recurrence [24].

Similar to HPV, EBV has evolved a strategy to avoid immune surveillance by downregulation of TAP. EBV expresses a protein, vIL-10, that is similar in sequence to human IL-10. vIL-10 downregulates TAP1 gene expression, thereby affecting the transport of peptides into ER [25].

B. Genetic Diseases

There is little knowledge about genetic TAP defects in humans. Bare Lymphocyte Syndrome (BLS) is characterized by a severe downregulation or deficiency of MHC class-I and/or class II molecules. Some studies revealed that the disease may be caused by a genetic deficiency of TAP2, which is mainly due to a premature stop codon resulting in a non-functional TAP complex [26].

C. Autoimmune Disease and Transplantation

Some patients with diverse MHC-linked autoimmune diseases, including type I diabetes, Sjogren's syndrome, Graves' disease and Haishimoto' disease, have a downregulation of mRNA levels for TAP1 and TAP2. These data suggest that defective transcription of TAP genes can contribute to reduced MHC class-I cell surface expression in autoimmune diseases [27]. The incidence and severity of acute rejection after renal transplantations seem to be influenced by TAP2 gene polymorphism. It appears that donors' APC expressing the TAP2*0103 allele have an attenuated efficacy in the presentation of allospecific antigens to the recipient's T cells[28].

D. TAP Deficiency and Tumor Development

Many tumors escape recognition by CTLs. In some cancers, including melanomas, this has been associated with ineffective antigen processing and presentation of tumor specific peptides due to low levels of MHC class-I molecules [29]. In murine as well as human cancers, a downregulation of TAP1 expression by an unknown mechanism or a mutation of TAP resulting in a loss or decrease in class I surface expression has been demonstrated [30]. In breast carcinomas, downregulation of TAP1expression was found in 44% of the lesions [31].

II.1.2.3. MHC class-II antigen processing pathway

MHC class-II antigen presentation is mostly utilized by professional antigen presenting cells to present exogenous peptides derived from captured proteins. Proteins are endocytosed and degraded by acid-dependent proteases in endosomes. The peptides are displayed on MHC class-II molecules.

MHC class II molecules are heterodimeric (αß) cell surface glycoproteins. In humans, there are three MHC class-II molecules (HLA-DR, HLA-DP and HLA-DQ). In mice, the coressponding molecules are H2A and H2-E. Newly synthesized MHC class II α and β chains assemble in the ER with a third glycoprotein, the invariant chain (Ii) forming Ii-MHC class II complex [32]. The cytoplasmic tail of Ii contains a motif that targets the Ii–MHC class II complex to the endosomal pathway. Ii–MHC class II complexes are rapidly internalized into specialized compartments of the endocytic pathway (MHC class II compartments), where peptide loading occurs [33]. Maturation of the early endosome leads to activation of lysosomal enzymes. During transport, Ii is proteolytically cleaved, yielding a nested set of Ii derived peptides, termed CLIP [34]. CLIP is subsequently exchanged for tightly bound antigenic peptides

derived from internalized antigens or endogenous proteins. Dissociation of CLIP and loading of peptides is mediated by the MHC-like molecule HLA-DM. Possibly by its preferential binding of the open, peptide-less conformation of MHC class II, HLA-DM then catalyzes peptide exchange, favoring more stable peptide-MHC complexes [35]. These peptide–MHC class II complexes then traffic to the plasma membrane. These peptides loaded on MHC class-II molecules and expressed on the cell surface are recognized by TCR of CD4+ T cells (fig. 4).

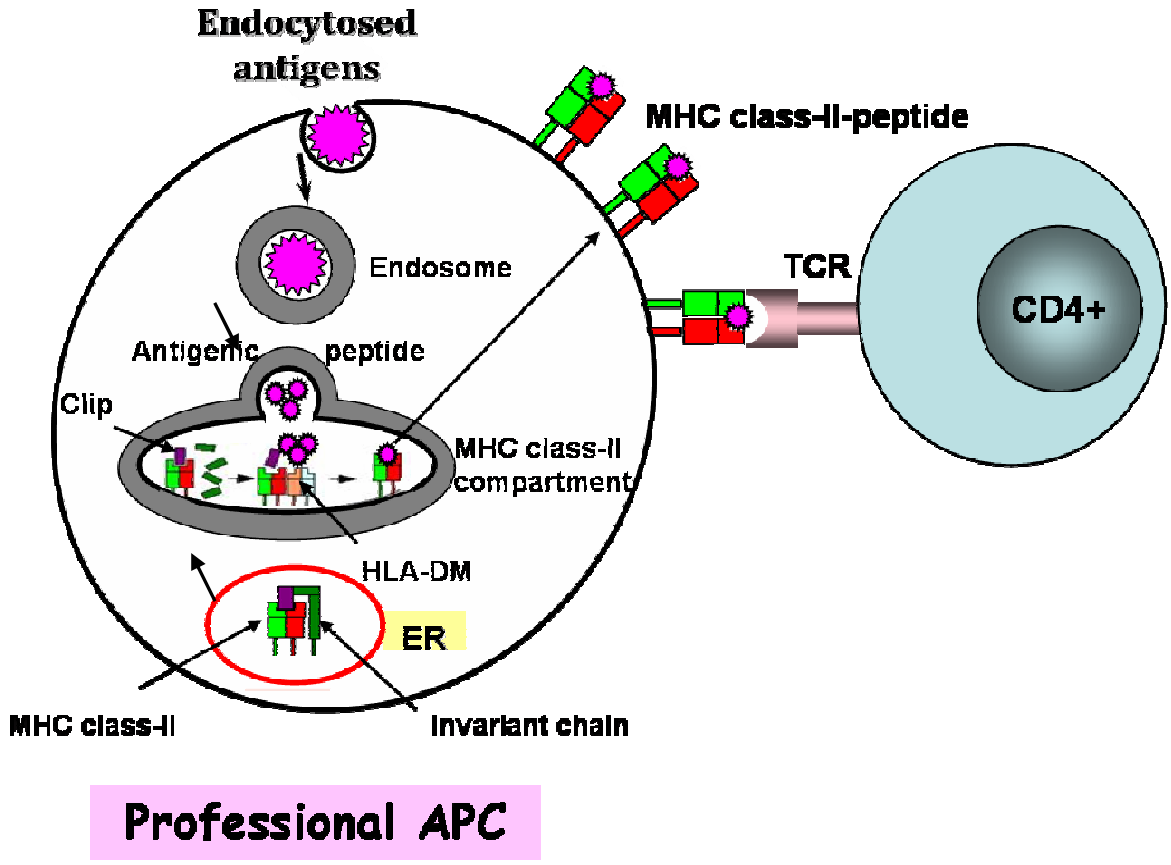


Figure (4): Overview of MHC II Antigen-processing pathway.

Notwithstanding the critical role of $CD8^+$ T cells, induction of tumor-specific $CD4^+$ T cells is also important not only to help $CD8^+$ responses, but also to mediate anti-tumor effector functions through induction of eosinophils and macrophages to produce superoxides and nitric oxide [36].

Recognition of the antigen, along with triggering of co-stimulatory molecules (B7-CD28) results in activation of antigen-specific CD4+ T cells, which leads to lymphoproliferation and cytokine secretion. Depending on several conditions (e.g. strength of antigen signalling, co-stimulation and cytokines secreted by APC) CD4+ T cells differentiate into either TH1 or TH2 type cells. TH1 cells secrete predominantly IFN-γ, which plays a role in the activation of cell mediated immune responses, culminating in activation of cytotoxic T lymphocytes. TH2 cells on the other hand secrete IL-4, which helps B cells to differentiate into antibody secreting plasma cells (figure 5) [36].

Most potential tumor antigens are not expressed on the surface of tumor cells and thus are inaccessible to antibodies [37]. However, tumor cells could be ingested by host antigen presenting cell (e.g. dendritic cells), where antigens are processed and displayed by the host APC's class I and class II MHC molecules. Therefore, eventual tumor antigens may be recognized by both CD8+ and CD4+ T cells. This process is called cross-presentation or cross-priming as one cell type (APC) presents antigen of another cell (tumor cell) and activates T lymphocytes specific for the second cell type [38]. Several other immune mechanisms may play a role in tumor rejection. Activated macrophages and natural killer (NK) cells are also capable of killing tumor cells in vitro [39].

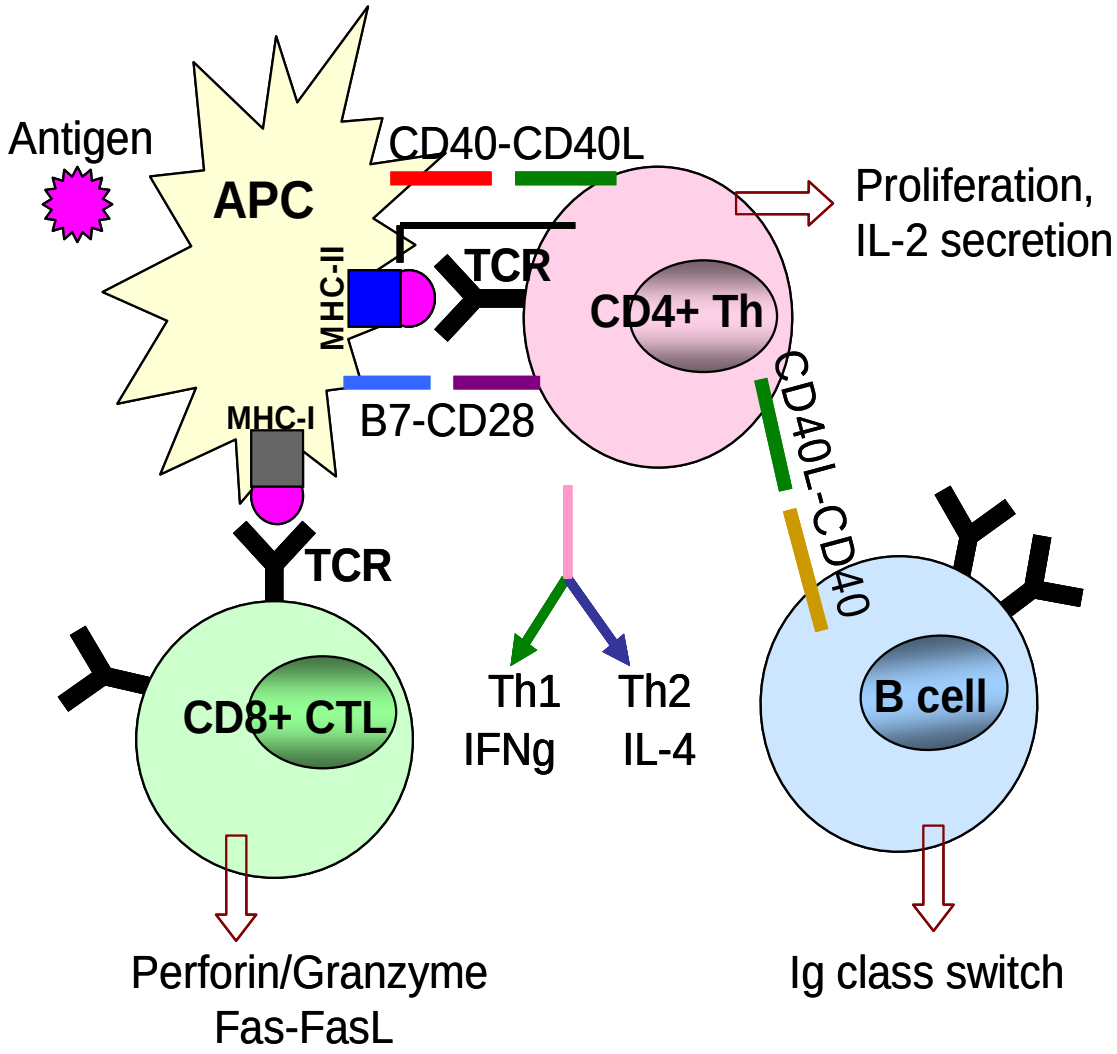
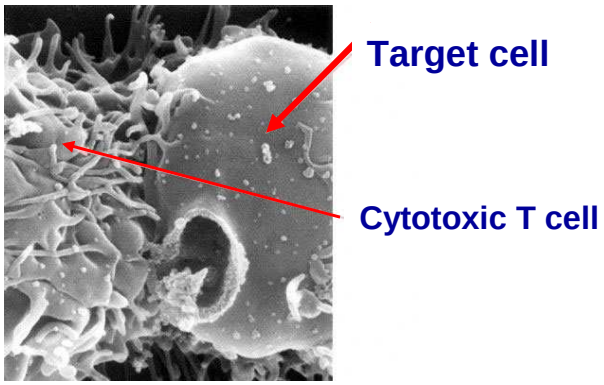


Figure (5): Antigen specific immune response. Antigen taken by APC is presented by MHC class-I to CD8+ cells and MHC class-II to CD4+ cells. Antigen recognition along with co-stimulatory molecules results in activation of CD4+ cells (proliferation and cytokine secretion). CD4+ T cells differentiate into either TH1 or TH2 type cells. TH1 cells secrete predominantly IFN-γ, which plays a role in activation of cytotoxic T lymphocytes. TH2 cells on the other hand secrete IL-4, which helps B cells differentiate into antibody secreting plasma cells.

After recognition of a specific epitope, the CTL bind firmly to the surface of the target cells. Then, the lysis of target cells proceeds through a sequence of programmed steps. CTL have been shown to be responsible for elimination of transduced cells in vivo by effector mechanisms involving Fas-FasL interaction and perforin-granzymes release. The combination of perforin and granzymes significantly increases the lytic ability of CTL (picture 1) [40].

Image figure (1): CTL binding to and destroying a tumor cell using perforin.
(ASM MicrobeLibrary)



Non mutated tumor associated antigens are relatively poorly immunogenic, since they are recognized as self proteins and they are accordingly tolerated. However, upon presentation by highly professional antigen presenting cells (APC) including mature dendritic cells (DCs), naïve or memory specific T cells have been shown to be expanded in response to antigenic stimulation. These lymphocytes usually carry T cell receptor of relatively low affinity, but they are still able to kill tumor cells expressing the antigenic epitopes in the context of appropriate MHC determinants.

Different signals are required for T cell activation (fig. 6). T lymphocytes have antigen specific receptors (TCR) that recognize MHC restricted epitopes derived from processed antigens. APC activate naïve T cells by presentation of antigen within MHC antigens, the primary targets for allo-recognition. This process requires binding of antigen/MHC complex to the TCR/CD3 complex. This event initiates a cascade of signalling events that begins with the activation of several cytoplasmic protein tyrosine kinases. Recruitment of the CD4 or CD8

associated tyrosine kinase, Lck, into the vicinity of TCR complex is believed to induce phosphorylation of CD3 proteins ultimately leading to downstream signal progression. However, in order not to lead to anergy, activation of T cells requires signals not only through the TCR (signal 1) but also through co-stimulatory molecules pathways (signal 2) such as CD28, CD2, CD30, CD44, CD154 and lymphocyte function-associated antigen-1 (LFA-1). After activation, a number of cell surface and soluble molecules including T helper 1 cytokines such as IL-12, which plays an important role in CTL activation, are known to further regulate the immune response (signal 3) [41].

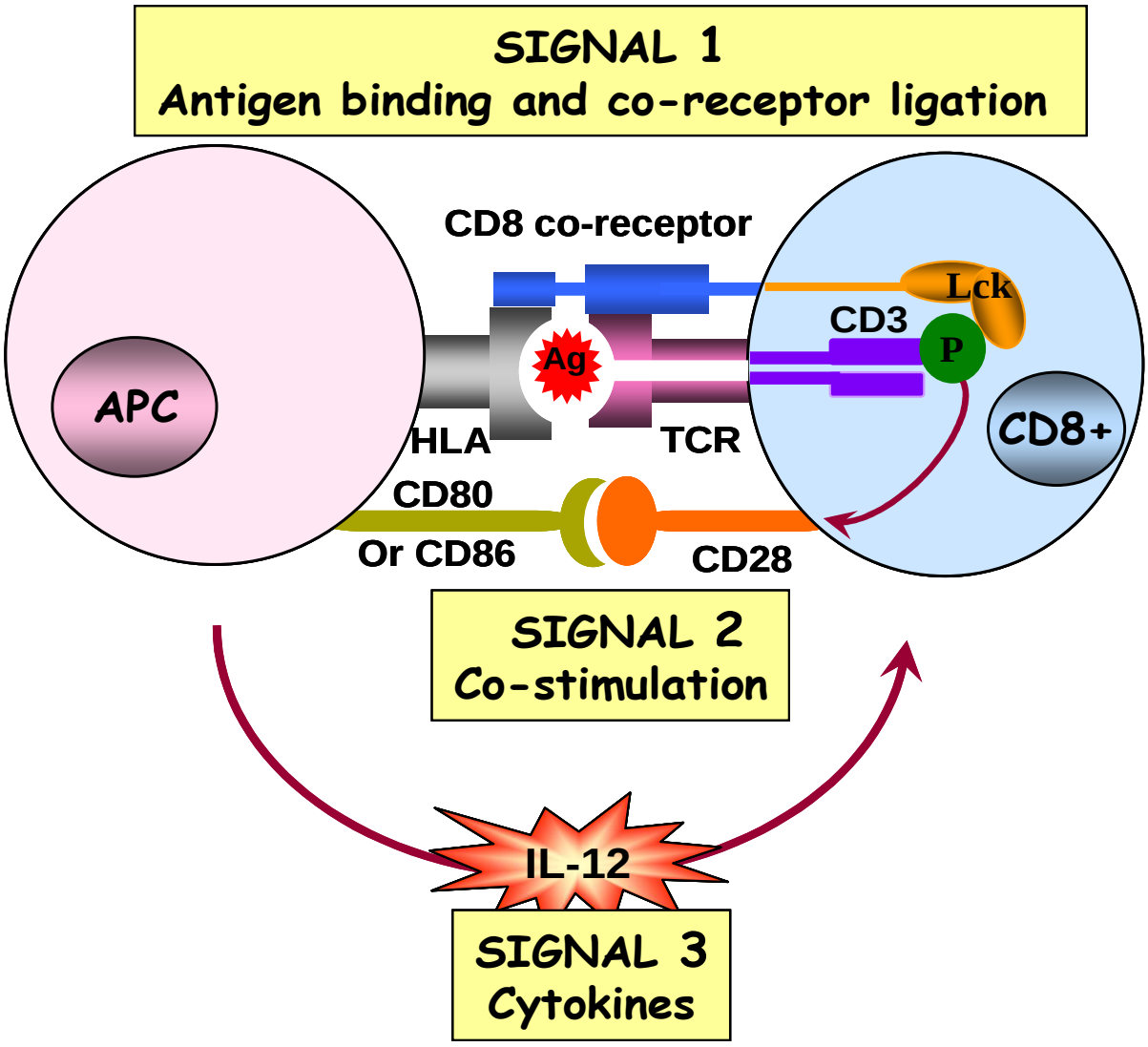


Figure (6): Requirements for T cell activation. T cell activation requires signalling through TCR (signal 1), co-stimulatory pathways (signal 2) and cytokine secretion (signal 3).

II.1.3. Immune Mechanisms of Tumor Rejection

The immune system has three primary roles in prevention of tumors. First, it can protect the host from virus induced tumors by eliminating infected cells or suppressing viral infections. Second, elimination of pathogens and timely resolution of inflammation can prevent the establishment of an inflammatory environment potentially conductive to tumorigenesis [42]. Third, the immune system can prevent the outgrowth of transformed cells or destroy these cells before they become harmful tumors. This mechanism called “immune surveillance” was first proposed in the 1950s by Burnet and Thomas [43].

The term cancer immune surveillance may no longer be appropriate to accurately describe the immune reaction against tumors as it was thought to function only at the earliest stage of cellular transformation. Rather, it was proposed to use a broader term “cancer immunoediting” to describe more appropriately the host defensive and tumor sculpting actions of the immune system that not only promote elimination of some tumors but also generate a nonprotective immune state to others [44].

Cancer immunoediting includes three processes (3Es). Elimination, that corresponds to immune surveillance. The elimination phase can be complete when all tumor cells are cleared or incomplete when only a portion of tumor cells are eliminated.

In case of partial tumor elimination, the theory of immunoediting is that a temporary state of equilibrium can then be established between the immune system and the developing tumor. During this period of Equilibrium, tumor cells either remain dormant or continue to evolve, accumulating further changes that may modulate the expression of tumor specific antigens [42]. The pressure exerted by the immune system during this phase can be sufficient to control tumor progression but if it fails to eliminate the tumor, the process results in the selection of tumor cell variants that are able to resist or suppress the antitumor immune response.

Escape is the process by which the immunologically sculpted tumor expands in an uncontrolled manner leading to development of clinically apparent tumors (fig.7) [44].

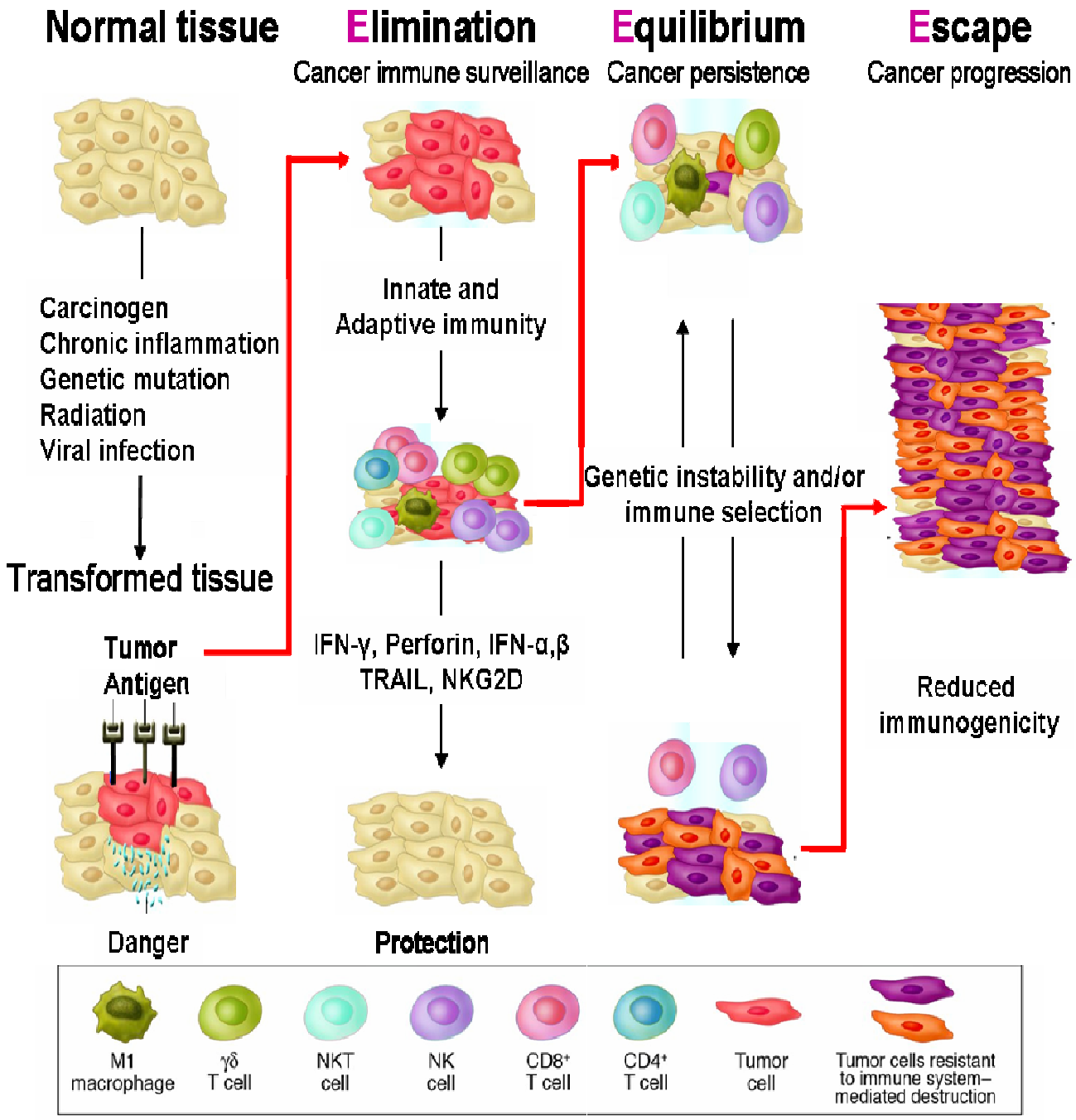


Figure (7): Tumor suppression by the immune system (cancer immunoediting). Cancer immunoediting is considered as a process composed of 3 phases: **elimination** or cancer immune surveillance; equilibrium, a phase of tumor dormancy where tumor cells and immunity enter into dynamic **equilibrium** that keeps tumor expansion in check; and **escape**, where tumor cells emerge that either display reduced immunogenicity or engage immunosuppressive mechanisms to attenuate antitumor immune responses leading to the appearance of progressively growing tumors (Modified from Smyth et al., 2006) [45].

A number of clinical observations have provided evidence supporting each of the distinct phases of human tumor immunoediting. The phenomenon of spontaneously regressing melanoma lesions accompanied by the clonal expansion of T cells is presently the strongest evidence for the elimination phase of cancer immunoediting in humans [46;47].

In animal models, immunization against malignant melanoma can cause vitiligo [48]. Occurence of vitiligo suggests the development of an antitumoral response and is commonly believed to be a positive prognostic factor [49-51]. The association between malignant melanoma and hypomelanosis is thought to be the consequence of the dualistic immune mediated response against antigens shared by normal melanocytes and malignant melanoma cells. In malignant melanoma associated vitiligo, infiltration by specific CD8+ T cells, directed against tumor melanocytic antigens, has been described [52;53].

Pharmacological immunosuppression to prevent transplant rejection is clearly associated with a heightened risk (3- to 100-fold increase) of developing certain types of malignancy [54]. These diseases are predominantly lymphomas; however, a range of solid tumors with no known viral association also occur with increased frequency. In addition to those tumors, a number of tumors (especially lymphomas) also occur most frequently in patients with primary and acquired immunodeficiencies [55].

Tumor infiltration by T cells, NK cells or natural killer T cells (NKT) has been associated with an improved prognosis for a number of different tumors [56-58]. Spontaneous tumor regression accompanied by lymphocyte infiltration has also been noted for a number of other tumor types [59-63].

Clinical evidence supporting the existence of the equilibrium phase of immunoediting is provided by a number of findings. First, the existence of an immune response to premalignant monoclonal gammopathy of undetermined

significance (MGUS) cells that eventually progress to multiple myeloma is consistent with the equilibrium phase, with the immune system controlling, but not eliminating, MGUS cells that eventually evolve and progress to malignancy [64]. Passive immunization with idiotype-specific antibody, in conjunction with either cytokine therapy or chemotherapy, can induce remission in some patients with low-grade B cell lymphoma [65]. Moreover, a role for the immune system in establishing long-term remission has also been suggested by studies of pediatric acute myeloid leukemia patients treated with either chemotherapy or chemotherapy combined with autologous bone marrow transplantation [66].

Clinical evidence also suggests that tumors can remain dormant in patients for many years, and cases of relapse after long periods (at least 10 years) of tumor remission have been noted [67-69], making immune control with subsequent escape an interesting possibility in these cases. Similarly, cases of transmission of tumors from organ donor to recipient have also been noted. In such cases, it is possible that the tumor was being held under control by an immunological mechanism in the donor and that transplantation of the organ into an immunosuppressed host allowed tumor outgrowth [70;71].

The escape phase is the best defined of the three phases in both mice and humans. For example, the growth of melanomas clearly results in the priming of a tumor-specific immune response, even though this response is often insufficient to completely eliminate tumors [72]. In the same way, the antitumor immune response seen in patients with paraneoplastic autoimmune syndromes (disease symptoms experienced in patients with tumors and caused by activation of an immune response specific for self antigens expressed on tumor cells) indicates that an ongoing immune response is, in many cases, insufficient to control tumor growth, even when it is sufficient to destroy normal self tissues [73-75]

II.1.4. Evasion of Immune Response by Tumors

A number of processes that dampen the immune response are exploited by tumors to escape immune surveillance. There are different mechanisms that contribute to tumor escape (Fig. 8). Many tumors down-regulate or eliminate their expression of MHC class-I molecules, which are necessary restricting elements for CTLs and whose absence renders the tumor cells invisible to CTLs [76;77]. Some tumors stop expressing the antigens that are the target of an immune attack. These tumors are called antigen loss variants [78]. Other tumors create an immunosuppressive milieu by secreting immunosuppressive cytokines such as IL-10 and transforming growth factor-b (TGF-ß) [79]. IL-10 inhibits antigen presentation and antigen-specific T cell expansion [80] but TGF-β directly inhibits T cell activation and proliferation [81].

T lymphocytes can also be rendered anergic by factors secreted in the tumor microenvironment. For example, indoleamine-2,3-dioxygenase (IDO) enzyme, which is involved in tryptophan catabolism, is overexpressed in defined tumor types. Depletion of tryptophan in the tumor microenvironment blocks activation and expansion of T cells which are dependent on tryptophan for cell cycle progression [82]. Tryptophan depletion may also promote apoptosis and/or induce T cell tolerance [83]. In addition, there is a family of receptors that are expressed on the T cells and act to downregulate T cell activation. This family includes: cytotoxic T-lymphocyte antigen 4 (CTLA-4), which binds to CD80 and CD86 on APCs, transmitting an inhibitory signal to T cells [84] and programmed death-1 (PD-1) which binds to programmed death ligand-1 (PDL-1) and PDL-2 on APCs. PD-1-PD-L1 interaction inhibits activation, expansion and acquisition of effector functions of T cells [85].

Another immunosuppressive mechanism in cancer is represented by the expansion of immature myeloid cell (iMC) populations which has been observed to be associated with profound suppression of T cell responses [86;87]. The

mechanism of iMC suppression appears to involve production of either arginase (ARG) enzyme [88] or inducible nitric oxide synthase (iNOS) by tumor cells [89]. Arginine depletion might lead to loss of T cell recognition of antigens and impaired T cell function [90].

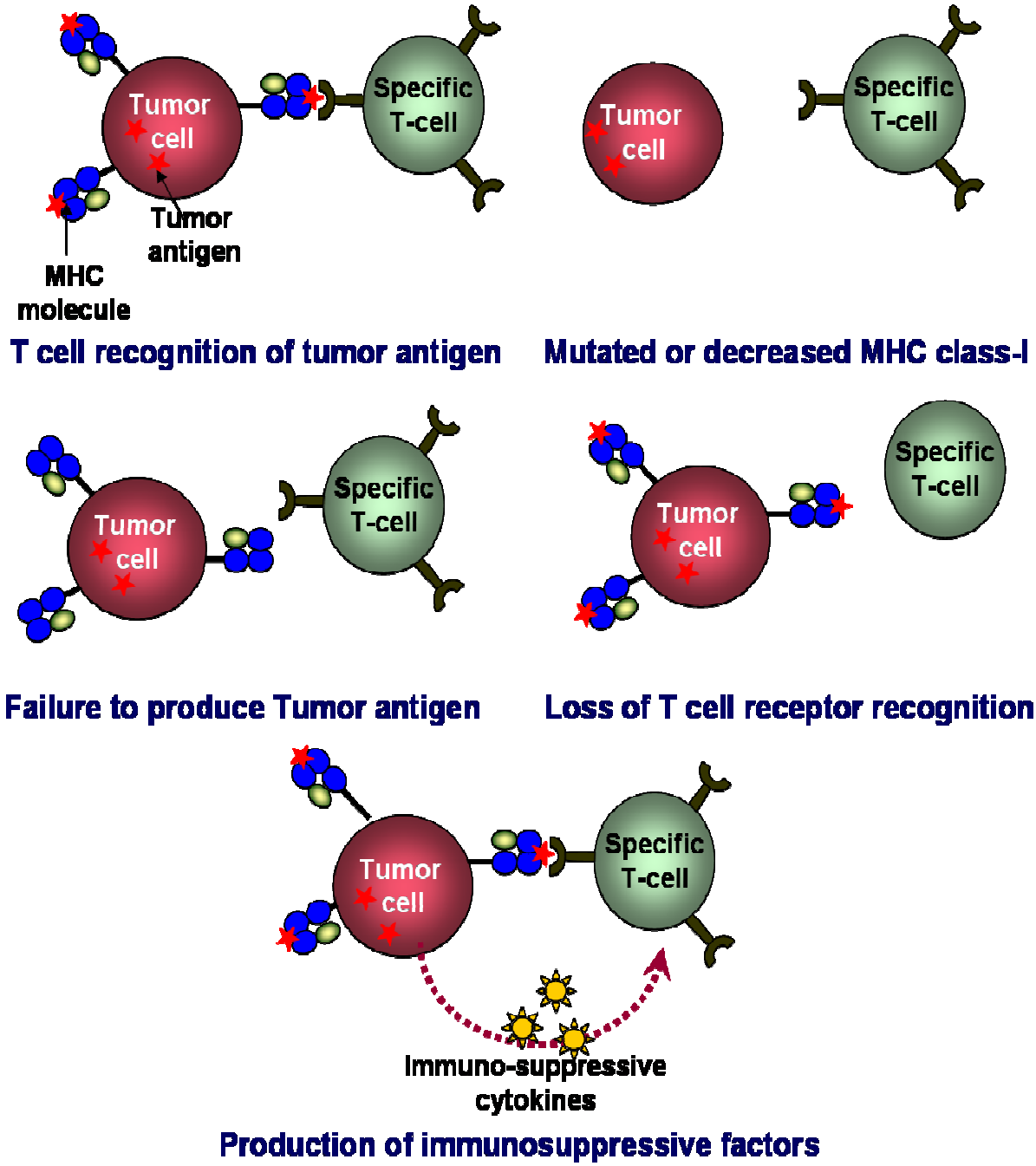


Figure (8): How tumors evade immune responses?
Antitumor immunity develops when T cells recognize tumor antigens and are activated. Tumor cells can evade immune responses by losing expression of MHC molecules, antigens or TCR or by producing immunosuppressive cytokines.

