Immunogenic Capacities of Recombinant Vaccinia

Virus Expressing CD154: Effects on CTL Priming

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<div align="right">Prof. Dr. Hans-Jakob Wirz, Dekan</div>

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ABBREVIATIONS

AB, Human AB serum
APC, antigen-presenting cell
CD154rVV, recombinant Vaccinia virus encoding CD154
Ct, cycle threshold
CTL, cytotoxic T cell
DNA, desoxyribonucleic acid
EBV-BL, Epstein-Barr-virus-transformed B lymphocyte
ELISA, Enzyme Link Imuno-Sorbent Assay
FCS, fetal Calf Serum
FITC, fluorescein isothiocyanate
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GM-CSF, Granulocyte macrophage-colony stimulating factor
HLA, human leucocyte antigen
iDC, immature dendritic cell
LPS, Lipopolysaccharides
MHC, Major Histocompatibility Complexe
MOI, Multiplicity of infection
ORF, Open Reading Frame
PBMC, peripheral-blood mononuclear cell
PBS, phosphate buffer saline
PE, phycoerythrin
PHA, phytohemagglutinin
qRT-PCR, quantitative Real-Time-Polymerase Chain Reaction
RNA, ribonucleic acid
rVV, recombinant Vaccinia virus
TAA, tumor-associated antigens
VV, Vaccinia virus
WT, Wild-Type strain of Vaccinia virus

Introduction
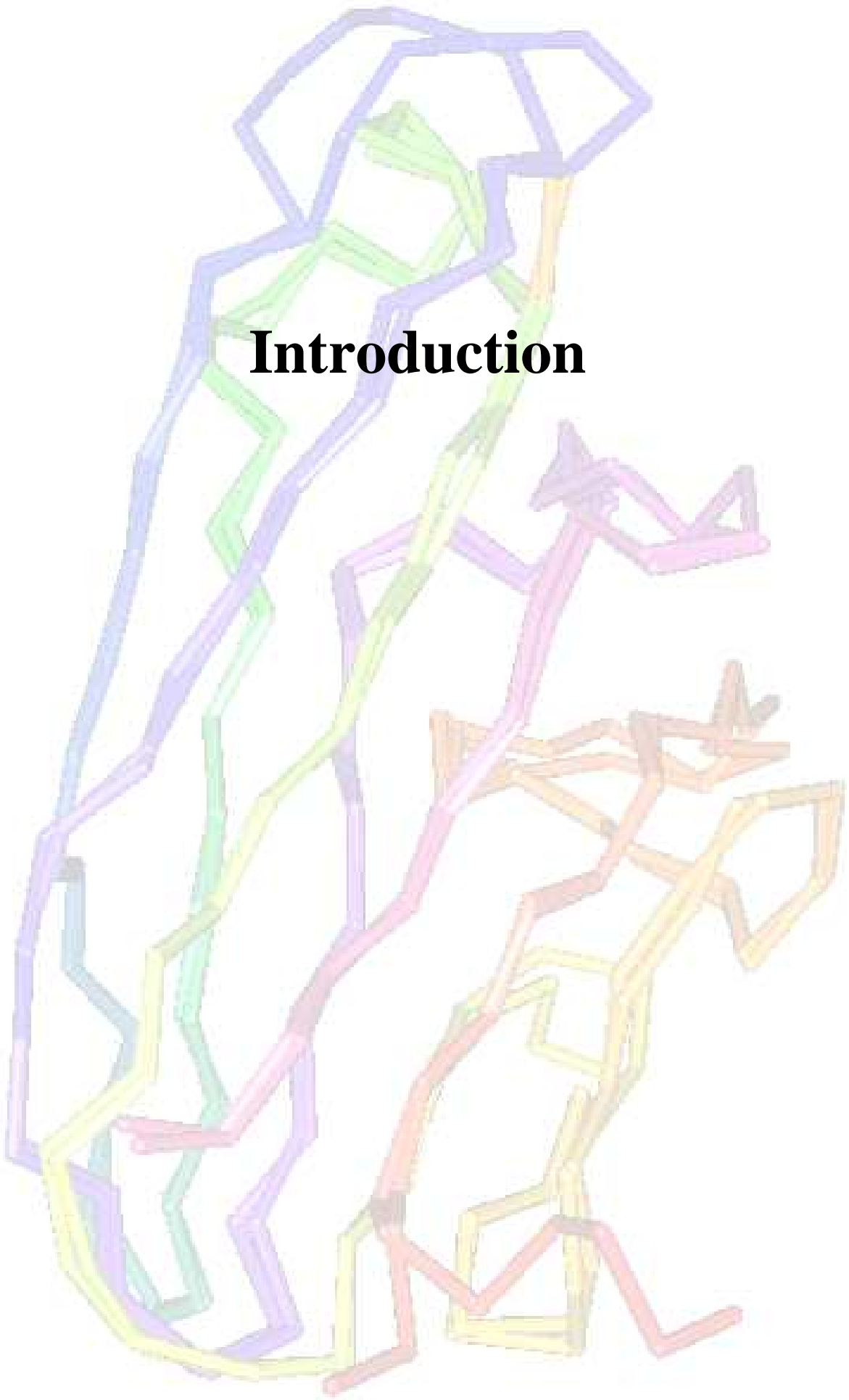
The main focus of our group is the development of an anti-cancer therapeutic approach through the generation of tumor specific CD8 cytotoxic T cell responses, using as principal vaccine vector, recombinant Vaccinia virus expressing different molecules important to generate a cellular immune response. In order to further investigate the capacity of those vectors to provide ligands for different stimulatory pathways relevant in the generation of CD8+ T cell responses, we designed a recombinant Vaccinia virus expressing CD154 which plays an important role in the activation of different help-dependent immune responses. Our aim is to evaluate the capacity of a recombinant Vaccinia virus expressing the CD154 to enhance CD8+ T cell activation, in order to have a higher Tumor Associated Antigen (TAA) specific response.

1. Cancer Immunotherapy

1.1. Melanoma as tumor model

Melanoma is a skin cancer resulting from malignant transformation of melanocytes. Melanocytes are located in the lower part of the epidermis (basal epidermal layer) of human skin and produce a pigment, the melanin, giving skin its color, whose production is increased following sun exposure. The melanomas are commonly classified depending on their anatomical location and stage of progression. This classification is also based on the presence of vertical growth, penetrating the upper and the underlying dermal layers. Primary melanomas are also detectable in districts other than skin where pigmented tissues are physiologically present, e.g. in the eye. Metastases may colonize skin and lymph nodes, or visceral sites like lung, liver, bone, brain and small intestine (Barnhill et al., 1993).

Historically the word "melanoma" was used in 1812 by a Parisian physician called René Laennec, who described a disease exhibiting a dramatic rise in appearance in Europe, with a persistent worldwide annual increase in incidence of 3-7% per year in Caucasian populations with light-colored skin (Laennec R.T.H., 1812, Extrait au mémoire de M Laennec, sur les mélanoses, Bull. Ec. Soc. Med. 24). Later in 1857, William Norris published "Eight cases of melanosis with pathological and therapeutical remarks on the disease" (Norris W., 1857, Eight cases of Melanosis with Pathological and Therapeutical Remarks on that Disease, Longman: London). But, the one who first suggested that sunlight could play a role in melanoma formation was VJ Mc Govern in 1952 (McGovern V.J., 1952, Melanoblastoma, Med. J. Aust., 139-142).

Between the early 1960s and the late 1980s, the incidence of melanoma and the corresponding trend in mortality increased at a rate of 3-7% per year in populations of European origin. Increased incidence was continuous in all age groups, although showing moderation or cessation in younger people in more recent times (Armstrong et al., 1994). This increased incidence most for the thinnest melanomas and least for the thickest, like the moderation in mortality, is likely due to earlier diagnosis and better detection. Tumor thickness is the strongest predictor of survival with an almost linear correlation to the risk of death for tumor thickness up to 6 mm with no further increase in mortality for higher levels (Garbe et al., 1992).

The usual risk factors for melanoma are represented by a blue eyes phenotype combined or not with blond or red hair, the presence of freckles on the skin and also the inability to tan, sun sensitivity, light complexion, and personal history of non-melanoma cutaneous cancer (Evans et al., 1988). Another well known risk factor is the number of nevi on the skin, directly correlated with the risk of malignant transformation (Garbe et al., 1994).

In particular, nevi need to be diagnosed following a precise classification taking into account asymmetry (not round or oval form), fuzzy margin, presence of different color in a same mole like brown, gray, black, red and blue, a size bigger than 5 mm or a growing nevus, and the convexness of the palpable mole.

However, the most critical process increasing the risk of developing melanoma is represented by intermittent and intense sun exposures for example during annual holidays in sunny areas with consequent sunburns in a high-risk phenotype, especially in childhood where UV radiation should be reduced (Langley et al., 1997). On the other hand, outdoor activities in childhood should be encouraged because they are associated with a lower risk of melanoma formation (Kaskel et al., 2001).

The genetic background underlying susceptibility to melanoma was also addressed. A recent study demonstrated that individuals living in Australia carrying a germ line mutation in CDKN2A gene, encoding the key melanoma suppressor protein p16INK4A, have a higher risk for melanoma. Mutated p16INK4A seems to be the most common cause of inherited susceptibility to melanoma (Bishop et al., 2002; Hussussian et al., 1994; Kamb et al., 1994).

Because the main factor implicated in malignant transformation is an excessive exposure to ultraviolet radiation among Caucasians (MacKie, 1998), molecular events following UV radiation of skin were investigated. The UV radiation wavelength are divided in three groups: UVA, B and C. UVC (200-280 nm) is negligible in skin cancer development since it is prevented from reaching the surface of the earth by the atmospheric ozone layer that blocks UV light below approximatively 300 nm. UVA (320-400 nm) have longer wavelength and penetrate deeper into the skin than UVB, and pass through glass windows. Nevertheless, they are thousand times less effective at causing sunburns than UVB radiation. The UVB (280-320 nm) are considered to represent the most carcinogenic wave band. Indeed, nucleic acids and proteins absorb light within the UVB range, peaking at 260 nm and 280 nm respectively. This absorption of UVB by DNA causes damages that, if not repaired, can induce mutations causing skin cancer. The major UVB-induced photoproducts in DNA are the 6-4 photoproducts generated between adjacent pyrimidine residues, and pyrimidine dimers, formed specifically between adjacent thymidine (T) or Cytosine (C) residues (Figure 1). This latter damage is more carcinogenic than 6-4 photoproducts because repaired less efficiently (Matsumura et al., 2002).

a Formation of a cyclobutane pyrmidine dimer

b Formation of a (6-4) photoproduct
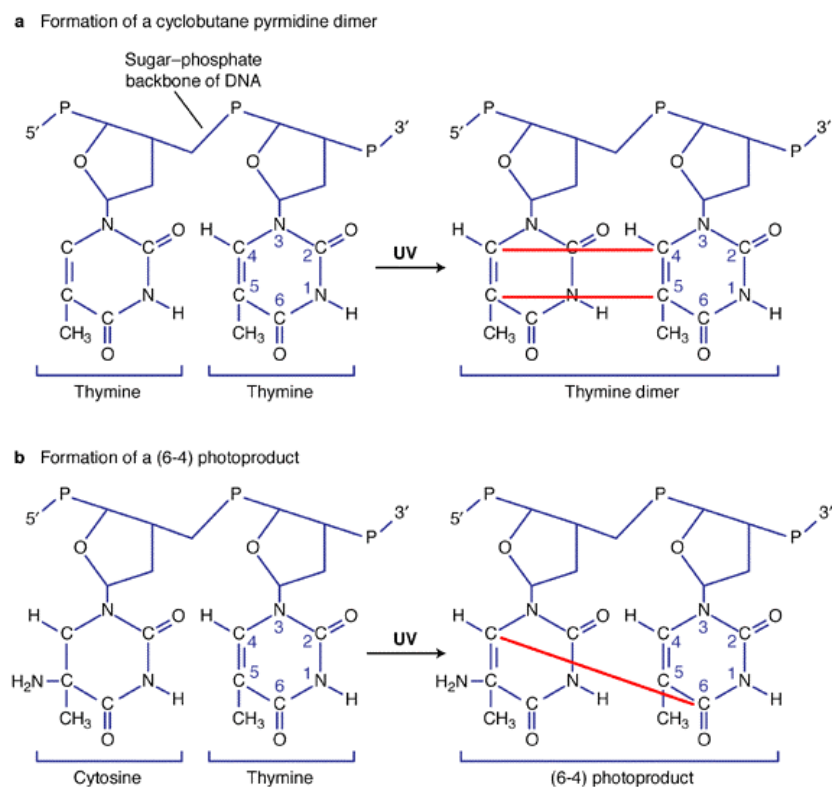
Figure 1. Structure of the major UV-induced photoproducts in DNA.
Absorbtion of UV light by DNA induces mutagenic photoproducts or lesions in DNA between adjacent pyrimidines [thymine (T), cytosine (C)] in the form of two main types of dimers. (a) Two adjacent T molecules (shown here), or an adjacent T and C residue, can be converted to a T–T or C–C cyclobutane pyrmidine dimer (CPD), respectively. The double bonds between C-4 and C-5 carbon atoms of any two adjacent pyrimidines become saturated to produce a four-membered ring. (b) In the other type of dimer, (6-4) photoproducts are formed between the 5' C-4 position and the 3' C-6 position of two adjacent pyrimidines, either between TC (shown here) or CC residues.

Treatments of melanoma is based on surgery to remove the tumor of all stages, chemotherapy, external and internal radiation therapy and biological therapy aiming at stimulating the ability of the immune system to fight cancer. Primary melanoma have excellent long-term prognosis following surgical removal. On the other hand, metastatic tumors have a severe prognosis with a median survival lower than 12 months and a 5 year survival of 5%. Thus, novel therapeutic approaches are urgently required for patients with metastatic melanoma. A promising approach may be represented by the use of agents that stimulate a tumor-specific immune response in combination with non specific immune modulators.

1.2. Immunotherapeutic approach to stimulate tumor-specific response

TAA provides the basis for specific cancer vaccines (Brasseur et al., 1992; Dudley et al., 2003; Lethe et al., 1997; Rosenberg et al., 1988; Topalian et al., 1990; Traversari et al., 1992; Vose et al., 1985; Yron et al., 1980).

Human antigens can be classified in three groups. The first group is composed by the so called differentiation antigens expressed in melanoma and normal melanocytes. Tyrosinase (Robbins et al., 1994), MART-1/Melan-A (Coulie et al., 1994), gp_{100} (Adema et al., 1993) and TRP-2 (Noppen et al., 2000) belong to this group. A second group includes several families of antigens, so called cancer/testis antigens, expressed in cancers of different histological origin and testis, for example MAGE. MAGE genes are silent in all healthy adult tissues except testis and placenta (Takahashi et al., 1995). A third group is represented by antigens specific for individual tumors, resulting from mutations, deletions or recombinations. Accordingly, the clinical relevance of these antigens is usually limited to individual patients and are consequently not the most appropriate in our purpose.

Cancer cells are by definition poor immunogens. However, it is possible to use, as vaccines, synthetic peptides whose sequences correspond to epitopes of TAA recognized by T lymphocytes. Notably, the clinical use of peptides alone is limited by their rapid proteolytic digestion, leading to limited bio-availability and poor immunogenicity. Optimal CTL activation depends on efficient antigen presentation by Major Histocompatibility Complex (MHC) molecules loaded through the class-I endogenous pathway of antigen processing (Maffei et al., 1997; Townsend et al., 1986; van Endert, 1999).

There are different signals required for T cell activation. The primary mediators of immune reactivity are T and B lymphocytes. T lymphocytes have antigen-specific receptors that recognize MHC restricted epitope derived from processed antigens. Antigen-presenting cells (APC) activate naïve T cells by presentation of the antigen within major histocompatibility complex (MHC) antigens, the primary targets for allo-recognition. This process requires binding of the antigen/MHC complex to the TCR/CD3 complex. This event initiates a cascade of signaling events that begins with the activation of several cytoplasmic protein tyrosine kinases. Recruitment of the CD4 or the CD8 associated tyrosine kinase, Lck,

into the vicinity of the TCR complex is believed to induce phosphorylation of CD3 proteins ultimately leading to downstream signal progression. However, in order not to lead to anergy, the activation of the T cells requires signaling not only through the T cell receptor (TCR; signal 1) but also through co-stimulatory pathways (signal 2). The candidate co-stimulatory molecules required for complete T-cell activation are T-cell surface molecules such as CD28, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD30, CD44 and CD154. Each has the ability to augment the T-cell proliferative response to antigenic stimuli. It is likely that each of these acts through different mechanisms, some delivering co-stimulatory signals to the T-cell, some enhancing adhesion to APC, and still others mediating homing to target tissues (Lenschow et al., 1996). After activation, a number of cell surface and soluble molecules are known to regulate further the immune response (signal 3), like for instance T helper 1 cytokines such as IL-12 which plays an important role in CTL activation (Figure 2).
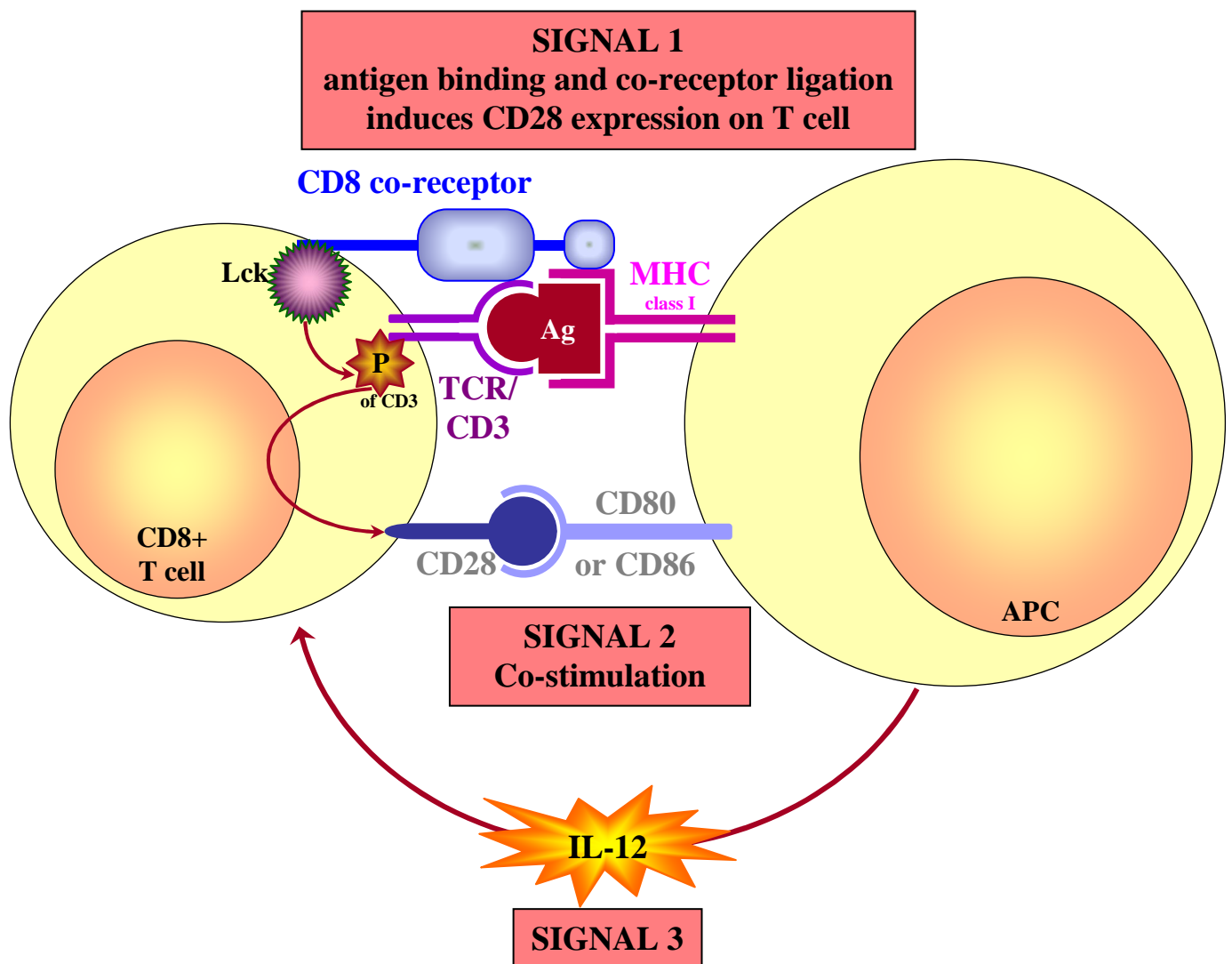
Figure 2. Requirements for T cell activation.
T-cell activation requires signaling through the T-cell receptor (TCR) (signal 1) and co-stimulatory pathways (signal 2): for instance, antigen/MHC binding to the TCR and CD28 interaction with CD80 or CD86 expressed on the surface of APC.

Even if the idea of therapeutic anti-cancer vaccination is built on the theory that the immune system can control cancer, there are still some major obstacles: heterogeneity of tumor cells leading to the possible immune escape of tumor cells by down-regulation or loss of β2-microglobulin, HLA Class I or TAA. Nevertheless, this approach is really promising and seems powerful.

2. CD40/CD154 pathway

2.1. CD40

2.1.1. CD40 discovery

CD40 was first described in 1985 as a putative 50 kD antigen associated with human urinary bladder carcinoma. This protein was also expressed on B lymphocytes and on some malignant B cells, especially in chronic lymphocytic leukemia, hairy cell leukemia and immunocytoma (Paulie et al., 1985).

2.1.2. CD40 gene

CD40 gene (11031 bases) maps to human chromosome 20q12-q13-2 (Banchereau et al., 1994) (Figure 3).
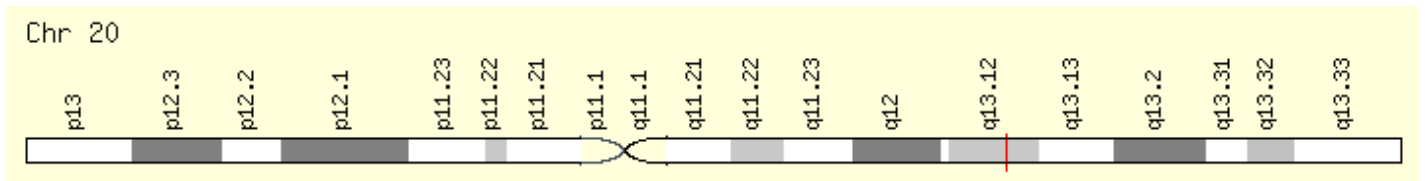
Figure 3. Chromosomal location of CD40 gene (according to GeneLoc and/or HUGO, and/or LocusLink (NCBI build 31))
CD40 geneon chromosome 20: start at 45,385,328 bp from pter, end at 45,396,915 bp from pter, size: 11,587 bases, orientation: plus strand.

CD40 expression is controlled by post-transcriptional regulation through alternative splicing and at post-translational level. The CD40 gene contains 9 exons: the first ATG is located in exon 1 and the stop codon for translation in exon 9. Five alternative CD40 isoforms were identified by analyzing expression levels of CD40 mRNA by RT-PCR: CD40 type I to V. Structure of CD40 isoforme mRNA are summarized in Figure 4 (Tone et al., 2001).

A

B

Figure 4. Structures of CD40 mRNAs isoforms.
(A) CD40 mRNA comprises sequences included in exons 1-9. Position of the first ATG and the stop codon for translation of CD40 are indicated.
(B) Five alternative CD40 isoforms were identified by analysing expression levels of CD40 mRNA by RT-PCR: CD40 type I to V. Positions of alternative splicing are indicated by thick solid lines. The alternative splice site in exon 8 (5 bp downstream of the 5' end of the exon 8) is indicated by dotted line. Position of Stop codons for translation are indicated by arrows heads.

2.1.3. CD40 protein

CD40 is a 277 amino-acids (30619 kDa) (Figure 5) glycoprotein (Armitage et al., 1992) of 45-50 kD, member of the Tumor Necrosis Factor Receptor (TNFR) superfamily, expressed on the surface of specific cell types (Banchereau et al., 1994). The five mRNA isoforms are translated into five protein isoforms (Tone et al., 2001), as shown in Figure 6. Authors suggested that type I isoform normally signals through its endodomain resulting in define IL-12p40 gene expression, as oppose to type II and IV isoforms lacking this signaling endodomain, which might operate as dominant negative inhibitors by competing for CD154.

MVRLPLQCVLWGCLLTAVHPEPPTACREKQYLINSQCCSLCQPGQKLVSDCTEFTETECL
PCGESEFLDTWNRETHCHQHKYCDPNLGLRVQQKGTSETDTICTCEEGWHCTSEACESCV
LHRSCSPGFGVKQIATGVSDTICEPCPVGFFSNVSSAFEKCHPWTSCETKDLVVQQAGTN
KTDVVCGPQDRLRALVVIPIIFGILFAILLVLVFIKKVAKKPTNKAPHPKQEPQEINFPD
DLPGSNTAAPVQETLHGCQPVTQEDGKESRISVQERQ

Figure 5. CD40 sequence (according FASTA data base).
CD40 is a 277 amino-acids (30619 kDa) protein glycosylated member of the Tumor Necrosis Factor Receptor superfamily.
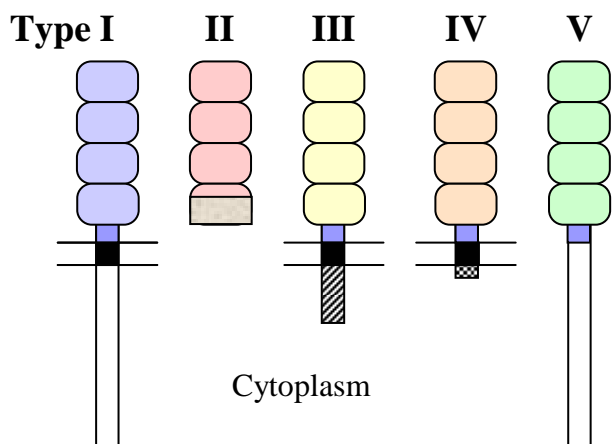
Figure 6. Structures of CD40 isoforms. Structure presumed from amino acid sequences

We now know that CD40 is a surface molecule expressed not only on B lymphocytes and on some carcinomas cell lines, but also on some epithelial cells (Armitage et al., 1992), dendritic cells (Moodycliffe et al., 2000), follicular dendritic cells, hematopoietic progenitor cells (Banchereau et al., 1994), vascular endothelial cells, fibroblasts and most of all, monocytes and macrophages (Maruo et al., 1997; Stout et al., 1996b; Stout et al., 1996a). CD40 expression is reviewed in the Table 1 (Grewal et al., 1998; Schonbeck et al., 2001a).

Cell type	Human	Mouse
B cells	+	+
Dendritic cells	+	+
Follicular dendritic cells	-	+
Monocytes	+	+
Macrophages	+	+
Thymic epithelium cells	-	+
Hematopoietic progenitor cells	+	-
Carcinomas	+	-
Endothelial cells	+	+
Mast cells	+	+
Fibroblasts	- or +	+
Smooth muscle cells	+	-
Eosinophils	+	
Basophils	+	
Epithelial cells	+	
Keratinocytes	+	
T cells (CD4$^+$, CD8$^+$, CD4$^+$/8$^+$, TCR$^+$)	+	

Table 1. CD40 expression

Like other receptors of the TNFR superfamily, CD40 presumably acts as a trimer receptor. Indeed, it has been demonstrated that, upon CD154 binding on its extracellular domain, CD40 forms at least a trimeric complex that is the minimal multimeric form sufficient to activate NF-$_\kappa$B (Ni et al., 2000; Pullen et al., 1999).

CD40-initiated signals may result in multifaceted physiologic outcomes. In particular, this receptor activates signal transduction pathways other than NF-$_\kappa$B, like JAK/STAT, JunNterminal Kinase and Janus kinase. Indeed, Jak3, which is constitutively associated with CD40 by a proline-rich sequence in the membrane-proximal region of CD40, is activated by CD40 ligation and plays an important role in CD40-mediated function. This activation occurs through protein tyrosine phosphorylation (which is necessary for Jak3 as well as STAT3 activation), even if CD40 lacks in intrinsic tyrosinase kinase activity (Hanissian et al., 1997; Leo et al., 1999).