II.2. CANCER VACCINES

For many years, the treatment of cancer has been based on surgery, chemotherapy, and irradiation. However, new biological therapies are being developed. Cancer vaccines represent a promising type of biological therapy including a number of innovative treatments that are likely to become important in treating cancer. Similar to vaccines against infectious agents, the aim of cancer vaccines is to stimulate the immune system to recognise cancer cells and destroy them [91].

Vaccines intended to prevent or treat cancer appear to have safety profiles comparable to those of traditional vaccines [92]. However, the most commonly reported side effect of cancer vaccines is inflammation at the site where the vaccine is injected into the body. Reported symptoms include redness, pain, swelling, heightened temperature, itchiness, and occasionally a rash [93].

II.2.1. Cancer Immunotherapy

The potential of cancer immunotherapy was first documented by William Coley in 1890, when he was trying to replicate the fever and ‘cytokine storm’ that he had witnessed following septicemia when he observed the regression of some tumors. Bacterial products (Coley's toxins) were administered for advanced inoperable cancers with dramatic responses [94]. Today, we understand that the components of bacterial extract stimulated the immune response unspecifically.

In general terms, tumor immunotherapy refers to the use of elements of the body's natural immune system to fight cancer. Patients typically associate the term vaccine with the prevention of infectious diseases, such as measles. Recent research has indicated that the vaccine approach may also be useful in the prevention and treatment of cancer.

Tumor vaccines typically include tumor antigens that can be used to stimulate an immune response. However, limited success has been achieved with traditional immunotherapy, as cancer cells tend to evolve mechanisms that evade immune detection. A wide array of gene therapy techniques are being used to overcome these limitations [95].

Different types of vaccines are used to treat different types of cancer. Typically, patients receive tumor vaccine therapy on an out-patient basis. Vaccines are delivered through an injection into the skin or directly into the tumor. Recent clinical trials of second and third generation vaccines have shown encouraging results with a wide range of cancers, including lung cancer, pancreatic cancer, prostate cancer and malignant melanoma [96].

Vaccines to prevent infectious diseases are prophylactic or preventive vaccines. In contrast, cancer vaccines are expected to be mainly therapeutic (attacking a tumor which has already developed). However, some cancer vaccines, known as *cancer preventive vaccines* are designed to prevent cancers induced by infectious agents. For example, HPV16 and HPV18 together account for 70% of cervical cancers, and a vaccine developed against these two strains shows great promise [97]. *Cancer therapeutic vaccines* are intended to treat already existing cancers by strengthening the body's natural defenses against cancer. Designing these vaccines presents more challenges than preventive vaccines against infectious diseases as tumor antigens are mostly self antigens so inducing strong and long term immunological responses against tumor antigens often correlates with producing autoimmunity [98].

II.2.2. Immunotherapeutic Approaches: Melanoma as Model for Cancer Vaccine

Skin cancer is currently considered as a global epidemic. Primary melanoma is a malignant tumor of melanocytes and, less frequently, of retinal pigment epithelial cells (of the eye). Metastases may colonize skin and lymph nodes, or visceral sites like lung, liver, bone, brain and small intestine [99].

Melanoma is considered as the fourth common malignancy in Western countries. Worldwide, its incidence is increasing steadily at a yearly rate of 3-5% [100]. In Europe, the incidence in average risk regions has tripled for males and females within two generations, reaching 10.3 and 13.3 per 100,000 per year, respectively [101]. Identified melanoma risk factors are Caucasian skin types, childhood sun exposure, sunburn and intense intermittent sun exposure, typical of leisure activities [102].

Malignant melanoma has always been regarded as an immunogenic tumor, as regression zones within tumoral lesions can be observed frequently together with a dense infiltration of T cells possibly resulting from recognition of tumor-associated antigens either on antigen-presenting cells or on the surface of tumor cells by T lymphocytes [103]. Vitiligo is generally considered as a sign of good prognosis in melanoma. This observation suggests that vitiligo might result from an antitumoral response directed against differentiation antigens shared by normal melanocytes and melanoma cells. The particularly high frequency of vitiligo in melanoma patients treated with recombinant cytokines [104;105] further supports this hypothesis, and it is indicative of the involvement of cellular immune effectors. Furthermore, an inverse correlation between prognosis and the degree of lymphocytic infiltration of the primary tumor suggest that the activation of anti-tumoral immunity might be beneficial in attempts to induce the regression of established tumors or to prevent recurrence.

Active antigen-specific immunotherapy (AASIT) is currently being investigated in a number of clinical centres as a treatment option for advanced-stage melanoma. A large number of melanoma TAAs have been molecularly characterized and are being used in vaccination trials in various molecular forms and according to various immunization protocols [4].

II.2.3. Recombinant Viruses as Cancer Vaccines Vectors

One advantage of virus based cancer vaccines is that they are self adjuvanted as they are able to induce the appropriate “danger signals”. A number of trials utilizing recombinant viruses expressing tumor antigens, some with immuno-stimulatory molecules, have been reported or are in progress [106]. For vaccination purposes, the ideal viral vector should be safe and enable efficient presentation of expressed antigens to the immune system. It should also exhibit low intrinsic immunogenicity to allow for its re-administration in order to boost relevant specific immune responses [1]. Many viral vectors have been used successfully including retroviruses, poxviruses, adenoviruses, adeno-associated viruses, herpes simplex viruses and alphaviruses.

Avipox, vaccinia and adenovirus vectors have been mainly used for immunotherapeutic approaches. The avipox viruses which infect birds, do not replicate in mammalian cells. Therefore, there is little induction of a neutralising antibody response which could limit the efficacy of multiple vaccinations. However, the avipoxviruses have been shown to induce antitumor T cell responses, when used to deliver the CEA tumor antigen [107].

Recombinant adenoviral vectors (r.AdV) are being considered as a cancer vaccine platform because they are very efficient at transducing target cells in vitro & in vivo and can be produced at high titres [108]. r.AdV encoding MART-1 or gp100 melanoma antigens have been used to vaccinate patients with metastatic melanoma [109].

Adeno-associated viral vectors are one of the most extensively studied and highly used vectors for gene therapy appraoches. Simplicity of design, lack of pathogenicity, low immunogenicity and safety have made these vectors attractive for clinical applications [110-112].

VV exhibits a broad host range, allowing infection of many laboratory animals. This makes VV easy to study in the laboratory in animal models, and preclinical results can be more readily translated into clinical trials. Recombinant poxviruses expressing immunomodulatory molecules together with specific antigens might represent powerful vaccines for cancer immunotherapy [1]. This is in contrast to, for example, human adenovirus, for which a lack of good animal models has remained a major obstacle. Development in recombinant DNA technology has made efficient manipulation of the VV genome a reality [113].

II.3. VACCINIA VIRUS

Vaccinia is a highly immunogenic virus capable of inducing strong humoral as well as cell-mediated immune responses [114;115]. VV represents a unique opportunity for cancer immunotherapy approaches. In the context of cancer therapy, VV has been used mainly as a delivery vector to deliver TAAs to elicit antigen-specific immune responses [3;106], or to deliver immune modulating genes such as cytokines and costimulatory molecules directly into established tumors to change the local microenvironment [116;117]. Furthermore, it can also be used as a replication selective tumor-specific oncolytic virus [118]. In vivo administration of vaccinia virus appears to naturally possess an intrinsic ability to selectively infect cancer cells and generate antitumor immunity [119]. Oncolytic VV may also be prepared ex vivo by infecting tumor cell lines to form VV oncolysates with augmented immunogenicity and then administered in vivo [120]. Indeed, a number of cancer vaccines based on VV vectors have shown promising results in preclinical animal models and numerous clinical trials [117].

II.3.1. Properties

Vaccinia virus is a member of the genus Orthopoxvirus of the family Poxviridae. Poxviruses comprise a large family of viruses characterized by a large, linear dsDNA genome, a cytoplasmic site of replication and complex virion morphology. The best characterized member of the poxvirus family is variola, the causative agent of smallpox. The laboratory prototype virus used for the study of poxviruses is vaccinia; this virus was used as a live, naturally attenuated vaccine for the eradication of smallpox in the 1970s [121].

II.3.1.1. Origin

Vaccinia virus is closely related to the virus that causes cowpox. The precise origin of VV remain obscure due to the lack of record-tracking as the virus was repeatedly cultivated and passaged in research laboratories for many decades. The most common note is that vaccinia virus, cowpox virus and variola virus were all derived from a common ancestral virus [122].

II.3.1.2. History

The original vaccine for smallpox, and the origin of the idea of vaccination, was cowpox, reported on by Edward Jenner in 1798. The Latin term used for cowpox was *variolae vaccinae,* essentially a direct translation of "cow-related pox". That term lent its name to the whole idea of vaccination. When it was realized that the virus used in smallpox vaccination was not, or was no longer, the same as the cowpox virus, the name ` vaccinia ` stayed with the vaccine-related virus [123].

II.3.1.3. Taxonomy

VV is the most studied virus of the poxviridae family. The poxviruses represent a family of large DNA viruses that replicate in the cytoplasm. They are subdivided into the entomopoxvirus (EnPV) and chordopoxvirus (ChPV) subfamilies (*Entomopoxvirinae* and *Chordopoxvirinae*), which infect insects and chordates, respectively. The ChPVs are further divided into eight genera (*Avipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Capripoxvirus*, *Suipoxvirus*, *Leporipoxvirus*, *Yatapoxvirus* and *Parapoxvirus*), whereas the EnPVs are divided into three genera (*A*, *B* and *C*). Several strains of vaccinia virus are existing, some are replicating (Copenhagen, Wyeth, WR, Lister and NYCBOH), others are in contrast highly attenuated strains unable to replicate or replicating poorly in human cells (MVA, NYVAC, ALVAC and TROVAC) [124].

II.3.1.4. Morphology

Both the morphogenesis and structure of poxvirus virions are unique among viruses. Poxvirus virions apparently lack the symmetry features common to other viruses such as helical or icosahedral capsids or nucleocapsids. Instead poxvirus virions appear as “brick shaped” or “ovoid” membrane bound particles with a complex internal structure.

To exit from the cell, viral particles are propelled by a mechanism involving the cytoskeleton of the infected cells. The first indication that VV was able to interact with the cytoskeleton during its complex assembly process came from high voltage electron microscopy studies which showed virus particles at the tips of large microvilli-like projections in infected cells [125]. Indeed, VV induces the nucleation of actin tails from outer membrane surrounding the intracellular enveloped virus (IEV) [126].

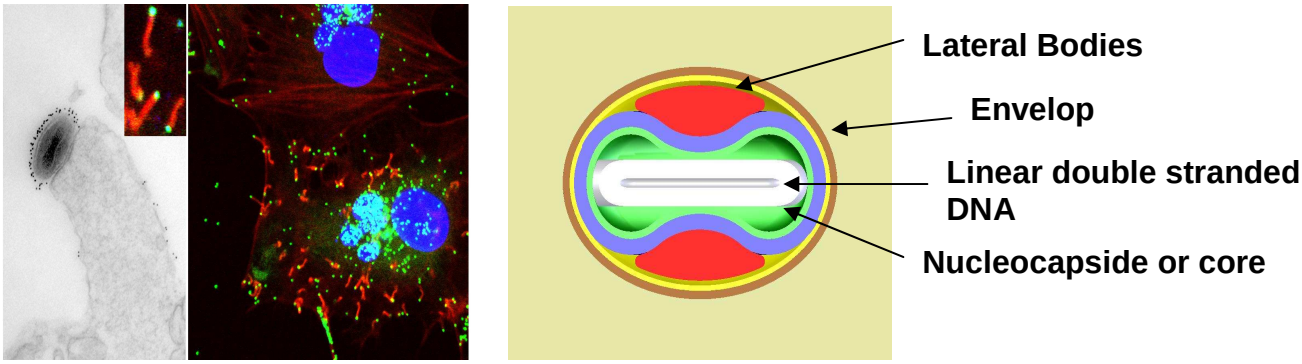


Figure (9): Structure of Vaccinia virus particle A. Virus particles labelled with a green fluorescent protein can be studied by modern microscopy techniques by Live Cell Imaging or fixed and processed for confocal and electron microscopy. Blue areas are cell DNA and viral DNA, the red filaments are actin tails which propel the green virus particles away from the cell surface [127]. **B.** the virus appears as a slightly flattened barrel with overall dimensions of approximately 360 x 270 x 250 nm. It is encased in an outer membrane that contains a lipid bilayer. Within the membrane is the core, which is also barrel shaped and contains two indentations, one on each of the largest surfaces. Filling the spaces between the core wall and the membrane that are created by the indentations in the core are “lateral bodies”.

In figure 9, the virus appears as a slightly flattened barrel with overall dimensions of approximately 360 x 270 x 250 nm. The particle is encased in an outer membrane, which itself consists of two component domains. The outermost membrane domain is 9 nm thick and the innermost membrane domain is 5 nm thick. The membrane contains a lipid bilayer, which probably corresponds to the inner membrane domain. Within the membrane is the core, which is also barrel shaped with two indentations, one on each of the largest surfaces. The core is defined by a core wall, which is also comprised of two layers. Filling the spaces between the core wall and the membrane that are created by the indentations in the core are “lateral bodies” [128].

II.3.1.5. Nucleic acid

Vaccinia virus genome is a linear double stranded DNA molecule characterized by a natural cross-linking at both termini of the two DNA molecule strands, essentially resulting in a single stranded circular DNA molecule [129]. The total genome length of the Copenhagen strain of VV is 192 kbp with a relative purine or pyrimidine bases composition of 66.6% A/T. 198 protein-coding regions “major” and 65 overlapping “minor” regions were identified, for a total of 263 potential genes [130]. In addition, the VV genome contains very long inverted terminal repeats (ITR) which are identical but oppositely oriented sequences at both ends of the genome. ITRs are important features required for VV DNA replication. A central region of the genome is highly conserved between different Orthopoxviruses. In contrast, the ends are hypervariable [129]. VV genes are largely nonoverlapping, which makes it relatively easy to manipulate the VV genome.

II.3.1.6. Routes of infection of poxviruses

Poxviruses can infect their host by different routes: through the skin by mechanical means, via respiratory tract (e.g. Variola virus infection of humans),

or by oral route [131]. Because one early gene of VV encodes a polypeptide termed viral growth factor (VGF) [132], with structural and functional homology to epidermal growth factor (EGF) and TGF-α [133], It has been suggested that the epidermal growth factor receptor (EGFR) is a receptor for vaccinia virus. However, the expression of VGF by vaccinia virus or EGFR by the target cells influenced neither virus adsorption to cells nor penetration. These results indicate that the EGFR is not a receptor for vaccinia virus [134].

II.3.1.7. Poxviruses replication

Poxviruses are unique among DNA viruses in that they reside exclusively in the cytoplasm of the host cell, where they replicate DNA, synthesize mRNA, and assemble progeny virus. This apparent autonomy from the nucleus is possible because these viruses encode many of the proteins that function in nucleic acid biosynthesis, including a DNA polymerase, RNA polymerase, transcription factors, and a nearly complete repertoire of mRNA modification enzymes [135].

Vaccinia virus coordinates its progression through its replicative cycle by expressing individual proteins at specific times. The temporal regulation of gene expression is controlled at the level of transcriptional initiation. The multisubunit viral mRNA polymerase, which structurally resembles its cellular counterparts, is responsible for all mRNA synthesis. Virus-encoded transcription factors are required for transcription of the early, intermediate, and late classes of gene promoters which are activated in that order. The factors required for activation of each class are products of the preceding class, establishing a cascade for gene activation [136].

All poxviruses replicate in the cytoplasm of infected cells by a complex, but largely conserved, morphogenic pathway (figure 10). Replication of vaccinia virus DNA occurs very efficiently within infected cells. It typically begins 1-2 hours after infection and results in the generation of 10,000 genome copies per

cell within hours of infection, of which half are ultimately packaged into infectious virions [137;138].

Two distinct infectious virus particles, the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV) can initiate infection [139]. The IMV and EEV virions differ in their surface glycoproteins and in the number of wrapping membranes [140].

The general scheme of VV replication (fig.10) can be summarized in five steps. (i) The binding of the virion to the cell membrane and is determined by several virion proteins and by glycosaminoglycans (GAGs) on the surface of the target cell or by components of the extracellular matrix. Fully permissive viral replication is characterized by three waves of viral mRNA and protein synthesis (known as early, intermediate and late), which are followed by morphogenesis of infectious particles. (ii) The transcription of early genes under control of early promoters begins few minutes after release of the core in the cytoplasm of infected cells. During this early infection phase, early RNA is transcribed by the virion associated RNA polymerase. (iii) Two to five hours after infection, the core liberates the viral DNA for cytoplasmic DNA replication and intermediate transcription occurs. (iv) Late RNA is then transcribed under control of late promoters. (v) The last step of the replication leads to morphogenesis of new viral particles by assembling viral proteins and the newly synthesized DNA [129]. The initial IMV is transported via microtubules (not shown in the figure) and it is wrapped with Golgi-derived membrane, after which it is referred to as an IEV. The IEV fuses to the cell surface membrane to form cell-associated enveloped virus, which is released to form free EEV. The EEV might also be formed by direct budding of IMV, therefore bypassing the IEV form [141].

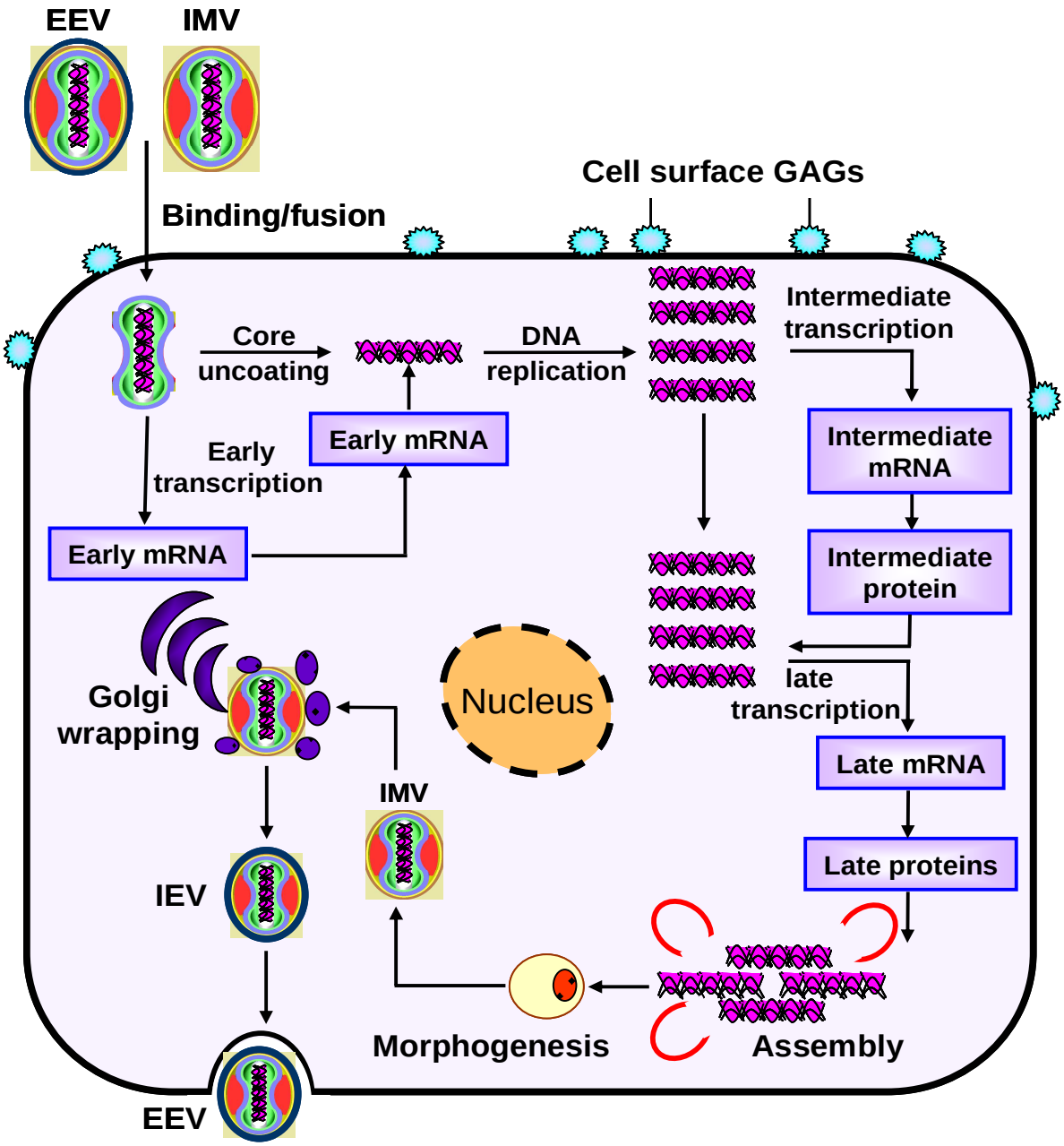


Figure (10): Poxvirus replication cycle. Two distinct infectiousvirus particles, the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV) — can initiate infection. The binding of the virion is determined by several virion proteins and by glycosaminoglycans (GAGs) on the surface of the target cell or by components of the extracellular matrix. Fully permissive viral replication is characterized by three waves of viral mRNA and protein synthesis (known as early, intermediate and late), which are followed by morphogenesis of infectious particles. The initial intracellular mature virus (IMV) is transported via microtubules (not shown in the figure) and is wrapped with Golgi-derived membrane, after which it is referred to as an intracellular enveloped virus (IEV). The IEV fuses to the cell surface membrane to form cell-associated enveloped virus (CEV; not shown), which is either extruded away from the cell by actin-tail polymerization (not shown) or is released to form free EEV. EEV might also form by direct budding of IMV, therefore bypassing the IEV form. Poxviruses also express a range of extracellular and intracellular modulators, some of which are defined as host-range factors that are required to complete the viral replication cycle.

II.3.2. Advantages of VV as a Delivery Vehicle for Cancer Immunotherapy

Although the global eradication of smallpox in the early 1980 was made possible by vaccination with VV, this virus is no longer needed for smallpox immunization, but now serves as a useful vector for expressing genes within the cytoplasm of eukaryotic cells. As a research tool, recombinant vaccinia viruses are used to synthesize biologically active proteins and analyze structure-function relations, determine the targets of humoral- and cell-mediated immunity, and investigate the immune responses needed for protection against specific infectious diseases. Upon generation of data on safety and efficacy, recombinant vaccinia and related poxviruses became candidates for live recombinant vaccines and for cancer immunotherapy [142]. The advantages of VV as a vector are outlined below.

II.3.2.1. Cytoplasmic Replication

VV replication occurs exclusively in the cytoplasm. This facilitates introduction of foreign genes into the viral genome by marker transfer and also the radiolabeling, detection, and isolation of proteins expressed by recombinant viruses. Furthermore, there is no risk of integration into the host cell genome and/or phenotypic transformation [113].

II.3.2.2. Wide Host Range

VV has a wide host range, capable of infecting almost all human cell types with high efficiency. It replicates in both primary cell cultures and many different cell culture lines isolated from virtually any animal species. VV also grows in almost all types of experimental animals commonly used in the laboratory [143].

II.3.2.3. large Viral Genome

The 192 kb genome of VV readily tolerates both large insertions of foreign DNA and deletions of viral sequences to further expand the quantity of insert. The ability to accommodate and express at least 25 kb of foreign DNA sequence by the virus is an important factor to be considered to construct a polyvalent vaccine [144].

II.3.2.4. Viral Transcriptional Machinery

VV transcribes its genome by using unique viral enzymes, viral transcription signals, and ancillary transcription factors [145]. Foreign transcripts will be capped and polyadenylated by VV enzymes and will serve as efficient messages for the translation of relatively high levels of the foreign protein within the infected cell.

II.3.2.5. Safety

VV was the first viral vaccine used by Edward Jenner to prevent smallpox. Thus, we might consider that it has been in clinical trials since 1798 [146]. Although complications such as postvaccination encephalitis or progressive VV infections can occur in immunocompromised recipient, overall VV is quite safe and effective vaccine, as evidenced by its successful use to eradicate smallpox from the human population globally.

II.3.2.6. Cost

Because of its broad host range, VV can easily be grown to high titers in a variety of cell lines or animal hosts [146]. It is cost effective to deliver as a vaccine as it is cheap to be produced. It is “off the shelf” reagent.

II.3.2.7. Stability

The VV virion is very stable, maintaining infectious titer while frozen for many years. Furthermore, VV particles can be stored as dry powder for prolonged periods, rehydrated, and inoculated with only minimal losses in infectivity, thus permitting easy transport and clinical application [147].

II.3.2.8. Ease of Administration

Classical intradermal administration of VV-based vaccine does not require the same level of medical training as an intravenous injection.

II.3.2.9. Replicating VV as Oncolytic Agent

Efficient replication, cell lysis, broad host range and spread, remarkable safety along with natural tropism of VV for tumor tissues, make vaccinia virus a very attractive vector for developing oncolytic viruses. Genetic modifications of VV have been designed to create oncolytic vectors that favour the natural tropism for tumor cells. One approach is to delete viral genes that are critical for efficient viral replication in normal cells but dispensable in tumor cells. For example, a recombinant VV with thymidine kinase gene (TK) deletion has demonstrated enhanced tumor selectivity over normal tissues [148]. VGF is expressed early during VV infection cycle and is secreted from infected cells. It binds growth factor receptors on surrounding resting cells and stimulates cell proliferation. Recombinant VV with double deletion of TK and VGF was found to have markedly enhanced tumor specificity [149].

II.3.3. Limitations of Vaccinia Virus as a Vector

Despite those benefits mentioned before, VV used as a gene therapy vector (for delivery of tumor antigens and immunoregulatory molecules), has encountered limited clinical success in cancer therapy. Among the possible issues, the high immunogenicity of the virus, which limits the possibility of repeated injections [150]. Immunodominance and immunoprevalence of viral

antigens may also represent a problem in the competition with the weaker recombinant tumor antigens for the cellular responses [151;152].

II.3.3.1. Long term memory response to vaccinia virus

Vaccinia virus replicates in the cytoplasm of infected cells and it is not thought to persist or become latent after the acute phase of infection. However, long-lived vaccinia virus-specific memory cytotoxic T-cells were identified in adults who had been immunized against smallpox as children.

Some authors observed that the capacity of VV to induce an immune response against heterologous proteins could be greatly impaired in recipients who had immunity against VV as vaccination with vaccinia had eradicated smallpox in 1980. Initially, vaccinia virus-specific T cells were detected in peripheral blood mononuclear cells while screening for human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses in HIV-1-seropositive subjects. These individuals had not had contact with VV since their primary immunization in early childhood. Several vaccinia virus-specific CD4+ T-cell clones were derived from these donors and characterized. Healthy, HIV-1-seronegative donors who had been immunized against smallpox many (35 to 50) years earlier were also screened for VV -specific T-cell immunity and significant CD8+ and CD4+ T-cell responses to VV was found after in vitro stimulation, indicating that these memory cells are maintained in vivo for many years [153]. It was concluded that specific VV T-cell immunity can persist for up to 50 years after immunization against smallpox in childhood in the presumed absence of exposure to the virus.

Antiviral antibody response remained stable between 1-75 years after vaccination [154]. The human CD8+ T-cell response to vaccinia is robust at early times after vaccination and can be very diverse within an individual [155]. Several candidate immunodominant antigens, containing multiple epitopes, have

been described. These antigens and epitopes should be useful in evaluating modified poxviruses being developed as vectors for heterologous antigens [156].

II.3.3.2. Immunodominance of poxviral-specific CTL

Many recombinant poxviral vaccines are currently in clinical trials for cancer and infectious diseases. However, these agents did not succeed to generate T cell responses specific for recombinant gene products at levels comparable with T cell responses associated with natural viral infections. The recent identification of vaccinia-encoded CTL epitopes allows the simultaneous comparison of CTL responses specific for poxviral and recombinant epitopes [157].

Harrington et al., had developed a simple intracellular cytokine staining (ICS) assay using VV-infected syngeneic cell lines expressing MHC class I and class II proteins to quantitate VV-specific CD8 and CD4 T-cell responses. Using this assay, they monitored the magnitude and duration of T-cell responses to the vector (VV) and also to the foreign epitope following infection of mice with r.VV expressing the $NP_{118-126}$ CTL epitope of lymphocytic choriomeningitis virus (LCMV). They also proved that VV specific effecter CD8+ and CD4+ T cells are able to produce IL-2, IFN-γ and TNF-α in response to vaccinia virus. |They found that the total number of CD8 T cells responding to $NP_{118-126}$ were about 20- to 30-fold lower than the number responding to the VV vector [151].

These results demonstrate that immunodominant vaccinia-specific CTL responses limit the effectiveness of poxviruses in recombinant vaccination strategies and that more powerful priming strategies are required to overcome immunodominance of poxvirus-specific T cell responses

Although vector specific immune responses, especially CD4+ T cells, may initially be beneficial for the induction of CTL responses against transgenes, they may also prevent multiple use of the same vaccine [158]. In

particular, responses against the vector and its immunodominant epitopes may out-compete the anti-tumor response specific for recombinant antigens [151;159].

These limitations of using VV as a vector were the initiative issues addressed by the hereby presented project which is designed to overcome the problem of long lived memory immune response against VV and counteract the immunodominance of VV epitopes.

II.4. IMMUNOMODULATIONS OF CANCER VACCINES

A key limitation of tumor immunotherapy arises from the fact that the host is tolerant for most tumor self antigens and T cells exhibit low avidity for these antigens. Breaking this tolerance is therefore a major goal of immunotherapy and requires the development of novel strategies for modulating the antitumor immune response through identification of immune adjuvants that can activate and amplify these residual low-avidity tumor-reactive T cells. The pattern and duration of immune responses associated with these new modalities differ from those associated with cytokines and cytotoxic agents. In addition, vaccines are being developed that may ultimately target TAA in combination with these immunomodulatory therapies [160]. Limited number of adjuvants is licensed for use in humans due to their potential severe side effects [161].

Strategies that enhance numbers and the effector functions of T cells have been demonstrated according to the three different signal stages needed to activate T cells: antigen, costimulation and cytokine environment.

II.4.1. Antigen Formulation (first signal)

Binding of antigen/MHC complex to the TCR/CD3 complex provides the first signal for activation of naïve T cells. Different approaches have been used to provide the antigen and optimize the formulation. From the simple cells lysate to complex nanoparticles, we will here describe only the option that we have been using for vaccinia vector.

- Minigene Formulation for Specific HLA Restriction

As CTL recognize antigens as short peptides presented by MHC class-I molecules, characterization of these antigen fragments denominated cytotoxic T cell determinants or epitopes, has allowed the design of immunization strategies based on the use of subunit vaccines [162]. In the case of tumor cells, many epitopes belonging to different tumour antigens and different HLA restrictions

have been characterized during the last years [163]. Whereas corresponding synthetic peptides are simple to obtain and to be used, their relative lack of intrinsic immunogenicity and instability render this approach in human application relatively inefficient if not properly adjuvanted. On the other hand, the formulation of these epitopes via intracellular expression, based on plasmid DNA or viral vectors, of minigenes encoding for these specific peptides has been successfully developed and clinically applied.

- Induction of Multiepitopic Immune Responses against Tumor

A common feature of “fast growing” entities such as cancer and viruses is that, facing a monovalent immune response recognizing a single antigen, they rapidly select variants that are no longer expressing or presenting the recognized epitope. In order to decrease chances of rapid variant selection, but also allowing larger spectra of immune responses, strategies combining several antigens have been formulated. Thus, the use of multiple TAA epitopes is an attempt to circumvent antigen expression heterogeneity and to limit immune escape [3;164].

- Targeting the Endoplasmc Reticulum

To facilitate the entry of the antigenic epitope into the endoplasmic reticulum, a sequence coding for adenovirus E3/19K leader peptide was added. Infection with r.VV expressing ER-targeted minigene elicited a stronger CTL response as compared to non-targeted or addition of exogenous peptide. These ER-targeted minigene formulations, for specific HLA restrictions, enable bypassing of a number of antigen processing steps, therefore avoiding limiting factors and ultimately result in an overall increased surface presentation of antigenic peptides within HLA molecules. Their capacity to generate epitope specific immune responses is thereby enhanced, as compared to vectors encoding the full antigen (figure 11) [165].