The cytoplasmic region of CD40 bears two major signaling domains (Hostager et al., 1996; Inui et al., 1990). Accumulating evidence suggests that CD40 signaling requires the association of either or both domains with binding proteins TRAFs (TNFR-associated factors). The TRAF family consists of six known members, of which TRAF1, TRAF2, TRAF3 and TRAF6 directly, and TRAF5 probably via TRAF3/TRAF5 hetero-oligomers, associated with CD40 (Schonbeck et al., 2000; Schonbeck et al., 2001b). Stability of TRAF3 may regulate the CD40-mediated activation of NF-$_\kappa$B, suggesting proteolysis of TRAF3 as a requirement for the CD40 mediated activation of this transcription factor (Annunziata et al., 2000). Signaling pathway is summarized in figure 7 (Xu et al., 2004).
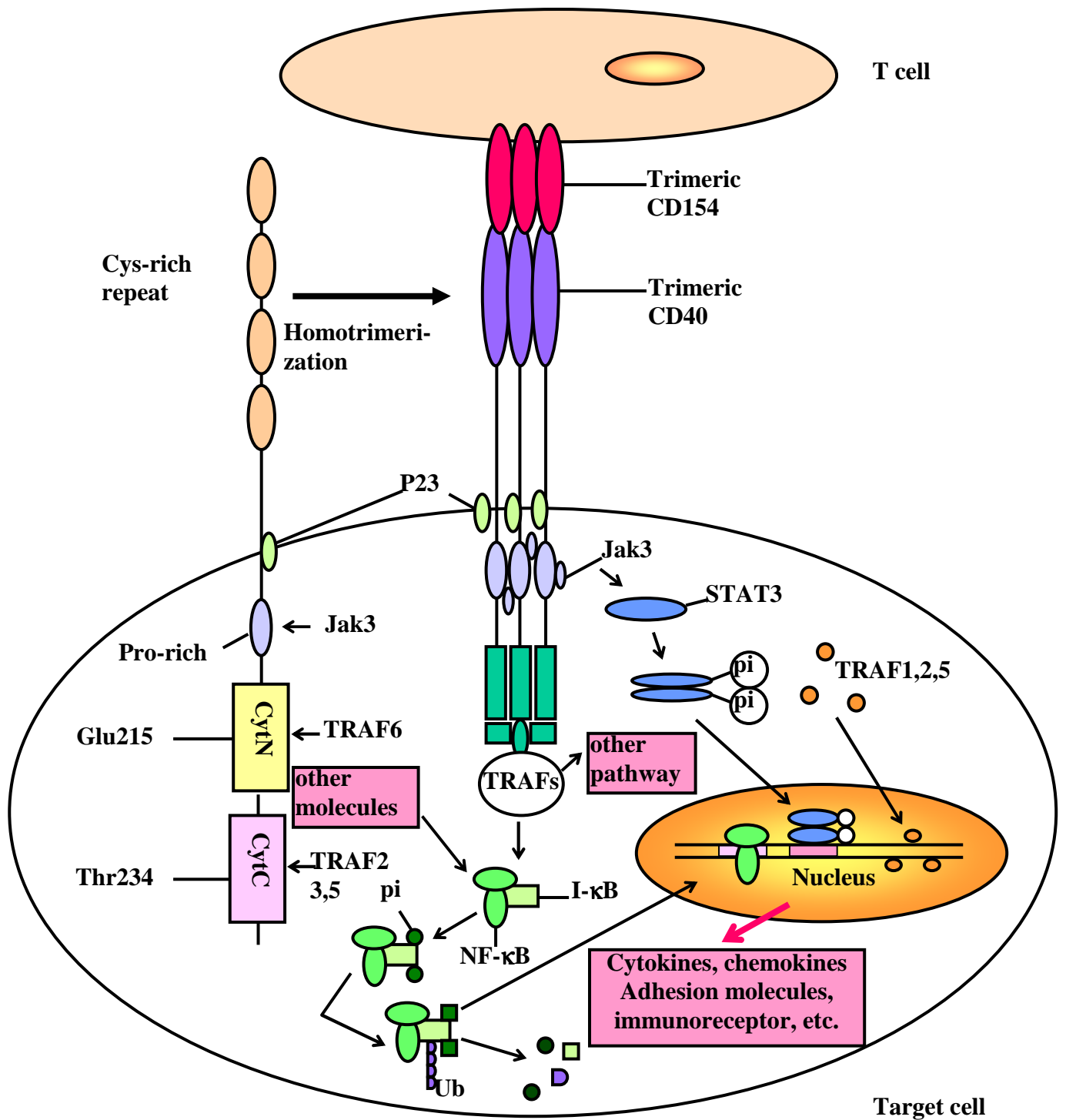
Figure 7. The role of CD40/CD154 interaction in cell immunoregulation.
Once interacted with CD154, CD40 activates a secondary messenger by JAK/STAT pathway either by recruiting TRAFs which can activate the NF-$_\kappa$B pathway or even by other unknown pathways.

2.2. CD154

2.2.1. CD154 discovery

The CD154, also called CD40 ligand, gp39 or TRAP, was isolated and characterized in 1992 simultaneously by three different groups, as a ligand for CD40.

A first group cloned a murine ligand for CD40 expressed on the cell surface of activated T cells and mediating B cell proliferation in the absence of co-stimulus as well as IgE production in the presence of IL-4 (Armitage et al., 1992). A second group identified a cell surface protein of approximately 39 kDalton expressed by activated T cells, a type II membrane protein with homology to Tumor Necrosis Factor (TNF) binding CD40 (Hollenbaugh et al., 1992).

A third group cloned TRAP (TNF-related activation protein), a ligand for CD40 expressed on human T cells: a cDNA clone was isolated from a collection of T cell activation genes, and the structure features predicted a type II transmembrane protein also compatible with a secreted form. Human TRAP was highly similar to a murine CD40 ligand (82.8% cDNA identity and 77.4% protein identity between the murine CD40 ligand and the human TRAP) and bound a soluble CD40 construct (Graf et al., 1992).

2.2.2. CD154 gene

CD154 gene (12180 bases) maps in the q26.3-q27.1 region of the X chromosome (Figure 8) (Banchereau et al., 1994; Graf et al., 1992). Interestingly, since this gene contains a potential NF-κB binding site within its promoter at positions –1190 to –1181, regulation of its expression in primary human T cells is under NF-κB control (Srahna et al., 2001).
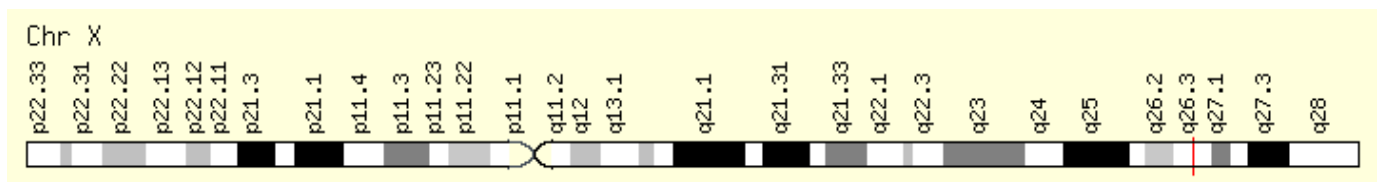
Figure 8. Chromosomal location of CD154 gene (according to GeneLoc and/or HUGO, and/or LocusLink (NCBI build 33))
CD154 gene on chromosome X: start at 133,675,149 bp from pter, end at 133,687,329 bp from pter, size: 12,180 bases, orientation: plus strand

Human CD154 sequence is represented by a 1.8 kb mRNA containing an ORF coding for 261 amino-acids highly homologous to the mouse CD154 cDNA coding region. The 3' non-coding region of the human CD154 mRNA contains AUUU repeats as well as a long stretch of 33 CA repeats (Gauchat et al., 1993), confering instability to mRNA (Shaw et al., 1986).

This mRNA instability is comparable to that of IL-2 mRNA (Ford et al., 1999; Rigby et al., 1999). p25, a protein capable of binding the CD154 3'UTR contributes to CD154 mRNA instability. Another protein, p50, binding to a distinct site in CD154 3'UTR, is also involved in CD154 mRNA turnover. These proteins contact directly U and/or C in the conserved region (nucleotides 293 to 986) of the human CD154 3'UTR.

CD154 mRNA can be stabilized by PMA, which modulates the binding activities of p25 and p50, or by prolonged T cell activation. Decay of CD154 mRNA throughout T cell activation is uncoupled from T cell costimulation by CD28 signaling.

Recently in 2003, regulation of CD154 mRNA stability by polypyrimidine tract binding protein (PTB) was exposed (Figure 9) (Hamilton et al., 2003). Indeed, CD154 3'UTR contains a novel cis-acting element whose function is determinate by the binding of PTB and PTB-T, an alternatively spliced PTB isoform. Importantly, p25 and p40 CD154 3'UTR binding proteins are related to PTB.

All these mecanisms are excluded in our construct as UTR regions are not present. This confers to CD154 mRNA expressed by recombinant Vaccinia virus more stability, resulting in more protein translation.
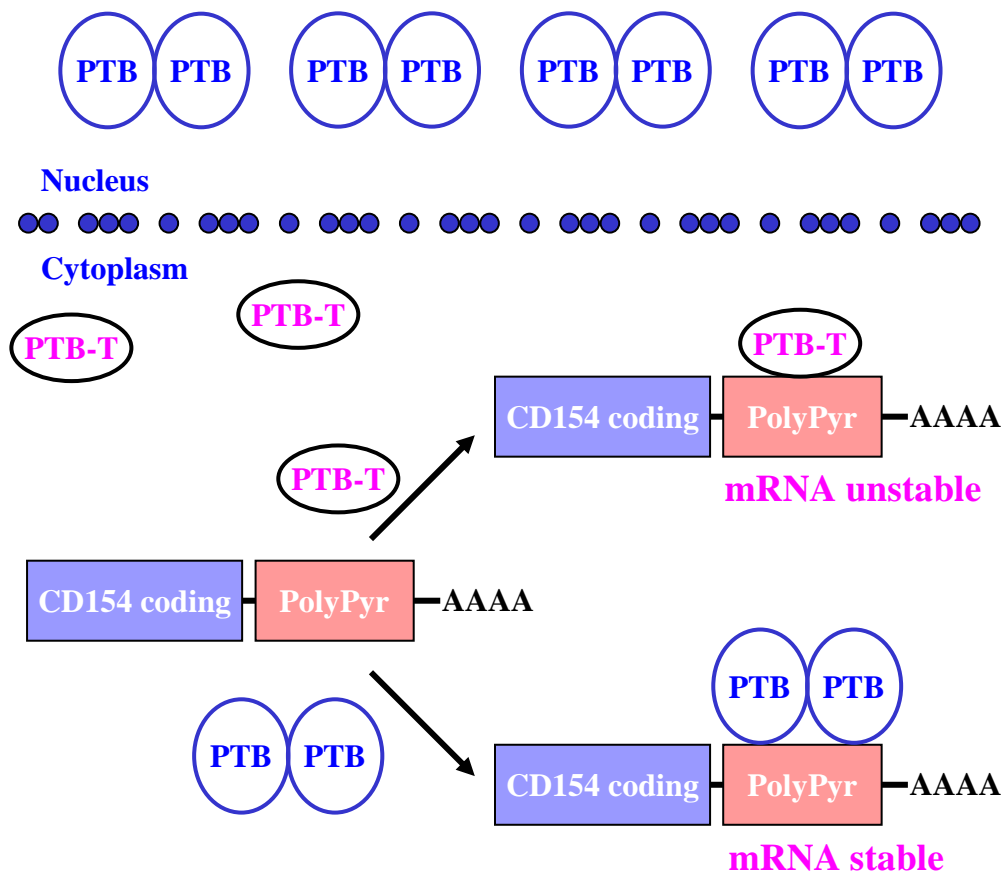
Figure 9. Model of CD154 mRNA stability regulation by PTB and PTB-T binding to the polypyrimidine-rich region in the 3' UTR.
PTB-T and PTB compete for binding to CD154 3'UTR and determine changes in mRNA stability. PTB is shown as a dimeric molecule that is primarily nuclear. PTB-T, which lack both the nuclear localization sequence and dimerization domain of PTB, is shown to be predominantly cytoplasmic and monomeric.

2.2.3. CD154 protein

Human CD154 is a 261 amino-acids (29273 kD) type II membrane protein glycosylated (Gauchat et al., 1993) to an approximate MW of 33 to 39 kD. CD154 is expressed by activated T cells (Hollenbaugh et al., 1992) (Figure 10) and belongs to the TNF superfamily (Banchereau et al., 1994), showing 23.4% homology with TNF-α and 20.7% with TNF-β. Human CD154 has 5 cysteines as opposed to 4 cysteines for the mouse CD154. It consists of 22-residues intracellular N terminal domain, a short transmembrane segment, a relatively long, 65-residue extracellular 'stalk' and a globular TNF-like extracellular domain of about 150 residues at the C-terminal end (Karpusas et al., 1995). The transmembrane segment deduced from the hydrophobicity plot is followed by a protease cleavage site, which can represent a signal domain.

MIETYNQTSPRSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHRRLDKIEDERNLH
EDFVFMKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDIMLNKEETKKENSFEMQKGDQNP
QIAAHVISEASSKTTSVLQWAEKGYYTMSNNLVTLENGKQLTVKRQGLYYIYAQVTFCSN
REASSQAPFIASLCLKSPGRFERILLRAANTHSSAKPCGQQSIHLGGVFELQPGASVFVN
VTDPSQVSHGTGFTSFGLLKL

Figure 10. CD154 sequence (according FASTA data base).
CD154 is a 261 amino-acids (29273 kDa) glycosylated member of the Tumor Necrosis Factor superfamily.

Crystal structure of a soluble extracellular fragment of human CD154 was determinated in 1995 (Karpusas et al., 1995) (Figure 11). The CD40 binding site consists of a groove formed between the monomers and a mixture of both hydrophobic and hydrophilic residues on the surface of the binding site. Structural domains of CD154 implicate multiple functions (Andre et al., 2002a), for instance, by the presence of a Lysine-Arginine-Glutamic acid (KGD) motif.
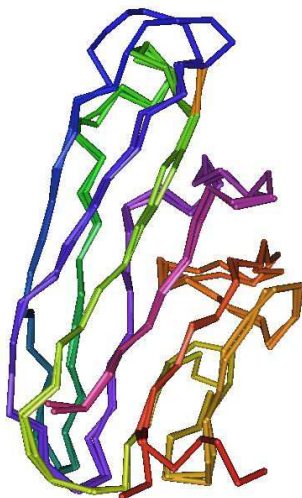
Figure 11. Crystal structure of a soluble extracellular fragment of human CD154 to 2 Å resolution.
CD154 folds as a sandwich of two β sheets with jellyroll or Grey key topology. Molecule dimensions: 25å x 30å x 50å. The overall fold is similar to that of TNF-α and lymphotoxin-α.

Interestingly, it seems that CD154 molecules form trimers similar to those observed in the crystal structure of TNF-α and LT-α. Moreover, clustering of CD154 was demonstrated to be required for functional contact with CD40. CD154 clustering is mediated by an association of the ligand with p53, a translocation of acid sphingomyelinase to the cell membrane, and activation of the acid sphingomyelinase. The ensuing production of ceramide appears to promote the formation of ceramid-enriched signaling platforms that serve to cluster the CD154. Finally, it was demonstrated that CD40 clustering depends on reciprocal clustering of CD154 (Figure 12) (Grassme et al., 2002).
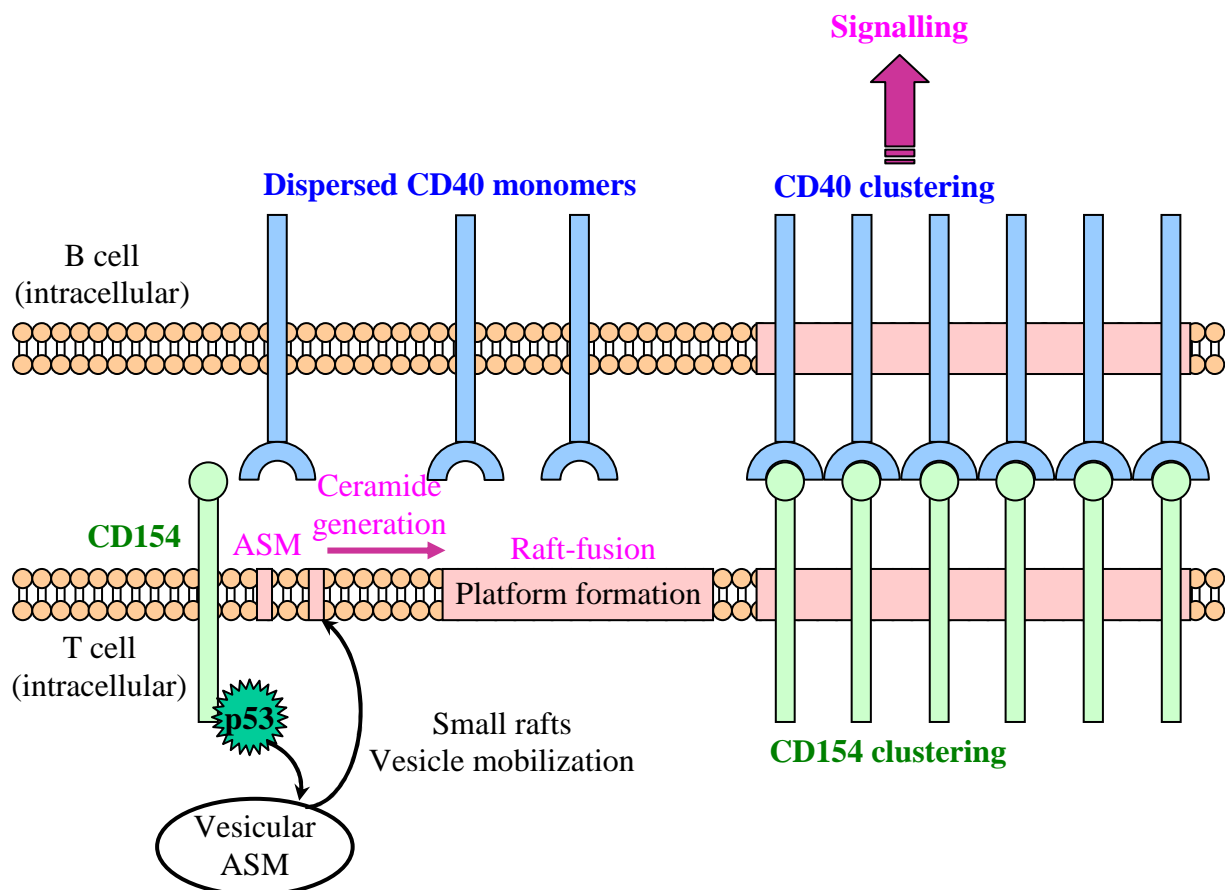
Figure 12. Clustering of CD154 is required for the clustering of CD40.
CD154 clustering is mediated by an association with p53, a translocation of acid sphingomyelinase (ASM) to the cell membran, and activation of ASM and the formation of ceramids. Ceramides appear to modify preexisting sphingolipid-rich membrane microdomains to fuse and form ceramid-enriched signaling platforms that serve to cluster the CD154, which functions as a prerequisite for CD40 clustering and, finally, CD40-dependent signaling (Grassme et al., 2002).

CD154 is mostly expressed on CD4+ T cells but also on some CD8+ T cells as well as basophils and mast cells (Banchereau et al., 1994). CD154 is also expressed on many other cell types reviewed in the table 2 (Grewal et al., 1998; Schonbeck et al., 2001a). Following T cell receptor triggering of CD4+ T cells *in vitro*, CD154 surface expression peeks at 8 hours following activation. Subsequently, the surface expression of CD154 is rapidly down-regulated and is undetectable by 24 hours following stimulation (Yellin et al., 1994b).

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Cell type	Human	Mouse
Activated CD4+ T cells from spleen and blood	+	+
Activated Th1 and Th2 clones	-	+
Antigen primed lymph node cells	-	+
PMA activated CD8+ T cells	+	-
CD8+ T cell clones	-	+
γδ TCR+ T cell clones	-	+
NK cells	-	+
Monocytes	+	+
Macrophages	+	
Fetal thymocytes	-	+
Small intestine	-	+
basophiles	+	-
Mast cells	+	-
Eosinophiles	+	-
Activated dendritic cells	+	-
Endothelial cells	+	
Smooth muscle cells	+	
Platelets	+	
B cells and B cell lines (probably not physiological)	+	-

Table 2. CD154 expression

2.2.4. Soluble form of CD154: sCD40L

CD154 also exists in a soluble form (sCD40L). CD154 is cryptic in unstimulated platelets but is expressed on the platelet surface within seconds after *in vitro* activation and during the process of thrombus formation *in vivo* (Henn et al., 1998). CD154 is then cleaved over a period of minutes to hours, generating a soluble fragment (sCD40L), released into the circulation (Aukrust et al., 1999) that remains trimeric (Andre et al., 2002a). sCD40L has the same tumor necrosis factor homology domain allowing binding to CD40 and the same

Lysine-Arginine-Glutamic acid (KGD) motif, which remains part of the sCD40L cleavage product (Andre et al., 2002b).

Its physiological role is, like TNF-α and IL-1, to induce endothelial cells to secrete chemokines and to express adhesion molecules, thereby generating signals for the recruitment and extravasation of leukocytes at the site of injury (Henn et al., 1998). The sCD40L released from platelets during thrombosis plays a key role in different steps of atherosclerotic lesion progression by inducing the production and release of pro-inflammatory cytokines from vascular cells and matrix metalloproteinases from resident cells in the atheroma. In thrombosis, sCD40L stabilizes platelet-rich thrombi, and in restenosis, it inhibits the re-endothelialization of the injured vessel potentially leading to the activation and proliferation of smooth muscle cells. Studies on the cellular distribution of CD154 estimated that over 95% of sCD40L derives from platelets. This suggests that platelet stimulatory events must be considered in the biological and pathological context of CD154 function (Andre et al., 2002a).

2.2.5. CD154 deficiency: clinical consequences

Because interaction between CD40 and CD154 on activated T cells is critical for T-cell-driven isotype switching (Fuleihan et al., 1993), CD154 involvement in X-linked hyper-IgM syndrome was examined. This X chromosome-linked immunodeficiency is a rare inherited disorder (Hendriks et al., 1990) characterized by profound susceptibility to bacterial infections, increased susceptibility to opportunistic infections, very low or absent IgG, IgA and IgE, and normal to increased IgM and IgD serum levels (Callard et al., 1993; Korthauer et al., 1993; Ramesh et al., 1994). Pathologically, lymphoid tissues show disorganization of the follicular architecture and PAS positive plasmacytoid cells containing IgM. Lymph nodes lack germinal centers (Ramesh N, Geha RS, Notarangelo LD; New York: Oxford University Press, 1999, 233-40). Importantly, naturally occurring mutations of CD154 resulting in defect in CD154, induce a clinical severe immunodeficiency known as hyper-IgM syndrome or HIGM. Indeed, defective expression of the CD154 induces the failure of CD154 to interact with CD40 on functionally intact B cells, and this abnormality provides the molecular basis for immunoglobulin isotype switch defects observed in this disease (DiSanto et al., 1993; Fuleihan et al., 1993; Korthauer et al., 1993).

Multiple lines of evidence support the view that, both CD154 on platelets and sCD40L are raised in patients with acute coronary syndromes (Garlichs et al., 2001; Heeschen et al., 2003; Varo et al., 2003a), unstable angina (Aukrust et al., 1999) and myocardial infection (Garlichs et al., 2001; Schonbeck et al., 2001c; Varo et al., 2003a) as compared to patients with stable angina and controls. Patients with moderate hypercholesterolemia, a risk factor for cardiovascular disease associated with inflammation and hypercoagulability, show a significant increase of CD154 on platelets and in plasma or serum (Cipollone et al., 2002; Semb et al., 2003) and statin therapy was found to markedly decrease sCD40L levels (Cipollone et al., 2002). Cardiopulmonary bypass, inducing platelet activation and systemic inflammatory responses, causes an increase in plasma concentration of sCD40L, possibly contributing to thrombotic and inflammatory complications (Nannizzi-Alaimo et al., 2002). In addition, high levels of plasma sCD40L indicate a proinflammatory status in individuals with type 1 or 2 diabetes (Varo et al., 2003b), and blockage of sCD40L is known to prevent autoimmune diabetes (Balasa et al., 1997; Homann et al., 2002). Development of acute cerebral ischemia can also be mediated by the CD40 system. Indeed, patients with acute cerebral ischemia show upregulation of the sCD40L compared with controls (Garlichs et al., 2003). After adjustment for other risk predictors, sCD40L level could be used as a predictive factor in cardiovascular diseases, diabetes and stroke.

2.3. CD40/CD154 interactions (Three-dimensional structure of a CD40/CD154 interaction (McWhirter et al., 1999) is illustrated in Figure 13)
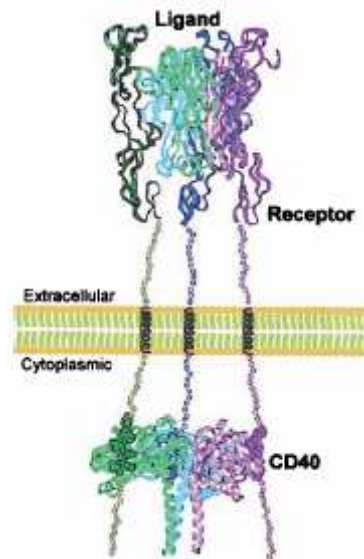
Figure 13. Cristallographic representation of a CD40-CD154 interaction
CD154 – promoted trimerization positions the CD40 intracellular domains to match the spacing between the receptor binding sites on the CD40 trimer.

2.3.1. Regulation of APC activity

Contact-dependent interactions between T cells and Antigen Presenting Cells (APCs) providing bi-directional stimulatory signals are required for the initiation of successful T cell mediated immune responses. Based on the common model of T-cell activation (Janeway, Jr. et al., 1994), once MHC antigen complexes are presented by APCs to naïve T cells, the first antigen signal is delivered via the T-cell receptor/CD3 complex. In order not to lead to anergy (state in which T lymphocytes are alive but incapable of proliferating or transcribing IL-2 gene in response to antigenic stimulation provided by APCs), but to T cell activation, this first signal must be followed by a second one passing through costimulatory molecules. Indeed, once primed, APCs express costimulatory molecules like for instance B7.1 (CD80) and B7.2 (CD86), providing CD28 mediated costimulatory signals required for full T cells activation (Figure 2).

Several studies have demonstrated that CD40/CD154 interaction was important in the induction of costimulatory activity on DCs and macrophages (Grewal et al., 1995; Grewal et al., 1996a). CD154 produces a prosurvival signal in DCs, and upregulates costimulatory molecule expression (CD80, CD86) enhancing antigen presentation by DCs. In turn, this interaction stimulates CD154+ helper and cytotoxic T cells by upregulating their IL-2 receptor expression (Roy et al., 1995; Sin et al., 2001). Following CD154 activation, the activated DCs promote cell-mediated immunity by an increased production of TNF-α and IL-12 activating neighboring DCs and T cells (Bleharski et al., 2001).

Similarly, CD40/CD154 interactions induce also dendritic cells to secrete an important set of cytokines and chemokines including also IL-8 and macrophage inflammatory protein-1 alpha (MIP-1α) (Dubois et al., 1998). Nevertheless, the major interest in our context remains the effect of CD40 ligation on IL-12 secretion: IL-12 itself up-regulates CD154 expression on T cells and synergizes with IL-2 and with other co-stimulatory interactions including CD80 and CD86, to maximize CD154 expression (Peng et al., 1998), IL-12 is a chemotactic molecule for macrophages (Ha et al., 1999), enhances CD8+ T cell homeostatic expansion (Kieper et al., 2001), and, in the presence of antigen, acts directly on the naïve CD8+ T cells to promote clonal expansion and differentiation (Curtsinger et al., 1999), rendering IL-12 promising adjuvant for cancer vaccination (Portielje et al., 2003). Similarly, IL-15 expressed by DC, monocytes and macrophages (Fehniger et al., 2001; Jonuleit et al., 1997), particularly after CD40 ligation on DC (Kuniyoshi et al., 1999) can be of major interest in our purpose: indeed, IL-15 is a potent chemoattractant for T cells (Wilkinson et al., 1995), promotes long-term survival of anti-tumor cytotoxic T lymphocytes (Lu et al., 2002), and stimulates proliferation of human memory T cells. In the same way, GM-CSF secreted by monocytes and macrophages is induced by CD40 ligation (Aldinucci et al., 2002) and is crucial for DC development (Inaba et al., 1992).

One major issue of CD40/CD154 interactions is the potential effect on apoptosis. Indeed, CD40 ligation inhibits spontaneous DC apoptosis (Ludewig et al., 1995), but also Fas/CD95-mediated apoptosis (Koppi et al., 1997).

DC culture on CD154-transfected fibroblastic cells up-regulates the expression of *bcl-2* and, concomitantly, renders them virtually resistant to Fas-induced apoptosis. This suggests that upon encountering antigen-specific T cells, DC may become resistant to Fas-induced apoptosis (Bjorck et al., 1997).

Finally, CD154 was demonstrated to inhibit tumor growth in a colon carcinoma model, but most of all, over-expression of CD154 on these tumor cells *in vitro* significantly decreased the level of tumor-induced DC apoptosis in a IL-12 independent manner (Esche et al., 1999).

2.3.2. CD40/CD154 and B cells activation

CD154 expressed on activated helper T cell membranes induces a number of B cell activation events. For instance, soluble CD40 inhibits T-dependant B cell proliferation (Lane et al., 1992), and a soluble fusion protein of CD40 and human immunoglobulin, CD40-Ig, inhibits entry of B cells into cell cycle, proliferation, and differentiation by activated Th1 and Th2, indicating that CD154 membrane protein expressed on activated helper T cells transduces T helper dependent B cell activation (Noelle et al., 1992). B cell proliferation induced by CD40 synergizes with IL-4 receptor signaling (Brines et al., 1993). Nevertheless, CD40 ligation is necessary but not sufficient for the delivery of T help to B cells (Poudrier et al., 1994), meaning that B cells require a second signal besides CD40/CD154 to drive proliferation. By contrast, sCD40L alone is able to provide co-stimulatory signals to B cells (Hollenbaugh et al., 1992).

CD40 signaling implication in B cell activation is also demonstrated by Lymphotoxin-α (LT-α) expression in CD40 activated B cells. LT-α is a member of the TNF superfamily playing a role in CD40 mediated B cell activation (Worm et al., 1994).

On the other hand, in particular situations, B lymphocytes can play an important role in antigen presentation (Constant et al., 1995; Kupfer et al., 1987; Rivera et al., 2001). Induction of optimal levels of T cell priming to an antigen requires the involvement of Ag-specific B cells, in particular through the interaction of CD154 expressed on activated CD4+ T cells with CD40 on the surface of naïve B cells. This interaction results in rescuing B cells

from apoptosis induced by Fas or by surface IgM cross-linking by antigen (Lederman et al., 1994; Tsubata et al., 1993; Valentine et al., 1992). Then, it up-regulates the co-stimulatory molecules CD80 and CD86 interacting with CD28 and CTLA-4 on the surface of activate T cells (Kaneko et al., 1996; Ranheim et al., 1994; Yellin et al., 1994a), providing more effective T cell costimulation (Ranheim et al., 1993). Finally, this interaction also increases the expression of other cell surface activation molecules like for instance CD23, CD54, CD95, and of adhesion molecules optimizing the effectiveness of B cell-T cell interaction. Indeed, signaling through CD40 on B cell surface, activates the CD1a/CD18 (LFA-1) intracellular adhesion system in B cells (Barrett et al., 1991). Furthermore, it induces LT-α secretion and promotes B cell growth and differentiation to plasma cells (Hanissian et al., 1997; Ranheim et al., 1993; Schattner et al., 1995; Worm et al., 1994).

2.3.3. CD40/CD154: Induction of Immuno-modulatory molecules

Interleukin-6 (IL-6) is the major cytokine mediating antigen-driven B cell differentiation. CD154 may trigger IL-6 secretion by monocytes resulting in increased Ig secretion (Urashima et al., 1996). This effect is accompanied by an antibody isotype switching, as described above. Similarly, CD40 ligation induces TNF-α secretion that stimulates IgM (Hostager et al., 2002) and favors IL-10 secretion (Rogers et al., 2003).

On the other hand, stimulation of CD40 with purified soluble CD154 also induces pro-inflammatory responses in human monocytes, including secretion of IL-1β and IL-8. This suggests that ligation of CD40 on human monocytes may enhance or prolong inflammatory responses. This concept is reinforced by a study in which monocyte stimulation with a soluble murine CD8/human CD154 fusion protein increases the expression of cell-surface proteins including CD54, MHC class II, CD86 and CD40, demonstrating once again that CD40 plays a crucial role in the regulation of monocyte function, and in promoting inflammatory responses (Kiener et al., 1995).

Ligation of CD40 on endothelial cells and smooth muscle cells induces the expression of leukocyte adhesion molecules such as vascular cell adhesion molecule-1 VCAM-1, E-Selectin, and intracellular adhesion molecules ICAM-1 (Hollenbaugh et al., 1995; Karmann et al., 1995; Yellin et al., 1995), whereas CD40 ligation on macrophages triggers LFA-1 and also ICAM-1 expression (Kiener et al., 1995). In addition, ligation of CD40 on endothelial cells, smooth muscle cells, macrophages and T lymphocytes triggers expression and release of chemoattractants such as IL-8 (Simonini et al., 2000), MIP-1α (Macrophage Inflammatory Protein), MIP-1β, RANTES (Regulated on Activation, Normal T cell Expressed and Secreted), SDF-1 (Stromal cell-Derived Factor 1), and MCP-1 (Monocyte Chemotactic Protein) (Abi-Younes et al., 2000; Denger et al., 1999; Kornbluth et al., 1998; Nanki et al., 2000; Thienel et al., 1999). These chemokines probably attract and direct T lymphocytes and macrophages, thus sustaining inflammation (Schonbeck et al., 2001).

More important in our context, CD40/CD154 interactions, in combination with IFN-γ, foster Th1 immune response. Indeed, CD40 signaling appears to suffice for the induction of Th1-dominated, and the suppression of Th2-dominated, immune responses *in vitro* and *in vivo* (Ruedl et al., 2000). This function might involve suppression of IL-4 and induction of IL-12p40 expression by endothelial cells, smooth muscle cells and macrophages (Lienenluke et al., 2000; Mosca et al., 2000). IL-12p40, dimerized with the IL-12p35 subunit, induces the production of IFN-γ, a cytokine that not only directly promotes Th1 responses, but further elevates CD40 levels, suggesting a positive feedback loop. IL-12 synergizes, probably via enhanced expression of CD40 on monocytes and of IL-12 receptor on T lymphocytes, in the production of IFN-γ with IL-15, which is a potent stimulator of T-lymphocyte proliferation, thus accentuating Th1-predominant immune responses (Avice et al., 1998; Musso et al.,

1999). Notably, IL-15 also increases the expression of CD154 on T lymphocytes (Mottonen et al., 2000; Skov et al., 2000)

2.3.4. CD40/CD154: Role in development of B cell and CD8+ T cell memory

CD40/CD154 interaction is involved in the development of B cell and CD8+ T cell memory. Indeed, mice lacking either CD40 or CD154, are not able to drive IgG, IgA or IgE antibody responses, but most of all, they are unable to generate germinal centers, where memory B cells develop. That indicates that costimulation of T cells through CD154 is critical for their differentiation into cells helping in the generation of B cells memory and of a mature antibody response (Foy et al., 1994; van Essen et al., 1995).

CD40 ligation on APC leads to the secretion of IL-15, a potent chemoattractant for T cells (Wilkinson et al., 1995), which stimulates the proliferation of human memory (CD45RO+) CD4+ and CD8+ T cells (Kanegane et al., 1996). Thus, CD40/CD154 interaction may play an important role in the development of T cell memory. This hypothesis was confirmed in a study in which CD40 was capable of costimulating CD4+ T cell proliferation particularly in the CD45RO+ subset (Rogers et al., 2003). In addition, antiviral CD8+ T cell memory responses were defective in CD154-deficient mice (Borrow et al., 1996), suggesting a requirement of CD154-mediated signal in the establishment or maintenance of CTL memory.

2.3.5. CD40/CD154 interactions and T cell activation and proliferation

T cells can be modulated to different extents by different ligands, ranging from activation of signaling cascades, to cytokine secretion or target cell killing, and to T cell proliferation.

CD154 ligation on CD40 induces the up-regulation of CD80 and CD86 molecules on antigen-presenting B cells that subsequently deliver costimulatory signals necessary for T cell proliferation and differentiation (Grewal et al., 1996b; Hollander et al., 1996; Yang et al., 1996). These data opened the way for a novel strategy for the enhancement of T cell response *in vivo* based on CD40 mediated up-regulation of costimulatoty activity on APC, to fight infections and immunodeficiencies, and possibly to contrast tumor growth.

This conclusion was consolidated by the fact that signaling through CD40 on the APC can replace CD4+ T-helper cells in priming of CD8+ CTL responses (Bennett et al., 1998; Schoenberger et al., 1998). CD40/CD154 interactions are therefore vital in the delivery of T-cell help for CTL priming.

Moreover, CD28 and CD154 were shown to play distinct and complementary roles in T cell activation, which may explain why blocking CD80/CD28 and CD154/CD40 interactions have an additive effect in inhibiting T cell responses, illustrating that CD28 is critical for initiating T cell responses, whereas CD154 is required for sustained T helper 1 responses (Howland et al., 2000).

The possible roles of CD40/CD154 interaction in T-cell priming were investigated in CD154-deficient mice (Grewal et al., 1997). Lack of *in vivo* T-cell priming in CD154-deficient mice was due to the limited expansion of antigen-specific T cells. Therefore, CD40/CD154 interactions may play a rate-limiting role in antigen-specific CD4+ T-cell priming and clonal expansion *in vivo*. Many studies have also focused on the important role of CD40/CD154 interaction in CD8+ T cell priming. Moreover, blockage of CD154 by *in vivo* administration of an anti-CD154 monoclonal antibody was shown to result in a profound inhibition of CTL priming that could be overcome by CD40 triggering. On the other hand, some results suggested strong CD8+ T cell priming activation following infection of CD154-

deficient mice with lymphocytic choriomeningitis virus, suggesting that, in this case, priming of CD8+ T cells was independent of CD40/CD154 (Borrow et al., 1996; Oxenius et al., 1996; Ribas et al., 2001).

In conclusion, it is known that the *in vivo* priming of CD8+ cytotoxic T lymphocytes (CTLs) responding to virally infected cells as well as allogenic cells or tumor cells generally requires the participation of CD4+ T-helper lymphocytes. The nature of this help has been further defined and shown to involve CD154 signals. For instance, CD154 up-regulation following MHC class II/peptide triggering on CD4+ T cells, results in the expression of CD154, which interacts with CD40 on DCs, resulting in increased MHC as well as co-stimulatory molecules, CD80 and CD86 (Caux et al., 1994). These events "licences" DCs to activate CD8+ precursors by efficient cross presentation of MHC/peptide complexes (Ridge et al., 1998). Moreover, blockade of CD40/CD154 pathway inhibits CTL priming demonstrating the important role of CD40/CD154 interactions for the delivery of T-cell help during CTL priming (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998).

2.3.6. Role of CD40/CD154 in human cancer

CD40 is constitutively expressed on a wide range of cell types, but also on many type of human cancers cells, such as bladder, ovarian, colorectal, liver, lung, pancreas, prostate, cervical, breast carcinoma, acute myelomonocytic leukemia, HIV-related lymphomas, Hodgkin's disease, low-grade B-cell malignancies and high-grade B-cell leukemia and lymphomas (Xu et al., 2004). This suggests that CD40 may play an important role in the process of tumor angiogenesis, development and progression. Indeed, CD40 is strongly expressed in the tumor endothelial vasculature of renal carcinomas (Kluth et al., 1997) and Kaposi's sarcoma (Pammer et al., 1996). In addition, CD154 produced a large spectrum of growth-regulatory effects on CD40-expressing tumor cells, and in some cases, such as in Hodgkin's disease (Clodi et al., 2002), CD40 activation by CD154 binding contributes to tumor survival and resistance to chemotherapy (Fluckiger et al., 1994; Johnson et al., 1993; Kluin-Nelemans et al., 1994).

In contrast, CD40 cross-linking induced cell cycle arrest in murine and human B lymphoma cells, which was critical for the induction and maintenance of tumor dormancy (Marches et al., 1995). Similarly, ligation of CD40 on human breast cancer cells (Tong et al., 2001) was found to produce a direct growth-inhibitory effect through cell cycle blockage and/or apoptotic induction with no overt side effects on their normal counterparts.

Agonistic anti-CD40 mAbs, recombinant CD154 and CD154-transfected cells were used in experimental therapy of human cancer. Systemic *in vivo* administration of agonistic anti-CD40 mAb into tumor-bearing mice resulted in tumor eradication by CD8+ T cells (van Mierlo et al., 2002). Similarly, combination of agonistic antibody to CD40 and IL-2 induced complete regression of metastatic tumor in mice (Murphy et al., 2003), and anti-tumor immune responses derived from transgenic expression of CD154 in myeloma cells were also demonstrated (Liu et al., 2002). CD154-transduced tumor cells were used as a vaccine against B-cell lymphoma in mice model (Briones et al., 2002). These approaches were also already used in human clinical trials. For instance, clinical effects of recombinant CD154 protein were observed in solid tumors and in non-Hodgkin's lymphoma. In phase I studies, soluble recombinant CD154 was administrated subcutaneously daily for 5 days, with encouraging anti-tumor activity including a long-term complete remission (Vonderheide et al., 2001; Younes, 2001). Similarly, an adenoviral vector encoding CD154 was well tolerated, in phase I clinical trials using infusion of transduced autologous leukemia cells (Kipps et al., 2000; Wierda et al., 2000): increased number of leukemia-specific T cells as well as reduction in leukemia cell count and lymph node size was demonstrated.

Potential side effects, however, should be carefully evaluated. For instance, efficacy of agonistic CD40-reactive mAb for clinical applications was limited by proinflammatory cytokine production by CD40-activated endothelial cells (Singh et al., 2001) and patients treated with recombinant adenovirus expressing CD154 transduced leukemia cells commonly experienced Influenza-like symptoms (Kipps et al., 2000). Nevertheless, CD154-based therapy alone or in combination with other therapies may offer an effective and safe strategy for the treatment of human cancers, auto-immune diseases and other CD40/CD154-associated diseases.

3. Vaccinia virus as cancer vaccine reagent

3.1. Properties

3.1.1. Taxonomy

Vaccinia virus is the most studied virus of the Poxviridae family. Although origine/natural host are not known, Poxviridae are divided in two subfamilies: Entemopoxviridae which are insect poxviruses and Chordopoxviridae which infect vertebrates. This last subfamily is divided in eight genus. The most common of them are *Avipoxvirus* including canarypox and fowlpox, *Parapoxvirus* and *Orthopoxvirus*. This last genus contains the most known and studied Poxviridae: cowpox, monkeypox, but most of all, Variola which causes smallpox and Vaccinia, used as a vaccine against smallpox. Several strains of Vaccinia virus are existing, some are replicating (Copenhaguen, Wyeth, WR, Lister, NYCBOH), others are in contrast highly attenuated strains unable to replicate or replicating poorly in human cells (MVA, NYVAC, ALVAC, TROVAC) (ICTVdB: universal virus database of the International Committee on Taxonomy of Viruses, Table 3).

Subfamilies	Genus	Members
Chordopoxviridae (vertebrate poxviruses)	*1. Orthopoxvirus*	Buffalopox, camelpox, cowpox, monkeypox, rabbitpox, raccoon pox, tatera pox, vaccinia, variola, vole pox
	2. Parapoxvirus	Chamois contagious ecthyma, Orf pseudocowpox, stomatitis papulosa
	3. Avipoxvirus	Canary pox, fowlpox, junco pox, pigeon pox, quail pox, sparrow pox, starling pox, turkey pox
	4. Capripoxvirus	Goatpox, sheeppox, lumpy skin disease
	5. Leporipoxvirus	Hare fibroma, myxoma, rabbit (Shope) fibroma, squirrel fibroma
	6. Suipoxvirus	Swinepox
	7. Molluscipoxvirus	Molluscum contagiosum
	8. Yatapoxvirus	Tanapox, Yaba
Entemopoxviridae (insect poxviruses)	*1. A*	*Melontha melontha*
	2. B	*Amsacta melontha*
	3. C	*Chironimus luridus*

Table 3. Taxonomic structure of Vaccinia virus.
Vaccinia virus belongs to the Poxviridae family, to the Chordopoxviridae subfamily, and more precisely, to the Orthopoxvirus genus.
(ICTVdB: universal virus database of the International Committee on Taxonomy of Viruses)

3.1.2. Morphology

Vaccinia virus particles are brick-shaped, measured 250 nm in diameter, are 250-300 nm long and 200 nm high. The core is biconcave flanked by two lateral bodies (ICTVdB, Figure 14, 15 and 16), and this complex is surrounded by one or two lipidic envelope.
To exit from cells, viral particles are propelled by a mechanism involving the cytoskeleton of the infected cells. The first indication that Vaccinia virus 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 (Strokes GV, 1976, J Virol, 18,636-643). Subsequent studies confirmed that these Vaccinia-tipped projections contained actin, as well as the actin cross-linking proteins α-actinin, filamin and fimbrin but not tropomyosin or myosin (Hiller et al., 1979; Hiller et al., 1981). Indeed, Vaccinia virus induces the nucleation of actin tails from outer membrane surrounding the intracellular enveloped virus (IEV) (Cudmore et al., 1996).

Figure 15. Electron micrograph of a thin-sectioned intra-cellular vaccinia particle (Virology, BN Fields and DM Knipe)
C: Core, L: Lateral body, E: outer membrane.

Figure 14. Vaccinia Virus particle
(Dr. Milan V.Nermut of the National Institute for Biological standards and control. Herts, U.K)
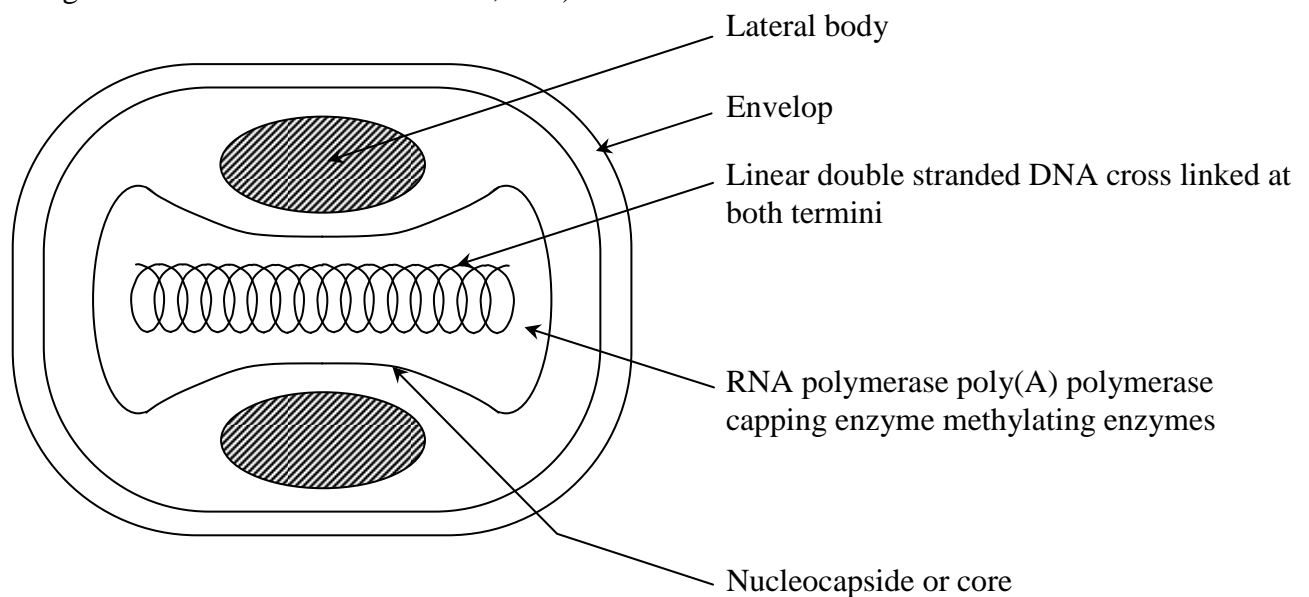
Figure 16. Schematic representation of a thin-sectioned intra-cellular vaccinia particle.

3.1.3. Nucleic acid

Virions contain one molecule of linear double stranded DNA characterized by a naturally cross-linking at both termini of the two DNA molecules strands (Wittek, 1982). The total genome length of the Copenhagen strain of Vaccinia virus is 191,636 bp with a relative purin or pyrimidin bases composition of 66.6 % A/T. 198 "major" protein-coding regions and 65 overlapping "minor" regions were identified, for a total of 263 potential genes (Goebel et al., 1990). In addition, the Vaccinia virus genome contains very long inverted terminal repeats of approximately 10 kbp which are further characterized by the presence of direct tandem repeats of a 70-base-pair sequence arranged in two blocks of 13 and 17 copies, respectively. A central region of the genome is highly conserved between different *Orthopoxviruses*. In contrast, the ends are hypervariable (Wittek, 1982).

3.1.4. Cellular receptor for Vaccinia virus?

Poxviruses can infect their hosts by different routes: through the skin by mechanical means, via respiratory tract (e.g., Variola virus infections of humans), or by oral route (Buller et al., 1991). Because one early gene of Vaccinia virus encodes a polypeptide related to EGF (Brown et al., 1985), the emergent question was whether EGF receptor (EGFR) may be a portal for infectivity. However, the expression of EGFR by target cells does not influence virus adsorption to cells or penetration (Hugin et al., 1994). Thus, the issue of an hypothetic receptor for Vaccinia virus remains to date open.

3.1.5. Poxvirus replication

Poxviruses replicate in the cytoplasm of infected cells and the cellular replication machinery is not essential since it is only present in the nucleus (Wittek, 1982). They encode most enzymes required either for macromolecular precursor pool regulations, or for biosynthetic processes (Buller et al., 1991). No origins of replication are known. Vaccinia virus follows then a "Self-priming" model for DNA replication summarized in Figure 17.
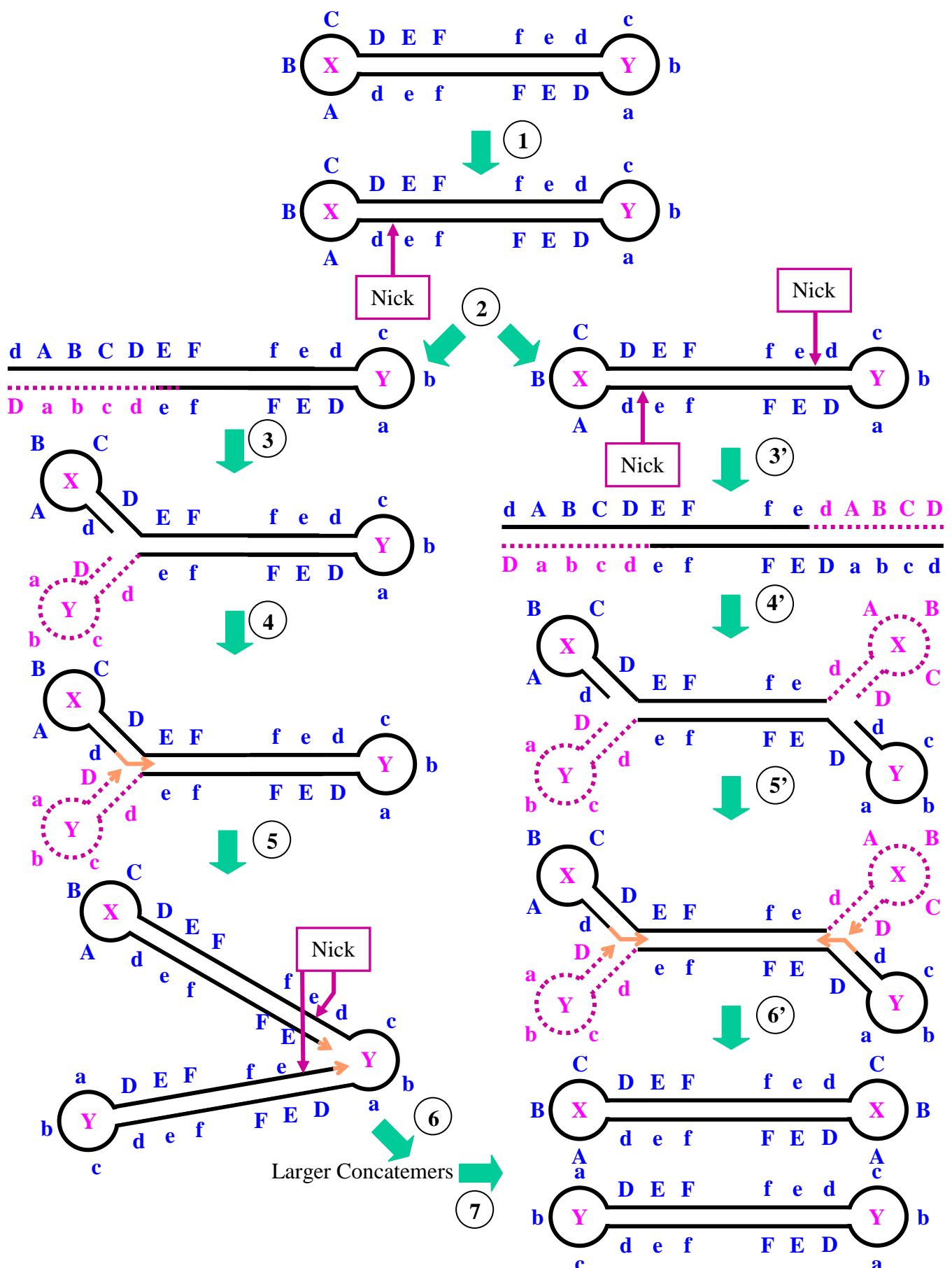
Figure 17. DNA replication in Vaccinia virus.

Poxvirus DNA replication occurs in the cytoplasm, no origins of replication are known, the cellular replication machinery is not essential since it is only present in the nucleus. Self-priming model: 1-6: Initiation at one end, formation of concatemers; 1-6': Initation at the both ends. X and Y represent inverted terminal repeats, two isomers are complementary. (inspired from K. Ballmer-Hofer lecture at the University of Basel, Switzerland).