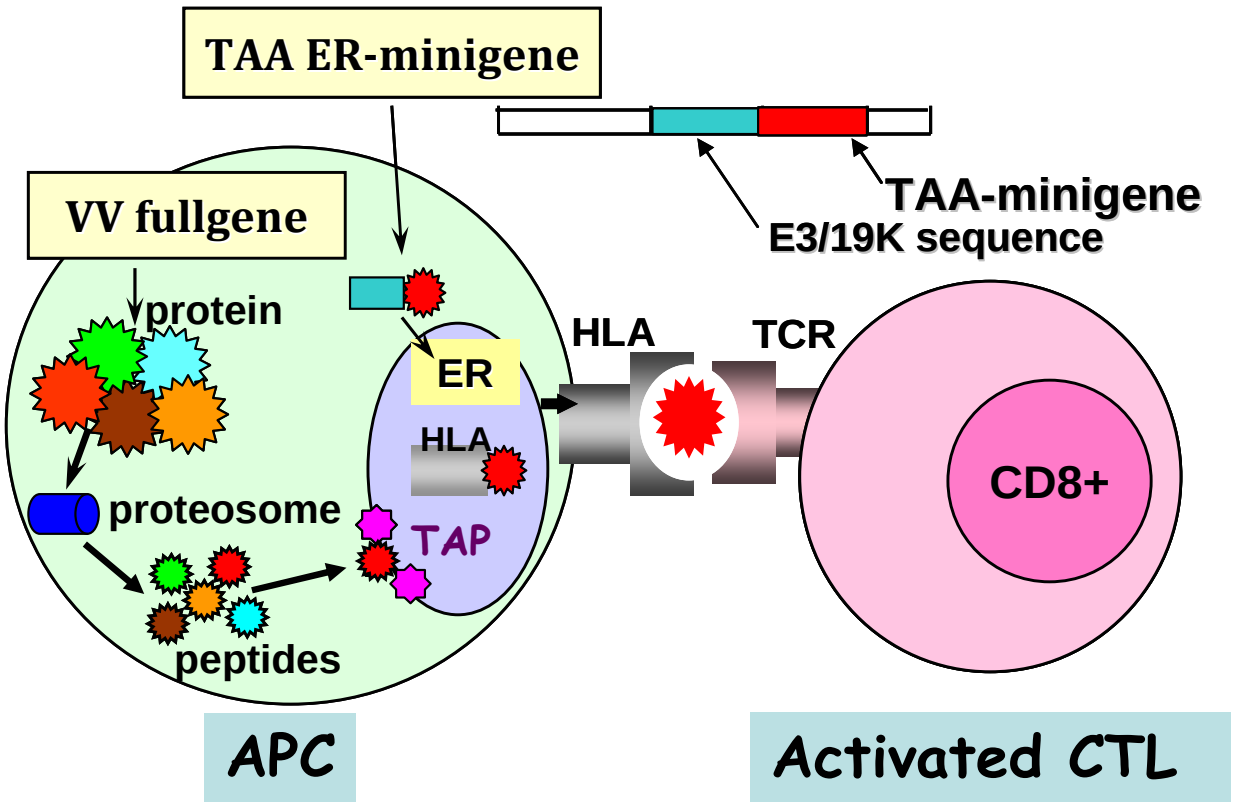


Figure (11): Minigene formulation.

TAA minigene is formulated in ER-targeted form which enables the encoded epitopes to pass directly to ER to be restricted by HLA molecule and bypass number of antigen processing steps. Viral proteins encoded from viral full genes undergo proteaomal degradation into small peptides that diffuse to TAP to be transported to ER, and then loaded on HLA molecule and recognized by specific T cell receptor (TCR).

- Epitope enhancement

Modification of the amino acid sequence of epitopes, commonly referred to as epitope enhancement, can improve the efficacy of vaccines through several means: (a) increasing affinity of peptide for MHC molecules [166]; (b) increasing TCR triggering [167]; or (c) inhibiting proteolysis of the peptide by serum peptidases [168]. Whenever the peptide sequence is altered, it is important to demonstrate that the T cells induced still recognize the native peptide sequence.

- Vectors

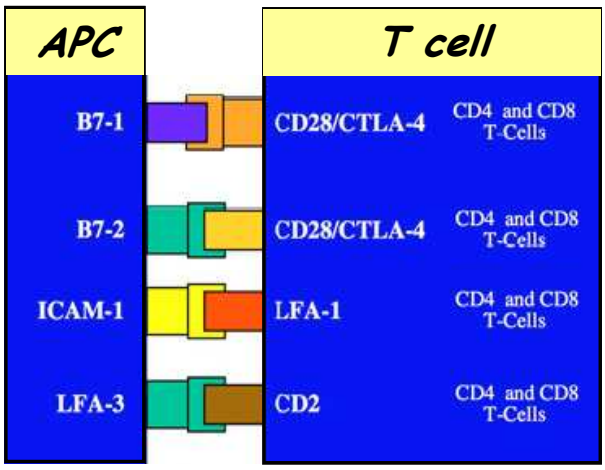
To increase the efficiency of antigen-dependent immune modulation, researchers started to investigate novel vectors for antigen delivery [169]. Viral vectors are potent gene delivery platforms because of their ability to efficiently infect host cells. Removal of the replicative and pathogenic ability of viruses, combined with their capacity to carry the therapeutic transgene and an ability to efficiently infect various mammalian cell types makes them amenable for use in gene therapy. However, the immune system has evolved to fight off invading pathogens, which makes viral vectors subject to immune responses that have to be either blocked or avoided to achieve therapeutic transgene expression [170].

Adamina et al., have developed liposomal vectors that protected tumor epitopes against peptidases [171], and then these vectors were refined into immuno-stimulatory reconstituted Influenza virosomes (IRIV) containing Influenza virus A derived hemagglutinin and neuraminidase [172]. It was shown that IRIV are able to improve the generation of CTL responses specific for encapsulated peptides in vitro [173] and in vivo [172;173].

II.4.2. T-Cell Co-stimulation (second signal)

T cells depend on signals additional to antigen recognition (Signal 2) to achieve full activation. The term costimulation usually describes the modification of T cell activation processes by the interaction of membrane-bound ligands with their T cell-expressed receptors. Costimulatory signals transduced via CD28 and TNFR family members (figure 12) play paramount roles in modulating innate, adaptive, and regulatory immunity [174]. Agonistic ligands for this class of immunomodulatory receptors have potential to serve as effective components of therapeutic cancer vaccines.

Figure (12): costimulatory molecules.



- B7 molecules

Interaction of the B7 molecules B7.1 (CD80) and B7.2 (CD86) on APC with CD28 on T cells is generally regarded as the primary costimulatory pathways involved in T cell activation. To enhance the immunogenicity of TAAs, transgenes for different T-cell costimulation molecules are placed into viral vectors along with the transgenes for the TAA [175]. The use of B7 co-stimulatory molecules as adjuvants in prime boost vaccination strategies has enhanced the generation of CTL when expressed along with TAA [3;176;177].

- ICAM-1/LFA-1

LFA-1 is the best known costimulatory member of the integrin family. LFA-1 is a key accessory molecule expressed on T cells that interacts with intercellular adhesion molecule 1(ICAM-1 or CD54) on APCs [178]. In the context of antitumor immunity, ICAM-1/LFA-1 interactions could be important for T cell priming by APCs as well as for transendothelial migration and tumor cell recognition at the tumor site, cytokine production and protection from apoptosis. A TRIad of COstimulatory Molecules (TRICOM; B7-1, ICAM-1 and LFA-3) has been shown to enhance T-cell responses to TAAs to levels far greater than any one or two of the costimulatory molecules in combination [179-183].

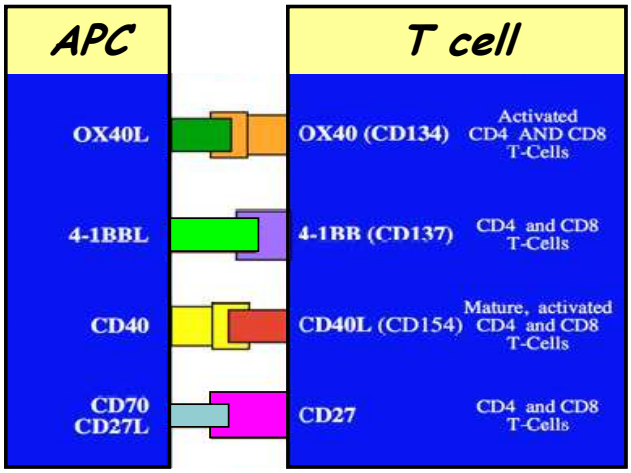
- CTLA-4 molecule

CTLA-4 is an inhibitory receptor on T cells that binds to B7 molecules with higher affinity than CD28. Engagement of CTLA-4, which is upregulated on activated T cells, counterbalances the activating effects of CD28 and leads to the inhibition of cell cycle progression and IL-2 production [184]. New immunotherapies targeting critical regulatory elements of the immune system may overcome tolerance and promote a more effective anti-tumor immune response. These include the use of monoclonal antibodies that block CTLA4 to prevent inhibitory signals that downregulate T-cell activation [185;186].

II.4.3. Costimulatory Molecules with Additional Functions (signal 3 and beyond)

After activation of T cells, a number of cell surface and soluble molecules are known to further regulate the immune response (signal 3). Certain costimulatory molecules, in contrast to B7, act predominantly on activated T cells. In particular, OX40L, 4-1BBL, CD70 and all members of TNF superfamily (e.g. CD40L), appear to have somewhat distinct roles as costimulators of activated CD4+ and CD8+ subsets of T cells (Figure 13). One of the suggested effects of these 'signal-3' molecules is to extend the lifespan of the stimulated effector cells by suppressing genes associated with apoptosis [187].

Figure (13): signal 3 costimulatory molecules.



- OX40L

Fowlpox viral vector expressing OX40L either alone (r.f-OX40L) or in combination with TRICOM costimulatory factors (r.f-TRICOM/OX40L) demonstrated that (a) OX40L plays a role in sustaining the long-term proliferation of CD8+ and CD4+T cells following activation, and (b) the anti-apoptotic effect of OX40L on T cells is likely the result of sustained expression of anti-apoptotic genes while genes involved in apoptosis are inhibited. In

addition, the use of r.f-TRICOM/OX40L both enhances initial activation and then further potentiates sustained activation of naive and effector T cells [188].

- 4-1BB ligand (4-1BBL)

4-1BB is an inducible costimulatory member of the TNFR family expressed on activated CD4+ and CD8+ T cells. Its ligand, 4-1BBL, is expressed on activated APC. 4-1BBL-mediated costimulation is highly effective in expanding and activating T cell memory responses to influenza virus and EBV and does so with faster kinetics than B7.1 [189].

- CD154 (CD40Ligand)

CD40 belongs to TNFR family and was first identified and functionally characterized on B lymphocytes. CD40 is expressed much more broadly on monocytes, dendritic cells, endothelial cells, and epithelial cells. In addition, the CD40-ligand (CD40-L/CD154), a member of the TNF family, is also expressed more widely on activated CD4+ T cells [190].

The interaction of DC expressed CD40 with CD40L on activated CD4 cells can profoundly enhance T cell responses, since CD40 signals result in the upregulation of MHC molecules and costimulatory molecules in DCs [191]. Thus the role of CD40L in T cell stimulation is indirect one through induction of T cell costimulatory ligands such as CD80 and CD86 on APC [192].

Recombinant VV expressing CD40L has provided an efficient adjuvance during the induction of CTL specific response for Mart-1/Melan-A_{27-35} TAA [193].

- Cytokines

Cytokines have become an integral part of cancer therapy and are also under trial together with cancer vaccines as post-surgical adjuvant therapies providing significant gains in long term survival rates.

IFN-alpha exhibits enhancing effects on T-cell and dendritic cell functions [194;195]. It is assumed that IFN-α has an antitumor and immunoadjuvant effect when used in combination with recombinant poxviruses [196]. Furthermore, immunization of stage IV melanoma patients with MART-1/Melan-A and gp100 peptides plus IFN-α resulted in induction of CTL immune response and activation of monocytes/dendritic cell precursors [197].

IL-2 is known to promote the expansion of TAA specific CTL. When IL-2 was paired with peptide vaccines in patients with resected stage III and IV melanoma, it appeared to boost the immune response to the vaccine [198;199].

After approval of GM-CSF use in stem cell and bone marrow transplantation, it was also suggested that GM-CSF might have application as immunotherapy in melanoma after surgical resection. The theory is that GM-CSF would activate antigen-presenting cells, and thus the ability to mount an immune response [200;201].

IL-2 and IL-15 appear to be comparably effective in the induction of CTL proliferation in response to MART-1/Melan-A_{27-35} targeted active specific immunotherapy [202].

- Toll like receptor (TLR) agonists

TLRs bind to one or more distinct pathogen-expressed molecules and can function as an “alarm signal” for the immune system, initiating appropriate host immune defenses. For example, TLR4 detects LPS which is specifically

expressed by Gram-negative bacteria. In response to LPS, TLR4 activation induces the secretion of proinflammatory cytokines and chemokines by host immune cells [203;204]. A synthetic agonist for TLR4, monophosphoryl lipid A, has been developed as a vaccine adjuvant [205].

TLR-9 recognizes unmethylated bacterial CpG-DNA and its clinical use is expected for cancer therapy as a potent inducer of a helper T cell 1 (Th1)-type T-cell response [206]. It was shown that TLR9 agonists stimulate dendritic cell maturation and ultimately induce a more effective immune response [207;208]. Early studies indicated that inserting model tumor-associated antigens into viruses, which contain TLR agonists, can augment their immunogenicity and function as tumor vaccines [209-211]. Recently, Speiser and colleagues have made efforts to use TLR agonists in conjunction with vaccination in patients with melanoma [212;213]. They found that combining TLR9 agonist CpG ODN 7909 (a 24-mer oligodeoxynucleotide containing 3 CpG motifs) with a Melan A/MART1 $_{26-35}$ peptide and incomplete Freund's adjuvant increased the number of MART1-specific T cells by >10-fold compared with vaccination without CpG [213].

Our group has generated recombinant vaccinia virus expressing antigenic epitopes derived from melanoma TAA. This r.VV is characterized by peculiar features. First, it encodes HLA-A0201 restricted **multiple epitopes** from three different melanoma differentiation antigens, Melan-A/Mart-1_{27-35}, $GP100_{280-288}$ and $tyrosinase_{1-9}$. Second, the antigenic epitopes are encompassed within a polypeptide including an adenovirus 19K derived leader sequence (MRYMILGLLALAAVCSA) driving the resulting recombinant products **directly into the ER**, thereby bypassing antigen processing steps. Third, genes encoding **CD80 and CD86 co-stimulatory molecules** (required for T cell activation) have been added to this vector. Following the *in vitro* demonstration of the vector (Penta-Mel-r.VV) efficacy [177;214], it was successfully tested in

phase I/II immunotherapy clinical trial for stage III and IV melanoma patients. Administration of this viral vector was based on intradermal (ID) injection followed by boosts with solution of the corresponding specific peptides [3].

The results of this first clinical study showed that despite a good immunogenicity of the viral vector, peptide ID injection were unable to properly boost the virally induced response. Therefore, we sought solutions to increase the potency of this protocol.

Studies with synthetic tumor antigenic peptides have demonstrated that induction of single amino acid substitution may dramatically increase their immunogenicity. Therefore, we also constructed a r.VV expressing tumor antigenic **peptide analogues** with appropriate nucleotide substitution which leads to improved antigen recognition and enhanced immunogenicity [215].

We have also developed a **r.VV expressing CD40L** and demonstrated its capacity to enhance APC immunogenicity for specific CD4+ and CD8+ T cell responses [193].

Finally, since lymph nodes are the primary site of immune reactions, it was suggested that intranodal administration might be more immunogenic than ID route for the induction of TAA specific immune responses [216] especially for soluble peptides which are rapidly degraded once injected in peptidases containing environment.

We therefore performed a second clinical trial based on the **intranodal** (IN) administration of our Penta-Mel-r.VV boosted by 3 IN injections of soluble peptides. Remarkably, CTL responses against at least one of the antigenic epitopes were detectable in the majority of patients and humoral responsiveness to VV vector was confirmed [217].

Moreover, some patients received “supplementary rec.VV immunization” on compassionate basis. These additional viral vector injections demonstrated an improved immune responsivness thus underlining the potential of prolonged immunization protocols with a viral vector. However, these multi-virus injections immediately raised the issue of immune-impairement due to anti-vector responses.

In the present study, we are addressing the issue of the possible limitations of using VV as a viral vector, due to prior systemic immunity and to immunodominance of VV antigens, resulting in reduced induction of immune response against weaker tumor antigens. We developed a r.VV expressing HSV-US12, which down-regulates MHC class-I antigen presentation by blocking TAP transport. The relevance of this viral vector, especially in the perspective of multiple-boost vaccine protocol for cancer immunotherapy, was hereby investigated.

II.5. INFECTED CELL PROTEIN 47 (ICP47) AS IMMUNOMODULATOR

HSV-I is a highly abundant human pathogen that achieves lifelong persistence in the ganglia of the nervous system. Upon exogenous stimuli, it can be repeatedly reactivated and infect related mucosal tissues leading to clinical symptoms. To escape immune surveillance, herpes simplex virus compromises the host's cytotoxic T lymphocyte response via different mechanisms. Among these mechanisms is ICP47 blockade of TAP function [218]. ICP47 represents the first natural inhibitor of an ABC transporter described so far [219].

II.5.1. US12 Gene

HSV has a number of genes devoted to immune evasion. US12 (also called alpha47) gene (fig. 14) encodes the small immediate-early regulatory protein ICP47, which inhibits antigen presentation in infected cells by specifically binding to and blocking TAP [220].

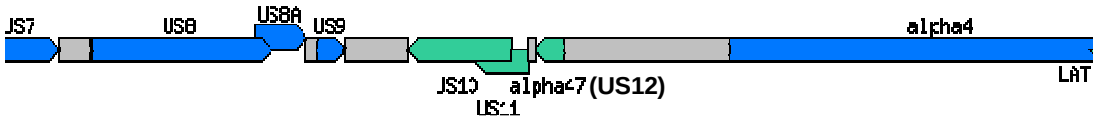


Figure (14): US12 gene

This presentation inhibition of viral and cellular antigens associated with MHC class-I proteins to CD8+ T-cells effectively decreases immune recognition and thus increases infective persistence [221].

II.5.2 Structure of ICP47

ICP47 is an 88 amino acid immediate early gene product (IE12) [218]. It is a membrane associated protein adopting an α-helical conformation. Functional studies with N- and C-terminally truncated variants of ICP47

demonstrated that the N-terminal domain of ICP47 is sufficient for TAP inhibition (fig.15: residues 1-53 in blue [219], 2-35 in yellow [222] and 3-34 in pink [223]). Moreover, by alanine scanning mutagenesis, three regions (residues 8-12, 17-24 and 28-31) were identified within the active domain of ICP47, which are critical for TAP inhibition [222].

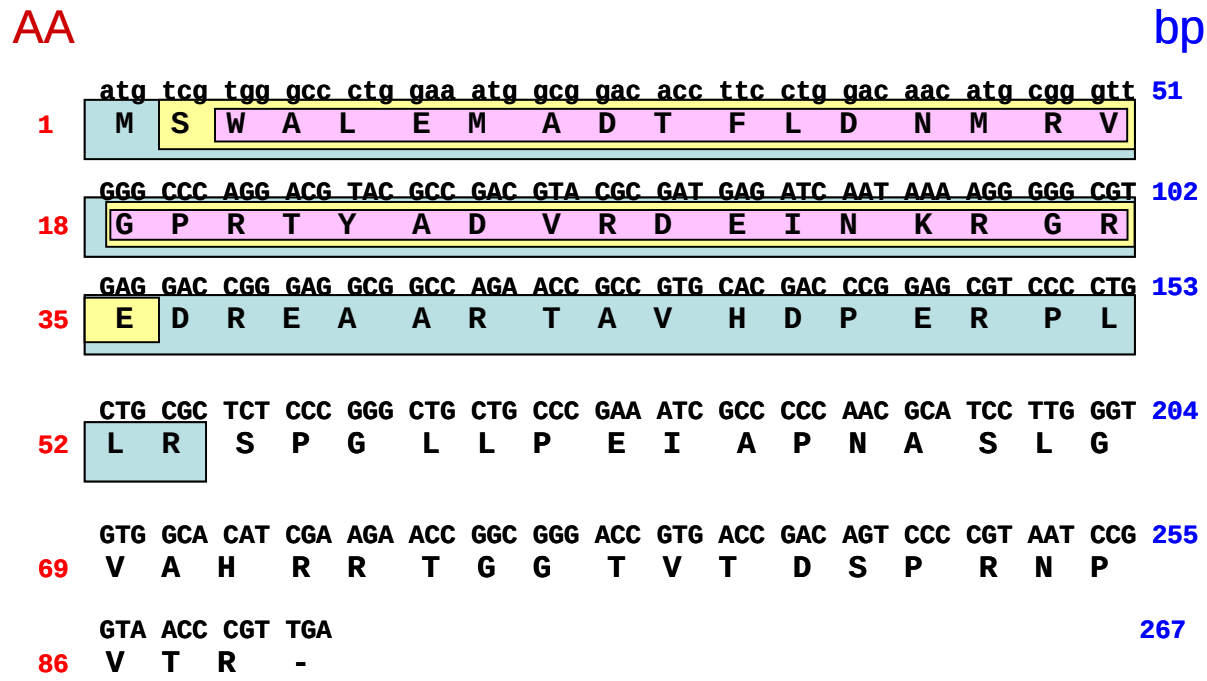


Figure (15): Active domain of ICP47 protein

Multidimensional solution NMR spectroscopy (fig. 16) indicates that the active domain of ICP47 adopts a helix-loop-helix conformation in the presence of detergent micelles.

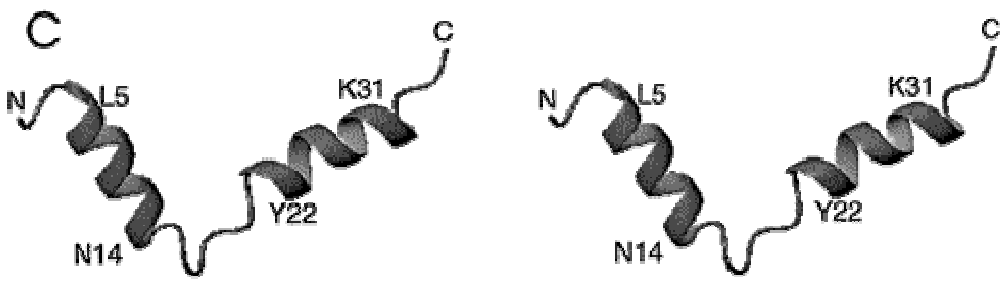


Figure (16): **Ribbon drawing of a single structure of ICP47.** AA (2−34) bound to SDS micelles [224].

The active domain of ICP47 appears to be mainly unstructured in aqueous solution [219]. In the presence of lipid like environment, an α-helical structure is induced, which is composed of two helical regions extending from residues 4-15 and 22-32 connected by a flexible loop. On the basis of these results, therapeutic drugs could be designed that are potent immune suppressors or that are applicable in novel vaccination strategies against HSV, restoring the ability of the immune system to recognize the infected cells [224].

II.5.3 Function of ICP47

TAP molecules possess a single peptide binding site shared between the two subunits TAP1 and TAP2 [225]. It has been demonstrated that ICP47 prevents peptide translocation into the ER lumen by specifically interacting with human TAP. Therefore, assembly and trafficking of MHC class-I molecules is impaired [226]. The active domain of ICP47 (residues 3-34) displays an identical ability to inhibit TAP function when compared to the full-length protein, illustrating preservation of the functional properties [223]. By binding with nanomolar affinity to the heterodimeric TAP complex, ICP47 blocks peptide but not ATP binding to the ABC transporter [218]. The active domain interacts with TAP at the subunit/membrane interface within the lipid head group region and blocks peptide translocation into the Endoplasmic Reticulum (ER) lumen (figure 17) [227]. In the membrane-bound state, ICP47 escapes proteasomal degradation, which otherwise occurs rapidly in the membrane-free state.

In the absence of a functional TAP transporter (within 3 h of infection), empty MHC I molecules are retained in the ER and ultimately directed to proteasomal degradation [21].

It was demonstrated that ICP47 binds with high affinity at least in part to the peptide binding site of TAP, thereby blocking the first and essential step in the translocation pathway [21]. Recently, a similar downregulation of peptide

transport activity was observed in bovine epithelial cells infected with bovine herpesvirus-1 [228]. The ICP47-TAP interaction is highly species specific, since the viral protein shows a more than 100-fold higher affinity for human than for murine TAP [218]. It has been further suggested that binding of ICP47 results in a conformational change of peptide transporter, which might also block TAP function.

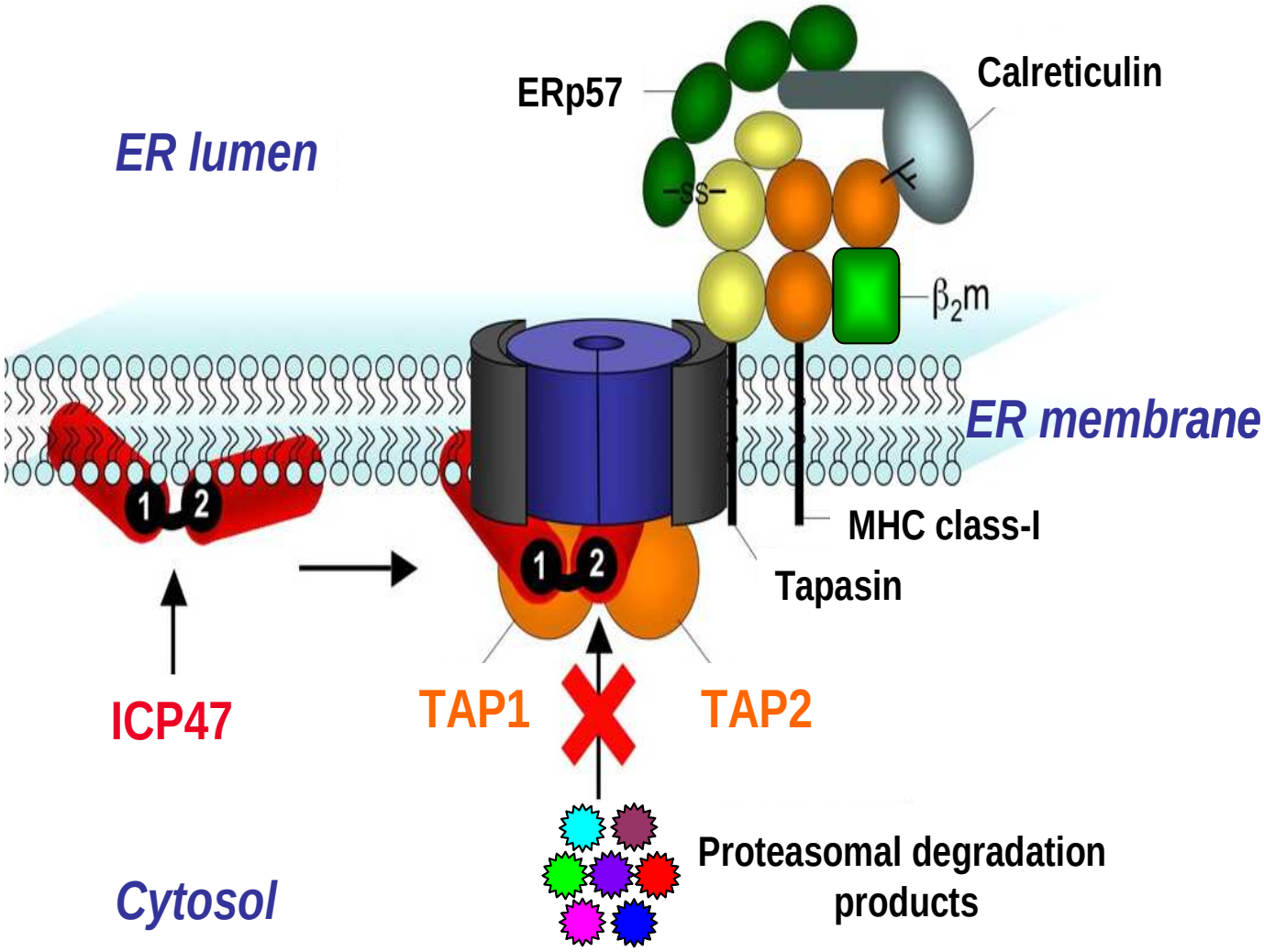


Figure (17): Model of the active domain of ICP47 in phospholipid bilayers.
After binding to the cytosolic face of the endoplasmic reticular (*ER*) membrane, ICP47 adopts a helix-loop-helix conformation. Subsequent association with the peptide-loading complex at the lipid-TAP interface blocks the peptide supply to MHC class-I molecules. The peptide-loading complex consists of the ATP-binding cassette halftransporter subunits TAP1 and TAP2, the adaptor protein tapasin, the MHC class-I heavy chain (*hc*), the noncovalently associated β-2-microglobulin ($\beta_2 m$), and several auxiliary factors (*e.g.* calnexin and the thiol-oxidoreductase ERp57) (Modified from Aisenbrey et al., 2006) [227].

II.6. RECOMBINANT VACCINIA VIRUS EXPRESSING ICP47 PROTEIN

Anti-vector immune responses are of major concern in clinical gene therapies. In recombinant virus mediated vaccination, this issue is even more critical since immunization procedure often require multiple injection of the vaccine. Reports from studies performed by using r.VV, suggest that previous immunization against the vector may result in impaired responsiveness against transgenic antigens, as compared to naïve recipient [158;229].

Competition between immunodominant epitopes [230] and relative responsiveness to vector and recombinant antigens [151] may play a role in this balance. Pre-existing cellular and humoral anti-vector immunity , which may be long lived for poxvirus [154], might relatively inhibit immunization although transgene CTL responses may still be possible [231], and have consistently been found in numerous vaccination protocols taking advantage of recombinant vaccinia vectors in pre-vaccinated patients [3;232].

Overcoming host CTL response and prolonging vector survival is now recognized as a major goal for many gene therapy models. Therefore, inserting the herpesvirus US12 gene into recombinant vaccinia virus vector which encodes TAA minigene may simultaneously decrease epitope competition and cellular anti-viral responses. In an APC infected with US12-recombinant vaccinia virus, the generation of most, if not all, epitopes drived from viral entire proteins following the classical MHC class-I processing, transport and presentation pathway, should be blocked. On the other hand, recombinant ER targeted vaccine epitopes should not be affected by ICP47 mediated blockade and may profit from reduced competition and more efficiently induce CTL response.

III.MATERIALS AND METHODS

III.1. MATERIALS

III.1.1. Cells

- CV-1 cells: are monkey African green kidney fibroblasts, ATCC CCL70, ECACC Ref N°: 87032605 grow rapidly and form monolayers of fibroblast like cells, cultured in DMEM 10% fetal culf serum (FCS).
- HeLa cells: are human epithelial cancer cells. They were a gift from Dr. Jantscheff (Department of Clinical Oncology, University of Basel) and are cultured in DMEM 10% FCS.
- EBV-BL: are human B lymphocyte transformed by Epstein-Barr virus, cultured in RPMI supplemented with 10% FCS.
- Na-8, D10 and WM115 MEL: are human melanoma cell lines. They are HLA-A*0201 positive, and are cultured in DMEM 10% FCS.
- PBMCs: are peripheral blood mononuclear cells from healthy donors, who are all HLA-A*0201 positive, cultured in RPMI 5% human serum. $CD8^+$, $CD4^+$ and $CD14^+$ cells are positively selected by magnetic absorbent cell sorting (MACS) technique using appropriate antibody-magnetic microbeads.

III.1.2. Viruses

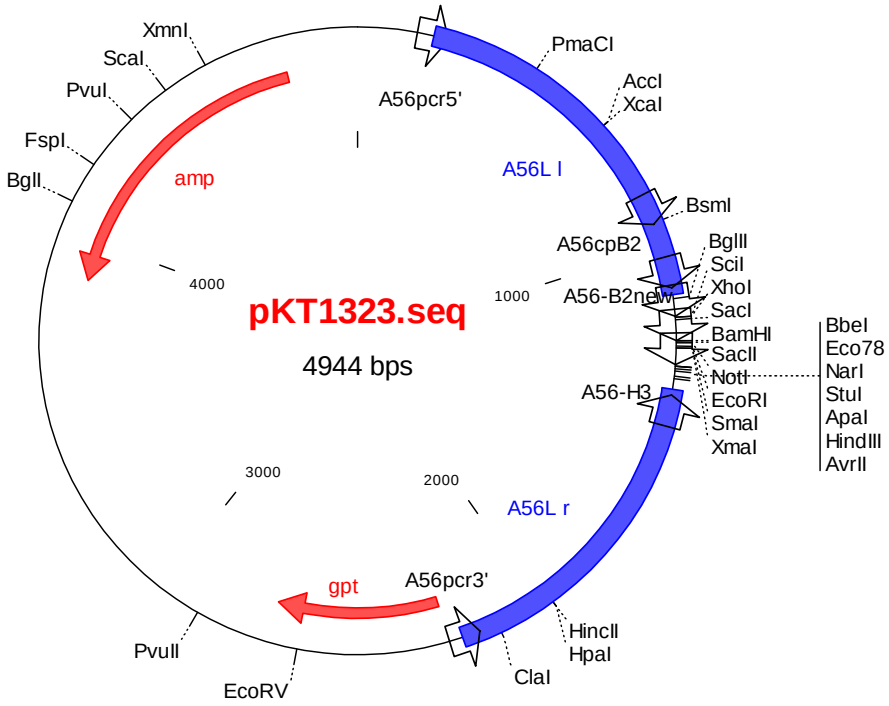
Vaccinia virus wild type and all derived recombinant constructs are based on the Copenhagen strain of Vaccinia virus (generously provided by Dr. R. Drillien, Strasbourg, France) All the recombinant vaccinia viruses used were produced according to Zajac et al., [233].

- r.VV-US12: recombinant vaccinia virus encoding the Herpes Simplex Virus US12 gene (codes for ICP47 protein) under early viral promoter control. US12 gene is inserted into the A56L locus of vaccinia virus genome by homologous recombination.

- r.VV-Mart: recombinant vaccinia virus encoding the minigene MART-1/Melan-A_{27-35} under early viral promoter control. MART-1/Melan-A is inserted in the I4L locus of vaccinia virus genome by homologous recombination.
- r.VV-Mart-US12: recombinant vaccinia virus encoding the minigene MART-1/Melan-A_{27-35} and HSV-US12 gene.

III.1.3. Plasmids

For the construction of recombinant virus, pKT 1323 plasmid (generous gift from Dr. K. Tsung, San Francisco, CA) was used. This plasmid (map 1) contains two homologous regions from A56R locus of the viral genome flanking the cloning site [234], in which the gene of interest is inserted under the control of a vaccinia specific early promoter and transcription termination signals.



Map (1): pKT for the construction of US12 encoding plasmid.
gpt gene is used as a transient marker for selection of recombinant vaccinia virus, and *amp* gene as selection marker for further tranfected bacteria. Short arrows indicate the location of oligonucleotides used for PCR or sequencing reactions.
amp = ampicillin resistance (β-Lactamase); gpt = guanine phosphoribosyl transferase, MPA resistance.

III.1.4. Media and Buffers

- DMEM 10% FCS: DMEM[1], 1% HEPES buffer[2] 1M, 1% Non Essential Amino Acids (MEM-NEAA)[2] (100x), 1% GlutaMAX™-I[2] (100x), 1%Sodium Pyruvate[2] (100x), 1% Kanamycin[2] (100x), 10% FCS.
- RPMI 5% HS: (complete medium): RPMI 1640 Medium[3], 1% HEPES buffer[2] 1M, 1% MEM-NEAA[2] (100x), 1% GlutaMAX™-I[2] (100x), 1%Sodium Pyruvate[2] (100x), 1% Kanamycin[2] (100x), 5% filtered human serum[4].
- CTL medium: RPMI 5% HS and IL-2 200unit/ml final.
- GM-CSF medium: RPMI 1640 Medium[3], 1% HEPES buffer[2] 1M, 1% MEM-NEAA[2] (100x), 1% GlutaMAX™-I[2] (100x), 1%Sodium Pyruvate[2] (100x), 1% Kanamycin[2] (100x), 10% FCS[2] and GM-CSF 50ng/ml[5].
- MACS buffer: PBS (BD nº349202), 0.5% FCS and EDTA pH 8, 0.5mM[1]

[1]Fluka, BuchsSG, Switzerland; [2]GIBCO, Paisley, UK; [3]Invitrogen, Carlsbad, CA; [4]Blood bank, University Hospital Basel, CH; [5]Novartis, Basel, CH.

III.1.5. Antibodies and MHC-multimers

- Mouse IgG antibodies to human HLA-ABC, HLA-A 0201 molecule, CD14, CD4, CD8, CD80, CD44 and HLA-DR molecule. Control IgG, R-PE, APC or FITC conjugated (BD PharMingen, Franklin Lakes, NJ).
- Soluble MHC-peptide pentamer Streptavidin R-PE conjugate, MHC allele: HLA-A 0201 (ProImmune, Oxford, UK): MART-1/Melan-A_{26-35}.
- Soluble MHC-peptide multimer Streptavidin R-PE conjugate, MHC allele: HLA-A 0201 (ProImmune, Oxford, UK): Vaccinia Virus $H3L_{184-192}$, $B22R_{29-37}$ and $C7L_{74-82}$.

III.1.6. Primers and Probes

β-actin

Pre-developed assay: (Applied Biosystem, Foster City, CA)

VV I3L [193]

Fwd CGGCTAGTCCTATGTTGTATCAAC TTC
Rev TGC AAATTTGGAATGCG
Probe FAM-AGAAGCCGTCTATGGAAACAT TAAGCACAAGG-TAMRA

GAPDH [235]

Fwd ATG GGG AAG GTG AAG GTC G
Rev TAA AAG CAG CCC TGG TGA CC
Probe FAM-CGC CCA ATA CGA CCA AAT CCG TTG AC-TAMRA

IFN-γ [236]

Fwd AGC TCT GCA TCG TTT TGG GTT
Rev GTT CCA TTA TCC GCT ACA TCT GAA
Probe FAM-TCT TGG CTG TTA CTG CCA GGA CCC A-TAMRA

IL-2 [237]

Fwd AAC TCA CCA GGA TGC TVA CAT TTA
Rev TCC CTG GGT CTT AAG TGA AAG TTT
Probe FAM-TTT TAC ATG CCC AAG AAG GCC ACA GAA CT-TAMRA

ICP47 (Inner)

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
ICP47 (Nest)

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

III.1.7. Chemicals

- Psoralen: (CN Biosiences, Nottingham, UK): 4 aminomethy- trioxsalen (trioxsalen: 4, 5, 8- trimethylpsoralen): $C_{15}H_{15}NO_3$.
- Paraformaldehyde 1%: (Polyoxymethylene, $(CH_2O)_n$, 30.03 D, Fluka Chemi AG, Buchs, Switzerland).

III.2. METHODS

III.2.1. Virological Methods

III.2.1.1. Cloning procedure for recombinant vaccinia virus preparation

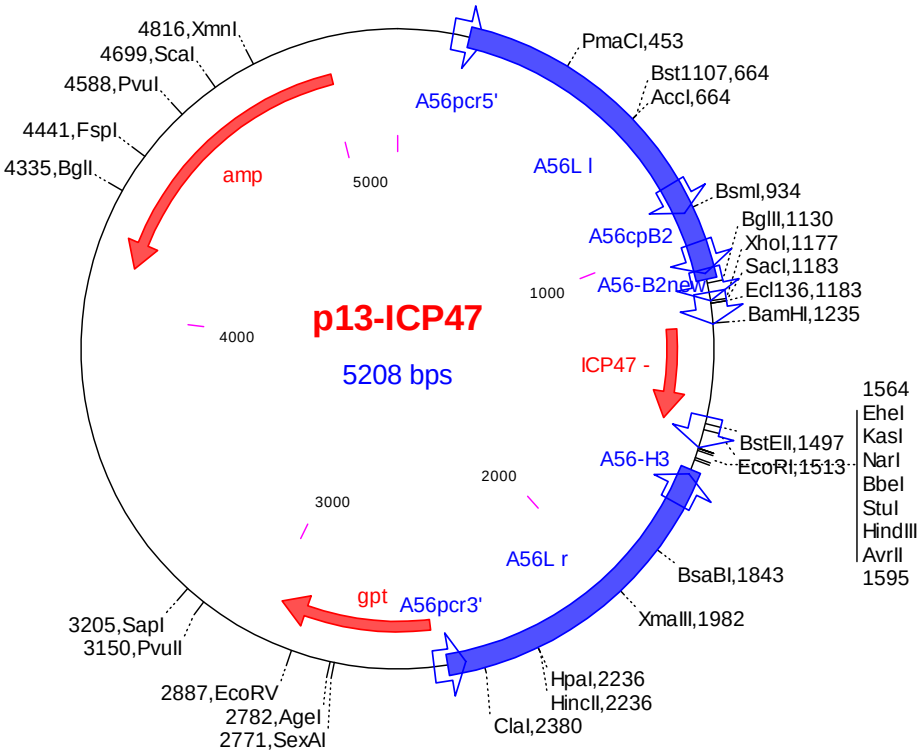
DNA samples of cerebrospinal fluid from HSV positive patients were kindly provided by Prof. M.E. Lafon, (Laboratoire de Virologie, University of Bordeaux, Bordeaux – France). US12 gene was amplified and isolated by nested PCR technique to reduce contamination in the PCR product.

Technical Procedure: the first round polymerase chain reaction (PCR) performed with 5µl DNA sample from HSV genome. After 15 cycles performed with the "nested" primers, 5µl of the PCR product are used to start the second run of 20 cycles using the set of "inner" primers. After amplification, the nested PCR product is run on 1% agarose gel. The size of the second round product is expected to be 267bp.

US12 gene is inserted into pKT1323 after plasmid digestion by BamHI and EcoRI restriction enzymes (map 2) (Promega, Madison, WI). Competent E. coli (Top 10; Invitrogen, Paisley, UK) are transformed by electroporation (25µF and 2.5kV; Gene Pulser apparatus; Bio-Rad Laboratories, Hercules, CA) following manufacturer's protocols and plated on LB agar (GIBCO, Paisley, UK) containing 100µg/ml carbenicillin (Fluka Chemie, Buchs, CH). After colony selection and amplification of bacteria in LB medium (GIBCO), plasmid DNA is isolated using the NucleoSpin® Plasmid Kit (Macherey-Nagel, Oensingen, CH). The insert presence is verified on 0.8% - 1% agarose gel (GIBCO) after restriction with HindIII and BglII restriction enzymes. Selected clones are also analysed by gene sequencing to verify the presence of US12 gene insert into pKT1323.

III.2.1.2. Transfection into viral vector

Recombinant vaccinia virus is generated by homologous recombination with co-transfectant (VV plasmid). This plasmid (map 2) contains multiple expression/ insertion cassettes containing early promoters [238] and a multiple cloning site with the VV early transcriptional termination sequence (TTTTTNT) located downstream. The expression/ insertion cassettes are flanked by sequences being identical to different viral loci and allow homologous recombination and production of rVV. The gene encoding the *Escherichia coli* guanine phosphoribosyl transferase *(gpt)* is used as a transient marker for selection of r.VV [239].



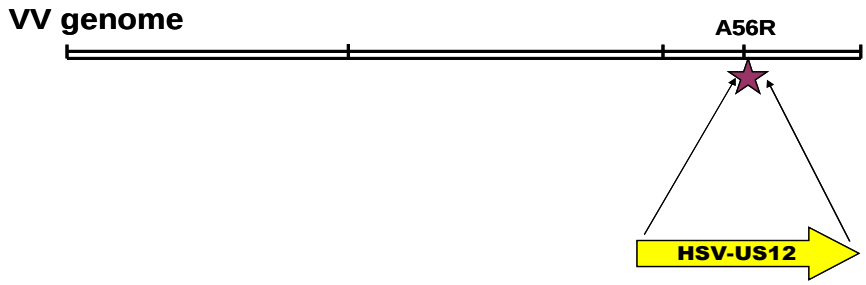
Map (2): US12 encoding plasmid
The plasmid contains two regions, homologous of a viral genome locus A56R; illustrated by blue boxes, flanking the cloning site. US12 gene was inserted into pKT1323 by BamHI and EcoRI ligation.

Subconfluent adherent CV1 cells are infected at m.o.i 0.1 for one hour at 37°C with one of the following viruses after sonication:

- Copenhagen wild type strain (WT) of vaccinia virus (generous gift from Dr. R. Drillen, Strasbourg, France) to generate r.VV-US12 (figure 18.A).
- Recombinant vaccinia virus encoding MART-1/Melan-A_{27-35}, produced according to Zajac et al [2] to generate r.VV-Mart-US12 (figure 18.B). MART-1/Melan-A_{27-35} is a TAP independent tumor antigenic epitope.

Lipofectamine™ Reagent (160µg/ml; Invitrogen, Carlsbad, CA) and the shuttle plasmid DNA (2-5µg) are premixed for 20 minutes at room temperature and added to the infection in presence of serum free DMEM medium. After four hours of incubation at 37°C DMEM-10% FCS is added to the reaction.

A.



B.

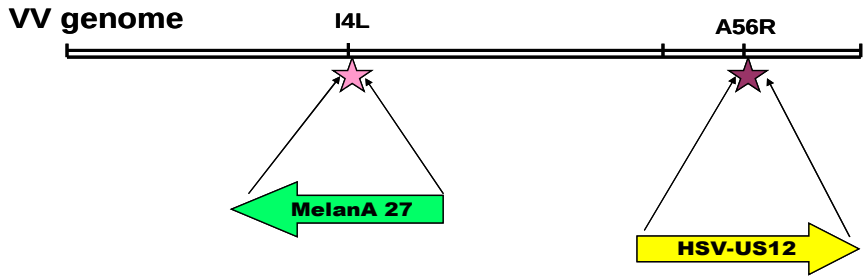


Figure (18): (A) Recombinant VV with **US12 gene**. **(B)** Recombinant VV with **Mart-1/Melan-A_{27-35} and US12 genes.**

Vaccinia undergoes homologous recombination (fig. 19) during replication in infected cells. This innate ability to recombine is used to introduce foreign DNA coupled to a vaccinia promoter, such as A56R locus into the viral genome. This recombination leads to insertion of the gene of interest (i.e. the foreign DNA) into the viral progeny. The usual target of insertion is a nonessential region, so that virus retains its ability to replicate independently and the system can be maintained. The estimated incidence of successful insertion is approximately 0.1% [142].

III.2.1.3. Recombinant VV selection with Mycophenolic acid inhibition/gpt expression

Recombinant viral clones are selected according to their transient expression of the E. coli gpt marker under the selective pressure of MPA, Xanthine and Hypoxanthine as described by Earl P.L. and Moss B. [240]. The mycophenolic acid (MPA) is an inhibitor of purine metabolism. It inhibits the enzyme inosine monophosphate dehydrogenase and thereby prevents the formation of xanthine monophosphate. This results in intracellular depletion of purine nucleotides and inhibition of cell growth [241]. This MPA was demonstrated to reversibly block formation of vaccinia virus plaque [142].

The inhibition of the de novo synthesis of purines by MPA can be overcome in cells that express the Escherichia coli gpt gene, which codes for the enzyme xanthine-guanine phosphoribosyltransferase (XGPRT), in the presence of xanthine and hypoxanthine in the growth medium [241].

The block of purine synthesis by MPA can also be overcome by a recombinant virus expressing the bacterial XGPRT (encoded by Escherichia coli gpt gene). Indeed, synthesis of XGPRT enables only the recombinant viruses to form large plaques in a selective medium containing MPA, xanthine and hypoxanthine [242].

All plaques picked at the first selection step contain recombinants. Any contaminating wild-type virus or an accidentally picked microplaque is removed by further rounds of plaque purification in selective medium and no background of spontaneously occurring gpt+ virus would be expected or has been observed. In addition, the gpt gene is incorporated into a plasmid vector that has a VV promoter and unique restriction endonucleases sites for insertion of the foreign gene. Because of VV derived flanking sequences, the entire selection-expression cassette is inserted as a unit into the VV genome by a single homologous recombination. Thus, all of the gpt+ recombinants analyzed also contained the foreign gene that had been inserted into the plasmid vector.

Technical Procedure: after complete infection of the cells (about two days), as monitored by cytopathic effect (CPE), viruses are harvested, sonicated and used for infection: 100µl of 10-3 and 10-4 of virus suspension are added to fresh subconfluent CV1 (non transfected WT virus served as control). For viral selection, a combination of the drugs, 25µg/ml MPA, 250µg/ml Xanthine and 25µg/ml Hypoxanthine (Sigma, St.Louis, MO), is added to the reaction and incubated at 37°C. Only recombinant virus expressing the enzyme ‘gpt’ can replicate in selective medium. Plaques are picked and resuspended in PBS. The selection of recombinant virus requires two to four rounds with selective pressure and two or three more rounds of plaques selection without pressure which enables either excision of the entire plasmid (obtained from WT virus) or excision of the plasmidic part resulting in the obtention of the recombinant virus.

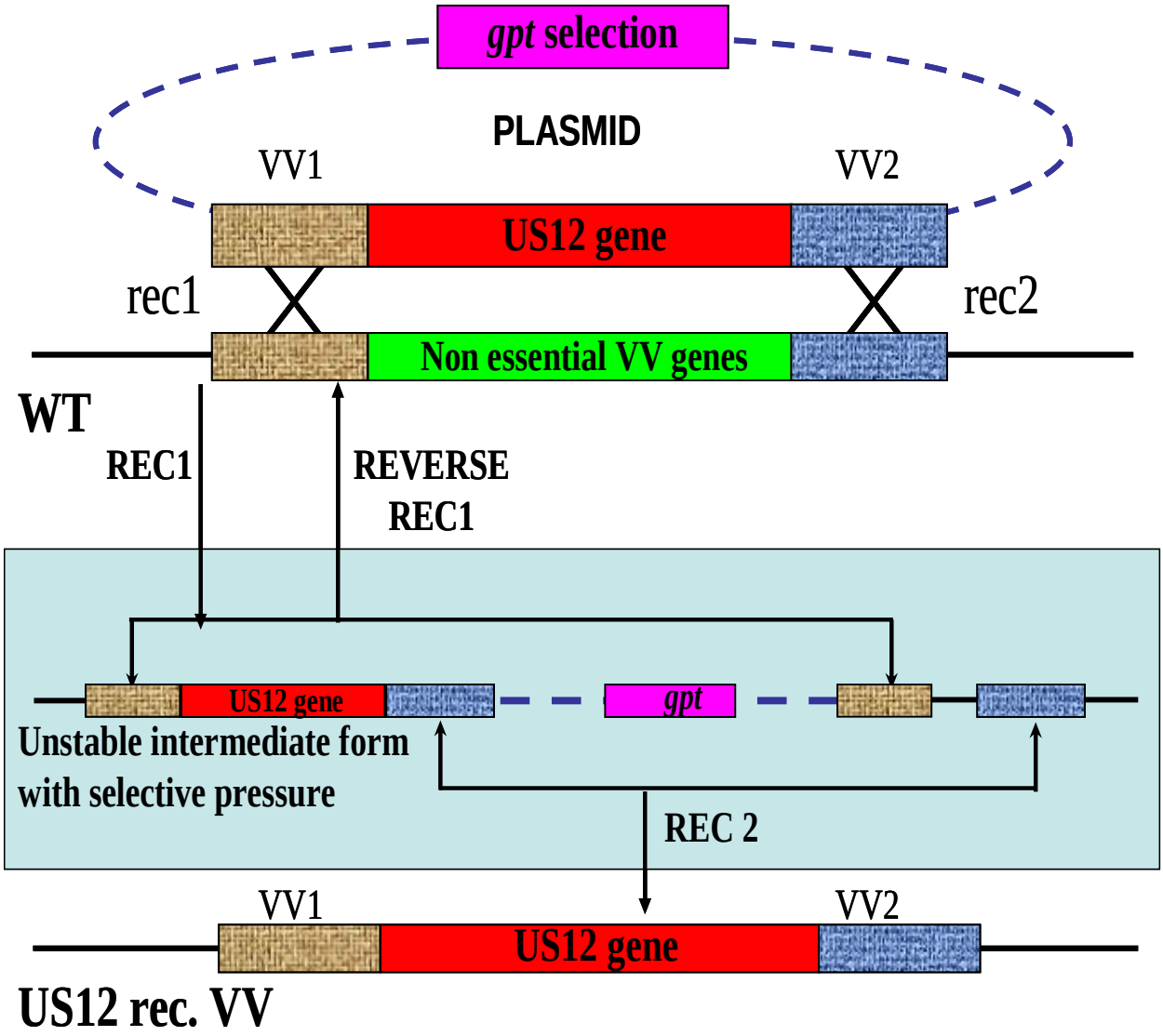


Figure (19): Principle of homologous recombination.

The cloning cassette is flanked by sequences identical to viral regions (VV1 and VV2) allowing homologous recombinations by genetic crossing-over leading to insertion of the gene of interest into the wild type genome of VV. During viral replication, a single homologous recombination event, e.g. with the region1 (rec1) generates a recombinant virus containing the entire plasmid whose presence in the viral genome is needed for selection under pressure by MPA. However, this intermediate recombinant form contains duplicate sequences and is therefore genetically unstable. Removal of selective pressure will allow the isolation of stable viruses derived from this intermediate form following recombination events either with the two VV sequences reverting to the initial form (WT) or following recombination with the second site (rec2) generating the stable recombinant virus (rec.VV-US12). The probability of each event is similar, thus the chances to have WT or recombinant virus are in theory 1:1.

To verify genomic insertion, viral DNA is purified from infected cells and PCR is performed with specific primers targeting the locus of the viral genome where the transgene is located. Primers A56cpB2 and A56pcr3` are used to PCR amplify the A56 R locus of the virus which is expected to have the US12 gene.

A56R-5': ACTCCACAGAGTTGATTGTA
A56R-3': GTATGTGACGGTGTCTGTAT

III.2.1.4. Amplification and cushion-concentration of vaccinia virus

Twelve 175 cm2 confluent flasks of CV-1 cells are infected with sonicated replicative virus at 0.01 multiplicity of infection (m.o.i.) until complete CPE occurs. After 4 days culture, 8ml TRIS 10mM are added on each decanted flask. Infected cells are detached by 1 cycle of freeze-thaw, collected and centrifuged (2.500 x g, 4min). Supernatant A is stored, while the pellet is resuspended in 5ml TRIS 10mM and sonicated to liberate the virus. After 4 minutes centrifugation at 2.500 x g, supernatant B is added to the viral solution A, while the pellet is resuspended in 3ml of TRIS 10mM, sonicated, and centrifuged (2.500 x g, 4 min). Supernatant containing the replicative viruses is added to the previous viral solution, distributed in the ultra-centrifuge tubes, 25 ml per tube. 10 ml sucrose 36% underlayer cushions are added in each tube. After 1 h 30 min ultra-centrifugation at 30 000 rpm, pellets are resuspended in TRIS 1mM, sonicated, aliquoted and stored at -20ºC.

III.2.1.5. Virus titration

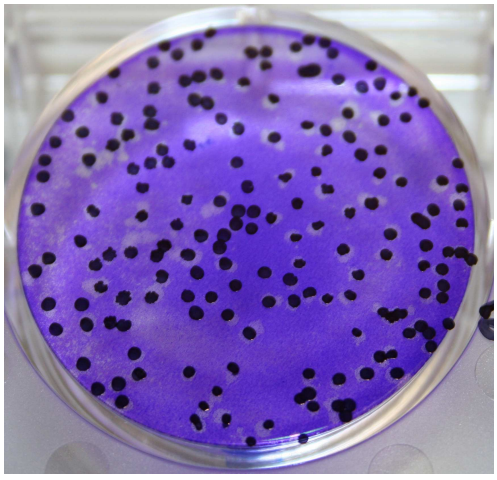
CV-1 cells are cultured in a 6 wells plate and are grown to sub-confluency. Replicative viral stock solutions are thaw, sonicated for 1 min and diluted in PBS from 10-1 to 10-8. After medium removal, 100µl of the 10-6to 10-8 dilutions are used to infect the wells in duplicates. The cells are incubated

at 37°C for one hour for viral adsorption with shaking the plate every 10 min. 2ml of medium is added in each well and the cells are incubated for 24 to 48 hours. As replicative vaccinia virus life cycle ends by lysis of the cells, one initial virus particle will induce after replication the formation of a visible hole in the cell monolayer. After medium is aspirated, cells are stained for 1min by 500µl of 0.1% violet crystal (Hexamethylpararosaniline chloride, C25H30CIN3, 407.98D (Fluka Chemie AG, Buchs, Switzerland) diluted in ethanol and the plaques are counted under light microscope. The viral stock solution concentration is calculated following the formula:

Viral concentration (pfu/ml) = number of plaques x 10 x dilution factor.

For estimation of the concentration of r.VV-US12 and r.VV-Mart-US12 viral stocks, the mean number of plaques in 2 identical wells is calculated and according to the dilution factor,

Concentration of r.VV-US12 was 1.9 x 10^9 pfu/ml (picture not shown) and of r.VV-Mart-US12 (picture 2) was 2.5 x 10^9 pfu/ml.



Picture (2): Titration of r.VV-Mart-US12.

Confluent CV-1 cells cultured in 6 well plates, were infected with 100µl replicative virus 10^{-6} ,10^{-7} or 10^{-8} diluted, and cells were stained after 24 hours infection, to visualize plaque forming units (pfu) induced by replication of the virus. As replicative vaccinia virus life cycle ends by lysis of infected cells, one initial virus will induce, after replication, the formation of a visible hole in the cell monolayer. For staining, medium was removed and cells were stained with 0.1 % violet crystal.

III.2.1.6. Vaccinia virus inactivation by psoralen and long-wave UV light

Although poxviruses are of low pathogenicity to humans, complete safety of vaccinia virus vector has to be ensured, especially in the view of potential vaccination of tumor bearing patients. Both recombinant viruses were treated with psoralen and long wave UV exposure. This treatment leads to cross-linking of the viral genomic DNA, preventing any possible replication of the virus. However, inactivated viruses are still able to infect cells and perform early genes transcription, which is independent of viral replication. This step required a precise monitoring of the inactivated viruses through characterization of the limited CPE of infected cells and a sufficient expression of viral early genes (evaluated by reverse transcribed real time PCR).

Vaccinia virus is diluted to a concentration of 5×10^8 PFU/ml in a Hanks´ Balanced Salts Solution (HBSS) (Invitrogen, Carlsbad, CA) containing 1 µg/ml Psoralen (CN Biosciences, Nottingham, UK). After 10 min incubation at room temperature, 1ml of the solution is irradiated in an uncovered 35-mm dish with 365 nm UV light for 10 min, applied energy 1.6 J (Stratalinker, Stratagene, La Jolla, CA) and aliquoted in 250µl vials.

In order to rapidly evaluate the extent of inactivation, CV-1 cells are infected with PLUV virus at different m.o.i. (from 0 to 20). CPE is evaluated under microscope after 24 hours of infection. The shape of non infected cells monolayer refers to 0% CPE. The global changes in the morphology of the culture determinate the percentage of CPE.

III.2.2. Cell Biology Methods

III.2.2.1. Cell isolation

III.2.2.1.1. Peripheral Blood Mononuclear Cells (PBMC) isolation

Anti-coagulated venous blood is diluted 1:2 in PBS and layered onto Ficoll. Lymphocytes and other mononuclear cells are isolated at the plasma-ficoll interface.

III.2.2.1.2. Cell sorting using MACS Magnetic MicroBeads

For CD14+, CD8+ or CD4+ cells separation, (following the manufacturer protocol) PBMC are magnetically labelled with MicroBeads specific for CD14, CD8 or CD4 (Miltenyi Biotec, Bergisch Gladbach, Germany), supplied as suspension and passed through a separation column placed in the magnetic field. The magnetically labelled cells are retained in the column while the unlabelled cells run through. After removal of the column from the magnetic field, the selected cells can be eluted as positively selected fraction, washed and resuspended in the appropriate medium.

III.2.2.2. Gene expression analysis

III.2.2.2.1. Total RNA isolation and DNA digestion

Cells (1×10^7 maximum) are lysed, Ethanol is added to provide appropriate binding conditions, and the sample is applied to RNeasy® mini column (Qiagen, Basel, Switzerland). RNA molecules > 200 nucleotides bind to the silica membrane, providing enrichment for mRNA since most RNAs are less than 200 nucleotides. The RNA extracted is treated with DNase I (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. DNase I is then inactivated by heating for 10 min at 65°C.

III.2.2.2.2. RNA Reverse Transcription

Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen, Carlsbad, CA) uses single-stranded RNA in the presence of a primer to synthesize a complementary DNA strand up to 7 Kb. Following the manufacturer's protocol, reverse transcription is performed and then M-MLV RT enzyme is inactivated by heating for 5 min at 95°C.

III.2.2.2.3. Gene Expression by quantitative Real-Time PCR (qRT-PCR)

qRT-PCR is performed using the TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Forster City, CA) and the appropriate primers and probes. The ABI prismTM 7300 sequence detection system (Applied Biosystems, Forster City, CA) is used. Data are collected and normalization of the samples is performed using β-actin or GAPDH as reference genes.

III.2.2.3. Flow Cytometry analysis

III.2.2.3.1. Characterization of surface molecules

MHC class-I (HLA-ABC and HLA-A2) surface presentation is characterized using monoclonal FITC-conjugate antibodies.

Technically, infected cells are stained with specific or control IgG antibodies, incubated for 45min at 4˚C in the dark, washed twice in cold PBS, fixed 1min in Paraformaldehyde 1%, resuspended in 200 µl PBS and analysed on a FACS Calibur® cytometer (Becton Dickinson, Franklin Lakes, NJ). Staining with antibodies for CD80, HLA-DR, CD44, CD14, CD8, CD4 (PE, FITC or APC conjugate antibodies) is also performed following the same protocol.

III.2.2.3.2. MHC-Multimer Staining For Visualization of CD8+ Cells

T-cell receptors have an intrinsic low affinity for their cognate MHC-peptide ligand. To identify specific TCR on the surface of CD8+ cells, one can use soluble multimeric MHC-peptide complexes

Principle: biotin is added to recombinant MHC peptide complexes which are assembled to form pentamers with avidin linked to a fluorochrome (PE). Only the T cells that have TCR capable of binding to the particular MHC-peptide combination of the pentamer are able to bind to the pentamer. Vaccinia virus $H3L_{184-192}$, $B22R_{29-37}$ and $C7L_{74-82}$ or MART-1/Melan-A_{26-35} peptide-MHC multimers (Proimmune, Oxford, UK) are used. The concomitant use of a monoclonal antibody that is specific for a T cell marker (anti-CD8) allows the detection of (CD8+) T cells specific for the peptide of interest (figure 20).

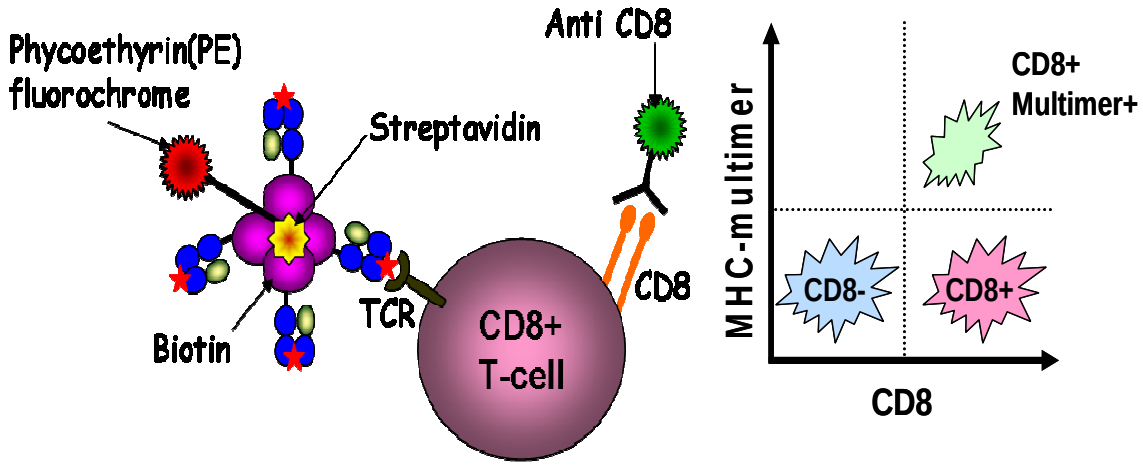


Figure (20): Principle of multimer staining for visualization of CD8+ cells

III.2.2.3.3. Cytokine Intracellular Staining

Principle: Intracellular cytokine staining relies upon the stimulation of T cells in the presence of an inhibitor (Brefeldin) of protein transport retaining the produced cytokines inside the cell. To analyze the effector function of antigen-specific T cells, the cells are first stimulated with antigen, followed by staining with antibodies specific for extracellular markers (such as CD8), then by membrane permeabilization and intracellular cytokine staining.

Technically, 10^6 cells are resuspended in 1ml CM with 5% HS and Brefeldin is added to a final concentration of 10µg/ml, then homogenized and incubated for 5 hours at 37˚C. After incubation, the cells are washed twice in MACS buffer and monoclonal antidody for the surface marker should be added at this step then the pellet is resuspended in 2ml paraformaldehyde 1% and incubated for 5 min at room temperature. After incubation, cells are washed once in MACS buffer then the pellet is resuspended in 500µl FACS^{TM} permeabilizing solution (BD nº340973) diluted 1/10 in H_2O. The cells are gently vortexed and incubated for 10 min at room temperature. 15µl of the antibody for intracellular cytokine is added and incubated for 30-45 min at 4˚C in the dark, washed twice in cold PBS, fixed 1 min in Paraformaldehyde 1%, resuspended in 200 µl PBS and analysed on a FACS Calibur® cytometer (Becton Dickinson, Franklin Lakes, NJ).

III.2.2.3.4. PI/Annexin Staining For Detection of Cell Viability

Annexin V-FITC Apoptosis Detection kit I (BD Pharmingen^{TM}, Franklin Lakes, NJ) was used for detection of cell viability.