The general scheme of Vaccinia virus replication can be summarized in five steps. First, the attachment of the virion to the cell membrane is followed by the release of the core into the cytoplasm of the infected cells. At this stage, the transcription of the early genes begins under the control of early promoters, characterized by complete sets of single nucleotide substitutions. They consist of a 16 base-pair critical regions, separated by 11 base-pairs of a less critical T-rich sequence (Davison et al., 1989a). During this early infection phase, early RNA are transcribed by the virion associated RNA polymerase (Wittek, 1982). This occurs a few minutes after infection and leads to early virion proteins production. Two to five hours after the beginning of the infection, the core which is now uncoated, liberates the viral DNA for the cytoplasmic DNA replication. Later in infection, late RNA are transcribed, under the control of late promoters. These consist of three regions: an upstream sequence of about 20 base-pairs, rich in T and A residues, separated by a spacer region of about 6 base-pairs from a highly conserved (-1)TAAAT(+4) element within which transcription initiates (Davison et al., 1989b). Late RNA, which often contain self-complementary sequences, are very heterogeneous in length because there are no late stop transcription signals (Wittek, 1982). These late mRNA lead to the production of late enzymes and virions proteins. These late virions proteins can be cleaved, glycosylated and phosphorylated by post-translational modifications. The last step of the replication leads to morphogenesis of new viral particles by assembling the early and late proteins and the newly synthesized DNA. The viral complex is then surrounded by lipidic envelopes. The first envelope consists, for some intracellular mature virus particles, in membranes derived from either the trans-Golgi network or tubular endosomes, resulting in intracellular enveloped virus which can be released upon lysis of the cell. A second enveloping process results from budding of intracellular mature virus particles through the plasma membrane (figure 18).
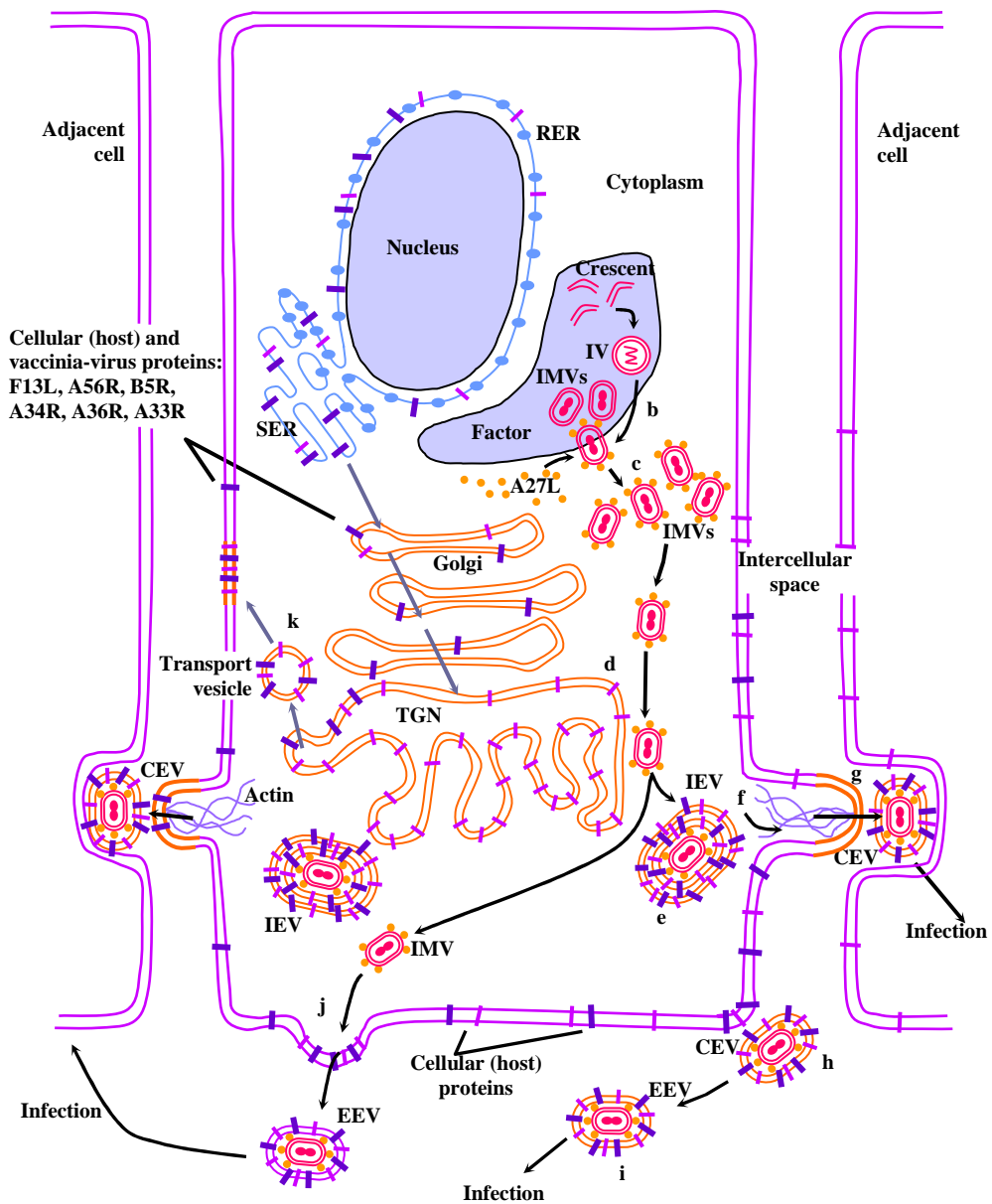
Figure 18. Assembly of Vaccinia virus in a human cell.
SER = smooth endoplasmic reticulum; RER = rough endoplasmic reticulum. The assembly of vaccinia virus is a complex process that results in the formation of two infectious forms of virus: intracellular mature virus (IMV) and extracellular enveloped virus (EEV). (a) Vaccinia-virus morphogenesis starts in cytoplasmic 'factories' with the formation of membrane crescents, which extend to form spherical immature virus (IV) particles. (b) IV particles are non-infectious until they undergo morphological condensation into 'brick-shaped' IMV particles. IMV particles recruit the virus A27L protein (p14 kDa) onto their surface. (c) During infection, most IMV particles remain within the cell until they are released by cell lysis. However, some IMV particles become enveloped by membranes derived from either the trans-Golgi network (TGN) or tubular endosomes (d) to form intracellular enveloped virus (IEV) (e). (f) Proteins within the outer membrane of IEV particles induce the polarised, unidirectional polymerisation of actin, which propels the particle towards the plasma membrane and assists the infection of neighbouring cells. (g) During this process, the outer membrane of the IEV particle fuses with the plasma membrane of the cell, exposing an infectious particle on the cell surface and leaving proteins that induce actin polymerisation within the plasma membrane. (h) If these particles remain attached to the cell they are called cell-associated enveloped virus (CEV); (i) however, if they are released from the cell they are called extracellular enveloped virus (EEV). (j) In addition to the classical mechanism of vaccinia-virus assembly described above, EEV particles can also form via direct budding of IMV particles through the plasma membrane. This alternative mechanism of EEV formation might be facilitated by the transport of viral glycoproteins from the TGN to the plasma membrane (k)

The morphogenesis ends about 20 hours after the beginning of the infection (Figure 19).
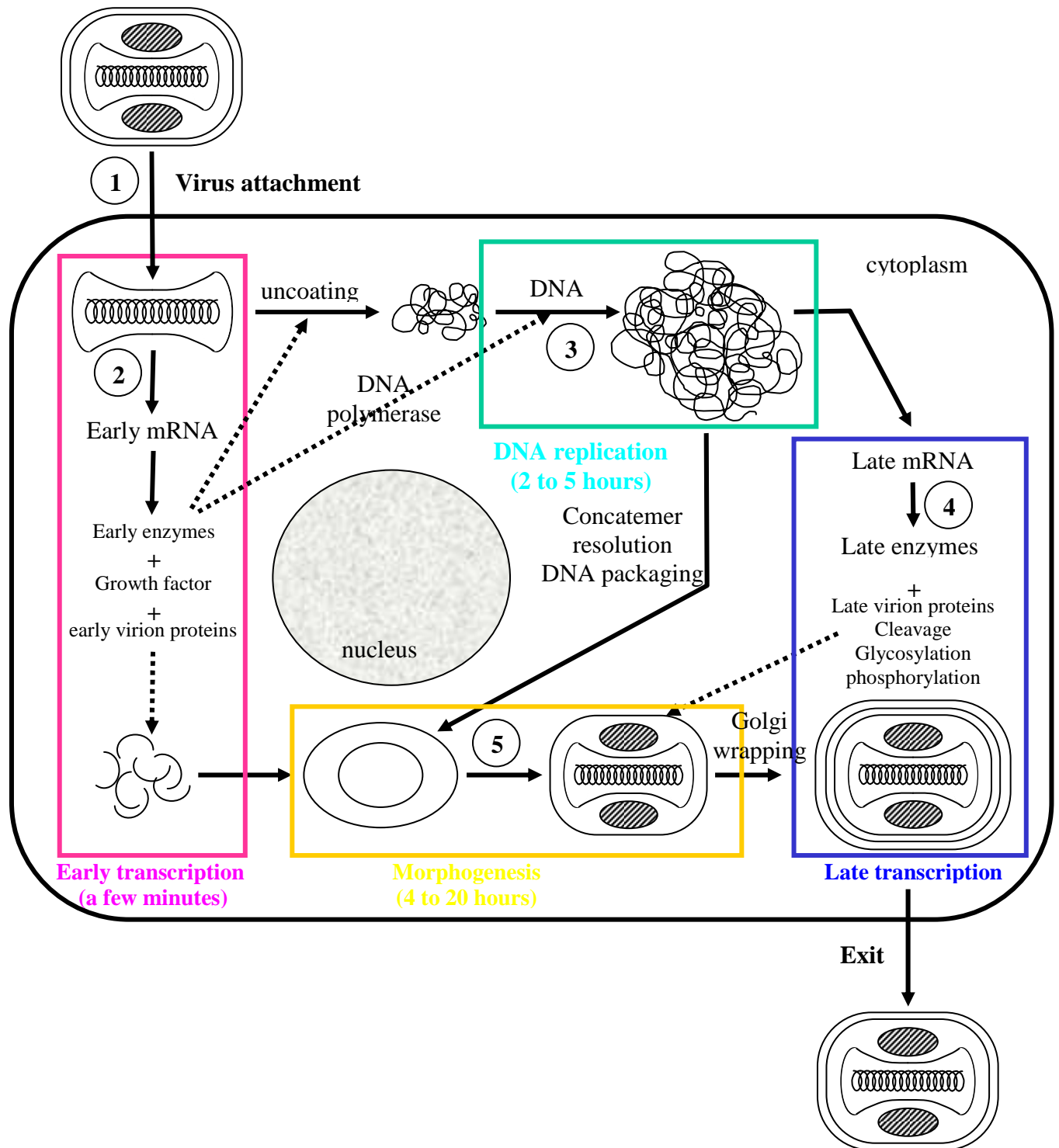
Figure 19. Infection cycle of Vaccinia virus.
1: attachment of the virion to the cell membrane, release of the core into the cytoplasme of the infected cell. 2: transcription of early genes. 3: DNA liberation from the core for the cytoplasmic DNA replication. 4: transcription of late genes. 5: morphogenesis of new viral particles (Moss, B., Fields, B.N., Knipe, D.M. and Howley, P.M., Poxviridae: the viruses and their replication, 1996, Lippincott-Raven, Philadelphia, Vol. 2, pp. 2637-2671).

3.2. Vaccinia virus as a vector for therapeutic vaccination

The global smallpox eradication in the early 1980 was made possible by vaccination with Vaccinia virus, the vaccine, which could easily be produced in regional centers, and used in remote regions of the globe (Paoletti, 1996).

In the first assay as cloning vector, a recombinant Vaccinia virus containing the thymidine kinase gene from Herpes simplex virus was generated, using *in vivo* recombination by co-transfection of eukaryotic tissue culture cells with cloned Bam-HI-digested thymidine kinase gene from Herpes simplex virus containing flanking Vaccinia virus DNA sequences (Panicali et al., 1982). Recombinant Vaccinia viruses were then developed as novel live vaccines (Smith et al., 1984). *In vivo* vaccination was then tested in the fox. Recombinant Vaccinia virus coding the rabies surface antigen gene was administrated to foxes, eliciting the production of titers of rabies-neutralizing antibodies equal or superior to those obtained with conventional vaccines and conferring complete protection to severe challenge infection with street rabies virus (Blancou et al., 1986). Twelve years after, oral vaccination of foxes against rabies using recombinant Vaccinia virus as vector has led to the elimination of sylvatic rabies from large areas in western Europe (Pastoret et al., 1998).

Vaccination using recombinant Vaccinia virus was also tested in human. Indeed, the first experimental immunization of human against the AIDS retrovirus was started in 1986, using recombinant Vaccinia virus expressing the complete gp160 env protein of HIV-1. This trial was the first report showing that immune state against HIV can be obtained in human (Zagury et al., 1988).

Mimicking the endogenous pathway of antigen presentation by MHC class-I molecules by infection by recombinant viruses encoding multiple TAA could elicit tumor specific cell mediated immune responses possibly enhanced by the use of cytokines, or co-stimulatory molecules such as CD80 and CD86 (Marti et al., 1997; Oertli et al., 2002).

Viral vectors, to be used in humans, must have an extremely safe profile. This becomes even more relevant when recombinant viruses are used in cancer patients, who frequently show impaired immune defenses, as a consequence of their disease and/or treatments. Furthermore, viral vectors should be able to infect both dividing and non dividing human cells and, particularly, human antigen-presenting cells such as dendritic cells or macrophages. Finally, viral vectors should, ideally, elicit limited anti-viral responses, thereby allowing for reiterated booster administrations (Bonnet et al., 2000).

Even if Vaccinia showed some virulence and side effects (cutaneous complications, encephalitis, pathologic for the central nervous system) depending on the immune status of the vaccinee, during anti-variolic program, it may be considered as a highly attractive vector for cancer gene therapy. It has a large genome that can be engineered by recombination for the insertion and simultaneous expression of multiple genes. It has the capacity to accommodate large size inserts of 30 kb or more (Flexner et al., 1988). Vaccinia virus infects a large variety of cells. Importantly, recombinant viruses must retain the capacity to be produced on appropriate cell substrates and in adequate yields in spite of their attenuation profile.

Vaccinia virus transcribes its genome by using viral enzymes and signals only. Recombinant foreign transcripts will be capped and polyadenylated by Vaccinia virus enzymes and will be translated into relatively high levels of recombinant protein within the infected cells.

Although naturally cytopathic, this viral vector can be engineered to be non cytopathic but still infectious (Peplinski et al., 1998). Indeed, the cytopathic effect (CPE) of infection with poxvirus in general and Vaccinia virus in particular includes the induction of early cell

rounding, damage of the host genome and RNA, inhibition of host protein synthesis, and eventually, death of the infected cells (Bablanian et al., 1978). To develop better viral vectors non replicating and less cytopathic, Vaccinia virus were generated by treatment of cell-free Vaccinia viruses suspension with psoralen and long-wave UV light (PLUV) which is known to target nucleic acid and introduce chemical cross-linking in the viral genome, even at the minimal required doses (0.3 μg of psoralen per ml and 10 min of long-wave UV irradiation (Tsung et al., 1996)). Indeed, in the presence of long-wave ultraviolet light, psoralen derivatives photoreact with the nucleic acids within intact viruses and cells. This photoreaction can leave protein antigens and other surface components relatively unmodified, while eliminating the infectivity of a wide range of infectious agents (Hanson, 1992). Even if the inactivation kinetics are non linear as a result of photodegradation of psoralens, this overview confirms that psoralen-long-wave UV-inactivated viruses are useful as non infectious antigens for use in immunoassays and as successful experimental vaccines.

Because the early phase of viral transcription is not affected by psoralen-mediated DNA cross-linking to the same degree as viral replication and late gene expression, the non-cytopathic VV thus generated are able to infect cells and express small but not large reporter genes under early viral promoters for at least 3 days efficiently. Indeed, majority of cells infected with PLUV-VV are not killed and are able to continue proliferation. The function of certain early viral proteins induce early CPE and death of infected cells, which are blocked by this treatment. Inactivation of a gene by cross-linking is directly proportional to the size of the target gene and the dose of cross-linking reagents: limited cross-linking was able to inhibit viral DNA replication, probably by blocking the movement of viral DNA polymerase along the genome (Tsung et al., 1996).

During a natural infection, Vaccinia virus life cycle remains completely within the cytoplasmic compartment of infected cells and ultimatly results with the cell lysis. Potential complications of integration into the host cell genome or phenotypic transformation can be excluded (Hruby, 1990). Furthermore, Vaccinia virus vectors are highly immunogenic, and induce humoral and cellular responses (Graham et al., 1991).

Vaccinia virus is easy to produce since it replicates in primary cell cultures and many different cell lines (L929, CV-1, Hela) isolated from virtually any animal species (Hruby, 1990). It is easy to inoculate (intradermal), has a high thermo-stability, and a transient expression (Jeremy W Fry and Kathryn J Wood, expert reviews in molecular medicine, 8 June 1999, Cambridge University Press). Furthermore, it can be dried down, rehydrated and inoculated with only minimal losses in infectivity (Hruby, 1990). Most importantly, a recombinant Vaccinia virus may represent an "off-the-shelf" reagent, stable, with maintained infectious titer while frozen for many years.

Based on all these characteristics, we chose Vaccinia virus as vector to engineer a cancer vaccine against melanoma. This virus was expressing two co-stimulatory molecules (CD80 and CD86) as well as three tumor associated antigens epitopes (gp$_{100}$, MART-1/Melan-A$_{27-35}$, Tyrosinase$_{1-9}$). The vaccine was successfully used in a phase I/II clinical trial in metastatic melanoma patients and demonstrated complete safety (Zajac et al., 2003). Remarkably, even if TAA specific responses were frequently induced by the viral administration, they tend to decrease over time. In order to improve the efficacy of this reagent, we tested the effect of CD154 expression by a recombinant Vaccinia virus in elicitation of tumor specific CTL responses *in vitro*.

4. Aim of a vaccine based on recombinant Vaccinia virus expressing the CD154

The main focus of our group is the development of anti-cancer therapeutic approach through the generation of tumor specific CD8 cytotoxic T cell responses. As principal vaccine

vector, we are using recombinant Vaccinia virus expressing different molecules important to generate a cellular immune response.

Recombinant poxviruses expressing immunomodulatory molecules together with specific antigens might represent powerful vaccines for cancer immunotherapy (Bonnet et al., 2000; Lattime et al., 1996). Furthermore, co-expression of CD80 and CD86 costimulatory molecules was demonstrated to increase the immunogenic capacity of a recombinant Vaccinia virus encoding melanoma tumor associated antigens (Mart-1/Melan-A$_{27-35}$) (Marti et al., 1997; Zajac et al., 1997). rVV encoding Mart-1/Melan-A$_{27-35,}$ gp$_{100}$ and Tyrosinase$_{1-9}$ Tumor Associated Antigens (TAA) and with CD80 and CD86 (Oertli et al., 2002; Spagnoli et al., 2002) has been successfully used in a phase I/II clinical trial (Zajac et al., 2003). Nevertheless, even if specific responses to TAA were often induced after vaccination, these responses were limited in time and intensity. Therefore, we hypothezised that the immunological effect on APC and T cells of CD154 expressed by a recombinant Vaccinia virus could eventually improve the intensity and duration of T cell response. The following work performed *in vitro*, will mostly address the first issue. Our aim is to evaluate the capacity of a recombinant Vaccinia virus expressing CD154 to enhance CD8+ T cell activation, in order to obtain highly specific and sustained responsiveness.

Materials and Methods
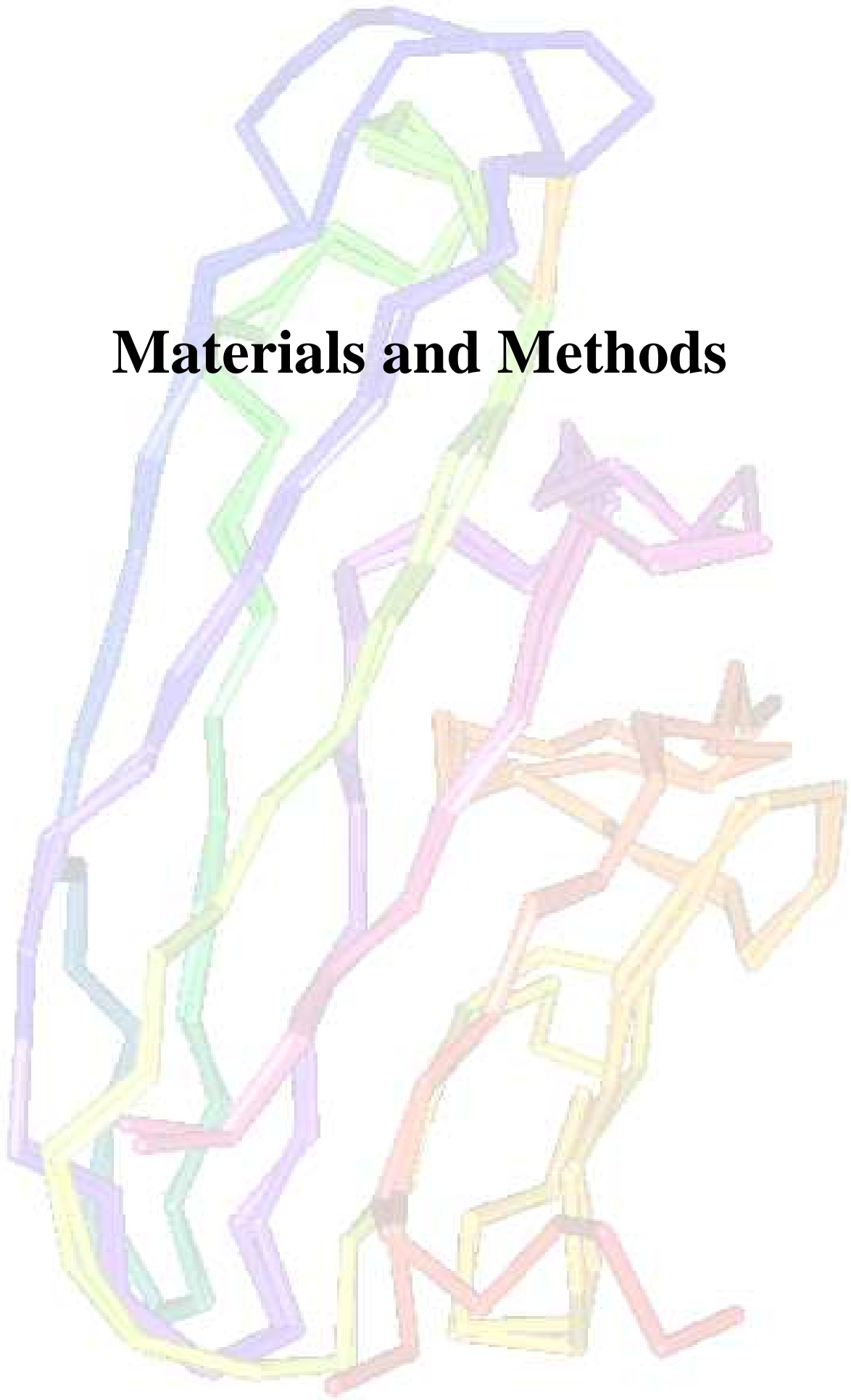
1. MATERIALS

1.1. Cells

- CV-1: Monkey African Green kidney fibroblast, ATCC CCL70, ECACC Ref N°: 87032605, grow rapidly and form monolayers of fibroblast like cells, cultured in DMEM 10% FCS.

- Vero: Monkey African Green kidney fibroblast-like, ECACC Ref N°: 84113001, cultured in DMEM 10% FCS.

- EBV-BL: B lymphocyte transformed by Epstein-Barr virus, cultured in CM 10% FCS.

- PMBC: Peripheral Blood Mononuclear Cell from healthy donor, cultured in CM 10% AB.

- Human primary skin fibroblasts generated in our lab as previously described, cultured in DMEM 10% FCS (Zajac et al., 1998).

1.2. Virus

These experiments were done using the Copenhague strain of Vaccinia virus (generous gift of Dr. R. Drillien, Strasbourg, France). All the recombinant Vaccinia viruses used were produced from this strain, and were elaborated in the ICFS, Kantonsspital, Basel, Switzerland (Zajac P et al.).

- WT: Wild-type Copenhague strain of Vaccinia virus, usually named as Control VV in our experiments.

- CD154rVV: recombinant Vaccinia virus encoding the CD154 under early viral promoter control. CD154 gene was inserted in the A56R locus of Vaccinia virus genome by homologous recombination.

1.3. Plasmids

pKT1323 (generous gift from Dr.K.Tsung, St Louis, MO) (Map 1).
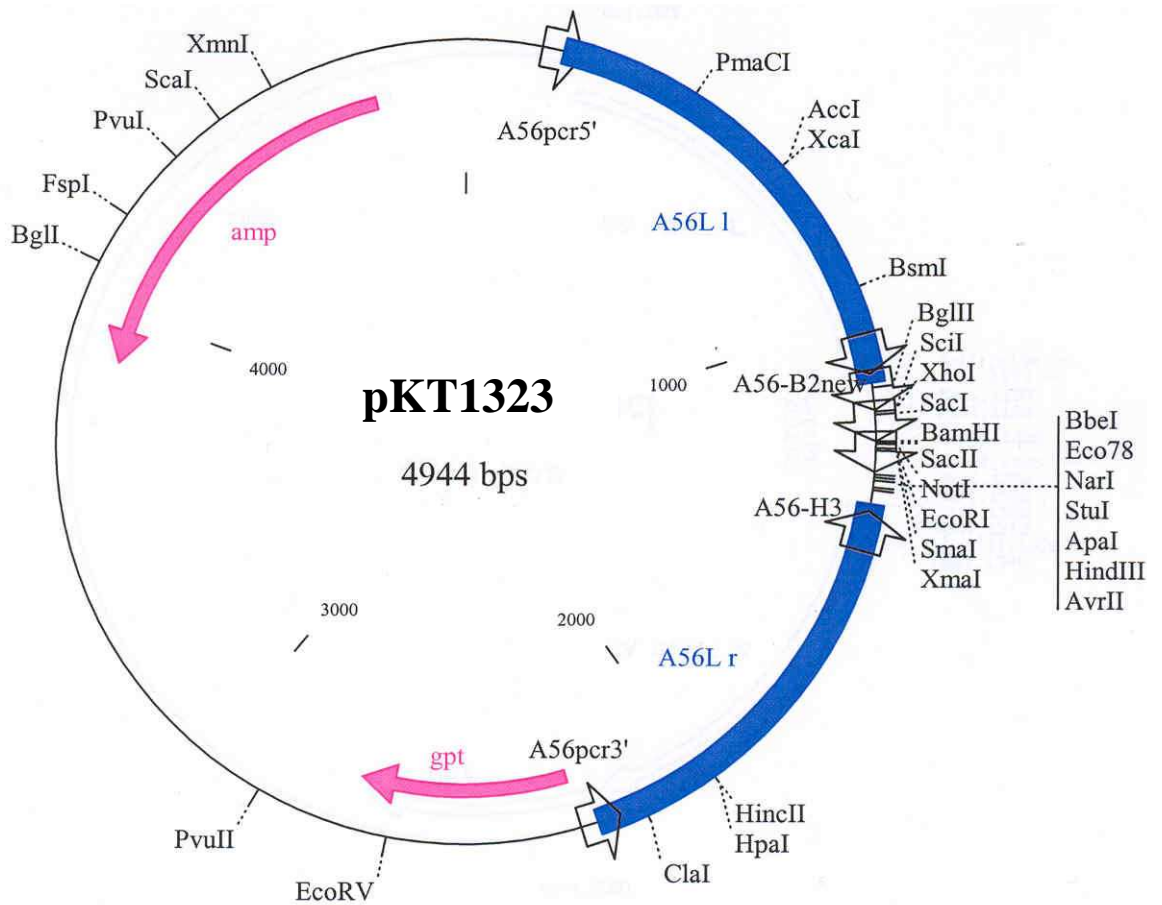
Map 1: pKT1323 plasmid for the construction of CD154 encoding plasmid.
(generous gift from Dr. K. Tsung (San Francisco, CA))
Escherichi coli guanine phosphoribosyl transferase (*gpt*) gene is used as a transient marker for selection of recombinant vaccinia virus, and *amp* gene as selection marker for further transfected bacteria.

pKT1323-CD154: pKT1323 plasmid carrying the CD154 gene (Map 2).
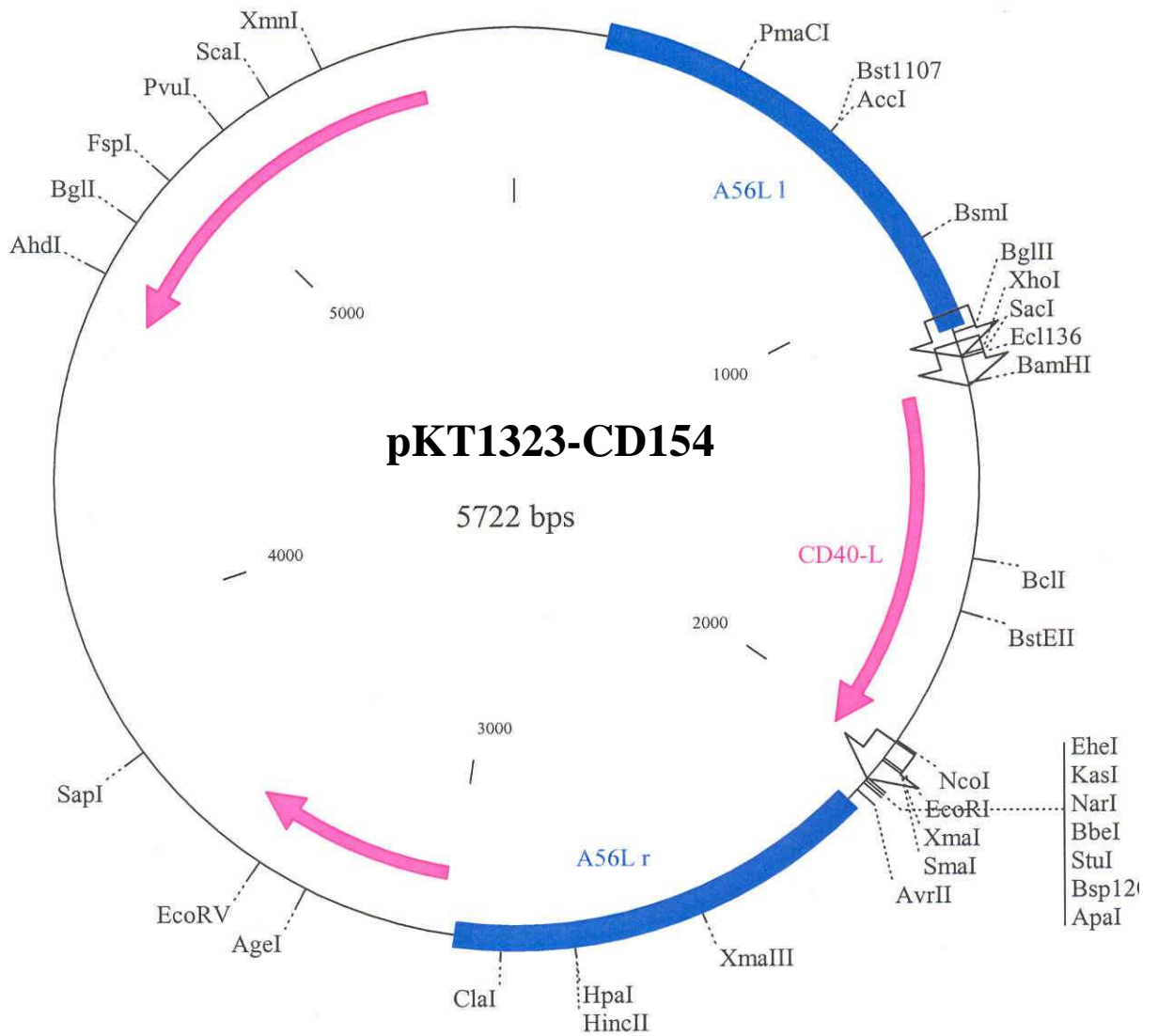
Map 2: CD154 encoding plasmid.
CD154 gene was inserted into pKT1323 by BamHI and EcoRI ligation

1.4. Buffers and media

1.4.1. Buffers

- HEPES Buffer Solution 1M (Invitrogen, Carlsbad, CA): 238.3 g/L, pH 7.2 to 7.5, pKa 7.3 at 37°C.