Principle: In apoptotic cell, phosphatidylserine (PS) is translocated from inner to outer leaflet of the plasma membrane, and is thereby exposed to the external cellular environment. Annexin V is a Ca^{2+} dependent phospholipid-binding

protein that has a high affinity for PS [243]. Since externalization of PS occurs in the earlier stages of apoptosis, Annexin V staining can identify apoptosis at an early stage. Annexin V is typically used with Propidium Iodide (PI), which is a vital dye excluded in viable cells with intact membrane (figure 21).

Technically, the cells are washed twice in cold PBS then resuspended into 1X binding buffer at a concentration of 1 x 10^6 cells/ml. 100µl of this solution is transferred into 5ml tube and 5µl of Annexin V and 5µl of PI are added. Cells are gently vortexed and incubated for 15 min at room temperature in the dark. 400µl of 1X binding buffer are added and cells are analysed by flow cytometry within 1 hour.

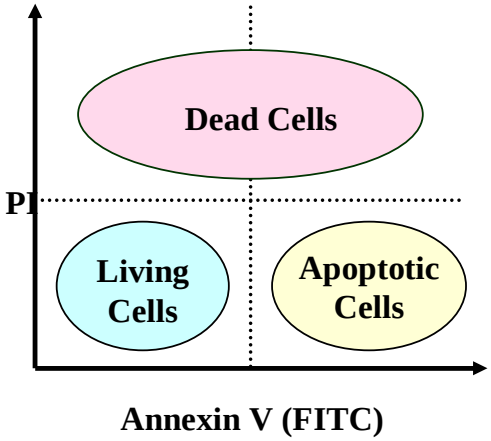


Figure (21): principle of PI/Annexin staining.

III.2.3. Immunological Methods

III.2.3.1. Generation of antigen specific CD8+ T cells

The capacity of r.VV-US12 to diminish the CTL response against vaccinia virus antigens by decreasing MHC class-I restricted presentation was tested in co-cultures of infected APCs with autologous CD8+ T cells. The capacity of r.VV-Mart-US12 to induce CTL response against MART-1/Melan-A27-35 was also similarly tested.

Technically, CD14+ cells from healthy donners (used as APC) resuspended in 200 µl, are infected with r.VV-US12, rVV-Mart-US12 or r.VV-Mart as control. Noninfected cells are used as negative control. The cells are cultured in CM-10% FCS with GM-CSF 50 ng/ml. After 36-48 hours, after taking part of infected cells for MHC class-I testing by FACS, the other part is co-cultured with autologous CD8+ and CD4+ T cells, separately. On day 8 and 15, CD8+ T cell cultures are stimulated with WT VV infected or MART-1/Melan-A_{26-35} peptide pulsed (20µg/ml) autologous CD14+ cells resuspended in CTL medium but CD4+ T cell cultures were only stimulated with WT VV infected autologous APC. After stimulation, antigen specific (VV or Mart) T cells were characterized by MHC peptide multimer staining or cytokine expression.

III.2.3.2. Measurement of cell proliferation using 3H-Thymidine incorporation

T lymphocytes proliferation is measured by incorporation of tritiated thymidine (3H-Thy) into the DNA of dividing cells, providing a measure of the rate of DNA synthesis by the entire cell population.

T lymphocytes primed with recombinant VV infected CD14+ cells, are cultured in a 96 well plate with flat bottom, in a final volume of 200 µl per well for 6 days. 3H-Thy (20 µl of a 1/20 dilution) is then added to each well and incubated for 18 hours at 37°C. The cells are harvested and lysed in a

Micro96TM Cell Harvester (Skatron, Sunnyvale, CA). Nucleic acids are sticking on a prewetted glass fiber filter. After three washes, the filter is dried and liquid scintillation cocktail is added (OPTI-FLUOR®, PerkinElmer, Boston, MA) then scintillation emission is measured.

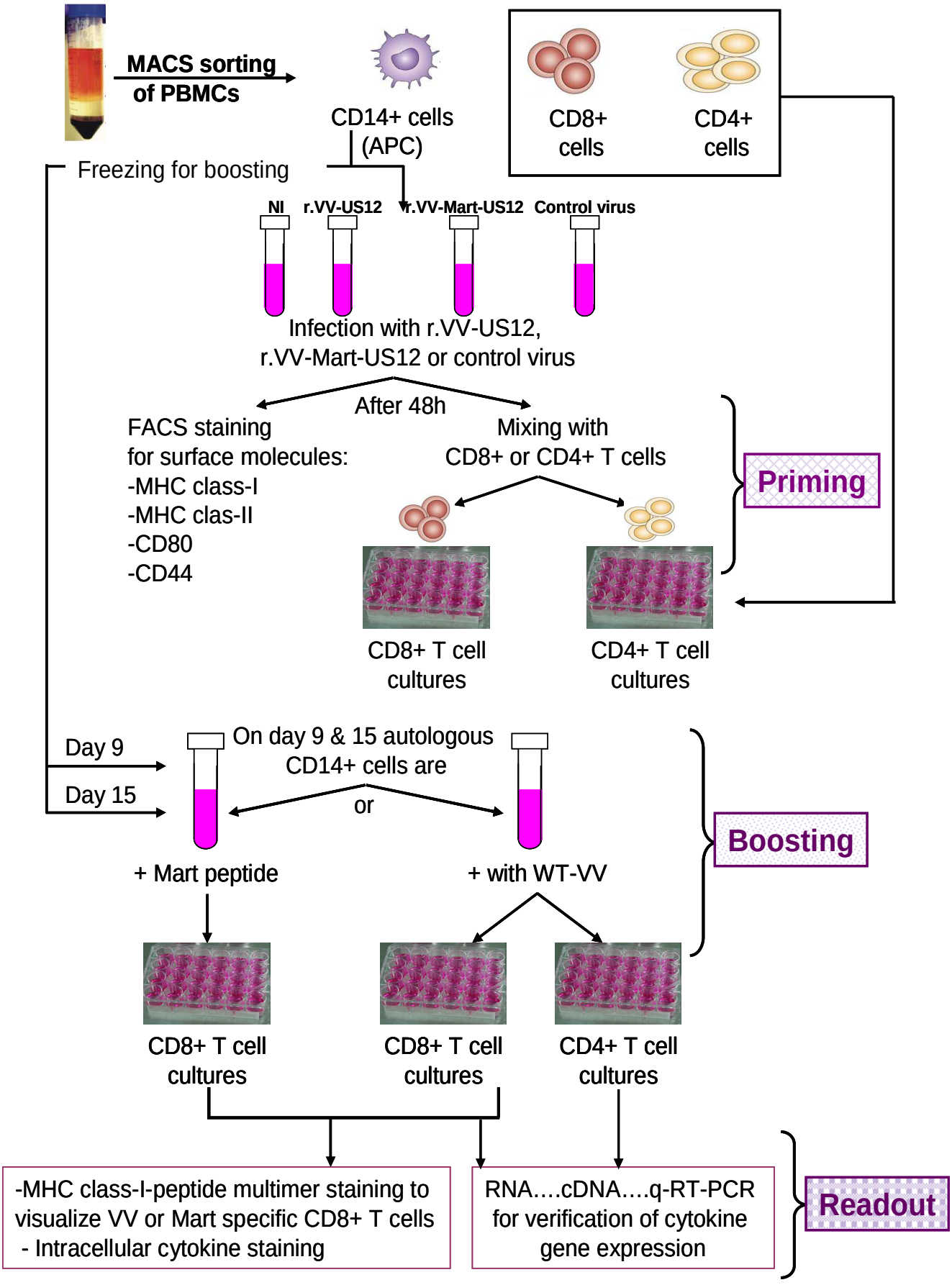


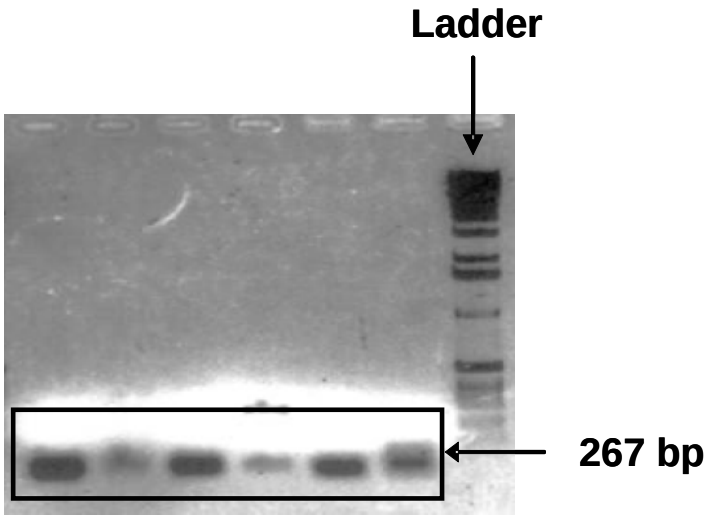
Figure (22): Working plan

IV.RESULTS

IV.1. CONSTRUCTION OF r.VV-US12 and r.VV-MART-US12

To construct recombinant vaccinia virus, a two-step procedure has been developed. In the first step, a plasmid containing the gene of interest, controlled by a vaccinia virus promoter and flanked by sequences derived from a non essential site on the viral genome, is generated. In the second step, the foreign genetic material is transfered into the viral genome by homologous recombination *in vivo* (as mentioned in Methods) [242].

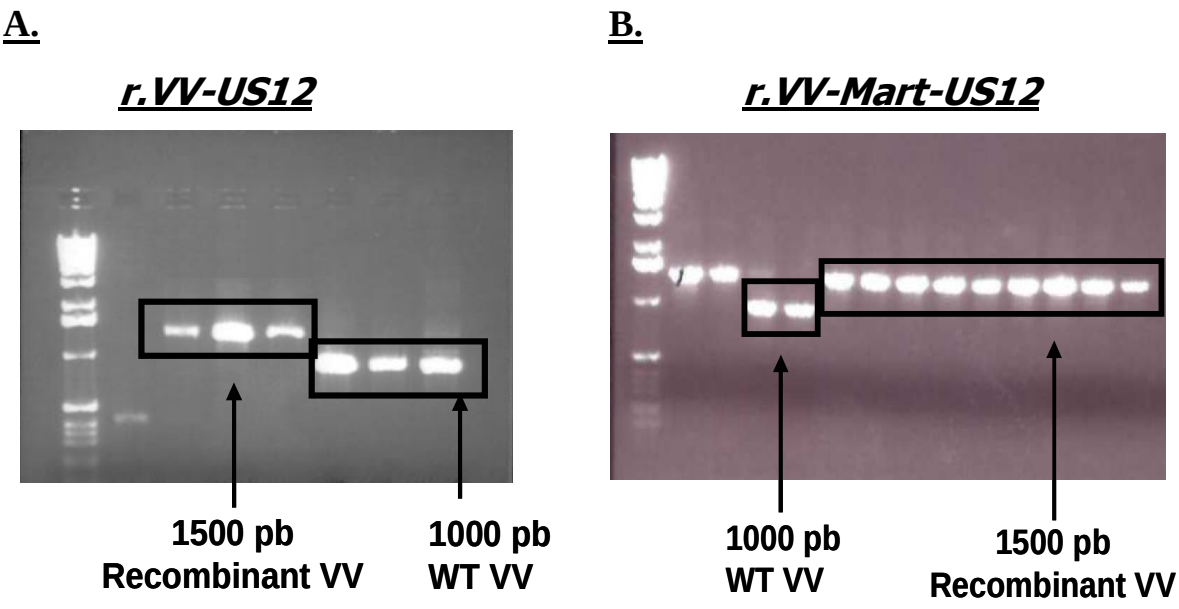
HSV-US12 gene was amplified by nested PCR from cerebrospinal fluid of HSV infected patients. A 267bp band corresponding to the US12 gene (picture 3) was purified and inserted into pKT1323 plasmid.



Picture (3): PCR amplification of US12 gene.
US12 gene was amplified from DNA extract from cerebrospinal fluid of HSV infected patients using nested PCR technique. The expected 267 bp bands corresponding to US12 gene were detected.

In order to obtain a recombinant vaccinia virus, the shuttle plasmid with the transgene US12 (pKT1323-US12) was transfected on an adherent cell monolayer (CV-1cells) infected with the parental virus WT VV or r.VV-Mart to generate r.VV-US12 or r.VV-Mart-US12 constructs, respectively.

To verify genomic insertion, viral DNA was purified from infected cells and the A56R locus of the virus genome was amplified. PCR amplicons´ length was analysed on agarose gel in order to distinguish the recombinant from the wild type genome. The expected band of 1 k.bp or 1.5 k.bp was detected in wild type or recombinant genome, respectively. As shown in picture 4, analysis of viral clones indicates that we obtained 3out of 6 and 11out of 13 recombinant viral clones for r.VV-US12 (panel A) and r.VV-Mart-US12 (panel B) respectively.



Picture (4): PCR amplification of A56R locus
To distinguish the recombinant from the wild type genome, viral DNA was purified from infected cells and PCR was performed with specific primers targeting the A56 locus of the viral genome. In wild type genome, the expected band is of 1 k.bp and for recombinant genome is 1.5 k.bp. (A) r.VV-US12. (B) r.VV-Mart-US12.

The recombinant vaccinia virus DNA was sequenced. The 1246-1513 sequence (expected US12 gene) was analysed and it was found to match the

theoretical sequence of US12 gene of human Herpes Simplex virus type I (Strain 17). However, 2 mutations were found at position 1396 and 1424. At position 1396, a silent mutation thymine (T) to cytosine (C) was detected as CCT and CCC code for the same AA (proline) at position 50. At position 1424, a missense mutation adenine (A) to guanine (G) was detected leading to a lysine instead of a glutamic acid at position 60 (figure 23).

As detailed earlier (chapter II.5.3), the active domain of ICP47 protein, located in the N-terminal region, is not affected by this missense mutation. Therefore, it was anticipated that these recombinant VV expressing ICP47 should be functional.

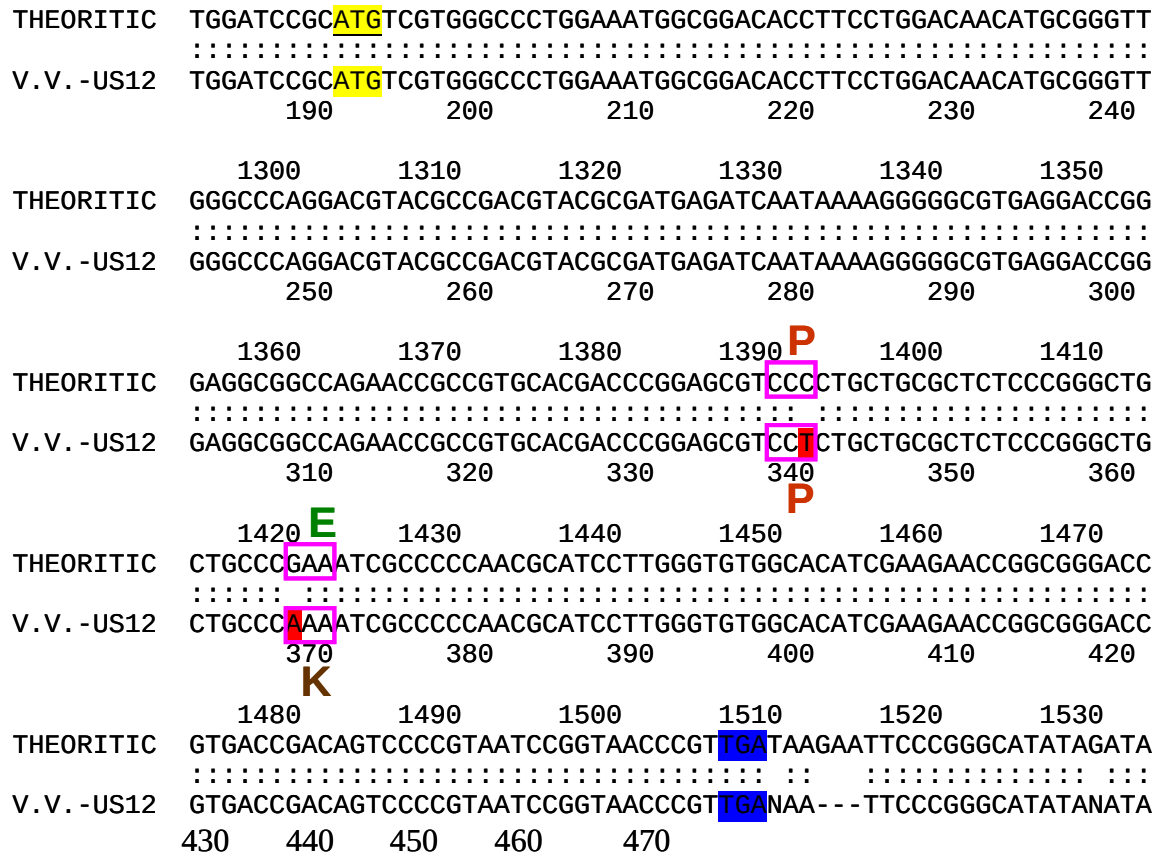


Figure (23): Matching theoretical sequence and r.VV-US12 genome for US12 gene.

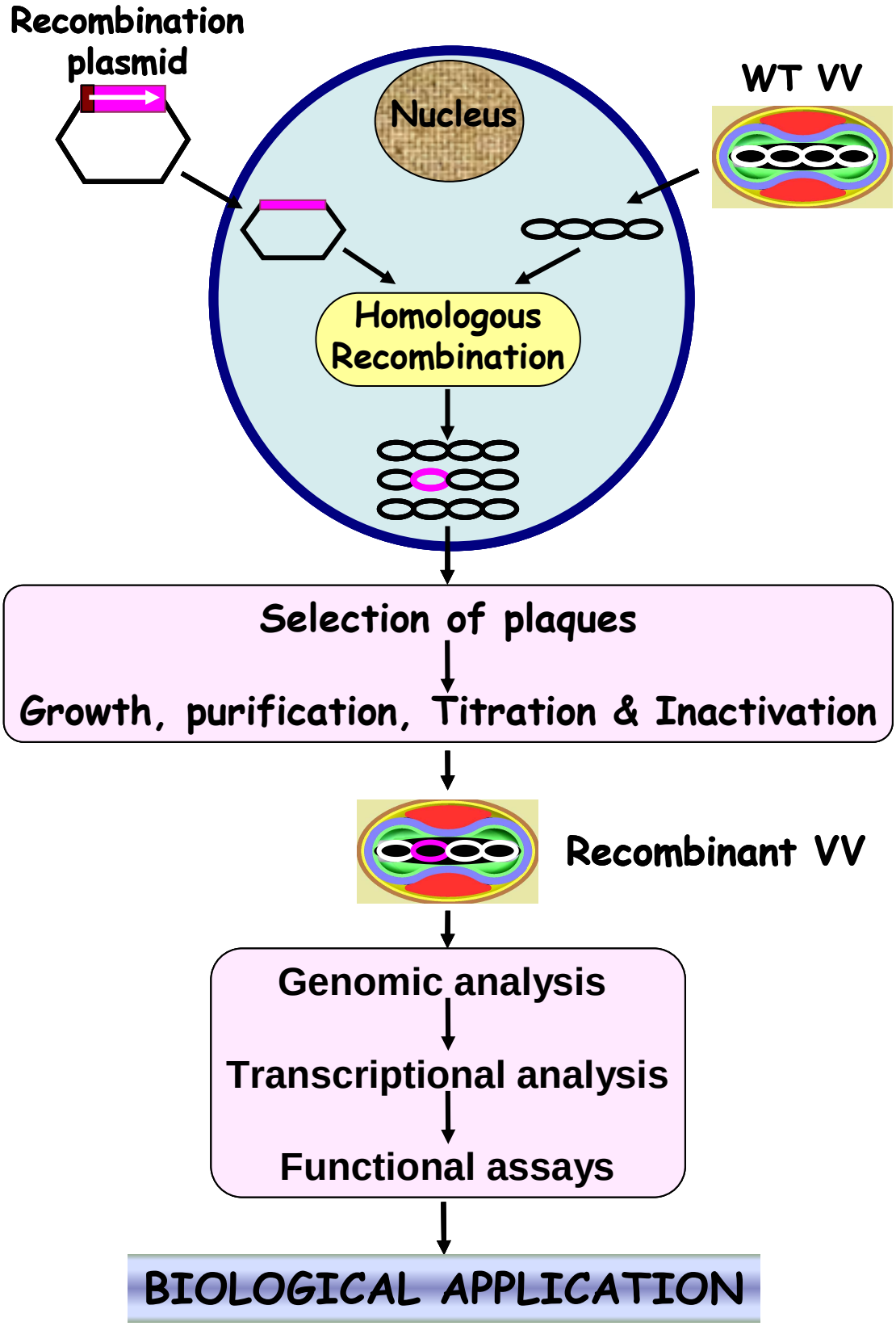


Figure (24): Summary of the construction and characterization of r.VV-US12.

IV.2. GENE EXPRESSION IN US12 RECOMBINANT VV INFECTED CELLS

The expression of US12 was first verified at the transcriptional level by infecting Hela cells with recombinant VV-US12. Infections were performed with replication-incompetent viruses treated with psoralen and long wave UV (PLUV). Adherent monolayers of Hela cells in 6 well plates ($1x10^6$ cells/well) were infected with WT-VV, r.VV-US12 and r.VV-Mart-US12 at different doses 2.5, 10 and 25 m.o.i.. 24 hours after infection, US12 gene expression was evaluated by reverse transcription qRT-PCR.

The results in figure 25 confirm that US12 gene is correctly inserted and expressed from VV genome of r.VV-US12 and r.VV-Mart-US12. This expression under vaccinia promoter is strong and dose dependent according to the concentration of the virus infecting the cells (m.o.i.).

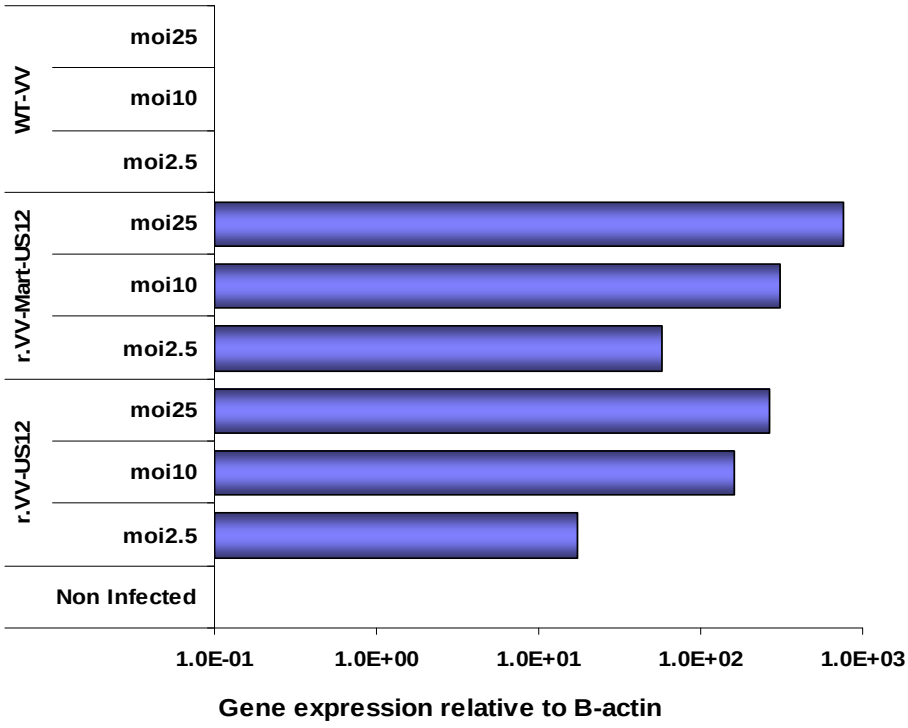


Figure (25): US12 gene expression in infected Hela cells.
1 x 10^6 HeLa cells were infected with PLUV r.VV-US12, r.VV-Mart-US12 or with wild type (WT) vaccinia virus at 2.5, 10, 25 m.o.i. and cultured overnight. Non infected cells were used as control. US12 gene expression was verified and data are expressed as ratio relative to a reference house keeping gene (β-actin).

Similarly, infected HeLa cells were used to compare the expression level of the transgene to that of vaccinia virus genes. These VV genes include Thymidine kinase enzyme (TK), I4L and I3L genes, which are three vaccinia virus early genes. I4L gene expression is not detectable in r.VV-Mart construct as the cassette containing the promoter and Mart insert was subcloned into the I4L locus of VV genome, thereby deleting I4L gene (figure26).

The results shown in figures 25 and 26 indicate that US12 gene expression in r.VV-US12 and r.VV-Mart-US12 infected cells, is comparatively as strong as natural early VV genes. This expression appears to be also comparable to the expression from WT infected cells confirming that psoralen and long wave UV treatment of the virus does not abolish the ability of the virus either to infect cells or to express its early genes.

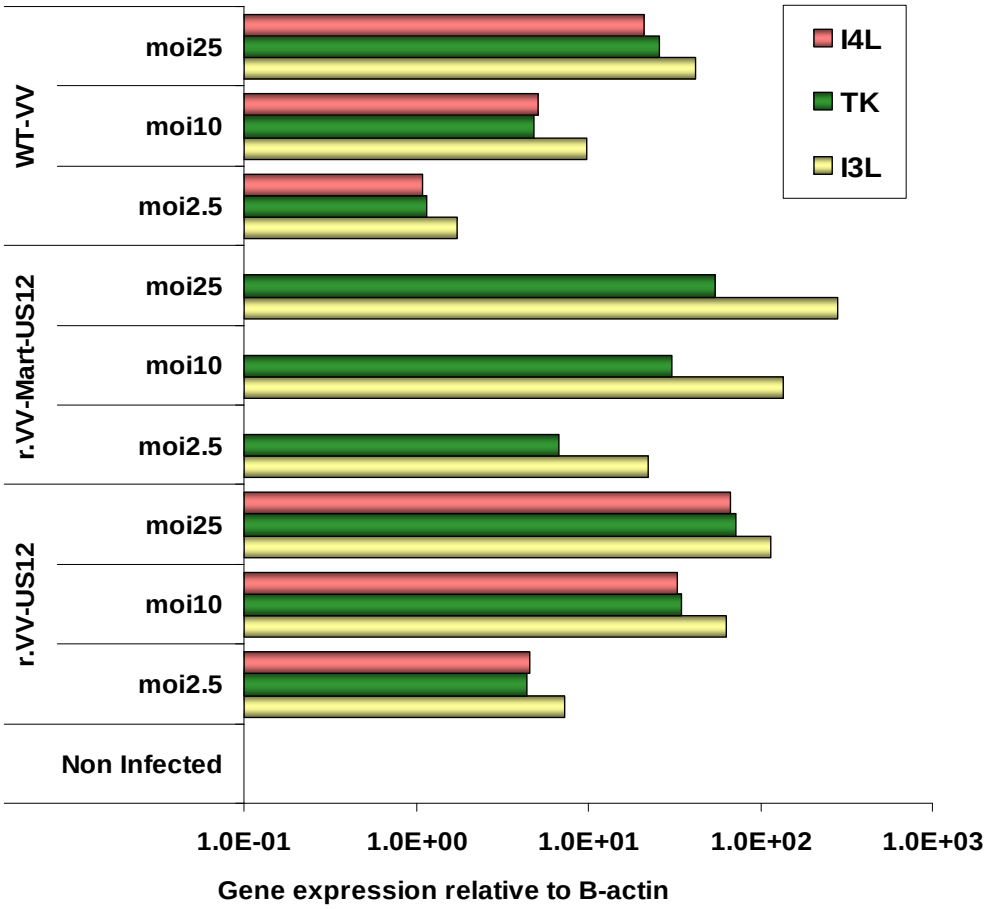


Figure (26): TK, I3L and I4L gene expression from infected HeLa cells.

IV.3. FUNCTIONAL ASSAYS OF r.VV-US12 AND R.VV-MART-US12

IV.3.1. Effect of ICP47 on the Expression of Surface Molecules

IV.3.1.1. Surface expression of total MHC class-I (HLA- ABC)

ICP47 protein blocks the transport of peptides, which results from metabolic degradation of proteins, into the ER. This blockade prevents the loading of peptides into the empty MHC class-I molecules located into the ER, thereby blocking the migration of new MHC class-I complexes to the cell surface, while the natural turnover through endocytosis of surface molecules will continue. Thus we expect that upon infection of cells with a functional ICP47 expressing VV, one should observe a decreasing amount of MHC complexes on the cell surface.

The effect of ICP47 on MHC class-I in r.VV-US12 or r.VV-Mart-US12 infected HeLa cells and EBV-transformed B cells were investigated using noninfected and WT infected cells as controls. 24 hours after infection, the cells were stained with HLA-ABC specific monoclonal antibody and analysed by FACS. As shown in figure 27, there is a significantly decreased intensity of MHC class-I surface expression on r.VV-US12 or r.VV-Mart-US12 infected cells as compared to noninfected or control virus infected cells. Of note, the downregulation of MHC class-I molecules in cells infected with WT-VV is consistent with previous observations which demonstrated that vaccinia virus infection can lead to a modest decrease in MHC class-I molecule surface expression on infected cells [244;245]. In this experiment, it appears that MHC class-I expression on the surface of r.VV-Mart-US12 infected cells is less decreased as compared to r.VV-US12 infected cells. r.VV-US12 infected cells display mean fluorescence intensity (MFI) representing 68% or 44% whereas r.VV-Mart-US12 infected cells display MFI of 75% or 54% in infected HeLa

cells or EBV-BL respectively, as compared to noninfected cells. This difference of about 10% might be due to a high expression of Mart epitope in the ER which is not affected by the TAP blockade and therefore would help to stabilize the HLA-A0201 molecule on the cell surface.

The formula used to calculate MFI ratio to noninfected cells is:

MFI ratio = (sample MFI – isotype MFI) x 100/ (noninfected MFI – isotype MFI)

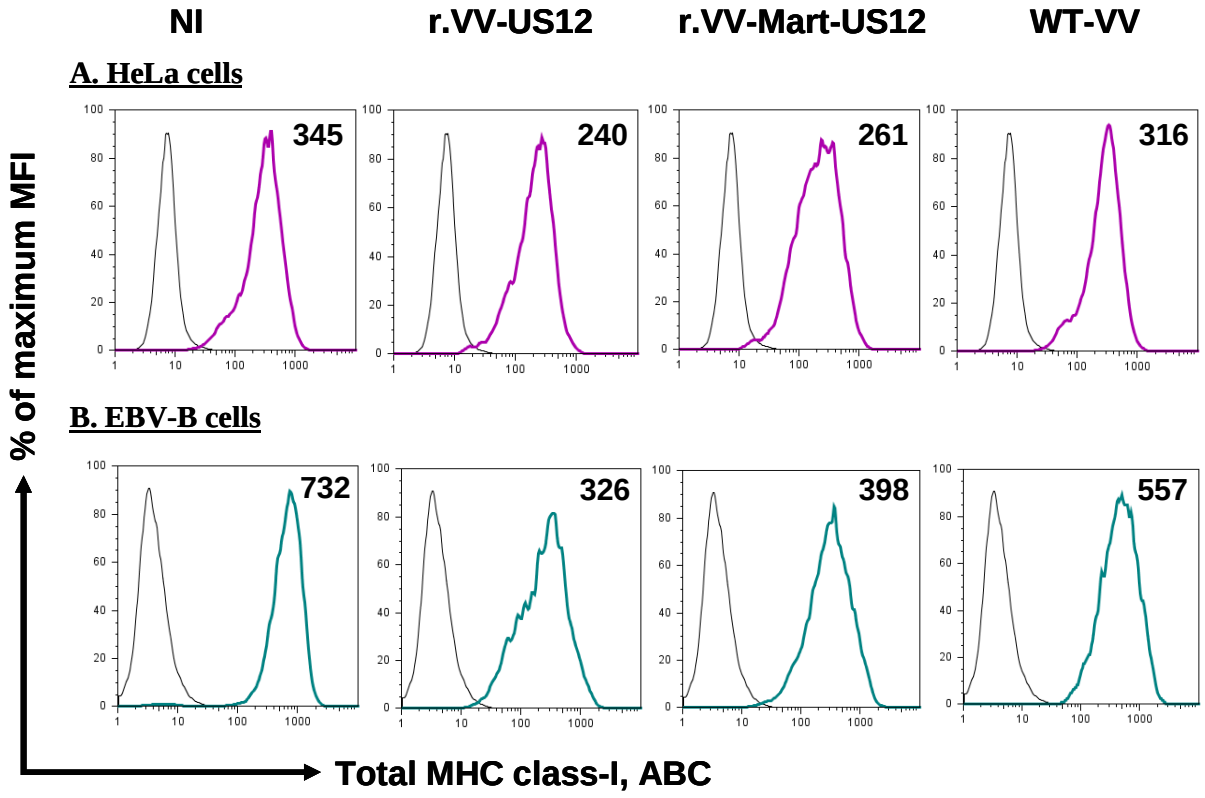


Figure (27): Flow cytometric analysis of MHC class-I in infected cells
(A) HeLa cells **(B)** EBV transformed human B cells. HeLa cells and EBV-BL cells were infected with r.VV-US12, r.VV-Mart-US12 or WT-VV control. Infections were performed with replication-incompetent (PLUV) virus at 10 m.o.i.. Cell surface expression of MHC class-I was verified 24h after infection by staining with a FITC-labelled monoclonal antibody specific for all MHC class-I A, B and C subgroups (thick line) or with isotype control antibody (thin line). Data are represented as mean fluorescence intensity (MFI).

IV.3.1.2. Surface expression of HLA-A2 molecules

MART-1/Melan-A_{27-35} is a short peptide encoded in r.VV-Mart-US12. It is an immunodominant non-mutated self differentiation epitope derived from a protein which is expressed by normal and malignant melanocyte. It is specifically binding to and presented by HLA-A0201 molecules on the cell surface.

To evaluate the effect of ICP47 in cells expressing TAP independent epitope, we infected EBV-BL from HLA-A2 positive healthy donor and different HLA-A2 melanoma cell lines (Na-8 and WM-115 cells) with r.VV-Mart-US12 and r.VV-US12.

Analysis of FACS results (figure 28) confirms the previous data with total HLA-ABC antibody as HLA-A2 surface expression compared to noninfected cells shows MFI of 30%, 48% and 69% in r.VV-US12 infected EBV-BL, Na-8 and WM115 cells, repectively. In presence of Mart epitope, HLA-A2 downregulation is “compensated”. In this case the MFI value is about 18-35% higher than in r.VV-US12 infected conditions. This measurable compensation of downregulation may imply that the HLA-A2 molecules loaded with MART-1/Melan-A_{27-35} peptide, in absence of TAP dependent peptides, represent a significant fraction of the total HLA-A2 molecules on the cell surface.

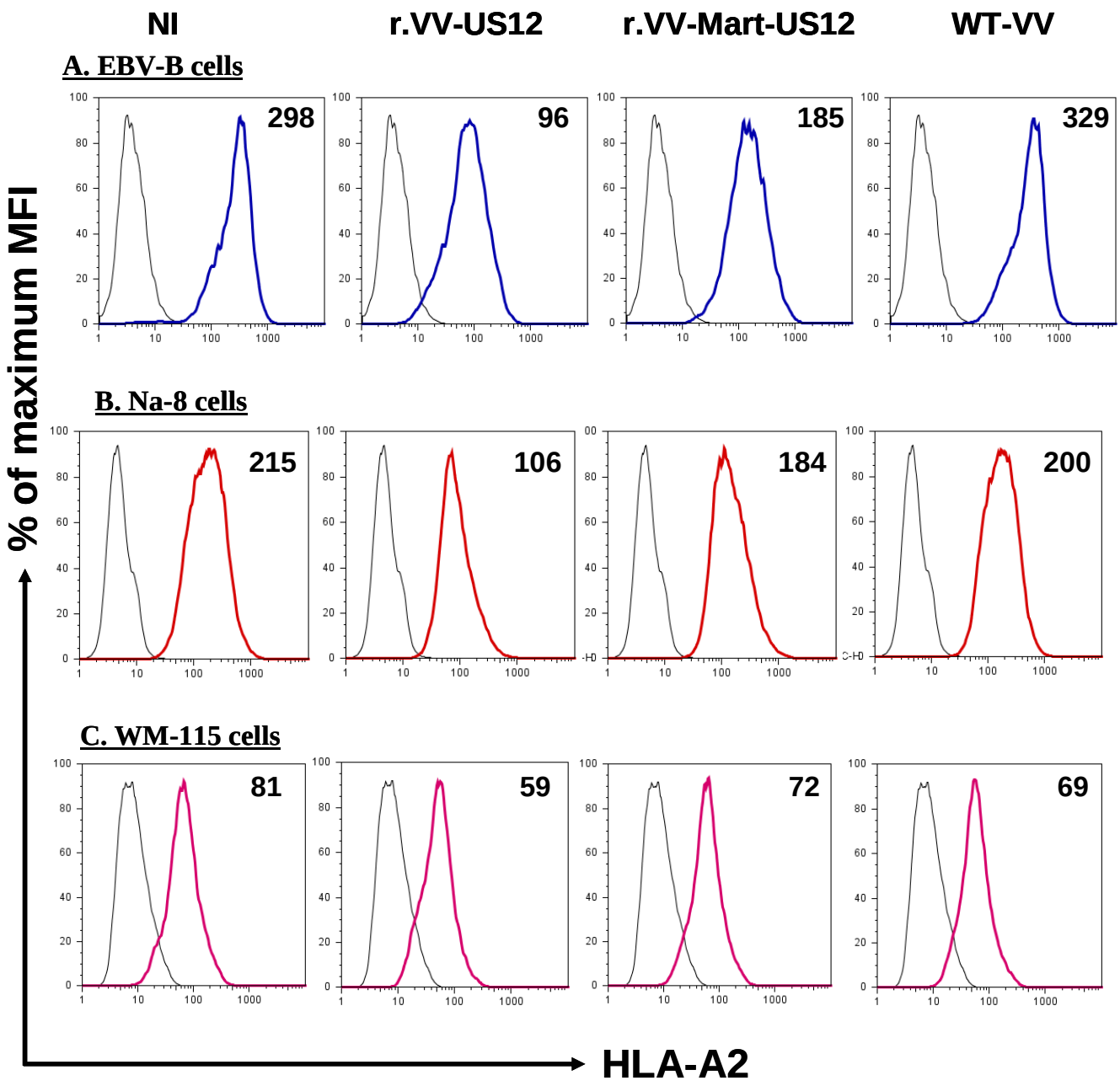


Figure (28): Flow cytometric analysis of HLA-A2 surface expression in infected cells
(A) EBV-BL **(B)** Na-8 cells **(C)** WM115 cells. These cells were infected with r.VV-US12, r.VV-Mart-US12 or control WT viruses. Infections were performed with replication-incompetent (PLUV) virus at 10 m.o.i. then cell surface expression of HLA-A2 was verified 24h after infection by staining with a FITC-labelled monoclonal antibody specific for HLA-A2 (thick line) or with isotype control antibody (thin line). Data are represented as mean fluorescence intensity (MFI).

IV.3.1.3. Analysis of MHC class-I surface expression kinetics

In the experiment shown in figure 29, we analysed the surface expression of HLA-A2 on Na-8 cells infected with r.VV-US12, r.VV-Mart-US12 or WT control VV at 10 m.o.i. (as compared to noninfected control) and at different time points (between 4h and 48h) after infection. The downregulation effect of ICP47 on MHC class-I surface expression (expressed as % compared to noninfected) starts to be measurable after 12 hours of infection and reaches a maximal effect (minimal level of surface MHC) after 36 to 48 hours of infection. In this experiment, the difference between r.VV-US12 and r.VV-Mart-US12 is significant and it is attributable to the expression of TAP independent Mart epitope.

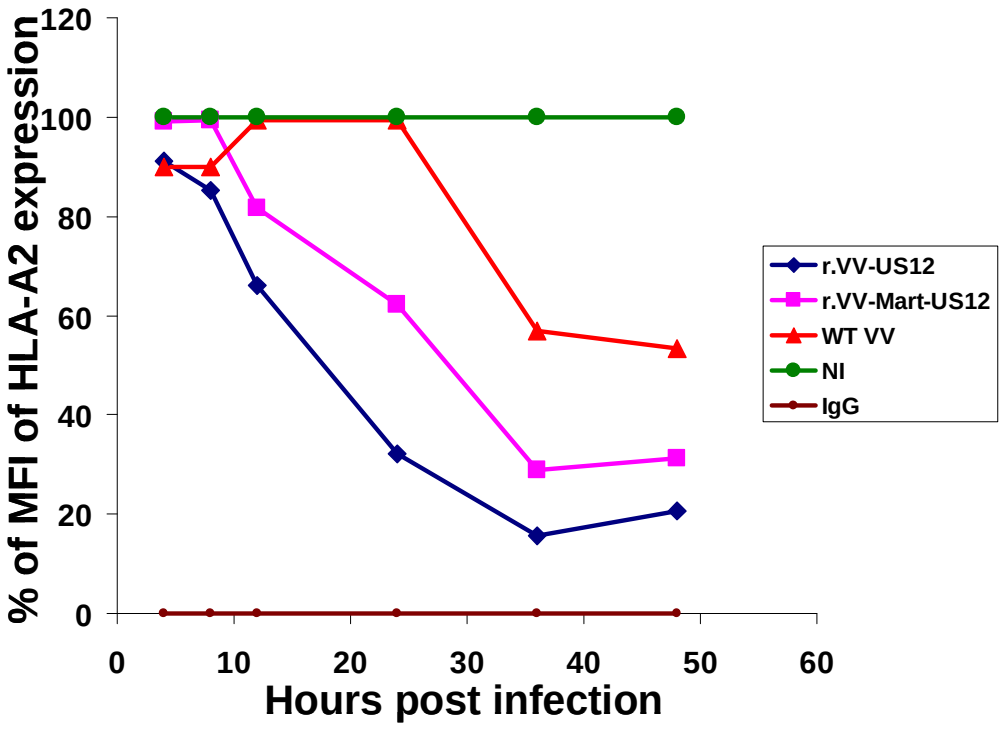


Figure (29): Kinetic analysis of HLA-A2 downregulation by ICP47 in infected Na-8 cells. 3 x 10^6 Na-8 cells were infected with PLUV r.VV-US12, r.VV-Mart-US12 or WT vaccinia virus (control VV) at 10 m.o.i. then at time points 4h, 8h, 12h, 24h, 36h and 48h after infection, 5 x 10^5 were stained for HLA-A2 FITC-labelled mAb and analysed by FACs. Percent of downregulation was calculated considering that noninfected cells MFI=100% and isotype stained cells as 0%.

Taking into account the results of this experiment and to confirm the significance of the difference of HLA-A2 surface expression between r.VV-US12 and r.VV-Mart-US12 infected cells, we monitored the capacity of ICP47 to downregulate MHC class-I and of the targeted MART-1/Melan-A_{27-35} peptide to compensate for this downregulation, 48 h after infection. Figure 30 shows the average from 6 different experiments performed on Na-8 cells and EBV-B cells from healthy donors. As compared to non infected cells, HLA-A2 surface staining in r.VV-US12 infected cells is decreased to 34% (+/- 2%) whereas it remains at the level of 75% (+/- 5%) in r.VV-Mart-US12 infected cells. The differences between r.VV-US12, r.VV-Mart-US12, r.VV-Mart and noninfected cells are highly significant (p value < 0.05). No significant difference is found here between r.VV-Mart infected and noninfected cells.

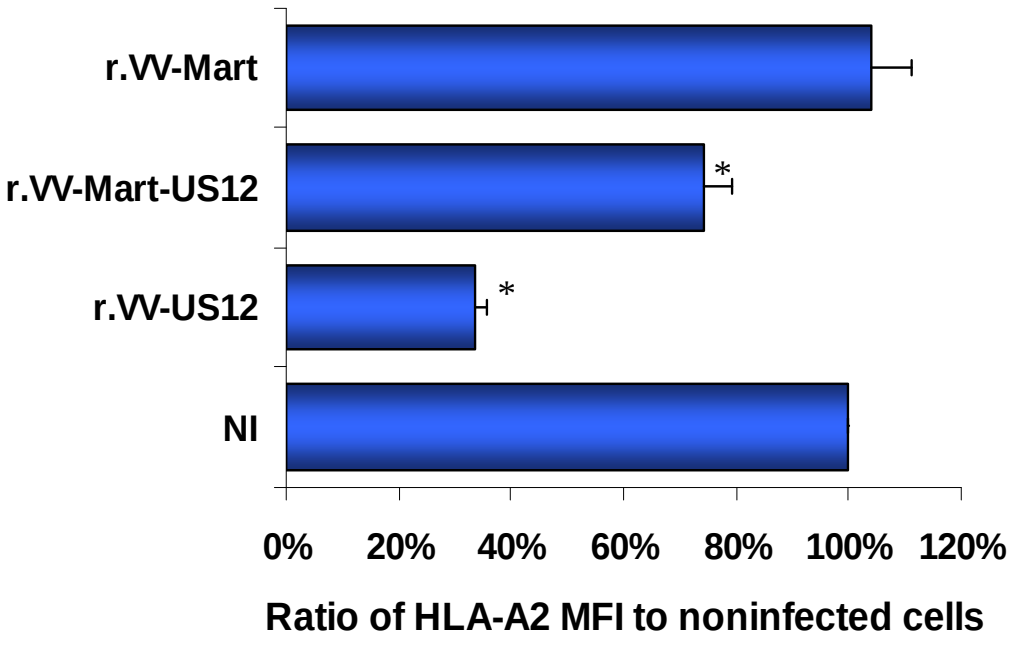


Figure (30): Downregulation of HLA-A2 surface expression in infected cells
Na-8 and EBV-BL cells were infected with PLUV r.VV-US12, r.VV-Mart-US12 or control r.VV-Mart viruses at 10 m.o.i. then cell surface expression of HLA-A2 was verified 48h after infection by staining with a FITC-labelled mAb specific for HLA-A2. Symbol (*) indicates significant ($p < 0.05$) differences as compared to all other samples (n=6).

IV.3.1.4. Effect of ICP47 on MHC class-II surface expression

Notwithstanding the critical role of CD8+ T cells, induction of tumor-specific $CD4^+$ T cells is important not only to help $CD8^+$ T cell response, but also to mediate anti-tumor effector functions through activation of eosinophils and macrophages [36]. Antigens uptaken by APC are processed and presented by MHC class-I to CD8+ T cells, and MHC class-II to CD4+ T cells. Recognition of the antigen, along with co-stimulatory molecules (B7-CD28) results in activation of antigen-specific CD4+ T cells, which leads to lymphoproliferation and cytokine secretion.

Therefore we verified that the downregulation of surface molecules following r.VV-US12 infection is limited to class-I molecules and does not affect other surface molecules. Figure 31 shows data from 3 independent experiments in which EBV-BL cells were infected with r.VV-US12 or r.VV-Mart -US12. Effects of ICP47 on MHC class-II (HLA-DR) were evaluated in comparison to uninfected cells and r.VV-Mart infected cells as controls. Flow cytometric analysis demonstrates stable expression of MHC class-II in r.VV-US12 or r.VV-Mart-US12 infected cells as compared to uninfected cells. In parallel, the HLA-A2 surface expression confirmed the downregulation in r.VV-US12 infected cells and the “compensated downregulation” in r.VV-Mart-US12 infected cells.

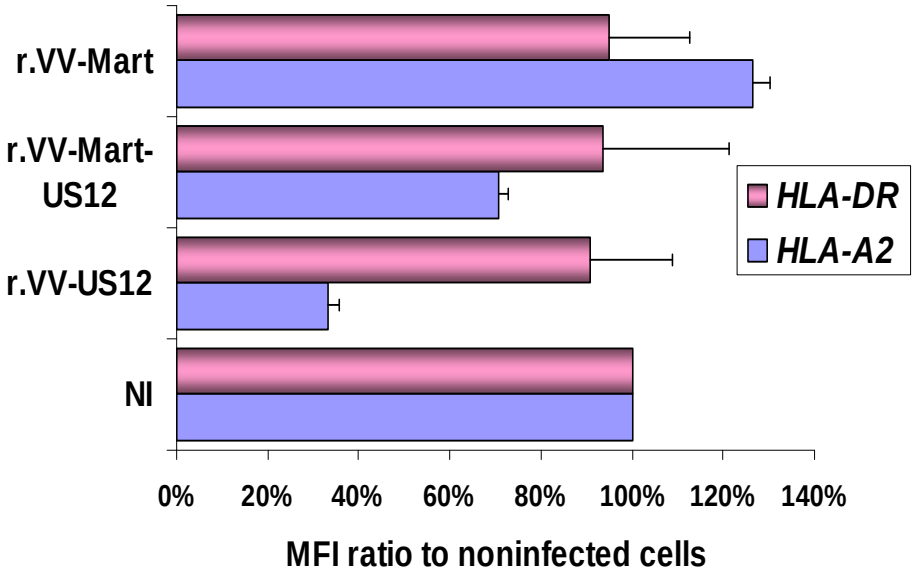


Figure (31): Effect of ICP47 on MHC class-II (HLA-DR) in viral infected cells.

1 x 10^6 EBV-transformed B lymphocytes were infected with PLUV r.VV-US12, r.VV-Mart-US12 or r.VV-Mart (control VV) at 10 m.o.i.. 48 h after infection, the cells were stained by FITC-labelled mAb specific for HLA-DR (n=3) and analysed by FACs. In parallel HLA-A2 surface expression was verified by specific APC-labelled mAb staining and FACS analysis. The results are expressed as percent of MFI considering noninfected cells as 100% expression.

IV.3.1.5. Effect of ICP47 on surface expression of co-stimulatory molecules

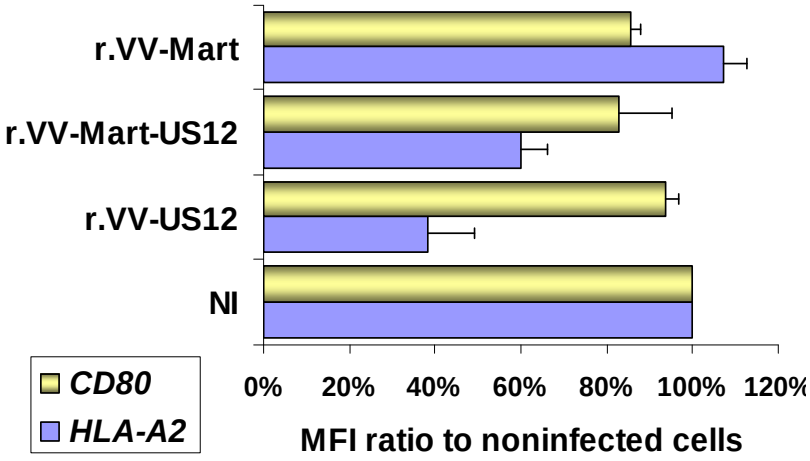
Co-stimulatory molecules play a decisive role during the generation of cellular immune response to antigenic challenges, steering it towards the induction of effector cells instead of tolerance [246]. The different stimulatory ability of APC not only depends on their cognitive signals, but also on the presence of costimulatory molecules [247].

Thus, dendritic cells, macrophages, and activated B lymphocytes with optimal expression of CD80 are efficient APC [248]. CD44 is a multifunctional adhesion molecule that has been shown to be a costimulatory factor for T-cell activation *in vitro* and *in vivo* [249]. Therefore we verified that the downregulation of surface molecules following r.VV-US12 infection did not affect these surface molecules. As a model of cells expressing those different molecules, we chose EBV-BL cell lines.

The average of different experiments in which, CD80 (B7.1) (fig. 32.A) (n=3) and CD44 (fig. 32.B) (n=2) were comparatively evaluated in noninfected, r.VV-US12, r.VV-Mart-US12 or r.VV-Mart infected cells, are represented in figure 32. Flow cytometry analysis demonstrated stable expression of CD80 and CD44 in r.VV-US12 or r.VV-Mart-US12 infected cells. In parallel, the HLA-A2 surface expression has confirmed the downregulation in r.VV-US12 infected cells and the "compensated downregulation" in r.VV-Mart-US12 infected cells.

Taken together, these data indicate that ICP47, expressed by PLUV rec. VV, is specifically downregulating MHC class-I and it is not affecting MHC class-II or other surface molecules

A. CD80 and HLA-A2



B. CD44 and HLA-A2

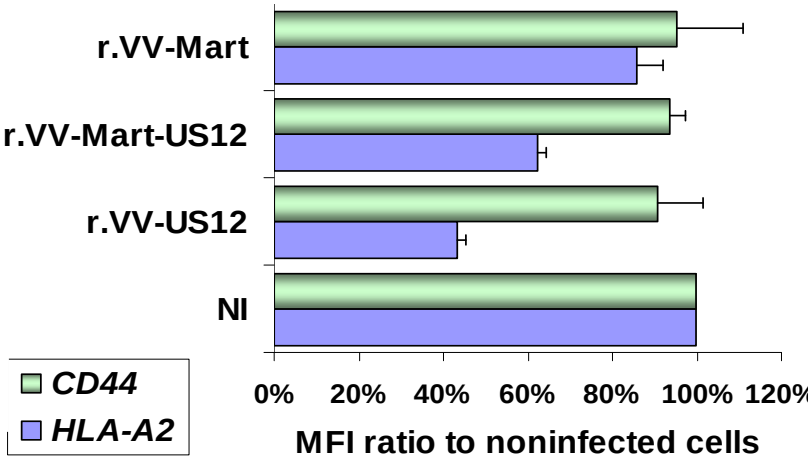


Figure (32): Effect of ICP47 on surface molecules, CD80 and CD44 in r.VV-US12 or r.VV-Mart-US12 infected cells. 1 x 10^6 EBV-transformed B lymphocytes were infected with PLUV r.VV-US12, r.VV-Mart-US12 or r.VV-Mart (control VV) at 10 m.o.i.. 48 h after infection, the cells were stained by PE-labelled mAb specific for **(A)** CD80 (n=3) and **(B)** CD44 (n=2) and analysed by FACS. In both panels, HLA-A2 surface expression is displayed. The results are expressed as percent of MFI considering noninfected cells as also 100% expression.

IV.3.2. ICP47 Modulation of MHC class-I Antigen Presentation

IV.3.2.1. Effect of r.VV with US12 on VV antigens specific T cell response

Decreased MHC class-I expression in r.VV-US12 infected cells, might result in a downregulation of their ability to present viral antigens to CD8+ T cells. Specific experiments were designed to address this issue.

CD14+ cells from healthy donors (n = 2) were infected with r.VV-US12, r.VV-Mart-US12 or WT-VV as control and co-cultured with autologous CD8+ and CD4+ T cells, separately. All cultures were re-stimulated on day 8 with WT-VV infected autologous CD14+ cells. On day 15 after the priming, specific multimer staining for a pool of common VV derived HLA-A2 restricted epitopes, including $H3L_{184-192}$, $B22R_{29-37}$ and $C7L_{74-82}$ [250;251] was performed. As shown in figure 33, cultures primed with r.VV-US12 or r.VV-Mart-US12 infected APCs induce 0.59% and 1.33% VV-multimer positive CD8+ T cells respectively, similar to 0.85% in noninfected culture, whereas WT-VV leads to the expansion of 4.25% antigen specific CD8+ T cells.

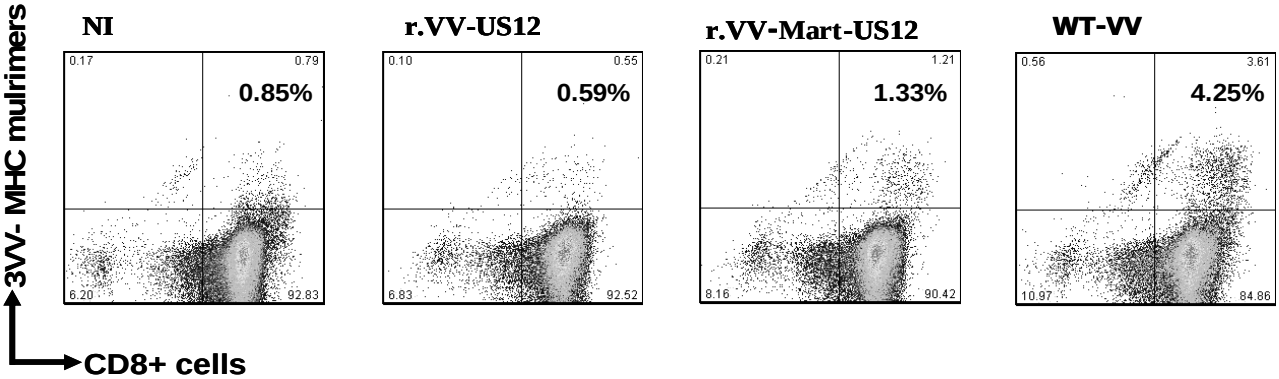


Figure (33): Inhibition of vaccinia virus specific CD8+ T cells response.
1 x 10^6 CD14+ cells from healthy donor were infected with PLUV r.VV-US12, r.VV-Mart-US12 or WT-VV (control VV) at 10 m.o.i. and 48h after infection, they were co-cultured with 1 x 10^6 autologous CD8+ T cells. Noninfected CD14+ cells were also used as control. On day 8, T cell cultures, were boosted with 5 x 10^5 autologous CD14+ cells, infected with WT-VV at 10 m.o.i.. FACs analysis of CD8+ T cells was performed 7 days after boosting by staining with APC-labelled anti-CD8 mAb and PE-labelled specific $H3L_{184-192}$, $B22R_{29-37}$, $C7L_{74-82}$ HLA-A2 multimers.

Antigen specific production of IFN-γ from the same cultures was characterized by intracellular staining. Figure 34 shows that CD8+ T cell cultures stimulated with r.VV-US12 or r.VV-Mart-US12 display 0.72% and 1.1% of CD8+ cells producing IFN-γ respectively as compared to 2.43% in cultures primed with WT VV or 0.63% in noninfected cultures. These results confirm that ICP47 ihibits the induction of MHC class-I VV antigen specific CD8+ T cell responses.

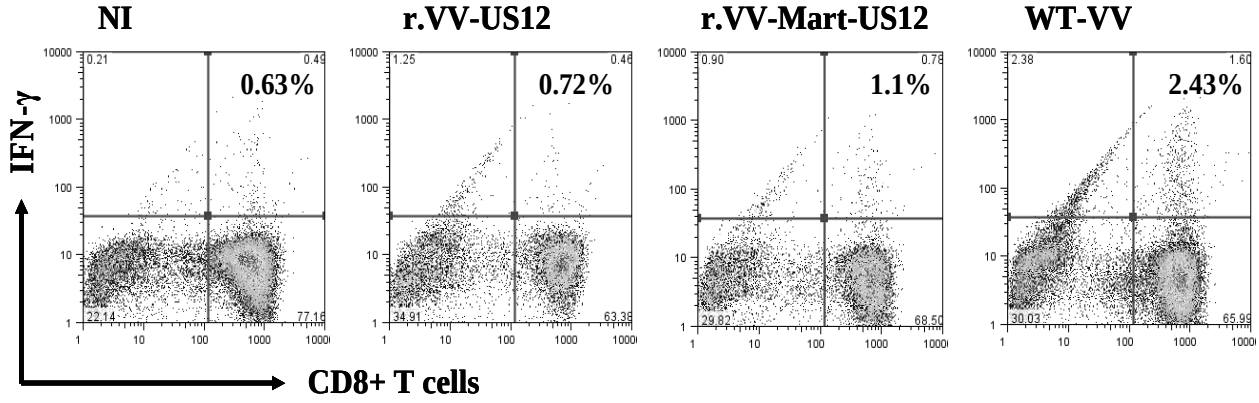


Figure (34): Cytokine evaluation of T cell response to VV antigens.
1 x 10^6 CD14+ cells from healthy donors were infected with PLUV r.VV-US12, r.VV-Mart-US12 or WT VV at 10 m.o.i. and co-cultured with 1 x 10^6 autologous CD8+ T cells. Noninfected cells were also used as control. On day 8 and 15 after priming, CD8+ cultures were re-stimulated with WT VV infected autologous CD14+ cells. On day 15, the cells were stained with PE-labelled anti-IFN-γ mAb and APC labelled-anti-CD8 mAb.

Similar experiments were performed with PBMCs from healthy donors to evaluate the effect of ICP47 not only on CD8+ T cells but also on CD4+ T cells. As read-out on day 15, a viral antigenic stimulation was performed (WT infected CD14+ cells) and cytokine (IFN-γ and IL-2) gene expression was analyzed by qRT-PCR, as ratio to the level measured in WT VV infected culture (100%).

In CD8+ T cell cultures, panel 35.A demonstrates that IFN-γ gene expression in cultures primed with r.VV-US12 or r.VV-Mart-US12 infected cells is 7% (+/-6.8%) and 24% (+/-12%), respectively, while cultures primed with noninfected APCs displayed only 1% of the control value.

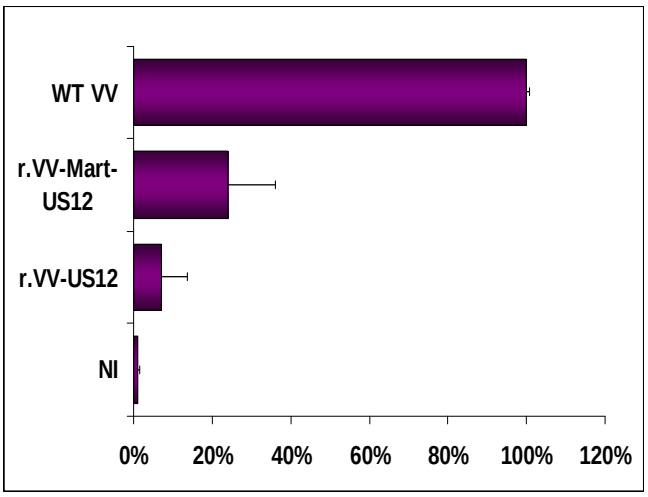
Panel 35.B shows IL-2 gene expression from the same CD8+ T cell cultures. Similarly to IFN-γ gene expression, CD8+ cell cultures primed with r.VV-US12 or r.VV-Mart-US12 infected APC give 27% (+/-2%) and 48% (+/-12%) of IL-2 expression respectively as compared to Control VV whereas, noninfected cultures display only 10% (+/-7%) of the control value.

Depending on a number of conditions (including strength of antigen signalling, co-stimulation and cytokines secreted by APC), CD4+ T cells differentiate into either TH1 or TH2 type cells. TH1 cells secrete predominantly IFN-γ, which plays a role in activation of cell mediated immune responses.

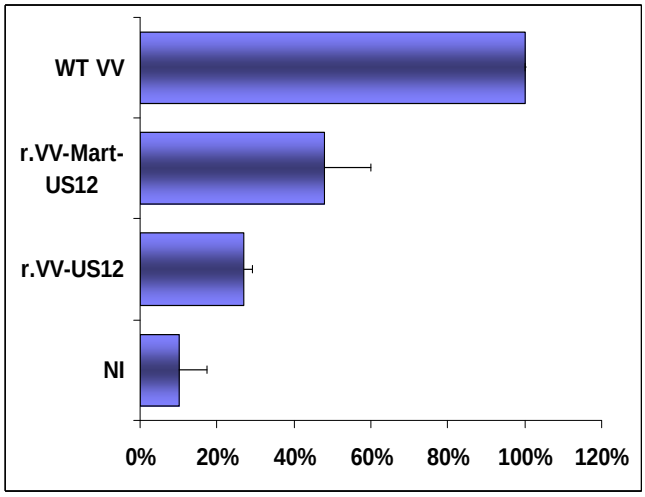
CD4+ T cells were primed with autologous CD14+ cells infected with r.VV-US12, r.VV-Mart-US12 or WT-VV as control. CD4+ T cell cultures were stimulated on day 8 and 15 with WT-VV infected autologous APC.

Panel 35.C, reports data from three independent experiments showing that although r.VV-US12 and r.VV-Mart-US12 infected APCs decrease activation of VV specific CD8+ T cells, these APCs triggered VV specific CD4+ T cells at least as efficiently as the WT control virus (143% +/-66% and 146% +/-42% of IFN-γ expression, respectively).

A. IFN-γ in CD8+ T cells



B. IL-2 in CD8+ T cells



C. IFN-γ in CD4+ T cells

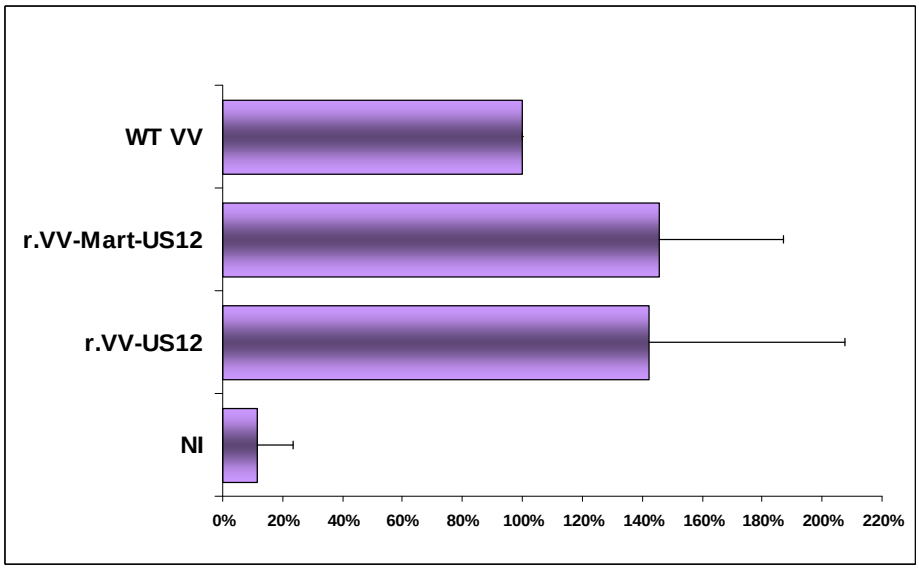


Figure (35): Inhibition of vaccinia virus CD8+ T cell antigens presentation as measured by cytokine gene expression. 1 x 10^6 CD8+ or CD4+ T cells from healthy donor were co-cultured with autologous 1 x 10^6 CD14+ cells infected with r.VV-US12, r.VV-Mart-US12 or control WT VV at 10 m.o.i. Noninfected cells were used as control. On days 8 and 15, all cultures were restimulated by 5 x 10^5 autologous CD14+ cells infected with WT VV at 10 m.o.i.. 24h after second stimulation, mRNA level were evaluated by qRT-PCR for **(A)** IFN-γ in CD8+ T cells, **(B)** IL-2 in CD8+ T cells and **(C)** IFN-γ in CD4+ T cells.