- MEM Non Essential AminoAcids (100x) without L-Glutamine (Invitrogen, Carlsbad, CA).

- Kanamycin solution (100x) (Invitrogen, Carlsbad, CA): 10 000 µg/ml kanamycin. Spectrum: Gram positive and gram negative bacteria.

- GlutaMAX™-I supplement 200 mM (100x) (Invitrogen, Carlsbad, CA).

- Trypsin-EDTA (1x) (Invitrogen, Carlsbad, CA): 0.5 g/L Trypsin (1:250), 0.2 g/L EDTA in Hanks' BSS, without Ca^{2+} and Mg^{2+}.

- Phosphate-Buffer Saline (PBS) (1x) (GIBCO ™).

- UltraPure™ 10x TAE buffer (Invitrogen, Carlsbad, CA): 400 mM Tris-acetate, 10 mM EDTA at pH 8.3.

- UltraPure™ 10x TBE buffer (Invitrogen, Carlsbad, CA): 1 M Tris, 0.9 M Boric acid, 0.01 M EDTA.

1.4.2. Media

- Dulbecco's Modified Eagle's Medium-low glucose (DMEM) (Fluka, BuchsSG, Switzerland).

- RPMI 1640 Medium (Invitrogen, Carlsbad, CA).

- CTL medium: RPMI 1640 Medium,1% HEPES Buffer 1M, 1% Non Essential AminoAcids (100x), 1% GlutaMAX™-I (100x), 1% Kanamycine (100x), 10% filtrated human serum AB, IL-2 200 unit/ml final.

- CM 10% AB (complete medium): RPMI 1640 Medium, 1% HEPES Buffer 1M, 1% Non Essential AminoAcids (100x), 1% GlutaMAX™-I (100x), 1% Kanamycine (100x), 10% filtrated human serum AB.

- CM 10% FCS: RPMI 1640 Medium, 1% HEPES Buffer 1M, 1% Non Essential AminoAcids (100x), 1% GlutaMAX™-I (100x), 1% Kanamycine (100x),10% FCS.

- DC medium: RPMI 1640 Medium, 10% FCS, IL-4 1000 unit/ml, rHuGM-CSF 50 ng/ml (LEUCOMAX®, 400 µg corresponding to 4.44 x 10^6 IU (Sandoz Pharmaceutical, Vienna, Austria)).

- DMEM 10% FCS: DMEM, 1% GlutaMAX™-I (100x), 1% Kanamycine (100x), 10% FCS.

- Freezing medium: RPMI, 40% FCS, 10% DMSO $((CH_3)_2SO$, 78.13 D (Sigma Chemical co., St Louis, MO)

- LB medium for agarose plate: LB Agar, powder (Lennox L Agar®, Invitrogen, Carlsbad, CA).

1.5. Antibodies and tetramers

- Soluble MHC-Peptide Tetramer streptavidin R-PE Conjugate, MHC allele: HLA-A 0201 (ProImmune, Oxford, UK): MART-1/Melan-A$_{37-35}$, EBV LMP2, EBV BMLF1, and CMVpp65 specific tetramer.

- Mouse IgG antibodies to human CD154, CD83, CD80, CD86, CD8, CD4, CD1a, CD14 and control IgG, R-PE or FITC conjugated (BD PharMingen, Franklin Lakes, NJ).

- ch5D12 (generous gift from M. de Boer, Pan Genetics B.V., Utrecht, The Netherlands): chimeric antagonist anti-human CD40 monoclonal antibody were the mouse variable regions were placed on a IgG4 human backbone.

1.6. Chemical

Psoralen: (CN Biosciences, Nottingham, UK): 4'aminomethyl-trioxsalen (trioxsalen: 4,5',8-trimethylpsoralen): $C_{15}H_{15}NO_3$.
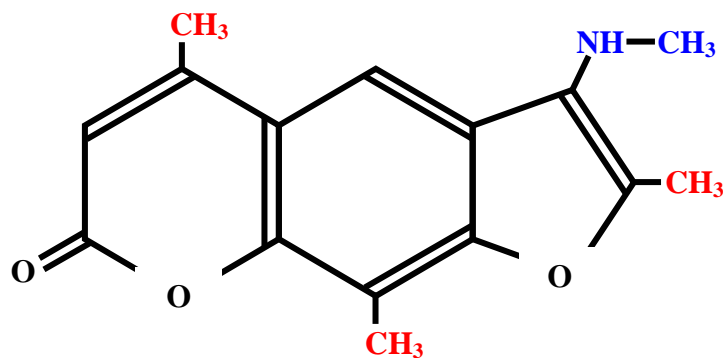
1.7. Primers and probes sequences 5'-3' for quantitative Real-Time PCR

vvI3L Fwd CGG CTA GTC CTA TGT TGT ATC AAC TTC
    Rev TGC AAA GAA TTT GGA ATG CG
    Probe FAM-AGA AGC CGT CTA TGG AAA CAT TAA GCA CAAGG-TAMRA

TNF-α Fwd CCC AGG GAC CTC TCT CTA ATC A
    Rev GCT ACA GGC TTG TCA CTC GG
    Probe FAM-CTG GCC CAG GCA GTC AGA TCA TCT T-TAMRA
    (Razeghi et al., 2001)

IL-2   Fwd   AAC TCA CCA GGA TGC TCA CAT TTA
            Rev   TCC CTG GGT CTT AAG TGA AAG TTT
            Probe FAM-TTT TAC ATG CCC AAG AAG GCC ACA GAA CT-TAMRA
            (Giulietti et al., 2001)

IL-12p40   Fwd   GCC CAG CTG CTG AGG AGA GT
            Rev   TGG GTG GGT CAG GTT TGA TG
            Probe FAM-ACG GCA TCC ACC ATG ACC TCA ATG-TAMRA
            (Yawalkar et al., 2000)

IFN-γ   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
            (Kammula et al., 1999)

GAPDH   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
            (Martin et al., 2001)

IL-6   Fwd   CAG CCC TGA GAA AGG AGA CAT G
            Rev   GGT TCA GGT TGT TTT CTG CCA
            Probe AGT AAC ATG TGT GAA AGC AGC AAA GAG GCA C-TAMRA

CD154   Fwd   GCT TTG AAA TGC AAA AAG GTG AT
            Rev   TTG TTT TAC TGC TGG CCT CAC TT

Il-15 and GM-CSF primers and probe: Assays-on-Demand, Gene Expression Products (Applied Biosystems, Foster City, CA). 18S ribosomal RNA primers and probe: TaqMan® Ribosomal RNA Control Reagents, VIC™ Probe (Applied Biosystems, Foster City, CA).

1.8. Primers and probes concentrations for quantitative Real-Time PCR

Gene	Primers and Probes concentration (nM)		
	Forward	Reverse	Probe
vvI3L	200	200	200
18S	50	50	12.5
GAPDH	100	100	200
CD154	100	100	0
IL-12p40	200	400	200
TNF-α	200	400	200
IL-2	200	400	200
IFN-γ	100	100	200
IL-6	200	200	200

2. METHODS

2.1. Cells isolation

2.1.1. Peripheral-blood Mononuclear Cells (PBMC) isolation on Ficoll gradient

Anticoagulated venous blood is layered onto ficoll (HISTOPAQUE®-1077 (Sigma-aldrich co., St Louis, USA), Polysucrose, 5.7 g/dl, sodium diatrizoate, 9.0 g/dl, density = 1.077 ± 0.001 g/ml). During centrifugation, erythrocytes and granulocytes are aggregated by polysucrose and sediment; whereas, lymphocytes and other mononuclear cells remain at the plasma-ficoll interface. Erythrocyte contamination is negligible. Most platelets are removed by low speed centrifugation during the washing steps. Venous blood is collected in preservative-free heparin tubes, should be processed within 2 hours, and diluted 1:2 depending upon absolute cell numbers to avoid gradient overloading.
Principle:
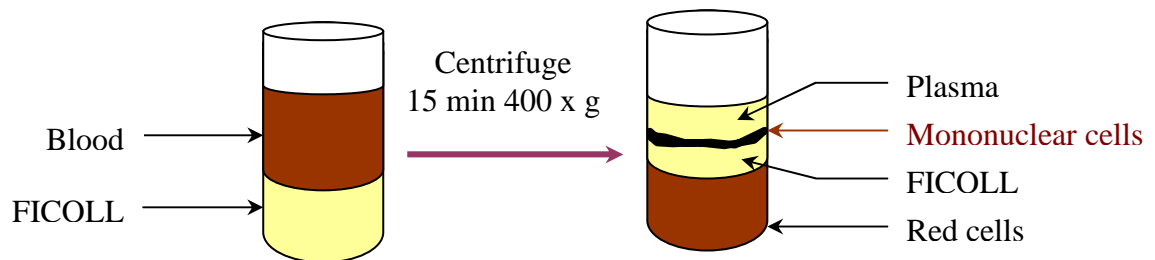
Technically, 15 ml blood is layered onto 12 ml ficoll and centrifuged 15 min at 400 x g at room temperature (lower temperatures may result in cell clumping and poor recovery). The opaque interface containing PBMC is collected, and cells are washed twice in one volume PBS by centrifugation (5 min, 250 x g). PBMC are resuspend in complete medium.

2.1.2. Cells sorting using MACS magnetic MicroBeads

For CD14+, CD8+ or CD4+ cells separation, PBMC are magnetically labeled with MicroBeads specific for CD14, CD8 or CD4 ((Miltenyi Biotec, Bergisch Gladbach, Germany), supplied as suspension containing 0.1 % BSA, 0.05 sodium azide, conjugated to monoclonal anti-human antibodies), and passed through a separation column which is placed in the magnetic field of a MACS separator (LS MACS separation columns, MidiMACS Separation Unit and MACS Multistand (Miltenyi Biotec, Bergisch Gladbach, Germany)). Columns capacity reaches a maximum of 2 x 10^9 total cells and 10^8 magnetically labeled cells. They have a hydrophilic coating allowing rapid filling. The column is washed with buffer before separation. The magnetically labeled cells are retained in the column while the unlabeled cells run through. After removal of the column from the magnetic field, the selected cells can be eluted as positively selected fraction.

Technically, PBMC (in suspension in PBS) are pelleted, resuspended in 5 ml MACS buffer (PBS 500 ml, FCS 0.5 %, EDTA 2 mM, filterted) and centrifuged 5 min at 400 x g. 50 µl MicroBeads per 10^7 total cells are added onto cells pellet, and gently mixed. After 15 min incubation at 4°C, cells are washed in MACS buffer and resuspended in 2 ml. 3 ml MACS buffer is applied on the column and let run through. This effluent is discard. Magnetically labeled cell suspension is applied on the column, and let run through. The effluent is collected as negative fraction. The column is washed twice with 3 ml MACS buffer and the effluent is collected with the same negative fraction. The column is removed from separator and placed

on a new collection tube. 5 ml of MACS buffer are applied twice to the column and the cells are flushed out using the supplied plunger. Cells are pelleted by 5 min centrifugation at 400 x g and resuspended in complete medium.

2.2. Cloning procedure for recombinant virus preparation

2.2.1. Plasmidic DNA digestion

1 µg plasmidic DNA is linearized for further cloning steps, by 1 hour digestion at 37°C, using 10 units of appropriate restriction enzymes and buffers.

2.2.2. Electrophoresis gel 1% agarose

1g UltraPure™ Agarose (Invitrogen, Carlsbad, CA) is dissolved in 100 ml TAE 1x modified buffer ((Invitrogen, Carlsbad, CA), 40 mM Tris-acetate, 0.571 ml Glacial acetic acid, 0.1 mM Na$_2$EDTA, pH 8.0, H$_2$O qsp 50 ml). This buffer is formulated with a lower concentration of EDTA than standard TAE in order to minimize any interference by the EDTA on enzyme activity required for downstream applications. The gel is supplemented with 10 µg/ml BET ((Fluka Chemie AG, Buchs, Switzerland), 3,8-Diamino-5-ethyl-6-phenylphenanthridinenium bromide, C$_{21}$H$_{20}$BrN$_3$, 394.31 D), and poured in an electrophoresis support. DNA samples are mixed with 5 µl gel loading buffer, loaded and electric field (100 mV) is applied. Results are observed under 365 nm UV light.

2.2.3. DNA extraction from agarose gel slice

The centrifugal filter device (DNA Gel Extraction Kit montage™ life Science kits (Millipore, Bedford, MA)) is designed to extract DNA fragments that are 100 to 10,000 bp in size from agarose gel slices in one 10 minutes spin.

Technically, the DNA band of interest is located in the gel using a 365 nm UV lamp. The gel slice (under 100 µl in volume or 100 mg in mass) containing the DNA is cut out, placed in the Gel Nebulizer, spined for 10 minutes at 5,000 x g. The extruded DNA passes through the microporous membrane in the sample filter cup and is collected in the filtrate vial.

2.2.4. Phenol/Chloroform extraction of nucleic acid: removal of contaminating proteins

Phenol ((Sigma Chemical co., St Louis, MO), C$_6$H$_5$OH, 94.11 D) is added to DNA solution (v/v), vortexed 1 min and centrifuged 1 min at 8,000 x g. The nucleic acids remain in the aqueous supernatant, whereas the proteins stay in the interphase, and the phenol in the organic lower phase. The supernatant is collected avoiding any contact with the proteic interphase. A second extraction is performed on the resting phenolic phase to get out DNA eventually still present after the first extraction (100 µl H$_2$O are added in the resting phenolic phase, vortexed 30 sec, centrifuged 1 min at 8,000 x g and the supernatant is collected as before). The two supernatants are mixed, and 1.5 volume of chloroform ((Sigma Chemical co., St Louis, MO), CHCl$_3$, 119.38 D) is added to remove any traces of phenol. After 30 sec vortex and 1 min centrifugation at 8,000 x g, the supernatant containing the DNA is collected for further uses.

2.2.5. Ethanol precipitation of DNA

For precipitation, 1/10 volume of NaAc 3M and 2.5 volume of ethanol 100 % are added on the DNA suspension, on ice. After homogenization, this mix is kept 15 min at – 70°C. After 15 min centrifugation at 4°C (500 x g), the supernatant is discarded and the pellet is dried. DNA pellet is then resuspended in 20 µl H$_2$O.

2.2.6. Ligation of a DNA fragment

The plasmid and the DNA fragment to insert were previously digested by the two restriction enzymes of interest and precipitated. Opened vector, insert, buffer and 1 unit of T4 DNA ligase (Invitrogen, Carlsbad, CA) are gently mixed. Simultaneously, a control ligation is done like previously but without insert, replacing it by the same volume of H$_2$O. These two ligations are incubated 2 hours at room temperature and used for transformation.

2.2.7. *Escherischia coli* transformation by electroporation

One aliquot of Top10 Electrocompetent *E. coli* ((Invitrogen, Carlsbad, CA), transformation efficiency $\geq$ 1 x 10^9 cfu/µg supercoiled DNA, genotype: F$^-$*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) stored at –70°C is thaw on ice. 1.5 µl of ligation is placed into an electroporation cuvette ((BIO-RAD, Hercules, CA), Gene Pulser® Cuvette, 0.2 cm) and 20 µl of *E. coli* are added onto the DNA. After 30 sec incubation on ice, the cuvette is perfectly dried and submitted to an electric choc (2.5 Volts, 200 Ω resistance, 25 µFD capacitance) in an electroporation machine.

The bacteria are resuspended in 600 µl LB medium (Lennox L Agar®, (Invitrogen, Carlsbad, CA)) and incubated 1 hour at 37°C. 200 µl of transformed *E. coli* are spread on a Petri dish (100 mm x 15 mm polystyrene bacteriological Petri dish, 56.5 cm^2 (BD Biosciences FALCON™, San Jose, CA)) containing LB medium and 1 unit/ml carbenicillin (α-Carboxybenzylpenicillin disodium salt, C$_{17}$H$_{16}$N$_2$Na$_2$O$_6$S, 422.4 g/mol. Spectrum: Gram-positive and -negative bacteria, Pseudomonas (Fluka, BuchsSG, Switzerland)). The petri dish is incubated over night at 37°C. Single colonies are picked up with a sterile tip and put in culture in 2 ml LB medium at 37°C overnight under agitation.

2.2.8. Plasmidic DNA isolation

Plasmidic DNA is liberated from *E. coli* by SDS/alkaline lysis. SDS precipitate and cell debris are pelleted by centrifugation, and supernatant is loaded onto the NucleoSpin® Plasmid column (Macherey-Nagel, Düren, Germany). Plasmidic DNA binds to the silica membrane. After one wash with ethanolic buffer, DNA is eluted under low ionic strength conditions with alkaline buffer (5mM Tris-Cl, pH 8.5).

Technically, *E. coli* (1-5 ml culture) are pelleted (30 sec, full speed), resuspended in 250 µl A1 buffer and lyzed by 250 µl A2 buffer (5 min, room teperature). Cells debris are precipitated by adding 300 µl A3 buffer and pelleted by centrifugation (10 min, full speed). The supernatant is loaded on the column and centrifuged (1 min, full speed). The column is washed with 600 µl ethanolic buffer and the silica membrane is dried by 2 min centrifugation at full speed. 50 µl alkaline buffer are added on the column. After 1 min incubation, plasmid DNA is eluted by 1 min centrifugation at full speed.

2.3. CD154rVV construction

2.3.1. PCR amplification of specific cDNA

Human PBMC from healthy donor were stimulated (PHA 5 µg/ml, 10 units/ml IL-2) for 24 and 48 hours. Cells were collected, washed and total RNAs were extracted. After DNase treatment and Reverse Transcription, CD154 cDNA was PCR amplified, using the following primers:

BamHI-CD154-5':

CTCGAG**GGATCC**ATGATCGAAACATACAAC
   │←**BamHI**→│

EcoRI-CD154-3':

C**GAATTC**TCAGAGTTTGAGTAAGCCAAAGGAC
 │←**EcoRI**→│

Technically, 2 µl cDNA were mixed with 2 µl PCR buffer 10x, 0.8 µl $MgCl_2$, 1.6 µl dNTP (2.5 mM each), 1 µl BamHI-CD154-5' 10 µM, 1 µl EcoRI-CD154-3' 10 µM, 1 µl TaqPolymerase and 10.6 µl H_2O. After PCR (5' at 95°C, (1' at 94°C, 2' at 72°C)$_{30x}$, 10' at 72°C, ∞ at +4°C), totality of PCR products were loaded on a 1% agarose gel.

2.3.2. Proteinase K treatment

In order to eliminate contaminating proteins, CD154 cDNA was treated with proteinase K (50 µl cDNA are incubated with 3 µl EDTA 100mM, 3 µl SDS 10%, 1.2 µl TRIS 0.5 M pH 8.0 and 6 µl Proteinase K at 0.5 mg/ml for 15' at 37°C). The enzyme was then inhibited at 65°C for 20'. After Phenol/Chloroform extraction, DNA encoding CD154 was ethanol precipitated.

2.3.3. Contruction of plasmid encoding CD154

CD154 cDNA fragment was digested for 1 hour at 37°C with *BamHI* (Cut: 5' G │ GATCC 3' (Invitrogen, Carlsbad, CA)), then with *EcoRI* (Cut: 5' G │ AATTC 3' (Invitrogen, Carlsbad, CA)) restriction enzymes. The digested fragment was isolated on a 1% agarose gel, extracted, precipitated and resuspended in 25 µl H_2O. Same digestion steps were performed on pKT1323 (Map 1): this vector was digested at first with *BamHI* (3 µl plasmid, 2 µl 10x Buffer supplied with the enzyme, 1.5 µl *BamHI* restriction enzyme, 15 µl H_2O: 1 hour at 37°C). Digestion was verified on 1 µl on an 1% agarose gel. On the previous digestion, we performed *EcoRI* digestion (previous 20 µl digestion, 1 µl 10x buffer supplied with the enzyme, 9 µl H_2O, 1.5 µl *EcoRI*: 1 hour at 37°C).

The double digestion was verified on a 1% agarose gel, by loading totality of the digestion mix. The open plamid was cut out from the gel, extracted, ethanol precipitated and resuspended in 25 µl H_2O. The open plasmid and the digested CD154 cDNA were ligated following the ligation protocol described in materiels and methods. In order to verify ligation efficacy, ligation product and native plasmid were digested by *SacII* (Cut: 5' CCGC │ GG 3'

(Promega, Madison, WI)). Indeed, only the native plasmid or the plasmid that did not integrate the insert will be cut by this enzyme (Map 1 and 2).

Electro-competent *Escherichia coli* were transfected following the described protocol, with this ligation product or with the native plasmid as control. As 100 µl of vector displayed as expected very few colonies (2 single colonies only), 100 µl of the ligation product displayed ≈ 700 colonies, whereas 10 µl of ligation product displayed 70 colonies. Nine of those colonies and 1 of the colonie of the control plate were picked up. These colonies were grown over night at 37°C under agitation in 2 ml LB medium. Plasmid were extracted from these bacterial cultures using DNA NucleoSpin® plasmid.

2.3.4. Directed Mutagenesis

In vitro site-directed mutagenesis is an invaluable technique allowing site-specific mutation for carrying out vector modification. The QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, Cambridge, UK) used in our context allows site-specific mutation in virtually any double-stranded plasmid, thus eliminating the need for subcloning into M13-based bacteriophage vectors and for ssDNA rescue. It requires no specialized vectors, unique restriction sites, or multiple transformations.

It is used to make point mutations, switch amino acids, and delete or insert single or multiple amino acids, using *PfuTurbo*® DNA polymerase, which replicates both plasmid strands with high fidelity (this enzyme has 6-fold higher fidelity in DNA synthesis than Taq DNA polymerase), and without displacing the mutant oligonucleotide primers.

The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by *PfuTurbo*® DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *DpnI*. The *DpnI* endonuclease (target sequence: 5'-Gm^6ATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to *DpnI* digestion. The nicked vector DNA containing the desired mutations is then transformed into XL1-Blue supercompetent cells. The high fidelity of the *PfuTurbo*® DNA polymerase, and the low number of thermal cycles contribute to the high mutation efficiency and decreased potential for generating random mutations during the reaction. The principle is summarized figure 20.

Step 1
Plasmid Preparation
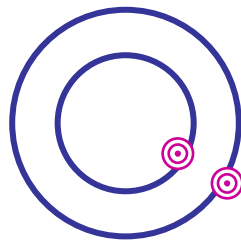
Gene in plasmid with
target site (◎) for mutation

Step 2
Temperature cycling
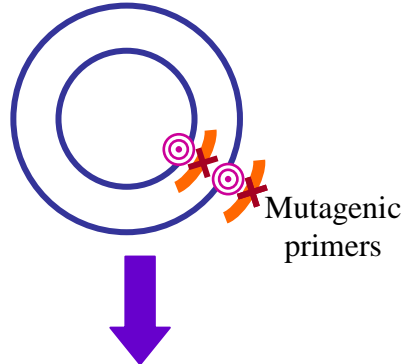
Denature the plasmid and anneal the
oligonucleotide primers (◣) containing
the desired mutation (✖)

Mutagenic
primers

Step 3
Digestion
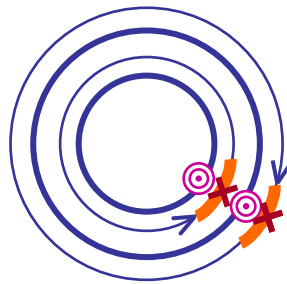
Using the nonstrand-displacing
action of *PfuTurbo*™ DNA polymerase,
extend and incorporate the
mutagenic primers resulting
in nicked circular strands

Step 4
Transformation
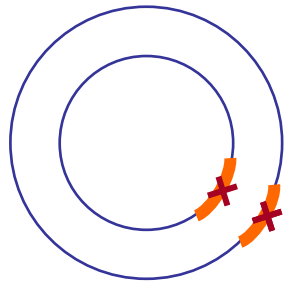
Digest the methylated, nonmutated
parental DNA template with *Dpn I*

Mutated plasmid
(contains nicked
circular strands

Transform the circular, nicked dsDNA
into XL1-Blue supercompetent cells

Legend
▬ Parental DNA plasmid
✖ Mutagenic primer
— Mutated DNA plasmid
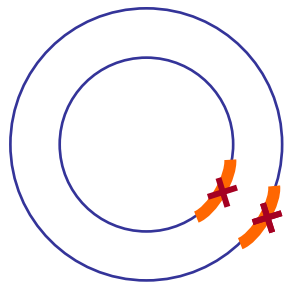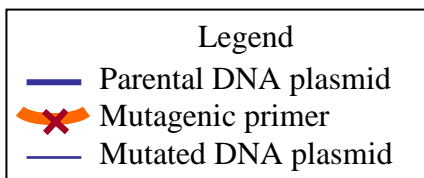
After transformation, the XL1-Blue
supercompetent cells repair the
nicks in the mutated plasmid

**Figure 20. Overview of the principle of the QuickChange™ Site-Directed
Mutagenesis Kit (Stratagene, Cambridge, UK)**

2.4. Virus preparation

2.4.1. Cell infection

Cells of interest are resuspended in medium complemented with FCS to avoid interactions with human anti-Vaccinia virus antibodies, present in human serum. Cells are counted and distributed in tubes or wells. Adherent cells are cultured 4 hours at 37°C. The excess of medium is aspirated and a minimal volume of medium is left on the cells to avoid drying. Cells growing in suspension are pelleted and resuspended in 200 µl medium. Viral solution is thaw and sonicated 1 min. The virus adsorb to the cells during 1 hour incubation at 37°C. Adherent cells are regularly slowly agitate to avoid cell drying. Cells are washed once with PBS and appropriate medium is added.

2.4.2. Recombinant Vaccinia virus construction

2.4.2.1. General principle

Recombinant Vaccinia virus are generated by homologous recombination with cotransfectant "VV plasmid".

This plasmid contains multiple expression/insertion cassettes containing early promoters (Davison et al., 1989a) 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 rVV (Falkner et al., 1990). This plasmide encoding the gene of interest is inserted by homologous recombination into the Wild Type strain (WT) of Copenhagen Vaccinia virus. Recombinant viral clones are selected according to their transient expression of the *E. coli* gpt marker under the selective pressure of mycophenolic acid (MPA), xanthine, and hypoxanthine as described (by Earl P.L. and Moss B. in "Current Protocols in Molecular Biology, pp 16.15.1-16.18.10. Greene Publishing, New York, 1995).

2.4.2.2. Vaccinia virus growth inhibition by mycophenolic acid (MPA)

The mycotoxin MPA (mycophenolic acid) is an inhibitor of purine metabolism. It inhibits the enzyme inosine monophosphate dehydrogenase and thereby prevents the formation of xanthine monophosphate. This results in the intracellular depletion of purine nucleotides and in an inhibition of cell growth (Mulligan et al., 1981). This MPA was demonstrated to reversibly block formation of Vaccinia virus plaque (Falkner et al., 1988). In addition, MPA is non mutagenic in the Ames test and in the related SOS test (Reiss, 1986): avoidance of mutagens should ensure virus stability.

2.4.2.3. Recombinant Vaccinia viruses selection system based on expression of the *Escherichia coli guanine phosphoribosyl transferase* (*gpt*) gene

The inhibition of the *de novo* synthesis of purines by MPA can be overcome by a cell that expresses 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 (Mulligan et al., 1981).

The block of purine synthesis by MPA can also be overcome by a recombinant virus expressing the bacterial XGPRT (encoded by the *Escherichia coli gpt* gene). Indeed, synthesis of XGPRT enabled only the recombinant viruses to form large plaques in a selective medium containing MPA, xanthine and hypoxanthine (Falkner et al., 1988).

In their experience, 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 was incorporated into a plasmid vector that has a Vacinia virus promoter and unique restriction endonucleases sites for insertion of a foreign gene. Because of Vaccinia virus-derived flanking sequences, the entire selection-expression cassette is inserted as a unit into the Vaccinia virus genome by homologous recombination. Thus, all of the Gpt[+] recombinants analyzed also contained the foreign gene that had been inserted into the plasmid vector.