IV.3.2.2 Effect of ICP47 on TAP dependent recombinant full antigens

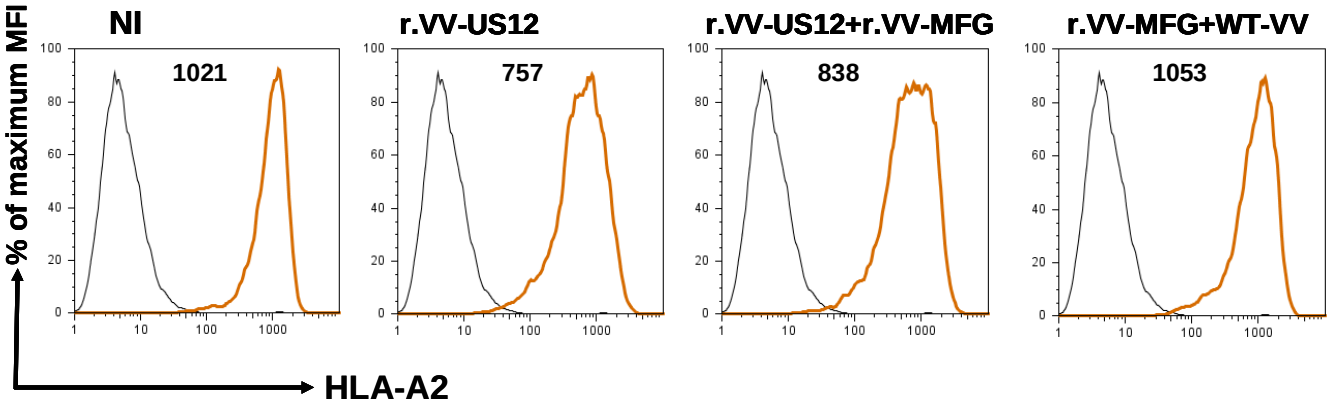
To confirm the necessity of rendering recombinant gene encoded antigen independent from TAP pathway, we also evaluated the effect of ICP47 on recombinant antigen expressed as full protein and restricted by MHC class-I molecules, thus requiring the complete pathway of antigen processing, transport and presentation.

In this experiment, APC (CD14+ cells) were co-infected with r.VV MART-1/Melan-A full gene (r.VV-MFG) together with either WT VV or r.VV-US12 at 5 m.o.i. for each virus or infected with r.VV-US12 at 10 m.o.i.. 48h after infection, a fraction of infected CD14+ cells was tested for HLA-A2 surface expression and the rest was co-cultured with autologous CD8+ T cells.

Results shown in figure 36 panel A confirm that co-expression of ICP47, simultaneously with Mart entire protein, reduced the mean value of HLA-A2 surface expression as compared to APC infected with r.VV-Mart alone. Of note, the apparent difference of HLA-A2 surface expression and Mart-MHC class-I pentamer staining between r.VV-US12 and r.VV-US12+r.VV-MFG, is probably due to the fact that in order to keep the same total viral infection dose for rVVUS12 alone, cells were infected with twice as much ICP47 expressing VV.

In panel B, the decreased expansion of MART-1/Melan-A_{26-35} specific CD8+ T cells from the condition co-expressing ICP47 with the antigen (2.7% vs 4.6%), confirms that this gene product decreases the presentation of MART-1/Melan-A_{27-35} epitope from the full protein.

A. HLA-A2 surface expression



B. HLA-A2 MART-1/Melan-A_{26-35} multimer staining

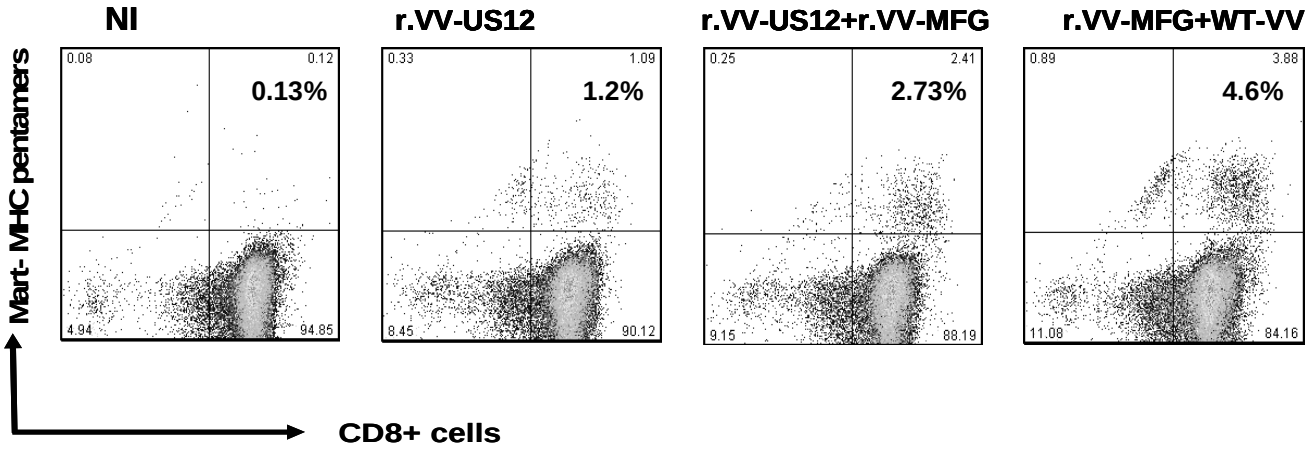


Figure (36): ICP47 diminishing MHC class-I presentation from MART entire protein.
1 x 10^6 initial CD14+ cells were infected with r.VV-US12 at 10 moi or co-infected with rVV-MFG (Mart full gene) together with either r.VV-US12 or WT VV at 5 m.o.i. for each. Noninfected cells were used as control. **(A)** cell surface expression of HLA-A2 was verified 48h after infection by staining with a FITC-labelled monoclonal antibody specific for HLA-A2 (thick line) or with isotype control antibody (thin line). **(B)** 8 days after priming, cultures were stimulated with 5 x 10^5 autologous CD14+ cells pulsed with MART-1/Melan-A_{26-35} peptide (20µg/ ml). FACs analysis of CD8+ T cells was performed on day 9 by staining with APC-labelled anti-CD8 mAbs and PE-labelled specific MART-1/Melan-A_{26-35}-HLA-A2 pentamers.

IV.3.2.3. CTL response to recombinant ER-targeted antigens

Following the results of VV antigen specific response, which confirmed that r.VV-US12 is able to reduce the CTL immune response to VV vector or to transgenic proteins, we assessed the immune response specific for ER-MART-1/Melan-A_{27-35} recombinant antigen. CTL response induced by the r.VV-Mart-US12 was monitored in stimulated peripheral blood CD8+ cells.

IV.3.2.3.1. Effect of ICP47 on ER- targeted recombinant epitope

In r.VV-Mart-US12 and r.VV-Mart constructs, Mart epitope is formulated in a TAP independent ER-targeted form (E3/19K-MART-1/Melan-A_{27-35}). This formulation for specific HLA restriction enables bypassing of a number of antigen processing steps, promoting the surface presentation of antigenic peptides within HLA-molecules [165] even in presence of ICP47.

To address the effect of r.VV-Mart-US12 on Mart specific CD8+ T cell stimulation, four independent "CTL priming" experiments were performed with PBMC from healthy donors. CD8+ T cell cultures were stimulated with autologous CD14+ cells either infected with r.VV-Mart (positive control), r.VV-Mart-US12, r.VV-US12 (negative control), or pulsed with MART-1/Melan-A_{26-35} peptide. Eight days after priming, CD8+ T cell cultures were re-stimulated with MART-1/Melan-A_{26-35} pulsed autologous CD14+ cells (see figure 22).

MART-1/Melan-A_{27-35} specific CD8+ T cells were characterized using MHC-pentamer. As shown in figure 37 panel A, cultures primed with r.VV-Mart-US12 infected APCs resulted in a significant expansion of MART-1/Melan-A_{27-35} specific CTL, similar to rVV-Mart alone (respectively 3.59% vs 3.6%) and expectably far more efficient than in control noninfected cells and r.VV-US12 primed cultures (0.59% and 1% , respectively).

Similarly, in panel 37.B showing another experiment, cultures primed with r.VV-Mart-US12 infected APCs induced a significant expansion of MART-1/Melan-A_{27-35} specific CTL 3.6% as compared to 5.27% in r.VV-Mart primed cultures and more efficiently than in cultures primed with Mart peptide pulsed APCs (1.48%).

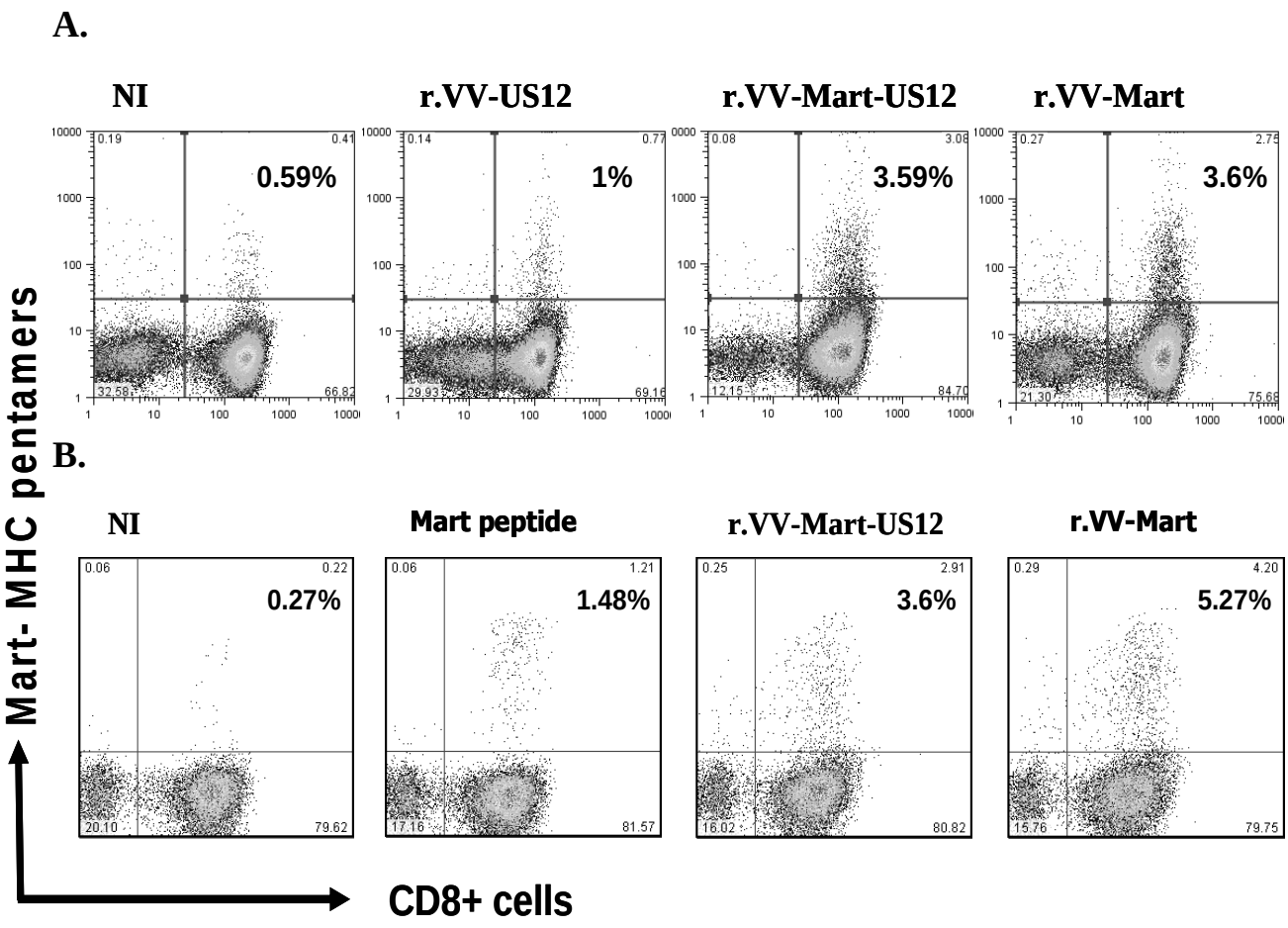


Figure (37): Induction of MART-1/Melan-A_{27-35} specific CD8+ Tcells.
(A) 1 x 10^6 CD14+ cells from healthy donor were infected with PLUV r.VV-US12, r.VV-Mart-US12 or r.VV-Mart at 10 m.o.i., co-cultured with 1 x 10^6 autologous CD8+ T cells. Non modified CD14+ cells were also used as control. On day 9 after priming, FACs analysis of CD8+ T cells was performed by staining with APC-labelled anti-CD8 mAbs and PE-labelled specific MART-1/Melan-$_{A26-35}$-HLA-A2 pentamers. **(B)** CD8+ T cell cultures were primed as indicated but in this experiment, the r.VV-US12 condition was replaced with CD14+ cells stimulated with MART-1/Melan-A_{26-35} peptide (20µg/ ml).

To further characterize the functional capacity of the expanded specific T cells, IFN-γ intracellular staining, following an antigenic stimulation, was performed. As shown in figure 38, cultures primed with r.VV-Mart-US12 infected APC display 2.55% IFN-γ positive cells as compared to 0.35%, 2.84% and 0.22% in cultures where APC were control noninfected condition, infected with rVV-Mart or r.VV-US12 respectively

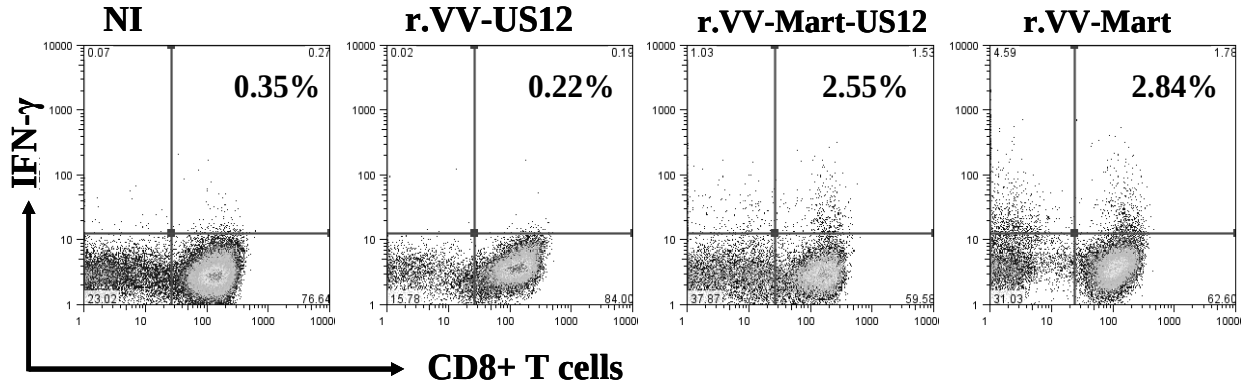


Figure (38): Cytokine evaluation of T cell response to MART-1/Melan-A_{27-35} antigen.

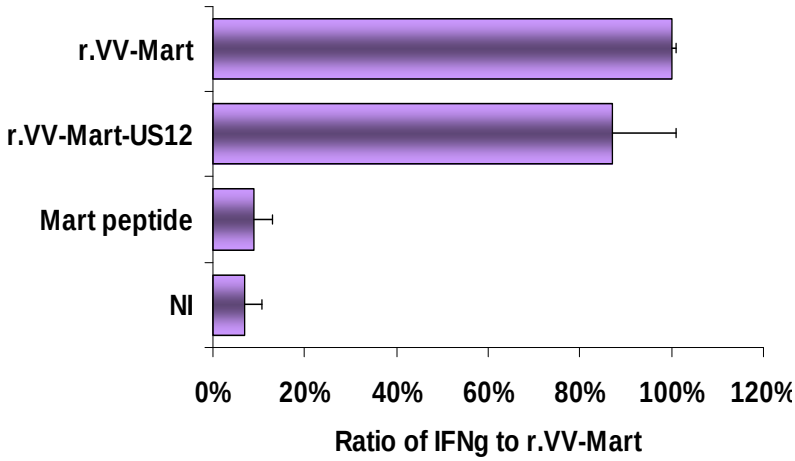
1 x 10^6 CD14+ cells from healthy donors were infected with PLUV r.VV-US12, r.VV-Mart-US12 or r.VV-Mart (control VV) at 10 m.o.i. and co-cultured with 1 x 10^6 autologous CD8+ T cells. Noninfected cells were also used as control. On day 9 and 15 after priming, 5 x 10^5 autologous CD14+ cells were stimulated with MART-1/Melan-A_{26-35} peptide (20μg per ml) and then all cultures were stimulated with these CD14+ cells. On day 15, the cells were stained with PE-labelled anti-IFN-γ mAb and APC-anti-CD8 mAb.

As an alternative test for the final readout after prime-boost strategy, we measured IFN-γ gene expression in CD8+ T cell cultures stimulated with different viral infected CD14+ cells (r.VV-Mart-US12 and r.VV-Mart) or with MART-1/Melan-A_{26-35} peptide. Lymphocytes were re-stimulated on day 9 and 15 using autologous Mart peptide pulsed CD14+ cells. Following the second stimulation, IFN-γ gene expression was monitored by qRT-PCR and expressed as ratio to the level measured in r.VV-Mart infected cultures (100%).

Figure 39 panel A, shows that cultures primed with r.VV-Mart-US12 display 87% (+/-14%) of expression as compared to r.VV-Mart. Only 9% (+/-4%) and 7% (+/-3.8%) IFN-γ expression could be detected in Mart peptide primed cultures and noninfected cultures respectively.

Similarly, IL-2 gene expression (panel B) from CD8+ T cell cultures stimulated with r.VV-Mart-US12 infected APC displayed 84% (+/-7%) as compared to r.VV-Mart stimulated cultures (100%).

A. IFN-γ expression



B. IL-2 expression

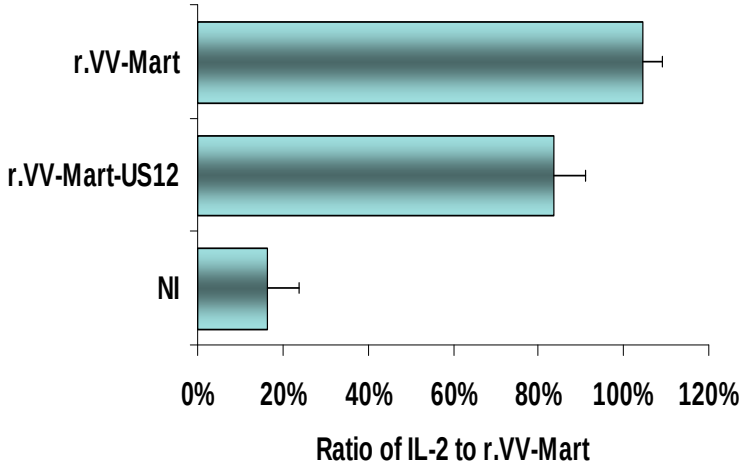


Figure (39): Induction of MART-1/Melan-A_{27-35} specific CD8+ T cells as measured by cytokine production. 1 x 10^6 CD8+ T cells from healthy donors were co-cultured with autologous 1 x 10^6 CD14+ cells infected with r.VV-Mart-US12 or r.VV-Mart at 10 m.o.i. or stimulated with MART-1/Melan-A_{26-35} peptide (20μg per ml). Noninfected cells were used as control. On day 9 and 15, all cultures were re-stimulated by 5 x 10^5 autologous CD14+ cells stimulated with Mart peptide. **(A)** IFN-γ and **(B)** IL-2 gene expression was verified by qRT-PCR and expressed as ratio relative to r.VV-Mart stimulated cultures.

Taken together, these data suggest that r.VV-Mart-US12 is able to induce MART-1/Melan-A_{27-35} antigen specific CTL immune responses as confirmed by specific MHC-pentamers staining and cytokines characterization. The expression of ICP47 together with Mart epitope in r.VV-Mart-US12 infected APC did not significantly diminish the presentation of ER-targeted Mart epitope. On the other hand, despite a low viral-epitopes immunocompetition and a visible fraction of HLA molecules loaded with Mart epitope, r.VV-Mart-US12 infected APCs did not display an enhanced stimulation of MART-1/Melan-A_{27-35} specific T-cells, as compared to APCs infected with the control vector expressing Mart epitope.

IV.3.3. Effect of ICP47 on MHC class-I Antigen Presentation in VV Presensitized PBMCs

The above experiments confirmed that rVV-MUS12 is able to prime-stimulate Mart specific CTL with a similar immunogenicity as compared to the control rVV-Mart.

In order to highlight *in vitro* the effect of ICP47 promoting the induction of rec.ER-epitope specific responses despite strong anti-vector immune responses, experiments of Mart specific CTL generation was performed with VV presensitzed PBMCs. This "*in vitro*" strategy should reflect more closely the *in vivo* conditions of pre-vaccinated patients or even multi-boosting protocols with a recombinant virus.

We selected two VV-vaccinated (57 and 44 years old) healthy donors. PBMCs were freshly isolated (figure 40) and CD14+ cells (APC) were infected with WT-VV before mixing with either autologous CD8+ or CD4+ sorted T cells. After 8 days, the cultures were primed, for MART epitope, with either rVV-Mart, rVV.Mart-US12 infected CD14+ cells. Here, the critical differential event for Mart specific CTL activation was related to the intensity of the APC-

clearing effect of the pre-existing VV CTL. Noninfected CD14+ and rVV-US12 infections were also performed as controls.

All cultures were then splitted in 2 (one for Mart-1/Melan-A_{27-35} and one for VV epitopes) and boosted twice on day 15 and 21 using either MART-1/Melan-A_{26-35} peptide pulsed or WT-VV infected autologous CD14+ cells. For the final readout, e.g. measuring of IFN-γ and IL-2 gene expression in CD8+ T cell cultures, effector cells were stimulated with rVV-Mart infected autologous APC, presenting all VV and Mart-1/Melan-A_{27-35} epitopes**.**

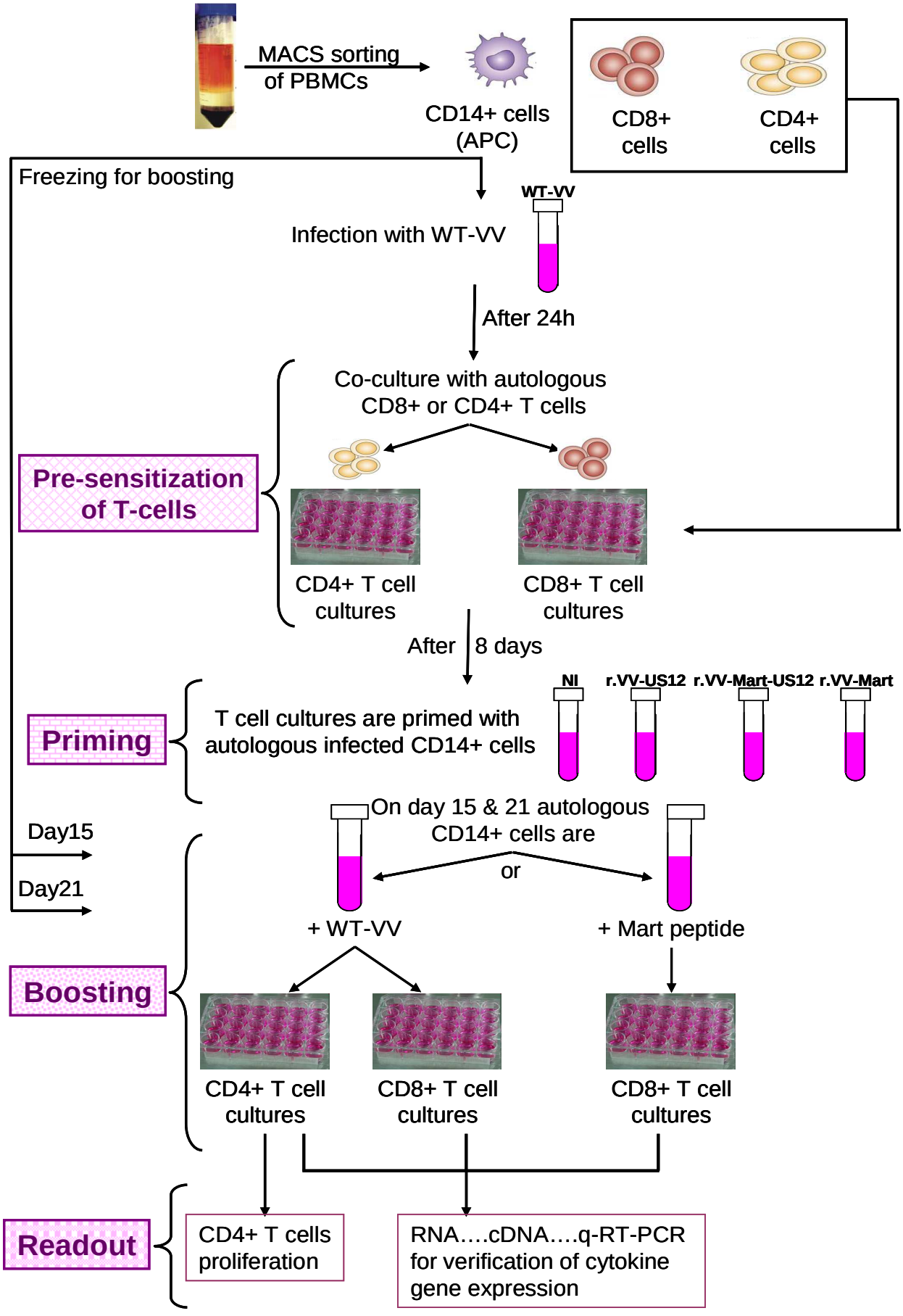


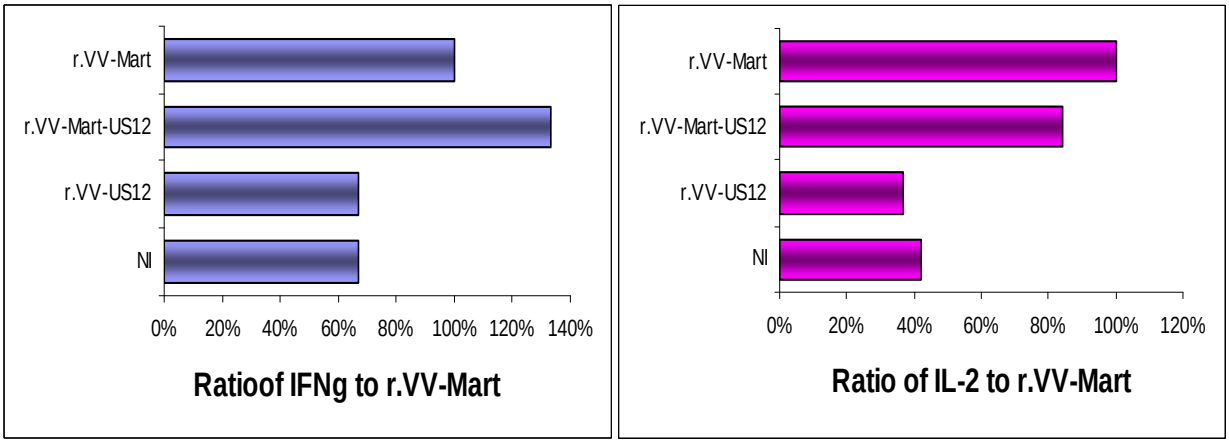
Figure (40): Presensitized PBMCs experiment.

IV.3.3.1. MART-1/Melan-A_{27-35} specific CD8+ T-cell response in VV presensitized PBMCs

Results in figure 41 show IFN-γ and IL-2 gene expression in "Mart boosted CD8+ T cell cultures" from both donors 1 and 2 (panels A and B respectively). Interestingly, gene expression in CD8+ T cell cultures primed with r.VV-Mart-US12 shows a significant increase for IFN-γ (700%) and IL-2 (350%) as compared to r.VV-Mart infected CD14+ cells, for donor B, who was revaccinated with the live virus only 15 years before. On the other hand, the ">50 years vaccinated" donor A displays a profile similar to the one observed previously in experiments performed without vv-presensitized with marginal differences with the rVV-Mart condition.

Thus, it appears that the decreased generation of antigenic peptides from VV proteins, due to ICP47, did play an "enhancing" role for Mart-1/Melan-A_{27-35} immunogenicity in the presence of pre-activated VV specific CD8+ T cell response.

A. Donor: 1



B. Donor: 2

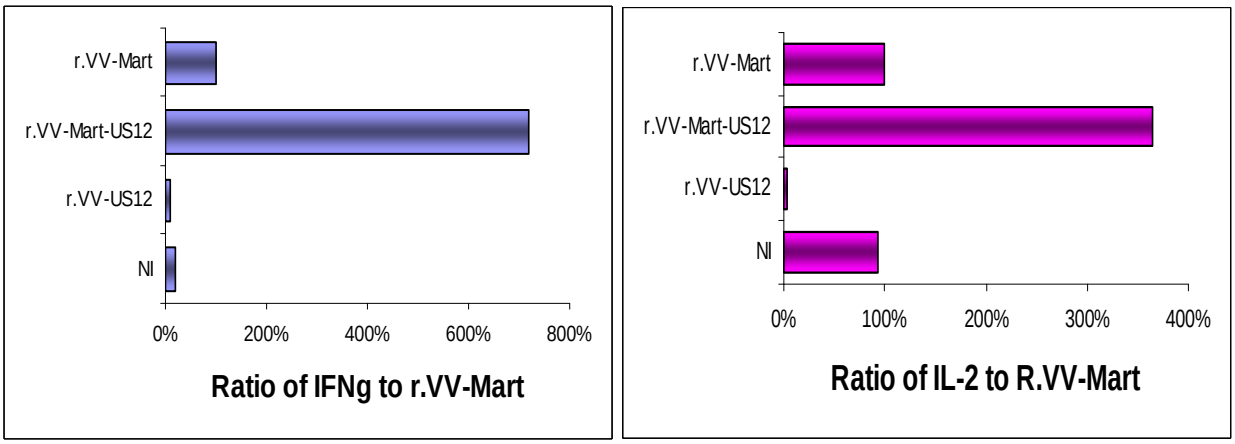


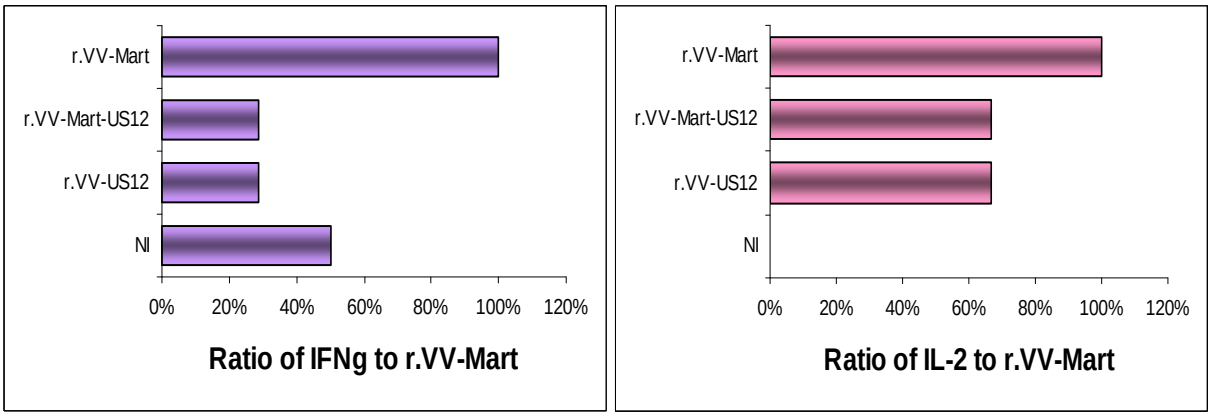
Figure (41): Cytokine gene expression in MART-1/Melan-A_{27-35} specific CD8+ T cell cultures from presensitized PBMC.

5 x 10^5 CD14+ cells were infected with PLUV WT-VV at 10 m.o.i. and mixed with autologous T cells. After 8 days, monocytes infected for 48h with r.VV-US12, r.VV-Mart-US12 or r.VV-Mart or noninfected (as negative control) were used to prime Mart specific CTL. On days 15 and 21 cultures were boosted with 5 x 10^5 autologous CD14+ cells pulsed with MART-1/Melan-A_{26-35} peptide (20 μg per ml). On day 27, IFN-γ and IL-2 gene expression, following rVVMart infected cells stimulation, was verified by qRT-PCR. Data are expressed as ratio to r.VV-Mart primed cultures.

IV.3.3.2. Inhibition of vaccinia virus specific CD8+ T cell response in VV presensitized PBMCs

The results shown in figure 42, from the "Vaccinia boosted T cell cultures" demonstrate that IFN-γ and IL-2 gene expression, in cultures restimulated on day8, with an ICP47 expressing virus (r.VV-US12 or r.VV-Mart-US12), is decreased (about 30% IFN and 60% IL2) as compared to that of the control r.VV-Mart. These data are confirming that ICP47 is able to diminish vaccinia viral antigens presentation leading to diminished VV antigens specific CD8+ T cell responses which is rapidly clearing the infected APC.

A. Donor: 1



B. Donor: 2

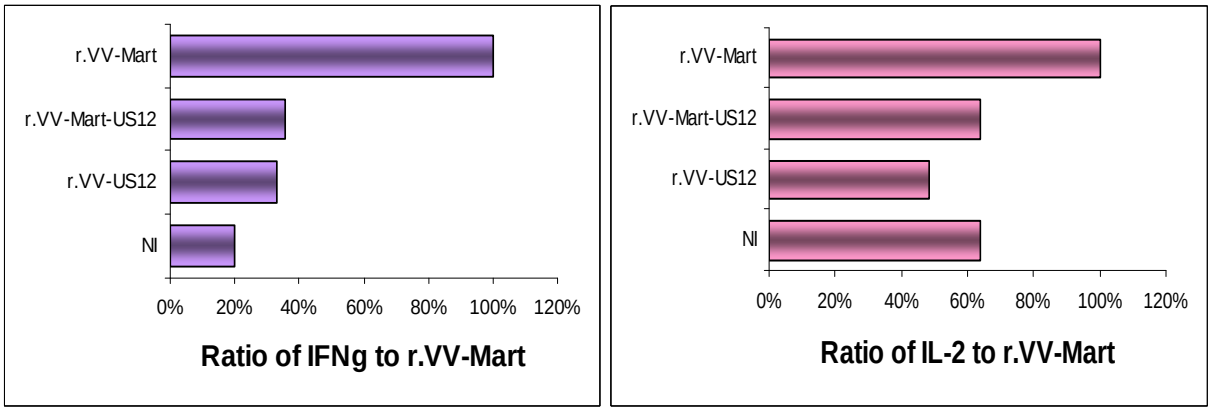


Figure (42): Cytokines gene expression in VV specific CD8+ T cells stimulated with VV from presensitized PBMC.

CD14+ cells were infected with WT-VV at 10 m.o.i and mixed with autologous CD8+ T cells. After 8days, cells restimulated with r.VV-US12, r.VV-Mart-US12 or r.VV-Mart at 10 m.o.i.. Noninfected monocytes were used as control. On day 15 and 21 all cultures were boosted for vaccinia antigens with autologous CD14+ cells infected with WT-VV. On day 27, following WT-VV infected cells stimulation; IFN-γ and IL-2 gene expression was verified by qRT-PCR and expressed as ratio to r.VV-Mart stimulated cultures.

IV.3.3.3. Induction of vaccinia virus specific CD4+ T cell response in VV presensitized PBMCs

We have previously shown that MHC class-II vaccinia virus specific CD4+ T cells from vaccinia immunized donors respond to VV by active proliferation [252]. In this study, we have also shown that expression of ICP47 does not affect the surface expression of MHC class-II molecule.

In this experiment with similarly VV-presensitized CD4+ T cells (only from donor 1), we analysed the effect of ICP47 encoding viruses stimulation (during the second stimulation) on CD4+ T cell cytokines and proliferative responses to viral antigen.

Figure 43 displays IFN-γ and IL2 gene expression following an antigenic stimulation of the different CD4+ Tcell cultures. Although, in the 2 conditions stimulated with US12 encoding virus, IL2 gene expression is detected slightly above 2 fold more as compared to the control virus condition, IFN-γ expression remains below this qRT-PCR threshold of 2 folds.

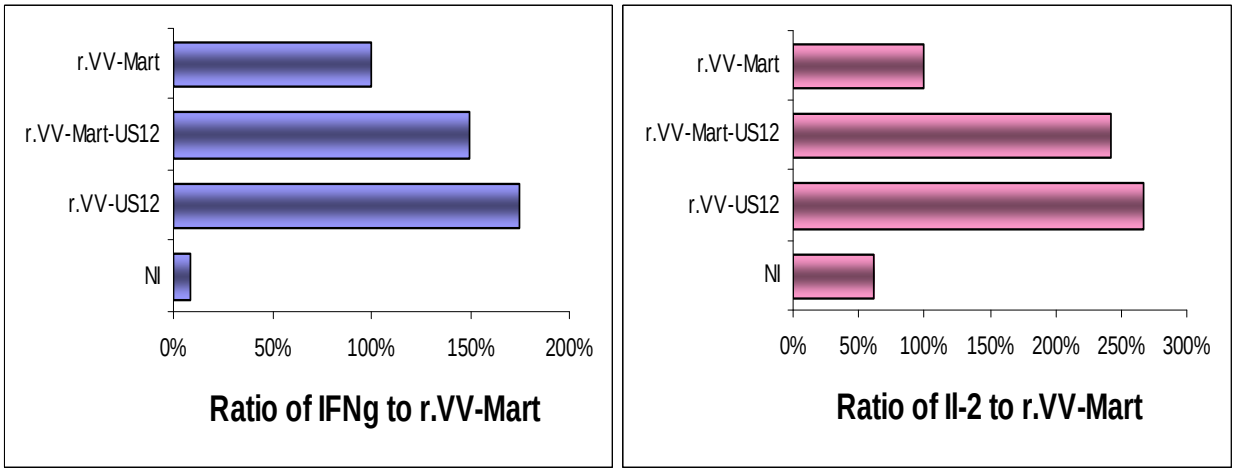


Figure (43): Cytokines gene expression in VV specific CD4+ T cells stimulated with VV from presensitized PBMC.
CD14+ cells were infected with WT-VV at 10 m.o.i and mixed with autologous CD4+ T cells. After 8days, cells restimulated with r.VV-US12, r.VV-Mart-US12 or r.VV-Mart at 10 m.o.i.. Noninfected monocytes were used as control. On day 15 and 23 all cultures were boosted for vaccinia antigens with autologous CD14+ cells infected with WT-VV. On day 24, following WT-VV infected cells stimulation, IFN-γ and IL-2 gene expression was verified by qRT-PCR and expressed as ratio to r.VV-Mart stimulated cultures.

As an alternative test, CD4+ T cells proliferation measuring the incorporation of tritiated thymidine into the dividing cells. Results shown in figure 44, demonstrate that CD4+ T cell proliferation following r.VV-US12 or r.VV-Mart-US12 stimulation is similar to that detectable in the control VV infected cultures.

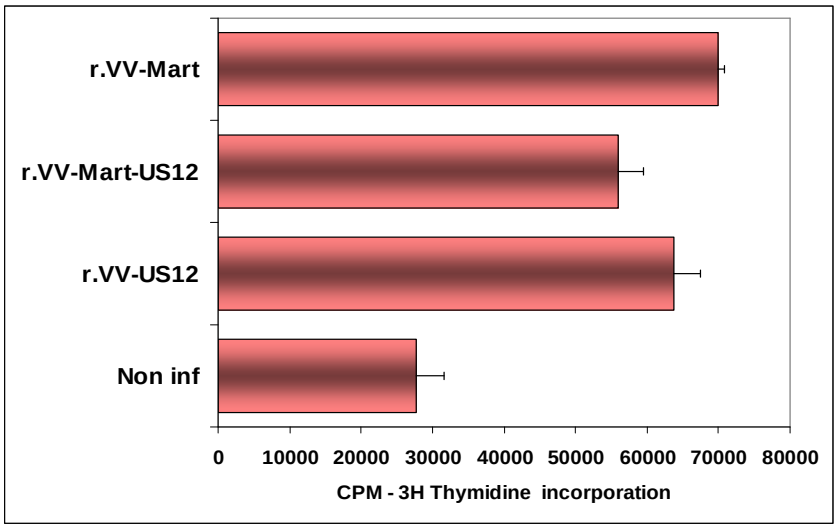


Figure (44): CD4+ T cell proliferation in response to VV antigens.
WT VV presensitzed CD4 cells were restimulated with PLUV r.VV-US12, r.VV-Mart-US12 or r.VV-Mart control virus at 10 m.o.i.. Cultures were boosted only once (on day 16) with autologous WT infected CD14+ infected cells. Proliferation of CD4+ T cells in the presence of autologous infected CD14+ cells was evaluated by thymidine 3H incorporation. Thymidine ^{3}H was added and cultures were re-incubated for 18h, then harvested, lyzed and washed. Liquid scintillation cocktail was added and scintillation emission was measured.

V. DISCUSSION

Despite the relative effectiveness of conventional chemotherapy and targeted agents, most patients with cancer experience tumor progression and ultimately die of their disease. Therefore, there is an urgent need for better treatments, not only for the advanced disease but also to prevent relapse. One promising approach seems to be the stimulation of tumor directed immune responses. For this aim, efficient presentation of immunogens to T cells, leading to generation of large numbers of TAA specific CTL, represents a critical issue for cancer immunotherapy.

The potential of immunotherapy was first documented by William Coley in 1890. Then it was generally ignored until the last part of the 20^{th} century, when studies of chemically induced tumors of inbred mice demonstrated spontaneous regression of melanoma fueled speculation that immune responses contributed to tumor regression [253]. In 1980, lymphocytes activated with lectins or IL-2 were demonstrated to target tumor cell in vitro [254;255].

In 1995, interferon α-2b became the first immunotherapy approved for adjuvant treatment of stage IIB/III melanoma by the United States Food and Drug Adminstration (USFDA) [256]. IL-2 was the second exogenous cytokine to demonstrate antitumor activity against melanoma and it was approved by the USFDA in 1998 for treatment of adults with advanced metastatic melanoma [160]. The discovery and cloning of a number of shared melanoma associated antigens or cancer germline antigens expressed specifically by solid tumors including melanoma, has spurred interest in peptide and protein vaccines [257]. (figure 45)

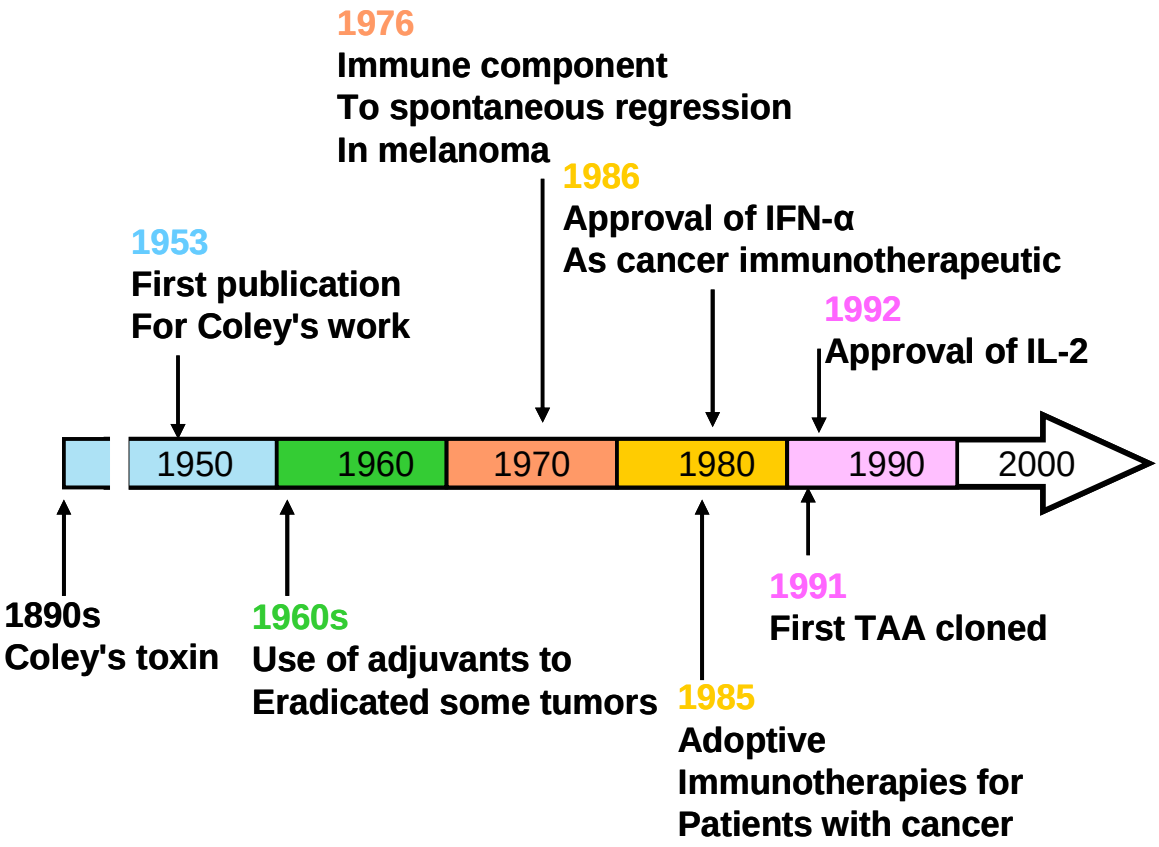


Figure (45): Key events in the history of cancer immunotherapy.

Despite the fact that melanoma may be a poor choice as a tumor model, due to its proclivity to rapidly grow and metastasize in a totally unpredictable manner, it has remained the "training ground" for a number of different experimental approaches, involving a wide range of technologies [258]. The limitations of immunotherapy for melanoma stem from tumor induced mechanisms of immune evasion that render the host tolerant of tumor antigens. Nevertheless, vaccines are being developed that may ultimately target melanoma either alone or in combination with immunomodulatory therapies.

Reproducible success for generating CD8+ T cell mediated vaccines has emerged from the use of recombinant viral vectors containing transgenes encoding antigens to which a CTL response is desired. Therefore, recombinant viruses rank among the most effective immunogens and appear to be of

particular interest in the development of TAA specific immunization procedures. In this approach, delivery of antigenic transgenes' products closely mimics the physiological endogenous production of MHC class-I ligands [259;260]. Furthermore and perhaps most importantly, such vectors may per se provide a typical danger signal, possibly activating APC and inducing high avidity CTL more effective at clearing tumors [261].

Immunization with recombinant viral vectors induces a strong and long lived CD8+ T cell response that is rarely matched in efficacy by other modes of immunization. Many recombinant poxviral vaccines, based on attenuated and nonattenuated strains of vaccinia, have demonstrated their safety and immunogenicity in many clinical trials [157;158;262]. This strong response to viral vectors may be a fortuitous by-product of the evolution of the innate immune system to combat viral infection and thus to recognize viral vectors as 'dangerous' [263]. Nonetheless, significant challenges remain prior to the successful use of recombinant viral vectors to induce therapeutic and protective CD8+ T cell response in human cancers.

Smallpox disease, caused by variola virus was eradicated in the 1970s by a worldwide vaccination with cross-protective vaccinia virus [264]. It was demonstrated that one round of vaccination with a live VV was sufficient to induce a long-lived (50 years) cellular immune response to several identical vaccinia epitopes. It was even claimed by Demkowicz et al., that human subjects with prior exposure to VV, years earlier, provide an excellent model for the study of human T cell memory [153]. It was also estimated by Hsieh et al that the significant T cell memory response to VV from successful vaccination may persist for about 20-30 years in the presumed absence of antigen [265]

In a previous randomized phase I trial reported by Cooney et al., when they immunized 35 healthy HIV-1 seronegative young adults with r.VV expressing gp160 envelope gene of HIV-1, it was found that individuals who

had been immunized as young children with VV had poor immune response to the HIV-1 gp160 antigen as compared with those who had no previous exposure to VV. The results from this trial suggested that long lasting immunity to VV had limited the replication of the recombinant vaccinia virus used for immunization [158].

Therefore, this strong immunogenicity, which was always taken as a positive feature, is paradoxically now considered as one major limitation. The potential use of recombinant viruses to produce protective immune response, especially for multiple boosting vaccine strategies, could be limited due to phenomenons, such as "immunodominance" and "viral vector clearance". The latter caveat is affecting the APC as effector CD8+ T cells, that recognize VV derived peptide/MHC complexes, may destroy the APC and this problem is more obvious upon increased numbers of subsequent boosts. Immunodominance of viral vectors, on the other hand, is manifested by a strong alternative CD8+ T cell response targeted to determinants expressed naturally by the vector that reduces or even prevents a CD8+ T cell response to the subdominant recombinant antigens.

Several recombinant vaccinia viruses showed that the CD8+ T cell response to foreign epitopes is coordinately regulated with the response to the VV vector but that the response directed against VV is much greater in magnitude than the response against the inserted recombinant epitope [151;157]. It is possible in experimental system to remove by mutation or deletion some immunodominant determinants in order to enhance the response to subdominant antigens [266;267]. However, this approach is not practical in a therapeutic setting, as there are no means to predict the immunodominance of antigens in an outbreed population [268].

Moreover, pre-existing memory against the virus backbone may limit its use as a viral vector for other vaccination purposes. Therefore using vaccine

constructs that possess limited replication within the host, such as modified VV Ankara, may be more efficient for vaccination purposes, because they are endowed with a reduced viral immunogenicity but can still prime protective immunity against the gene of interest [269-271].

Despite this caveat, numerous immunotherapy studies based on viral vectors have reported a successful transgene immunization. Nevertheless, the observed in vivo responses with “short” vaccination protocols are usually mild and transient. It became apparent that effective human cancer vaccines require prolonged boosting protocols. In order to avoid counter-productive response against single vector, different vaccine platforms expressing the target TAA were used in so called ″heterologous prime-boost” vaccine strategies and were shown to induce an enhanced immune response. In particular, we have previously reported the relative immune efficacy of a prime and boost vaccine regimen based on a non-replicating recombinant vaccinia virus encoding melanoma associated epitopes (GP100, Melan-A/Mart-1 and Tyrosinase) and both immunomodulatory molecules CD80 and CD86 which has been successfully used in a phase I/II clinical trial [3;214;272] and resulted in a substantial immunogenic response.

The application of non replicating vaccine was boosted with three injections of peptides (pept). Nevertheless, from both clinical trials based on this protocol (intradermal and intranodal administration), we could observe that peptide boost was far from the efficacy of the recombinant viral vaccine, as characterized by specific CTL responses. Following only two cycles of vaccinations (rec.VV-pept- pept- pept), the final level of CTL generated was not much different from the initial level. However, further injections (3, 4 or 5) of viral vaccine, were able to generate significant levels of specific CTL responses.

All these observations led us to conclude that the vaccination protocol should apply multiple boosting injections (more than 2) of the recombinant viral

vaccine. However, even if the recombinant antigen specific response was decreasing after only 2 vaccination cycles, the anti-VV-response (measured by antibodies) was increased after each r.VV boost.

Therefore, further investigations on recombinant vectors providing innovative pathways susceptible to enhance vaccine driven TAA specific CD8+ T cell response in cancer patients, without increasing the anti-VV vector response, might represent a crucial issue for cancer immunotherapy.

In this study, we hypothesized that, given the known role of TAP in antigen processing and presentation to be recognized by specific TCR, co-expression of a TAP blocker by recombinant VV vector would result in reduced presentation of VV derived peptides on the surface of the transduced cells. This immunomodulation should reduce the immunocompetition and render infected cells less vulnerable to cytolytic response of VV specific CD8+ T cells.

Therefore, we constructed and functionally characterized a r.VV expressing HSV-US12 gene (r.VV-US12), coding for ICP47, which specifically binds to the ABC transporter TAP and blocks binding of peptides generated by proteasomal degradation. This blockade subsequently inhibits their translocation into the ER and the loading onto empty MHC class-I molecules [273].

Based on this construct, we also engineered a novel reagent (r.VV-Mart-US12) simultaneously encoding both ER-targeted Mart-1/Melan-A_{27-35} antigen as minigene, and the HSV-US12 gene. Because of its ER targeting design, this model of HLA-A0201 TAA epitope is independent of processing and mostly of TAP translocation, thus expression and presentation to CD8+ T cells should be maintained despite TAP inhibition.

In this context, our strategy aims at reducing the immunodominance features of vaccinia virus for cancer immunotherapy, while maintaining high

levels of peptide-MHC complexes derived from recombinant foreign minigenes. The resulting viral vector should be more efficient at inducing CTL responses to recombinant targeted-antigens while possibly avoiding the need of complex protocol with heterologous prime-boost strategy.

Moreover, inactivation of viral replication by psoralen and long wave UV treatment, on one hand improves the safety and strongly diminishes cytopathic effect of our r.VV construct [234] and on the other hand, due to the inhibition of viral late transcription, considerably decrease the numbers of viral antigens expressed. Some studies showed that vaccinia virus specific CTL epitopes are mostly driven by early promoters [274;275] while humoral responses are mainly directed to viral proteins that are driven by late promoters [276]. Thus, in individuals pre-immunized against VV, our “classical” non replicating vectors, used in the two melanoma clinical trials [3;217], might have been recognized by pre-existing memory CTL but much less by pre-existing neutralizing Ab as compared to a replicating VV vector. Of note, confirming this hypothesis, the increased humoral responses following the 2 (or more) injections of non-replicating virus were detectable and significant but not very intense [252].

UV cross-linking affects genes proportionally to the gene length. Thus expression of minigenes, (about 100 bp), remains relatively spared by UV. It was also shown that PLUV treated VV elicits less inflammation but induces more foreign epitope specific CD8+ T cell response than untreated virus [268]. In the hereby presented study, US12 gene (267bp) expression was first characterized in cells infected with PLUV r.VV-US12 and was shown to be strong and viral dose dependent.

Replication incompetent r.VV-US12 is capable of decreasing cell surface expression of MHC class-I on infected human cells and decreases recognition by specific CTL.

These data are in agreement with published reports [277;278] showing that ICP47 binds to TAP and blocks peptide transport from the cytosol to ER which prevents MHC molecule loading and translocation to the cell surface [227;279]. Kinetics studies confirm that the maximum dowregulating effect, related to ICP47 expression and MHC turn-over was detected after 36h of infection as evaluated by flow cytometry on the cell surface. The intensity of MHC “downregulation” could reach 80% (compared to noninfected cells).

Most importantly, in all experiments evaluating the presence of MHC class-I on the cell surface following infection with the different viruses, the downregulation of MHC class-I is partially “compensated” in cells infected with r.VV-Mart-US12. This compensation is most probably due to the overexpression of Mart-1/Melan-A_{27-35} restricted epitope, stabilizing the HLA0201-peptide complex onto the cell surface and confirming the efficiency of the ER-targeting strategy which is independent of processing and TAP transport.

Generation of TAA specific CTL represents the main issue in cancer immunotherapy. To achieve this objective, different signals need to be provided by the antigen presenting cells. The first signal is represented by MHC class-I restricted antigenic peptides recognized by the T cell receptor. To avoid anergy and to promote T cell activation, this signal must be complemented by triggering co-stimulatory molecules, such as CD80 and CD86 as a second signal. This effect is eventually reinforced by third signals mediated by soluble factors such as T helper 1 cytokines (IL-2, IFN-γ, IL-12 and IL-15). In addition to professional costimulatory receptors, such as members of the B7 family, other molecules, such as CD44, [249] that have an adhesive function during DC-T-cell interactions have been found to modulate T-cell responses. Moreover, binding of CD4+ TCR by MHC class-II antigen peptide complex induces

activation and clonal expansion of antigen specific CD4+ T cells which are needed to induce efficient cellular and humoral immune responses.

The characterization of these molecules on r.VV-US12 infected APC (here CD14+cells) confirmed that, the downregulating effect of ICP47 on surface expression is specific to MHC class-I and it does not affect the expression of any other surface molecules tested so far which could play a role for activation of antigen specific CTL response. Similarly, we have shown that ICP47 does not downregulate the surface expression of MHC class-II molecules, which even appeared slightly upregulated. This maintenance of MHC class-II-peptide complexes inducing activation and proliferation of antigen specific CD4+ T cells is especially relevant as it represents the main pathway for the immunogenic adjuvant effect of the viral vector. Indeed, although VV specific CTL clearing effect is undesirable, the helper stimulation of VV specific CD4+ remains a key feature of the vaccine efficiency.

In order to formally demonstrate that the observed phenotype reflects the corresponding immunogenicity, we tested the capacity of our novel reagents to differentially induce antigen specific CD8+ T cells.

The results of CD8+ T cells stimulated with PLUV r.VV-US12 and r.VV-Mart-US12 infected monocytes, displayed a decreased antigenic responses towards vaccinia virus proteins, as verified by the characterization with 3 HLA-A2-multimers containing immunodominant viral epitopes $H3L_{184-192}$, $B22R_{29-37}$ and $C7L_{74-82}$ peptides. These data restricted to a single HLA and 3 epitopes, were also confirmed by the decreased levels IFN-γ and IL-2 cytokines gene expression in response to all possible epitopes presented by WT vaccinia infected monocytes.

ICP47 driven MHC class-I peptide decreased antigenicity from native proteins [280;281] was also demonstrated for a recombinant antigen using co-

infection of APC with r.VV-US12 together with r.VV-MFG (Melan-A/Mart-1 full gene). In these APC, similarly to viral antigens, Mart derived peptides follow the classical processing and MHC class-I pathway of antigen presentation. In presence of ICP47, we could observe a strongly decreased induction of Mart-1/Melan-A_{27-35} specific CTL response.

We then demonstrated that, using Mart-1/Melan-A_{27-35} as a model epitope of transgenic TAP-independent MHC class-I presentation, co-expression of ICP47 did not affect the generation of Mart-1/Melan-A_{27-35} specific CTL response as demonstrated by specific multimer staining and cytokine expression. These data confirmed that in TAP-blocked conditions, ER-targeted epitopes remain highly immunogenic and are able to induce specific CTL response.

Interestingly, as previously demonstrated for rVV-Mart, generation of Mart-1/Melan-A_{27-35} specific CTL is more efficient by using APCs infected with the novel r.VV-Mart-US12 than by using APC pulsed with the corresponding soluble peptide. Whereas large numbers of peptide-MHC complexes can also be obtained with the soluble peptide, these results clearly indicate that r.VV-Mart-US12 provides efficient adjuvance during the induction of CTL even in the limited cell conditions tested here (only CD8+ and CD14+).

The immunogenic advantage, related to co-expression of ICP47 with the recombinant epitope for CD8+ activation, is resulting from two different mechanisms acting in co-operation. First, the strong inhibition of antigenic MHC-peptides from VV proteins should decrease the induction of VV specific CD8+ T cell response. The removal of immunoprevalent and immunodominant competing determinants should lead to an increased frequency of CD8+ T cells specific for subdominant determinants [267;282;283]. Second, the decreased VV specific class-I presentation should also diminish the VV related cytolytic response and thereby will enhance the survival of infected APC needed for the recombinant antigen stimulation.

However, despite a successful demonstration of CD8+ T cell response to a minigene encoded epitope, we did not measure any increased immunogenicity, as compared to a control virus (without ICP47 block), in simple prime-boost-readout experiments. Enhanced priming could have been expected in view of the "hyper" saturation of a large number of HLA-A2 molecules with the over-expressed TAP independent epitope. Yet, the CTL response generated in this condition was not significantly different from the control ER-Mart rVV. We can speculate that for the limited amount of epitope-specific CD8+ T-cells to be expanded in our *in vitro* setting, the saturating amount of HLA-peptide on APC surface is already reached with the control rec.-virus despite the presence of TAP dependant competitive epitopes. Indeed, we previously published [165] that in APC infected with rVV-Mart, we reach stimulation plateau for specific CTL clones at very low multiplicity of infection (e.g: MOI 1 to 5).

Therefore, in order to be able to highlight in vitro the advantage provided by ICP47 block of vector epitopes, we investigated the differential priming for Mart epitope in conditions where VV-specific CTL driven APC clearance could become a limiting factor. In these conditions, we could indeed observe that, in presence of a 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.

Tumor specific CD4+ T cells have a critical role in helping cytotoxic CD8+ T cells to kill tumor cells [284]. It was also proposed that CD4+ T cells eliminate tumors through activation and recruitment of other effector cells, including macrophages and eosinophils [36]. In addition, defective immune response has often been attributed to lack of CD4+ T cell triggering, which would prevent the expansion of class I restricted CD8+ CTL [285;286].

Several studies suggest that cytokines, such as IFN-γ, secreted by type I (Th1) CD4+ T cells, might be involved in antitumor and antiangiogenic

activities leading to inhibition of tumor growth [287;288]. Moreover, IFN-γ was shown to be crucial for macrophage activation pathway that results also in inhibition of tumor cell growth [289].

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 preserved or even enhanced in cultures stimulated with r.VV-US12 or r.VV-Mart-US12 infected APC as compared to cultures stimulated with control virus. Our data clearly indicate that r.VV-US12 and r.VV-Mart-US12 provide efficient activation of VV antigen specific CD4+ T cells thereby enhancing their capacity to promote CD8+ T cells activation and expansion.

In addition to T cells, NK cells play a critical role in the control of viral infection [290]. NK cells autoreactivity is controlled by expression of inhibitory receptors interacting with MHC class-I molecules [291]. Studies in the human system indicate that KIR2D subfamily and CD94/NKG2a killer inhibitory receptors constitute the main receptors controlling NK cell autoreactivity [292]. KIR2D recognizes HLA-C molecules [293] and CD94/NK recognizes HLA-E molecules [294]. Because NK inhibitory receptors are engaged by membrane MHC class-I molecules, NK cells attack virally infected cells expressing abnormally low level of MHC class-I molecules on their surface [295]. Different studies of the response of isolated human NK cell clones to cells infected by HSV or HCMV were performed. Both viruses were found to induce NK cell cytotoxicity by downregulating HLA-C molecules engaged in triggering of killer inhibitory receptors. This conclusion was further substantiated by the finding that expression of viral genes, known to interfere with MHC class-I surface expression (US12 of HSV or US11 and US3 of HCMV), was sufficient to trigger the cytotoxicity of NK cell clones [296].

NK are also especially efficient in the rejection of tumors lacking MHC class-I molecules, including those with defects in the TAP protein [297]. It has

been shown that, in vivo, these tumors induce NK cell infiltration, cytotoxic activation and induction of transcription of IFN-γ in NK cells [298].

Capitalizing on these findings, we speculate that the significant MHC dowregulation following infection with ICP47 expressing rec.VV could be sufficient to mediate NK cell activation. Preliminary results indeed suggest an increased IFN-γ gene expression in co-cultures of NK cells and r.VV-US12 infected APC. This possible limitation of our novel vaccine platform should be further investigated to establish whether, if confirmed, NK activation by infected APC would lead to a decreased immunogenicity for the recombinant antigens. Nevertheless, as described above, NK cells are modulated by multiples signals and we can forsee that the co-expression of an NK-inhibitor ligand could protect ICP47-based viral vaccine.

Altogether, the results obtained with r.VV-Mart-US12 indicate that such viral vector is able to diminish viral immunodominant MHC class-I restricted antigens presentation and also to reduce rapid clearance of APCs during secondary boost due to strong antiviral response. Recombinant vaccinia virus co-expressing US12 and ER-Mart epitope confirms stable and increased immunogenicity of the recombinant epitope.

In clinical applications, this type of engineered vaccine may, at the same time, simplify the prime-boost protocols, as it would no longer require complex heterologous vaccine formulation and also allow increasing the possible number of boosts. Such vaccine strategy should elicit strong and long lasting tumor specific immune responses required to eliminate tumor burden as well as preventing delayed recurrence.

VI. CONCLUSION

Recombinant poxviruses expressing tumor associated antigens (TAAs) are currently being evaluated in clinical trials as an approach to treat several cancers. Possible resistance to recombinant viral vector is due to either prior systemic immunity to poxviruses or immunodominance of viral antigens which may reduce the induction of immune response against weaker tumor antigens. To address this issue, we developed a recombinant vaccinia virus expressing HSV type I protein ICP47. This protein downregulates MHC class-I antigen presentation by blocking the MHC encoded transporter associated with antigen processing (TAP), which translocates peptides, generated by proteasomal protein degradation, into the endoplasmic reticulum for loading onto MHC class I molecule.

Herpes simplex virus (HSV) US12 gene, coding for infected cell protein 47 (ICP47) was introduced into vaccinia virus and also into a recombinant vaccinia virus expressing MART-1/Melan-A_{27-35} as HLA-A201 ER-targeted epitope. Following infection with non-replicating recombinant virus, effect of ICP47 expression on cell surface MHC-class-I, MHC class-II and co-stimulatory molecules was characterized by antibody staining and FACS analysis. Human T-lymphocytes were stimulated in vitro with autologous CD14+ cells infected with r.VV-US12, r.VV-Mart-US12 or control virus. Proliferation of specific CD8+ and CD4+ for viral proteins and recombinant epitopes were monitored by MHC-multimer staining and interferon gamma (IFNg) expression analysis.

Recombinant vaccinia virus expressing the HSV-US12 gene confirmed a diminished class-I presentation and CD8+ recognition of native proteins while CD4+ helper-class-II stimulation is remained unaffected. Presence of ER- Mart-1/Melan-A_{26-35} minigene in r.VV-Mart-US12 construct appears to partially compensate for the surface MHC class-I molecule downregulation (due to

US12) and preserve a strong capacity to induce CTL response against the TAA epitope.

This type of ICP47 expressing viral vaccine could thereby profit from a diminished vector specific class-I recognition while conserving a strong immunogenic potential towards recombinant ER-targeted epitope. Such reagent could become of high relevance especially in multiple-boost vaccine protocol required in cancer immunotherapy.

Combined with the demonstrated potency of recombinant poxviruses expressing multiple antigens and co-stimulatory factors, we can foresee that the TAP-blocking strategy opens the way for a new generation of viral vector platform.

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