2.4.2.4. Technical procedure

Briefly, the plasmid containing the gene of interest is resuspended in 100 μl OPTI-MEM®I medium without serum (Invitrogen, Carlsbad, CA). 10 μl Lipofectamine (LIPOFECTAMINE™ Reagent (Invitrogen™ life technologies, Carlsbad, CA)) is combined with 100 μl of OPTI-MEM®I medium and mix gently with the plasmid suspension. After 30 min incubation at room temperature, transfection mix is diluted in 1 ml OPTI-MEM®I medium and put in a 35-mm dish onto 10^6 CV-1 cells infected with WT Vaccinia virus at 0.1 m.o.i.. Lysed cells and medium are harvested 48 to 72 hours after infection: $1/10^4$ of the viral solution is put onto 5×10^5 CV-1 cells in a 35-mm dish, for 1 hour at 37°C, and added with 2 ml DMEM 10% FCS, Xanthine (2,6-Dihydroxypurine, $C_5H_3N_4NaO_2$, 174.09 D (Sigma Chemical co., St Louis, MO)), Hypoxanthine (6-Hydroxypurine, $C_5H_4N_4O$, 136.11 D (Sigma Chemical co., St Louis, MO)), MPA (6-(4-Hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl)-4-methyl-4-hexenoic acid, $C_{17}H_{20}O_6$, 320.34 D (Fluka Chemie AG, Buchs, Switzerland)), for 2 days. Isolated plaques are selected under microscope and put in 1 ml PBS.

This insertion of the plasmid is "secured" by 2 to 3 cycles under selective pressure. The second part of recombinant Vaccinia virus isolation consists of similar cycles of plaques isolation but without pressure, which lead to either excision of the entire plasmid (obtention of WT virus) or excision of the plasmidic part resulting in the obtention of recombinant virus (see figure 21).
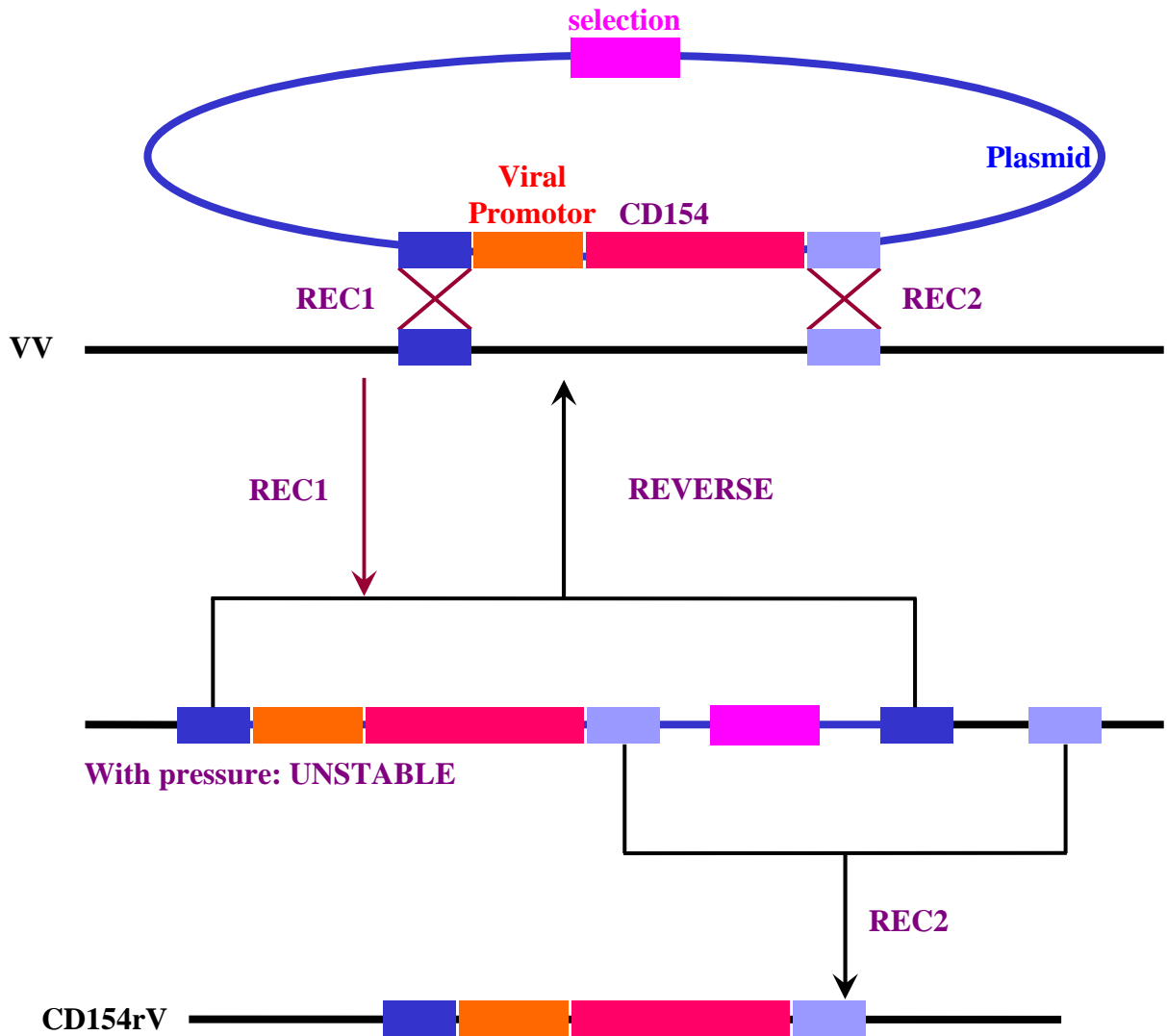
Figure 21. Principle of homologous recombination.

The expression/insertion cassette of the plasmid contains an early viral promoter as well as a Vaccinia virus early transcriptional termination sequence located downstream. This cassette is flanked by sequences being identical to different viral loci and allowing homologous recombination.

The gene of interest encoded by the plasmid is inserted by homologous recombination into the Wild Type strain (WT) of Copenhagen Vaccinia virus. This figure represents the first recombination possibility (beginning at REC1 site): the construction obtained by homologous recombination at the first site identical to a viral loci is unstable under selective pressure with mycophenolic acid (MPA), and can subsequently revert by recombination to the initial plasmid or to the plasmid encoding the CD154rVV by recombination at the second site (REC2).

The second recombination possibility (beginning at REC2 site) follows the same principle and ends to the plasmid encoding the CD154rVV by recombination at the first site (REC1) (not represented here).

2.4.3. Amplification and semi-purification of Vaccinia viruses

Twelve 175 cm^2 confluent flasks of CV-1 cells are infected with sonicated replicative virus at 0,01 m.o.i. leading to complete CPE. After 4 days culture, 8 ml TRIS 10mM are added on each decanted flasks. Infected cells are detached by 1 cycle of freeze-thaw, collected and centrifuged (2,500 x g, 4 min).

Supernatant A is stored, while pellet is resuspended in 5 ml TRIS 10mM and sonicated to liberate viruses. After 4 minutes centrifugation at 2,500 x g, supernatant B is added to the

viral solution A, while the pellet is resuspended in 3 ml of TRIS 10 mM, sonicated, and centrifuged (2,500 x g, 4 min). Supernatant containing the replicative viruses is added to the previous viral solution, distributed in Pollyallomer ultra-centrifuge tubes (1 x 3 ½ in. (Beckman Coulter, Fullerton, CA)), 25 ml per tube. 10 ml sucrose 36% underlayer cushions (β-D-Fructofuranosyl-α-D-glucopyranoside (= D(+)-Saccharose = α-D-Glc-(1→2)-β-D-Fru), $C_{12}H_{22}O_{11}$, 342.30 D (Fluka Chemie AG, Buchs, Switzerland)) are added in each tube. After 1 h 30 min ultra-centrifugation at 30, 000 x g, pellets are resuspended in TRIS 1 mM, sonicated, aliquoted and stored at –20°C.

2.4.4. Virus titration

CV-1 cells are cultured in a 6 wells plate and grown to subconfluency. Replicative viral stock solutions are thaw, sonicated 1 min, and diluted in PBS from 10^{-1} to 10^{-8}. After culture medium removal, 100 µl of the 10^{-6} to 10^{-8} dilutions are added to the wells. Virus adsorption to cells occurs during 1 hour incubation at 37°C. The plate is regularly slowly shaken every 10 minutes to avoid cells drying. 2 ml of medium are added in each well and the cells are incubated for 24 to 48 hours. Medium is aspirated, cells stained 1 min by 500 µl of 0.1% violet crystal (Hexamethylpararosaniline chloride, $C_{25}H_{30}ClN_3$, 407.98 D (Fluka Chemie AG, Buchs, Switzerland)) diluted in ethanol, and the plaques are counted under light. The viral stock solution concentration is calculated following the formular: viral concentration (pfu/ml) = number of plaques x 10 x dilution factor.

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

Vaccinia virus is diluted to a concentration of $5x10^8$ PFU/ml in a Hanks' Balanced Salts Solution (Invitrogen, Carlsbad, CA) containing 3 µg/ml Psoralen (CN Biosciences, Nottingham, UK). After 10 min incubation at room temperature, 1 ml of the solution is irradiated in an uncovered 35-mm dish with 365 nm UV light for 16 min, applied energy 1.6 J (Stratalinker, Stratagene, La Jolla, CA) and aliquoted in 250 µl vials.

In order to rapidly evaluate the extent of the inactivation, CV-1 cells are infected with PLUV virus at different m.o.i. (from 0 to 20). Cytopathic effect (CPE) is evaluated under microscope after 24 hours infection. The shape of the non infected cells monolayer referres to 0% CPE. The global changes in the morphology of the culture determinates the percentage of CPE. 100% CPE correspond to 100% cell displaying cytopathic morphology.

2.5. Gene expression analysis

2.5.1. Total RNA isolation

Cells ($1x10^7$ maximum) are lysed in denaturating guanidine isothiocyanate (GITC)-containing buffer inactivating RNases. 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 an enrichment for mRNA since most RNAs < 200 nucleotides (such as 5.8S rRNA, 5S rRNA and tRNAs).

Technically, cells are washed in PBS, pelleted, and lysed by 350 µl of RLT buffer containing 1% β-mercapto-ethanol. 350 µl of ethanol 70% are added and 700 µl of the sample are applied on the column. After centrifugation (8,000 x g, 15 sec), the column is washed with 700 µl RW1 buffer, and twice with 500 µl RPE buffer. The silica membrane is dried by centrifugation (2 min at 8,000 x g). The column is transferred to a collection tube and RNAs are eluted in 50 µl RNase-free water by centrifugation (8,000 x g, 1 min).

2.5.2. DNA digestion

1μg RNA is incubated with 1 unit DNase I (Deoxyribonuclease I, Amplification Grade (Invitrogen, Carlsbad, CA)) with the buffer from supplier, for 15 min at room temperature. DNase I is then inactivated by 1 μl of EDTA 25mM and by heating for 10 min at 65°C.

2.5.3. 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.

Technically, 1 μg of RNA (11 μl) is incubated 5 min at 65°C with 1 μl oligo(dT)$_{20}$ primer at 50 μM (Invitrogen, Carlsbad, CA) at 500 μg/ml or 1 μl random hexamers at 0.09 OD$_{260}$ units/ml (Invitrogen, Carlsbad, CA). This mix is quickly chill on ice. Reverse transcription is performed with 4 μl buffer from supplier, 1 μl dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP), 2 μl DTT 0.1 M ($C_4H_{10}O_2S_2$, 154.24 g/mol (Invitrogen, Carlsbad, CA)), and 1 μl (200 units) of M-MLV RT, by 1 hour incubation at 37°C. Enzyme is inactivated by 5 min heating at 95°C.

2.5.4. Gene expression by Real-Time qPCR

Real-time PCR reactions are performed in the presence of three sequence-specific oligonucleotides. Two of them serve as conventional PCR primers for the amplification of target molecules. The third fluolabeled oligonucleotide (probe) hybridizes within the amplified region: this probe is labeled with a reporter and a quencher dye in a distance of a few base pairs within the probe sequence. The reporter fluorescent dyes FAM (6-carboxy-fluorescein phosphoramidite, emission λ_{max} = 518 nm) and the quencher dye molecule TAMRA (5-carboxy-tetramethyl-rhodamine, emission λ_{max} = 582 nm) are incorporated at nucleotide nine of the 5' and 3' end of the oligonucleotide, respectively. In close steric proximity FAM works as a reporter while TAMRA is a quencher, greatly reducing the fluorescence emitted by the reporter dye. The 3' nucleotide of this oligonucleotide is blocked by phosphorylation, preventing elongation by Taq DNA polymerase. During PCR amplification, DNA polymerase extends the primers and displaces the probe from the template, hydrolyzing it through its 5'-exonuclease activity. Thus, reporter and quencher dyes are separated leading to a measurable increase of fluorescence. This emitted fluorescence is directly proportional to the number of template molecules in the tube. Threshold cycle (Ct) refers to the cycle when fluorescence signal exceeds the mean of background fluorescence plus ten times the standard deviation in cycle 3 to 15.

Quantification of cytokine gene expression was calculated by using the $2^{-\Delta\Delta C_T}$ method. This method uses a reference sample for comparison of every unknown sample's gene expression and gives similar results as the standard curve method (Livak et al., 2001; Malec et al., 2004; Nieman et al., 2004; Winer et al., 1999). Briefly, ΔC_T [C_T (gene of interest) – C_T (reference gene GAPDH)] is calculated for each sample and reference sample (condition infected with CD154rVV at lowest m.o.i.). $\Delta\Delta C_T$ [ΔC_T (reference sample) – ΔC_T (sample)] is evaluated, and relative quantification is calculated as $2^{-\Delta\Delta C_T}$. The results are expressed as a *n*-fold difference relative to the reference sample. A one-unit change in C_T reflects a two-fold change in mRNA content. Verification of equally purified and equally infected samples was performed respectively with vvI3L, a viral early gene (Rochester et al., 1998), and 18S genes.

Practically, Real-Time qPCR are performed in Thermofast® 96 wells plate (Abgene, Epsom, UK), using the TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Forster City, CA) and the ABI prim™ 7700 sequence detection system (Applied Biosystems, Forster City, CA). Stage 1 consists in 2 min at 50°C followed by stage 2: 10 min at 95°C. Stage 3 is repeated 45 times consisting in 15 sec at 95°C followed by 1 min at 60°C. Datas are collected at these two steps of stage 3 and analyzed. Normalization of sample was performed using GAPDH as reference gene.

2.6.   IL-12p70 detection by Enzyme Linked Immuno-Sorbent Assay (ELISA)

IL-12p70 ELISA is performed using Human IL-12(p70) BD OptEIA™ ELISA Set (Becton Dickinson, Franklin Lakes, NJ).

Serial dilutions of standard protein (500 pg/ml) is performed in Assay Diluent (NalgeNunc International, Rochester, NY) to 250, 125, 62.5, 31.3, 15.6, 7.8 and 0 pg/ml.

A 96 wells plate (Nunc-Immuno™ 96 MicroWell™ Plates (NalgeNunc International, Rochester, NY)) is coated with 100 µl per well of Capture Antibody 1:250 diluted in Coating Buffer 0.1 M (pH 9.5, 8.40 g $NaHCO_3$, 3.56 g Na_2CO3; q.s. to 1.0 l), sealed (Nunc Sealing polyester Tapes for ELISA plates (NalgeNunc International, Rochester, NY)) and incubated overnight at 4°C. Plate is washed 3 times with wash buffer (PBS with 0.05% Tween®20 (Sigma Chemical co., St Louis, MO)), blocked with 200 µl/well Assay Diluent, incubated 1 hour at room temperature, and washed 3 times more. Samples are diluted in Assay Diluent. 100 µl of each standard, sample, and control are put in duplicate in the plate. The plate is sealed and incubated for 2 hours at room temperature. Plate is washed 6 times. 100 µl of 1:250 diluted Detection Antibody (Biotinylated Anti-human IL-12(p70), is added to each well, the plate is sealed, incubated for 1 hours at room temperature, and washed 6 times.

To each well, 100 µl of 1:250 diluted Enzyme Reagent (Avidin-horseradish peroxidase conjugate) is added, the sealed plate is incubated 30 min at room temperature, and washed 6 times. 100 µl of substrate (BD Pharmingen™ TMB Substrate Reagent Set: Tetramethylbenzidine (TMB) and Hydrogen Peroxide (Becton Dickinson, Franklin Lakes, NJ)) is added to each well for 5 to 30 min incubation in the dark. The enzymatic reaction is stopped by 50 µl of Stop Solution (2N H_2SO_4). Absorbance is read at 450 nm within 30 min of stopping reaction.

2.7.   Phenotypic characterization of cells

2.7.1. Flow cytometry analysis

CD154 expression after cell infection was verified using anti-CD154 PE-conjugated antibodies (Immunotech, coulter company, Marseille, France). Infected cells were stained with specific or control IgG antibodies, incubated 45 min at 4°C in the dark, washed twice in cold PBS, fixed 1 min in Parafolmaldehyde 1% (Polyoxymethylene, $[CH_2O]_n$, 30.03 D, (Fluka Chemie AG, Buchs, Switzerland)), re-suspended in 200 µl PBS, and analyzed on a FACSCalibur® cytometer (Becton Dickinson, Franklin Lakes, NJ).

Staining with antibodies for CD83, CD14, CD1a, CD80, CD86 (PE or FITC-conjugated antibodies - PharMingen, San Diego, CA), were performed following the same protocol.

2.7.2. Tetramer analysis for direct visualization of CD8+ T cells

Peptide-MHC, ligand for a given population of T cells bearing TCRs with a corresponding specificity, are multimerized in soluble peptide-MHC tetramers: only the T cells that have TCR capable of binding to the particular MHC-peptide combination of the tetramer are able to bind the tetramer (Altman et al., 1996). The concomittent use of a monoclonal antibody that is specific for a T cell marker and conjugated with fluorescein isothiocyanate, for instance anti-CD8 monoclonal antibody FITC-conjugated, allows the detection of CD8$^+$ T cell specific for the peptide of interest (see figure 22).

MART-1/Melan-A$_{27\text{-}35}$ specific Tetramer (ProImmune, Oxford, UK - 1/100 diluted) followed by anti-CD8+ FITC-conjugated antibodies (PharMingen, San Diego, CA - 1/100 diluted) were used to identify MART-1/Melan-A$_{27\text{-}35}$ specific CD8+ T cells.
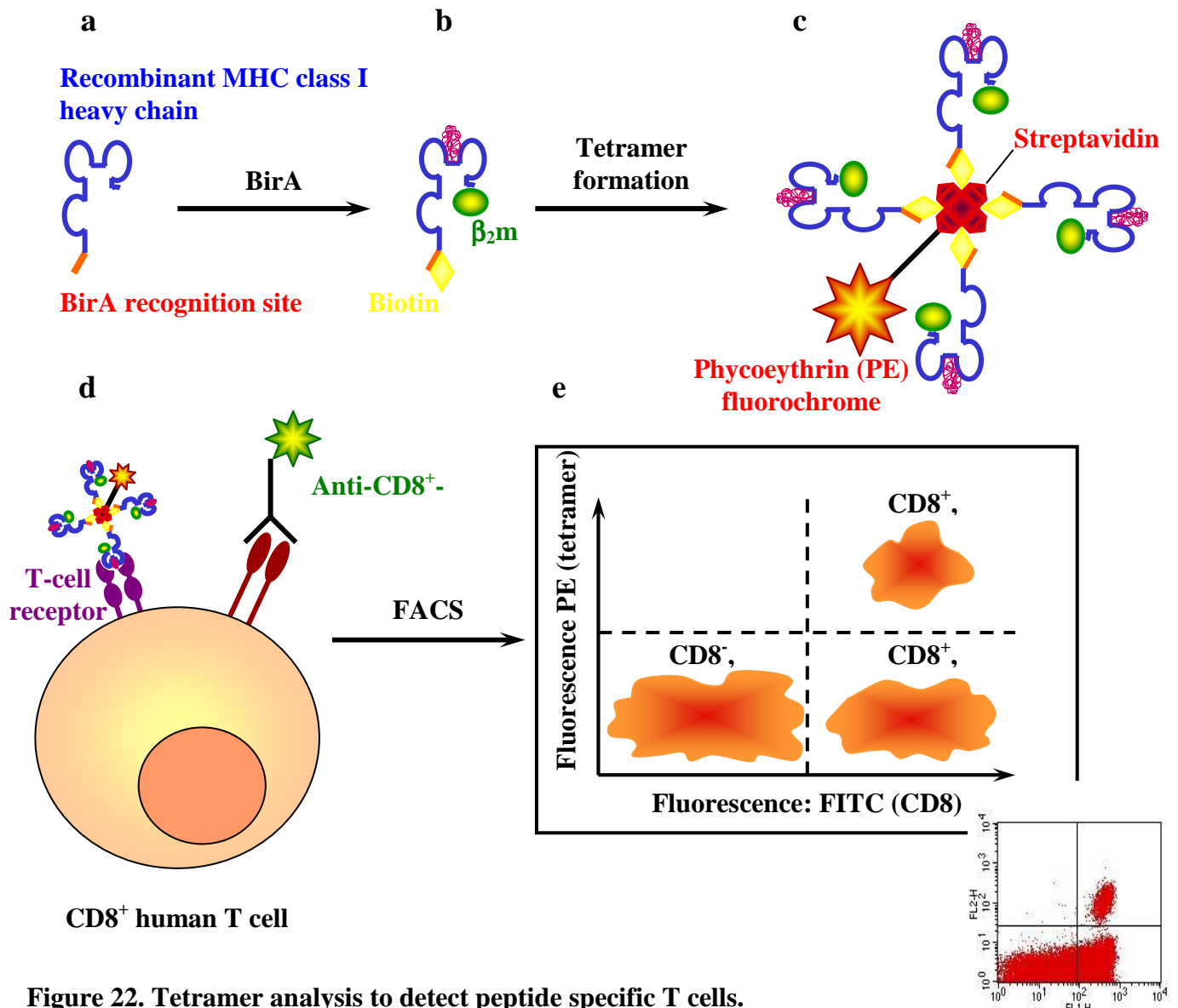
Figure 22. Tetramer analysis to detect peptide specific T cells.
(**a**) Soluble heavy chain of major histocompatibility complex (MHC) class I molecules are synthesized in *Escherichia coli*. (**b**) The molecules adopt an appropriate conformation following the addition of β_2 microglobulin (β_2m) and a synthetic peptide that represents the epitope that is recognized by the T-cell receptor of interest. This peptide is able to bind to the MHC molecule. In addition, the enzyme BirA is used to atach a biotin molecule to the specific BirA-recognition sequence, which has been incorporated into the carboxyl terminus of the MHC molecule. (**c**) Four MHC-biotin complexes are linked to a single streptavidin molecule, using the specific biotin-streptavidin interaction, to form a tetramer. The straptavidin molecule is tagged with a fluorochrome (e.g. phycoerythrin; PE). (**d**) Tetramers are mixed with the cell population that is to be analyzed. Only T cells that are capable of binding to the particular MHC-peptide combination that is present in the tetramer are able to bind the tetramer; thus, such cells will become labelled with the PE fluorochrome. A monoclonal antibody that is specific for a T-cell marker and is tagged with a different fluorochrome (e.g. fluorescein isothiocyanate (FITC)), can also be used. (**e**) The cells are then analyzed using flow cytometry; the proportion of the CD8+ T-cell population that stains positively with the tetramer can be determinated (top, right-hand quandrant).
Largely inspired from: Expert Reviews in Molecular Medicine, www-ermm.cbcu.cam.ac.uk, accession number: fig004jhc.

2.7.3. Cell apoptosis quantification by PI/annexin staining (Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen™, Franklin Lakes, NJ))

In apoptotic cells, phosphatidylserine (PS) is translocated from the inner to the 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 (Raynal et al., 1994). 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 in conjunction with Propidium Iodide (PI), which is a vital dye excluded in viable cells with intact membrane. For example, viable cells are Annexin V and PI negative, early apoptotic cells are Annexin V positive and PI negative, and late apoptotic or dead cells are both Annexin V and PI positive.

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

2.8. Induction of antigen-specific CTL by stimulation with CD154rVV of APCs from healthy donors

We tested the capacity of CD154rVV to enhance induction of MART-1/Melan-A$_{27-35}$-specific CTL in culture of infected APCs with autologous CD8+ T Cells. APC from healthy donors, CD14+ cells or iDC, were infected at different m.o.i. with CD154rVV or wild-type VV and co-cultured with autologous CD8+ T cells, as compare to APC cocultured with CD8+ T cells with MART-1/Melan-A$_{27-35}$ peptide (AAGIGILTV) at 10 μg/ml as antigen. After 1 hour adsorption, infected cells were washed and cultured at 37°C in complete medium with the autologous CD8+ T cells. After 8 days, half of the medium was replaced with fresh medium containing a final concentration of 10 units/ml IL-2. In order to minimize the induction of vaccine-virus-specific CTL and to optimally expand TAA-specific CTL, lymphocytes were restimulated on day 12 or 13, and 19 using 20 μg/ml peptide-pulsed and irradiated autologous EBV-BL re-suspended in complete medium supplemented with 10 units/ml IL-2. After supplementation of fresh medium and 10 units/ml IL-2 at day 22, cytotoxic activity was tested on day 26 of culture. MART-1/Melan-A$_{27-35}$-specific CTL phenotype was characterized by tetramer straining before boosts and cytotoxicity assay.

Similar experiments were performed using as antigenic peptide: EBV LMP2 (CLGGLLTMV), EBV BMLF1 (GLCTLVAML) or CMVpp65 (NLVPMVATV) peptide.

2.9. Measure of cell-mediated cytotoxicity using ^{51}Cr-release assay *in vitro*

Chromium-release assay is a standard assay to measure *in vitro* cytotoxic function (Brunner et al., 1968). Target cells radiolabeled with sodium chromate (Na$_2$^{51}CrO$_4$) which is taken up by live cells in its hexavalent form, are incubated with the test effector-cell population for a short period of 4 hours. The amount of ^{51}Cr released into the supernatant in its trivalent form is quantified to provide a measure of target cell lysis.

Technically, appropriate target cells, for instance EBV-BL, are washed in CM 10% FCS and the pelleted cells are labeled with 400 μCi sodium 51-chromate (Na$_2$^{51}CrO$_4$ half-life 27.7 days, (Amersham Biosciences, Buckinghamshire, UK)) in solution per 10^7 targets.

Targets are labeled for 1 hour in a 37°C, 5% CO_2, humidified incubator, with occasional shaking to inhibit pelleting. These targets are then washed two times in PBS and resuspended in 2 ml CM 10% FCS. Cells suspension is adjust to 2.5 x 10^4 / ml in CM 10% FCS. Effector cells, meaning CTLs are resuspended in 2 ml CM 10% FCS, and then adjust to their final plating concentration. Effector cells are added in the first raw of a 96 wells plate, and diluted 1:2 in the following wells in order to evaluate cytotoxicty at different Effector / Target ratio (E/T). 100 µl of target labeled cells are added in each well. Addition of a fixed number of target labeled cells on effector cells at multiple ratio achieves to various E/T ratio. The spontaneous release is measured in the wells containing only labeled target cells. The maximal release is obtained by complete lysis of labeled-target cells by HCl 1M addition. The plate is centrifuged 5 min at 150 x g , incubated at 37 °C for 4 hours.

Plate is then centrifuged and 30 µl of supernatants from each well are transferred into a lumaplate (Perkin-Elmer, Boston, MA). Release radioactivity is measured in a gamma-counter. Data are expressed as percentage of specific lysis of target cells ([(sample count – spontaneous count) x 100] / [maximal count – spontaneous count]).

2.10.   Cell proliferation assays using ^{3}H-Thymidine incorporation

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

CD14+ cells and T lymphocytes are cocultured in a 96 wells plate with flat bottom, in a final volume of 200 µl per wells. Cultured stimulated with PHA (4 µg/ml) are used as positive control. After 4 or 5 days incubation at 37°C, ^{3}H-Thy (20 µl of a 1/20 dilution) is added per well and reincubated for 18 hours. Cells are harvested and lyzed by an osmotic choc in a Micro96™ Cell Harvester (Skatron, Sunnyvale, CA). Nucleic acids are sticking on a prewetted glass fiber filter (Printed FiltermatA, Wallac, Turku, Finland). After 3 washes, the filter is dried, and enclosed in a plastic bag (Sample Bag, Wallac, Turku, Finland). Liquid scintillation cocktail is added (OPTI-FLUOR®, PerkinElmer, Boston, MA) and scintillation emission is measured.

2.11.   Lymphoproliferation assays using CFSE staining

The principle is summarized in figure 23 (Parish, 1999). Technically, lymphocytes are pelleted and washed once with PBS to remove all contaminating free proteins. The cells are resuspended in 1 ml PBS. 3 µl CFSE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA, SE;CFSE) mixed isomers, $C_{29}H_{19}NO_{11}$, 557.47 D (Molecular Probes, Eugene, OR)), stock at 5 mM are diluted to 1/2500 by addition of 7.5 ml PBS. 1 ml $CFSE_{1/2500}$ is added to 10^6 cells (in 1 ml PBS). CFSE dilution is finally 1/5000 (0.001 mM). Cells are incubated 10 min at 37°C in a water bath, and at least the same volume of blocking solution (complete medium) is added in order to block unbound CFSE. Cells are pelleted by centrifugation (5 min, 800 x g), washed 3 times with PBS, and finally resuspended in CM 10% AB medium for 5 days culture. Lymphocytes proliferation is evaluated by FACS analyses: at each cell division, the amount of CFSE initially present in the cells is divided by two between the daughter cells.
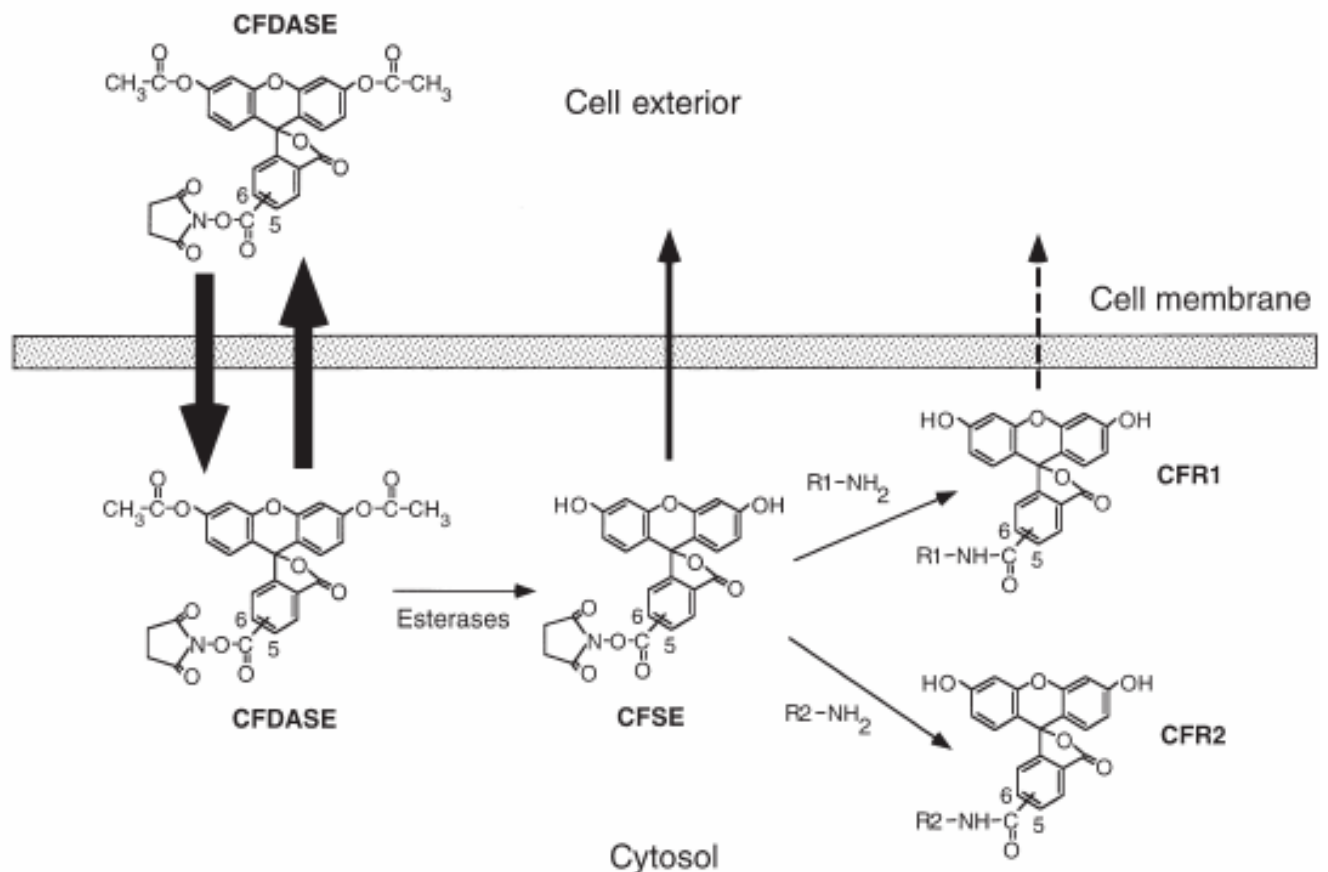
Figure 23. Principle of lymphoproliferative measurement using CFSE.
The non-fluorescent, highly membrane permeant, 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDASE) is readily taken up by cells, although its high lipophilicity also allows it to freely exit from cells. Intracellular esterases, however, can remove the two acetate groups from CFDASE to yield fluorescent 5-(and-6)-carboxyfluorescein succinimidyl ester (CFSE), which is much less membrane permeant and, therefore, exits from cells at a much slower rate. The succinimidyl moiety of CFSE is highly reactive with amino groups and can covalently couple 5-(and-6)-carboxyfluorescein (CF) to intracellular molecules. In some cases, CF covalently couples to intracellular molecules (R1-NH$_2$) to form conjugates (CFR1) that can still exit from the cell or are rapidly degraded. However, a proportion of CF becomes coupled to long-lived intracellular molecules (R2-NH$_2$) to form conjugates (CR2) that can not escape from the cell and thus, stable fluorescent labeling of cells is achieved. The principle is summarized in figure 6 (Parish CR et al., 1999).

Results
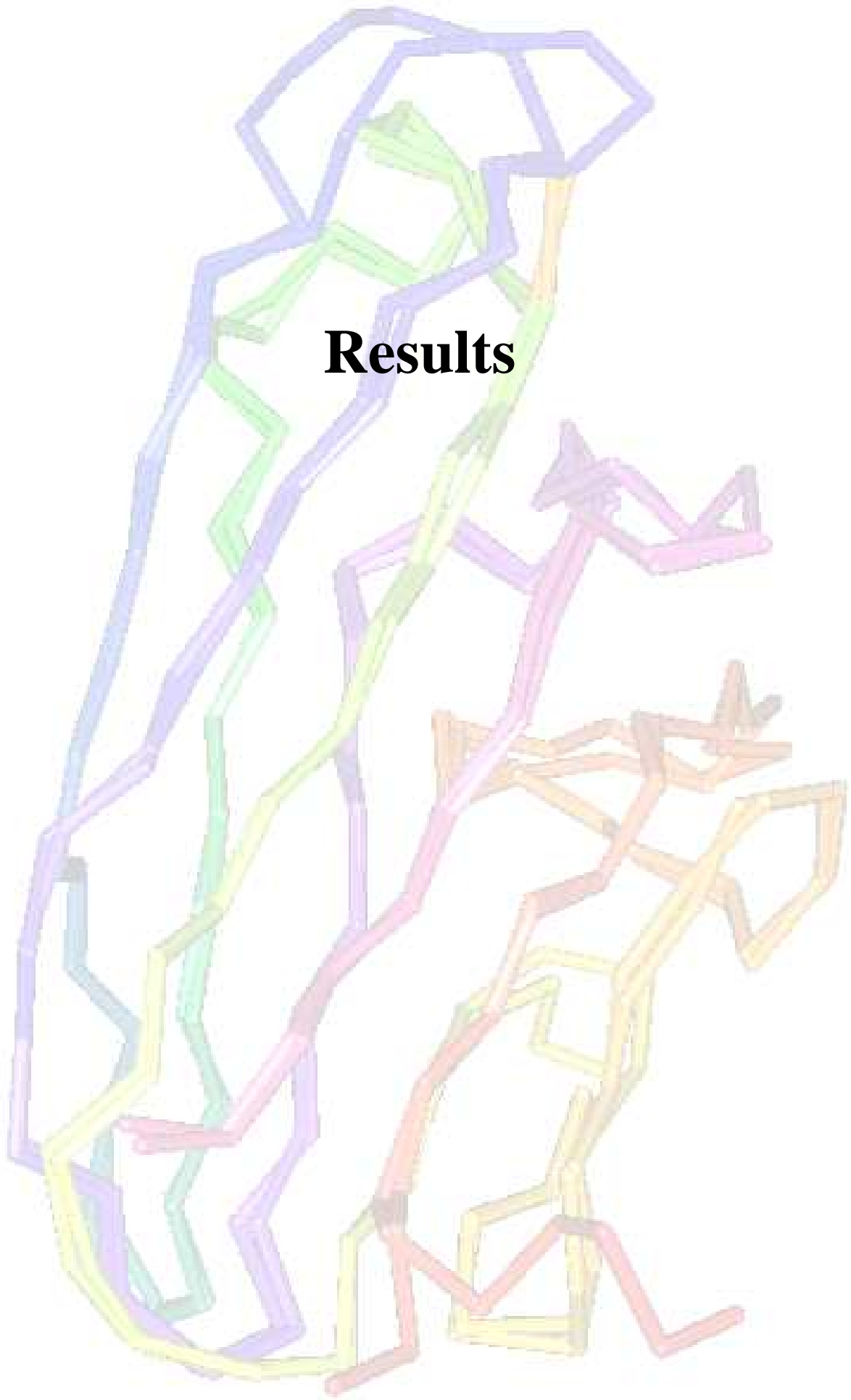
RESULTS

1. CD154rVV construction

A two-step procedure has been developed to construct recombinant Vaccinia viruses. In the first step, a plasmid containing a foreign gene 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 of the plasmid vector is inserted into the viral genome by homologous recombination *in vivo* (Falkner et al., 1988). Principle of this technology (Hruby, 1990) is summarized in Figure 24.
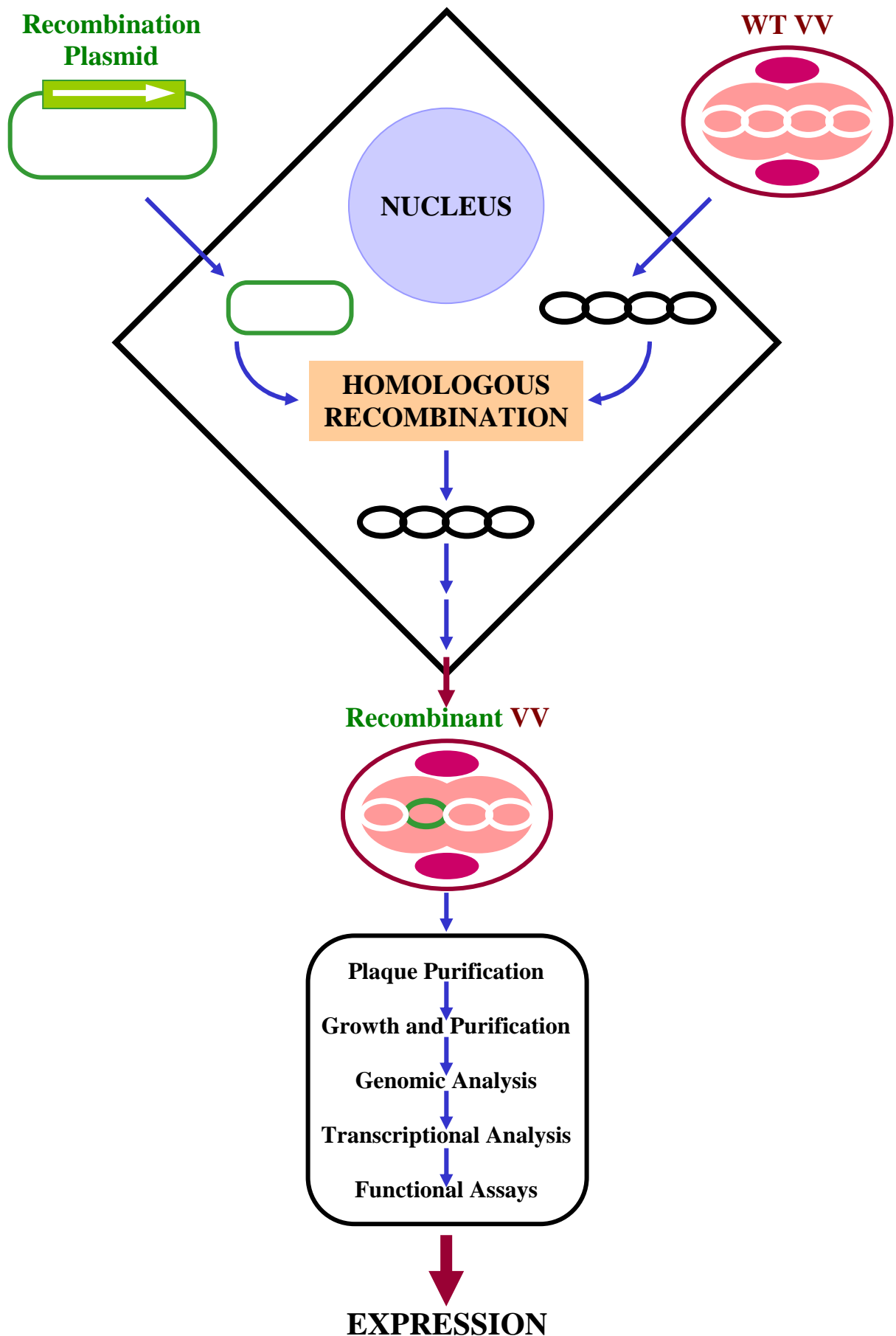
Figure 24. Principle of recombinant Vaccinia virus construction.
A foreign gene controlled by VV promoter and flanked by sequences derived from non essential site on the viral genome is encoded by a plasmid, and inserted into the viral genome by homologous recombination *in vivo*. Recombinant Vaccinia virus are selected, purified, grown and the presence of the transgene is verified by genomic, transcriptional and functional analysis.

Human PBMC from an healthy donor were stimulated by PHA (5 µg/ml) and IL-2 (10 U/ml) for 24 and 48 hours. Cells were collected, washed and total RNAs were extracted. After DNase treatment and Reverse Transcription, CD154 cDNA was PCR amplified (Photo 1).
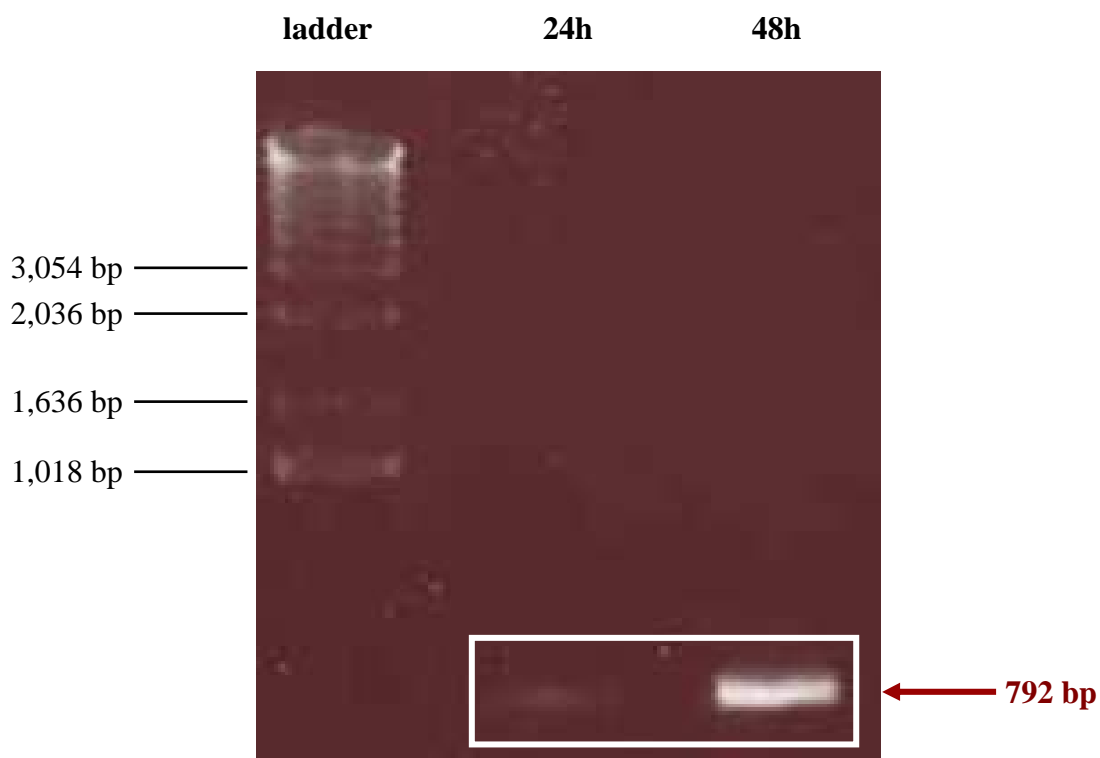
Photo 1: CD154 cDNA PCR amplification.
Human PBMC from a healthy donor were stimulated with PHA (5 µg/ml) and IL-2 (10 units/ml) for 24 and 48 hours. After RNA extraction, DNase treatment and Reverse Transcription, CD154 cDNA was PCR amplified and the expected 792 bp bands corresponding to the CD154 cDNA were cut out for the next steps.

CD154 cDNA was inserted by BamHI-EcoRI restriction into pKT1323 (generous gift from Dr. K. Tsung (San Francisco, CA)), between a Vaccinia virus early promoter and an early transcriptional termination sequence (Davison et al., 1989a). *Escherichia coli* guanine phosphoribosyl transferase (gpt) gene was used as a transient marker for selection of recombinant Vaccinia virus (Falkner et al., 1990). The plasmid was transformed in *E. coli* by electroporation.

Restriction analysis of 4 clones was performed by using either *BamHI + EcoRI* or *PvuII* (5' CAG ⏐ CTG 3', (Invitrogen, Carlsbad, CA)) restriction enzymes.
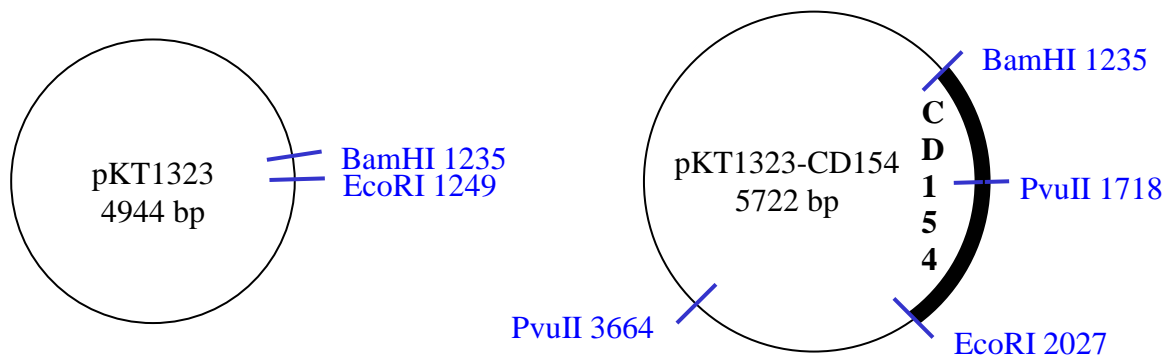
Selected clones were analyzed by restriction profile and sequencing confirmed the presence of CD154 insert. Nevertheless, the natural sequence of CD154 contains two TTTTTNT sequences, which are known to be STOP sequences for Vaccinia virus early transcription (Yuen et al., 1987). Point mutations in the these sequences may result in production of full-length early mRNAs in the absence of modifications of the amino acids sequence (Earl et al., 1990). Therefore, to maintain the expression of the entire CD154 protein, mutation of the two nucleotides sequences were performed as described below.

This basic procedure used for mutagenesis in plasmid sequence utilizes two synthetic oligonucleotide primers containing the desired mutations. In our case, as the two mutation points were close, we could design oligonucleotides with both desired mutations as shown below:

CD154-mutFor:

5'-GCA TGA AAA TAT TTA T GT ATT TAC TTA CTG TGT TTC TTA TCA C-3'

Ile

Nucleotide 1309: A instead of T

T was replaced by A because it conserved the resulting amino acid (Ile) and created a *SspI* site (5'AAT ⏐ ATT3').

CD154-mutRev:

5'-GTG ATA AGA AAC ACA GTA AGT AAA TAC ATA AAT ATT TTC ATG C-3'

Val

Summarize:

CD154mutFor

5'-AGC ATG AAA ATA TTT ATG TAT TTA CTT ACT GTG TTT CTT ATC ACC CAG-3'

3'-TCG TAC TTT TAT AAA TAC ATA AAT GAA TGA CAC AAA GAA TAG TGG GTC-5'

CD154mutRev
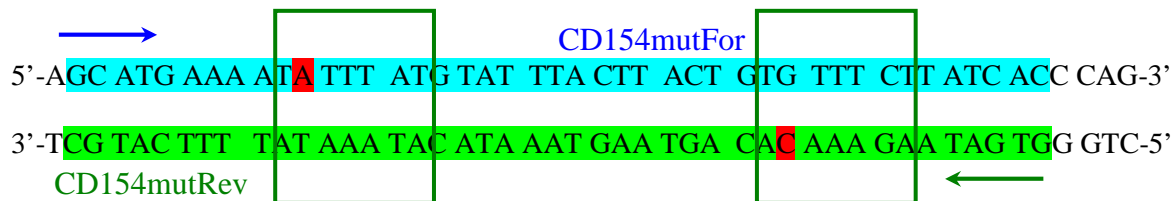
To generate a recombinant Vaccinia virus expressing CD154 (CD154rVV), the CD154 gene encoded by the mutated plasmid was inserted by homologous recombination into the Copenhagen Wild Type strain (WT) of Vaccinia virus (generous gift from Dr. R. Drillien, Strasbourg, France). Recombinant viral clones were selected according to their transient expression of the *E. coli gpt* marker, by several cycles of "plaque picking" under the selective pressure of MPA, xanthine and hypoxanthine (described in materials and methods). Additional selection rounds without pressure enable to eliminate the selection marker. Isolated plaques were amplified on confluent CV-1 cells and PCR tested to verify insertion. CD154rVV was then semi-purified from these cultures of infected CV-1 cells, on a 36% sucrose cushion. The resulting concentrated viral suspension was titrated (see one example of VV titration on Photo 2), and finally rendered replication incompetent by psoralen and long-wave UV treatment.
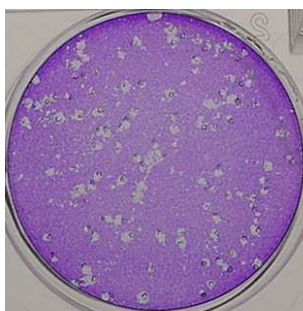
Photo 2. CD154rVV titration.
Confluent CV-1 cells cultured in 6 wells plates, were infected with 100 µl replicative virus 10^{-6} or 10^{-7} or 10^{-8} diluted, and cells were stained after 24 hours infection, to visualize plaque forming units (pfu) induced by the replication of virus. As replicative Vaccinia virus life cycle ends by the lysis of infected cells, one initial virus will induce after replication the formation of a hole visible in the cell monolayer. For staining, medium was removed and cells were stained with 0.1% violet crystal.

Viral concentration (pfu/ml) = number of pfu x 10 x dilution factor
Here, viral concentration = 123 x 10 x 10^6 pfu/ml

2. CD154 expression

2.1. CD154 protein expression on CD154rVV infected CV-1 cells

The capacity of the recombinant Vaccinia virus to express CD154 recombinant protein was verified by infecting constitutively CD154 negative CV-1 cells and using as controls non infected or Wild Type (Control VV) infected cells. Infections were performed either with

replicative virus at 5 m.o.i. or with replication-incompetent (psoralen long-wave UV inactivated: PLUV) virus at 20 m.o.i.. Cell surface expression of CD154 was verified by staining with an antibody specific for human CD154 and analyzed by flow cytometry.

As shown in figure 25, CD154 is indeed expressed on the surface of all CV-1 cells infected with replicative CD154rVV, whereas non infected cells and CV-1 cells infected with the replicative Control VV do not display CD154 expression. Similarly, CV-1 cells infected with PLUV inactivated CD154rVV express the recombinant CD154 protein while PLUV Control VV infected and non infected cells do not, thus demonstrating that expression of the transgene can be observed in high percentages of infected cells, although the psoralen long-wave UV treatment is affecting long gene expression (Tsung et al., 1996).
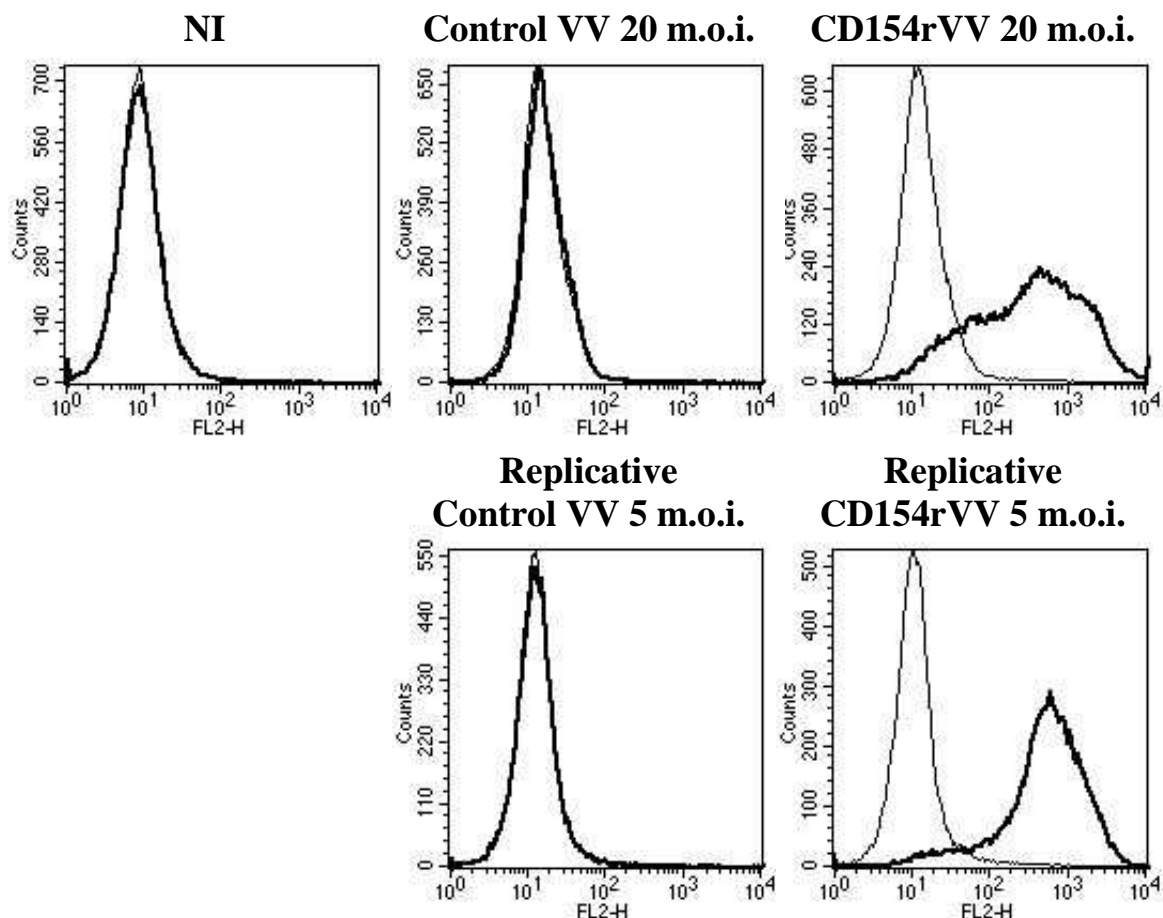
Figure 25. Flow cytometric analysis of the cell surface expression of CD154 in CD154rVV infected cells.
CV-1 cells (constitutively CD154 negative) were infected either with the replication-incompetent CD154rVV (PLUV) or with PLUV control VV at m.o.i. 20, or with the replicative CD154rVV or control VV at 5 m.o.i., and cultured overnight. Cells were stained with either PE-labeled anti-CD154 mAbs (**thick line**) or with Isotype control antibodies (thin line), washed, fixed with paraformaldehyde 1%, and subsequently analyzed by flow cytometry. Non infected (NI) CV-1 cells were used as negative control.

We also confirmed the relation between the dose of inactivated virus and the number of cells expressing the recombinant protein on their surface. As shown in figure 26, infection at a viral dose of 5 m.o.i. of CV-1 cells with PLUV CD154rVV, results in two populations, one clearly negative regarding CD154, and another one expressing the recombinant protein following to infection. With a higher viral dose (10 m.o.i.), the number of cells positive increases and reaches almost 100% of efficiently infected cells above 20 m.o.i..
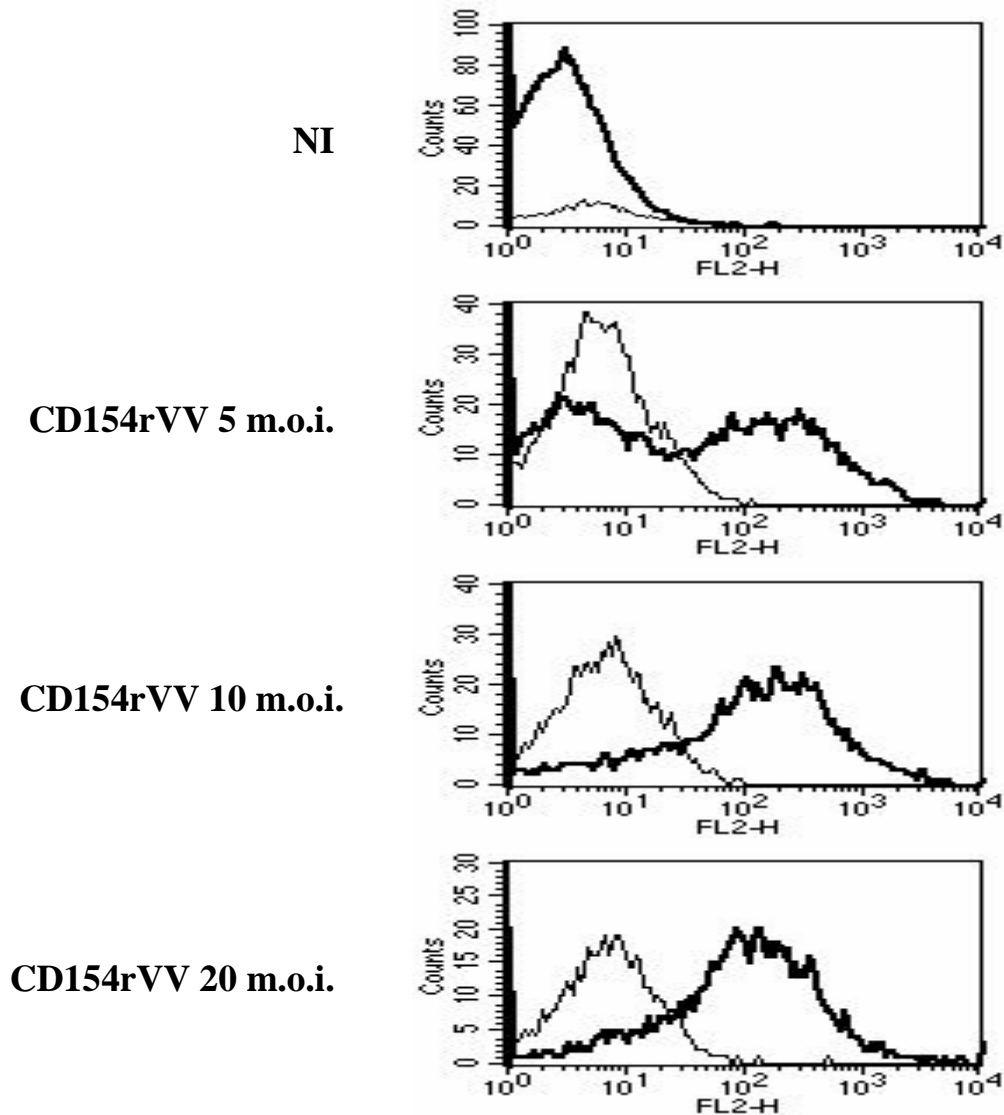
Figure 26. CD154 cell surface expression in CD154rVV infected cells as function of viral doses.
CV-1 cells were infected either with the replication-incompetent CD154rVV (PLUV) at 5, 10 or 20 m.o.i., and cultured overnight. Cells were stained with either PE-labeled anti-CD154 mAbs (**thick line**) or with Isotype control antibodies (thin line), washed, fixed with paraformaldehyde 1%, and subsequently analyzed by flow cytometry. Non infected (NI) CV-1 cells were used as negative control.

2.2. CD154 gene expression in CD154rVV infected monocytes upon 36 h infection

Following T cell receptor triggering on CD4+ T cells *in vitro*, CD154 surface expression is maximal from 8 hours following activation. Subsequently, the surface expression of CD154 is rapidly downregulated to background level within 24 hours following stimulation (Yellin et al., 1994b). In contrast, it is known that foreign gene expression induced by recombinant Vaccinia virus rendered replication-incompetent by psoralen and long-wave UV treatment, is sustained and still effective for at least three days after infection (Tsung et al., 1996). Because T cells are not susceptible to Vaccinia virus infection, we therefore comparatively verified the level of CD154 gene expression in infected CD14+ cells upon 36 hours infection, and in CD4+ T cells stimulated by culture with infected monocytes.

Figure 27 is displaying CD154 gene expression in infected monocytes and in total PBMC, upon 36 hours infection. CD4+ T cells cultured with autologous non infected PBMC are used to quantify the background level of CD154 gene expression in CD4+ T cells after 36 hours *in vitro* culture without any stimulation. As complementary control, we quantified CD154 gene expression in CD4+ T cells stimulated by culture with autologous PBMC, including monocytes infected with PLUV control VV. As expected, PLUV CD154rVV infected but not non infected and control VV infected monocytes, are expressing the CD154 recombinant gene, to a dramatically higher level (47.83 times more at m.o.i. 10) than the CD154 background level expressed in CD4+ T cells after 36 hours culture without any stimulation. These dose-dependent results are also observed in total PBMC including infected CD14+ cells, even if CD154 gene expression level is lower because of CD14+ cells dilution in total PBMC. On the other hand, CD4+ T cells cultured with total PBMC including control VV infected monocytes only displayed marginal CD154 gene expression upon 36 hours culture, since CD154 gene expression is similar to the background level of CD4+ T cells cultured without any stimulation.
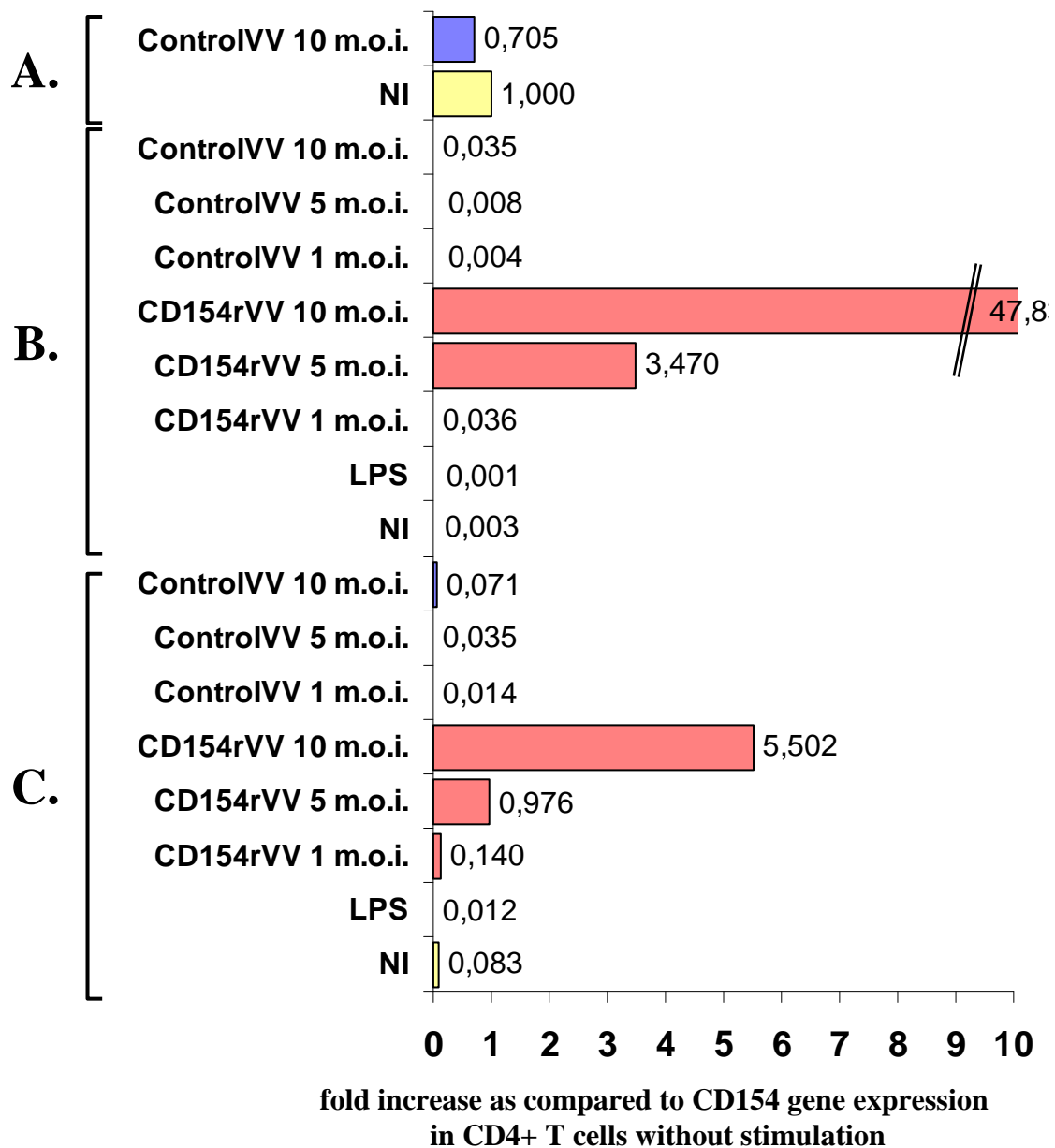
Figure 27. CD154 gene expression in PBMC upon CD14+ infection.
$4x10^6$ CD14+ cells were infected with the replication-incompetent (PLUV) CD154rVV or Control virus at 1, 5 or 10 m.o.i. and cultured with $10x10^6$ CD14-. Non Infected (NI) CD14+ and cells cultured with LPS (1 μg/ml) were used as controls. CD4+ and CD14+ cells were sorted after 36 hours co-culture using microbeads, and CD154 gene expression was analyzed by quantitative real-time PCR in **(A.)** CD4+ sorted cells cultured with infected CD14+ cells and the rest of the CD14- cells fraction, **(B.)** infected CD14+ cells alone and **(C.)** in total PBMC including infected CD14+. Data are expressed as fold increase as compared to CD154 expression in CD4+ cells culture with total non infected PBMC.

These results confirm that CD154rVV is expressing CD154 gene in infected cells, and that this expression is still significantly higher 36 hours after infection in infected monocytes than in stimulated CD4+ T cells. This prolonged CD154 expression may be an advantage in our context: the longer the monocytes are expressing CD154 upon CD154rVV infection, the longer this molecule should have a potential effect on APC activation by "cross self-stimulation" and T cell priming.

2.3. CD154 gene expression as activation marker for T cell stimulated by CD154rVV infected monocytes

Following T cell receptor triggering of CD4+ T cells *in vitro*, CD154 surface expression is maximal from 8 hours following activation and rapidly decreases to a background level. To verify if recombinant CD154 expressed by CD154rVV infected monocytes could, eventually, prolong CD4+ T cells activation, we also monitored CD154 gene expression in T cells following 36 hours culture with infected monocyte (Figure 28). Infected CD14+ cells fraction from PBMC were cultured with autologous CD14- cells fraction. After 36 hours culture, CD4+ and CD8+ T cells were sorted using micro-beads, and CD154 gene expression subsequently analyzed. CD4+ T cells cultured with autologous non infected PBMC were used to quantify the background level of CD154 gene expression after 36 hours *in vitro* culture without any stimulation.
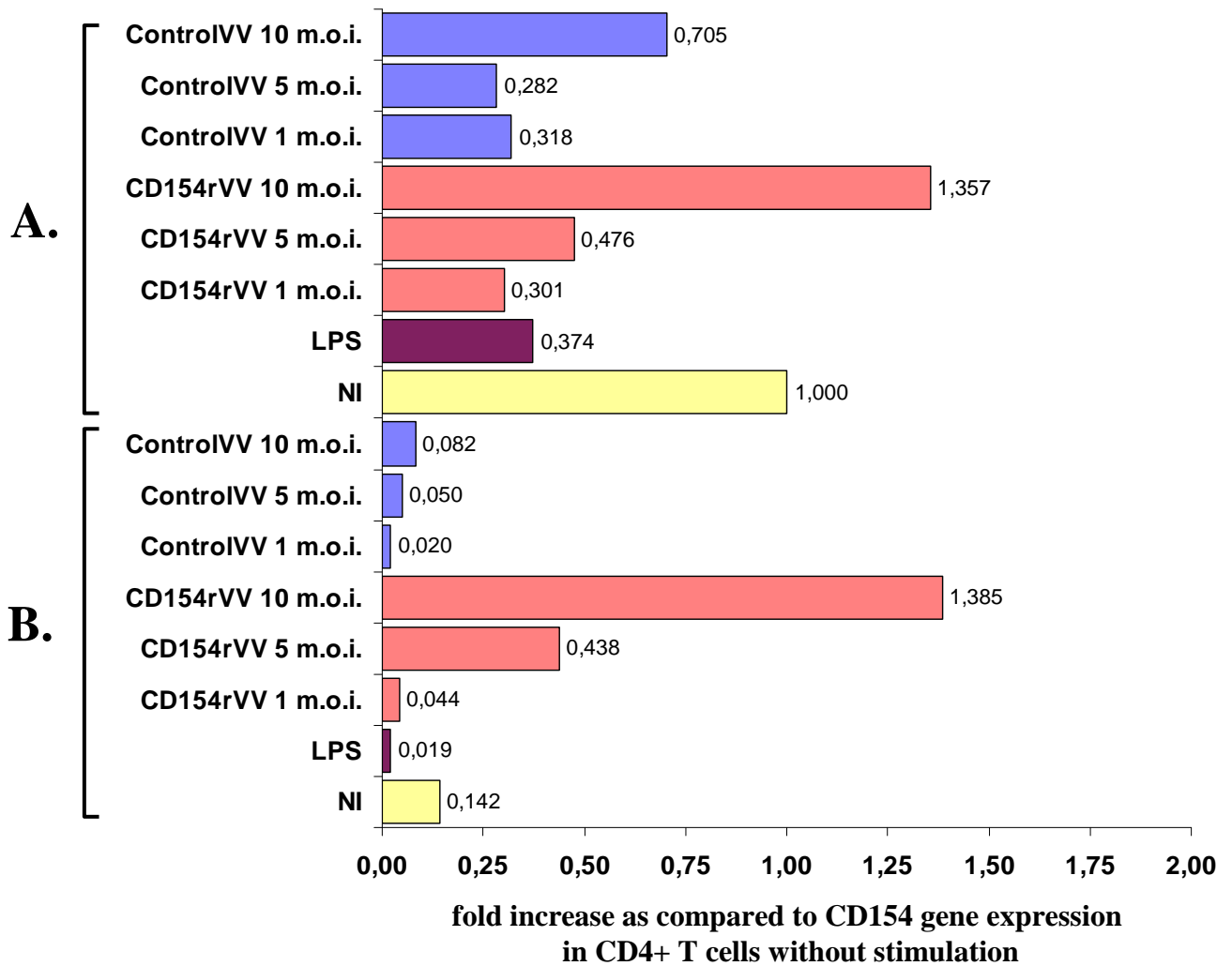
Figure 28. CD154 gene expression on T cells induced by culture with infected CD14+.
$4x10^6$ CD14+ cells were infected with the replication-incompetent (PLUV) CD154rVV or Control virus at 1, 5 or 10 m.o.i. and cultured with $10x10^6$ CD14-. Non Infected (NI) CD14+ and cells cultured with LPS (1 μg/ml) were used as controls. CD4+ and CD8+ cells were sorted after 36 hours co-culture using microbeads technology, and CD154 gene expression was analyzed by quantitative real-time PCR in **(A.)** CD4+ and **(B.)** CD8+ cells cultured with infected CD14+ cells and the rest of the CD14- cells fraction. Data are expressed as fold increase as compared to CD154 expression in CD4+ cells culture with total non infected PBMC.

Regarding CD4+ T cells activation upon 36 hours stimulation with infected monocytes, no major differences were observed in CD154 gene expression by CD4+ T cells cultured either with CD154rVV or Control VV infected monocytes. In addition, level of CD154 gene expression is similar to the one observed in non stimulated CD4+ T cells. It seems that recombinant CD154 expressed on CD154rVV infected monocytes is not prolonging CD4+ T cell activation to 36 hours, or at least, CD154 induction at their surface.

Interestingly, CD8+ T cells cultured with CD154rVV infected CD14+ cells for 36 hours, showed a marked CD154 gene expression induction as opposed to CD8+ T cells cultured with non infected or Control VV infected monocytes. It seems that CD154rVV infected monocytes are acting, for at least 36 hours, on CD8+ T cells, which respond by CD154 induction on their cell surface.

3. Impact of CD154rVV on Antigen Presenting Cells

3.1. Direct and indirect APC stimulation

CD154rVV impact on Antigen Presenting Cells (APC) was evaluated in two different culture setting.

Because T cells are not susceptible to Vaccinia virus infection, the first method consists in a direct APC infection: CD154 recombinant protein expressed on the surface of CD154rVV infected APC will bind to the CD40 expressed on the neighbouring APC. This binding results in APC stimulation, in a so called "cross self-stimulation".

However, intradermal injection of VV is likely to result in the massive infection of fibroblasts, present in a large numbers in these districts where comparatively few putative APC are present. Thus, we were also interested in indirect effects of fibroblasts infection on non infected APC. In this second setting, CD154 expressed on the surface of CD154rVV infected fibroblasts binds the CD40 expressed on APCs, resulting in an "indirect" APC stimulation.

3.2. Apoptosis induction upon PLUV Vaccinia virus infection

Since we are using Vaccinia virus as vector, we have to deal with some apoptosis induction, even if we are using PLUV inactivated viruses. Because it is known that CD154 is able to inhibit APC apoptosis, we investigated whether the recombinant Vaccinia virus expressing CD154 is inducing less apoptosis as compared to Wild Type virus used as control.

We first evaluated the impact of PLUV CD154rVV on infected CD14+ cells, since in the majority of our experiments, we used monocytes to demonstrate the effects of this virus on APCs. As shown in figure 29, even if we are using PLUV inactivated virus as vector, the Control virus increases to some extent the background amount of apoptotic and dead monocytes, as compared to non infected cells. This amount is nevertheless dramatically lower than the one observed in the presence of replicative virus (data not shown). Nevertheless, we showed that at same m.o.i., CD154rVV is inducing slightly lower apoptosis and necrosis of infected CD14+ cells. Even if non significant, these small differences were observed in the majority of the experiments performed.

A.

B.

Figure 29. Inhibition of apoptosis and cell death in infected CD14+ cells.
2×10^6 CD14+ cells were infected with the replication-incompetent (PLUV) CD154rVV or Control virus at 10 m.o.i. and cultured overnight. Non infected cells (NI) were used as control. Cells were stained with PI/Annexin V and the percentages of (**A.**) apoptotic and (**B.**) necrotic cells were evaluated by flow cytometry.

We then considered the impact of PLUV CD154rVV on iDC apoptosis. As shown in figure 30, iDC appear to be markedly more sensitive to infection than monocytes, since apoptotic and necrotic cells reached at 8 m.o.i. 59,69% of cells, as compared to 22,77% for CD14+ cells infected with the same virus at 10 m.o.i.. Consequently, we had to choose lower m.o.i. (<5) for the long-term experiments in which we were using only DC, to maintain a reasonable amount of infected cells alive. Nevertheless, as shown on this figure, similarly to infected CD14+, we demonstrated that PLUV CD154rVV was inducing slightly less apoptosis and cell death as compared to the control virus, especially at low m.o.i..

A.

Control VV 8 m.o.i.	9,49
Control VV 4 m.o.i.	7,74
Control VV 1 m.o.i.	7,09
CD154rVV 8 m.o.i.	7,74
CD154rVV 4 m.o.i.	7,08
CD154rVV 1 m.o.i.	4,29
NI	0,30

Percentage of apoptotic iDC

B.

Control VV 8 m.o.i.	50,20
Control VV 4 m.o.i.	33,65
Control VV 1 m.o.i.	12,24
CD154rVV 8 m.o.i.	44,19
CD154rVV 4 m.o.i.	23,52
CD154rVV 1 m.o.i.	6,17
NI	0,89

Percentage of necrotic iDC

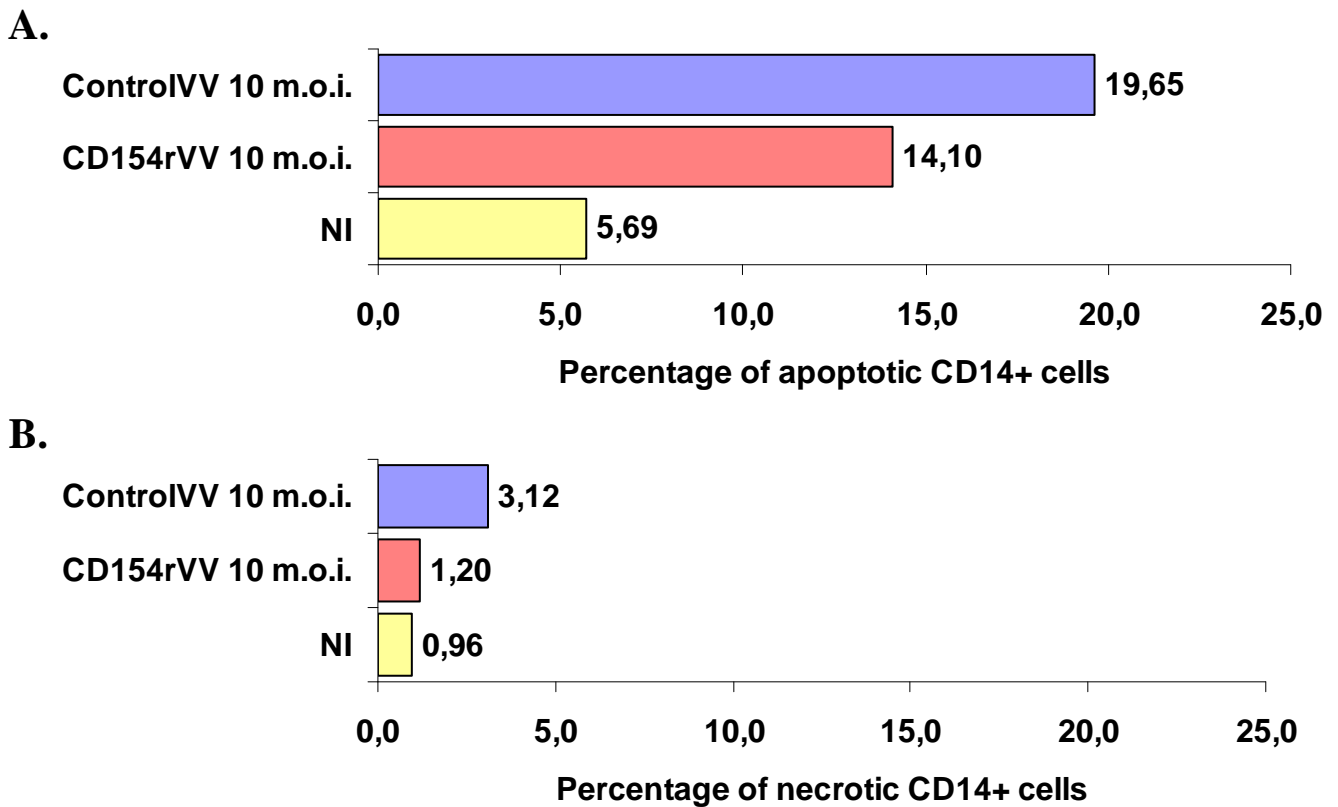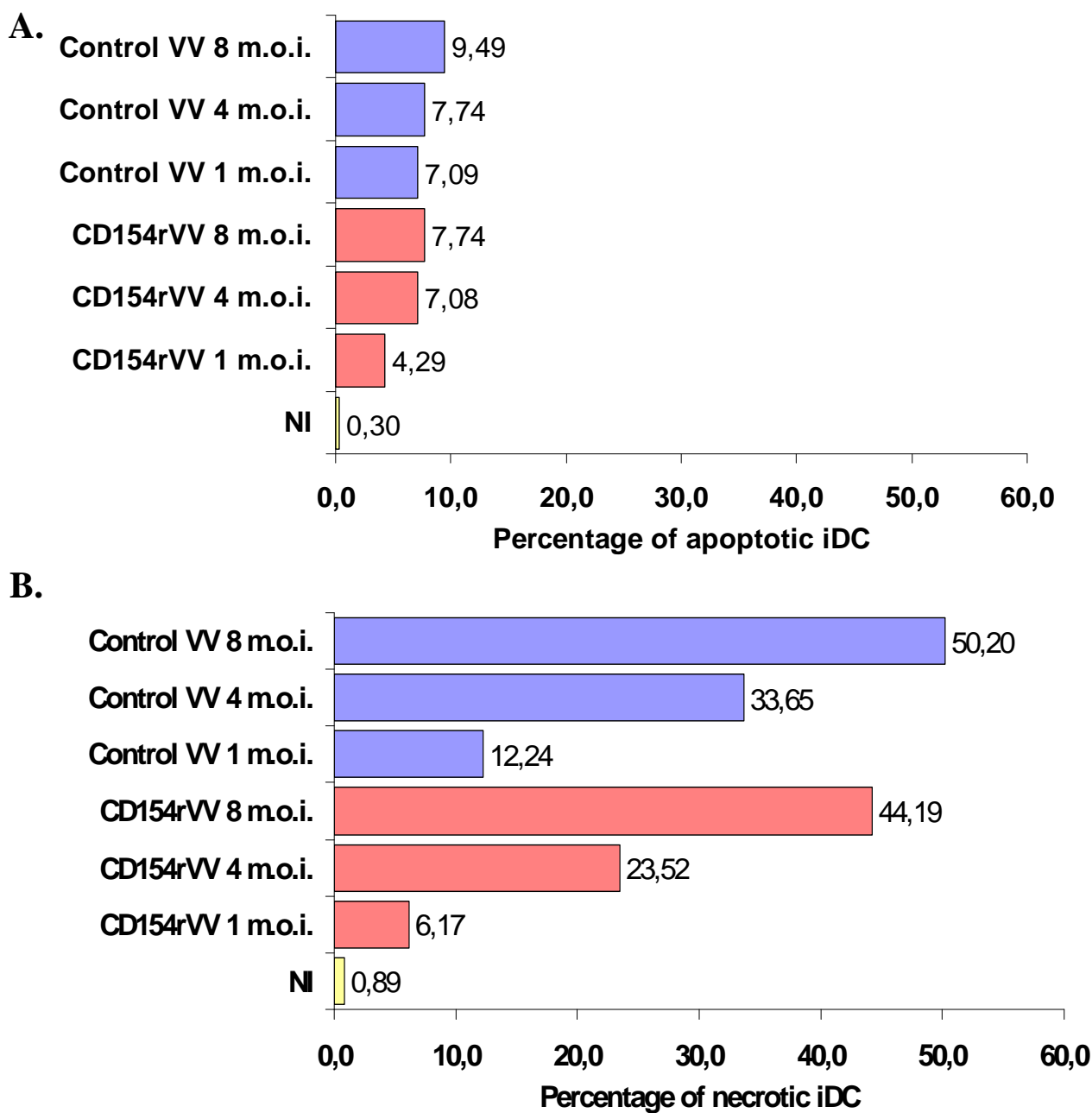**Figure 30. Inhibition of apoptosis and cell death in infected iDC cells**.
5 x 10^5 iDC cells were infected with the replication-incompetent (PLUV) CD154rVV or control virus at 1, 4 or 8 m.o.i. and cultured overnight. Non infected cells (NI) were used as control. Cells were stained with PI/Annexin V and the percentages of (**A.**) apoptotic and (**B.**) necrotic cells were evaluated by flow cytometry.

We also tried to evaluate the impact of PLUV CD154rVV on iDC comparing direct and indirect system in which non infected iDC were co-cultured with infected allogenic fibroblasts. As shown in figure 31, although background level of cell death in the absence of infection is, in this experiment, quite high (≈17 %), cell death following direct iDC infection was increased, as compared to non infected cells. Even if the difference is not significant, it seems that CD154rVV infected iDC die slightly less (25,94 %) than iDC infected with the Control virus (27,86 %). iDC death seems to decrease when cultured with infected fibroblasts as compared to the directly infected iDC (21,48 % with Control virus infected Vero versus 27,86 % for directly infected iDC). Even if not significant, this effect was even slightly more beneficial in the condition infected with CD154rVV (13,35 % of dead iDC).
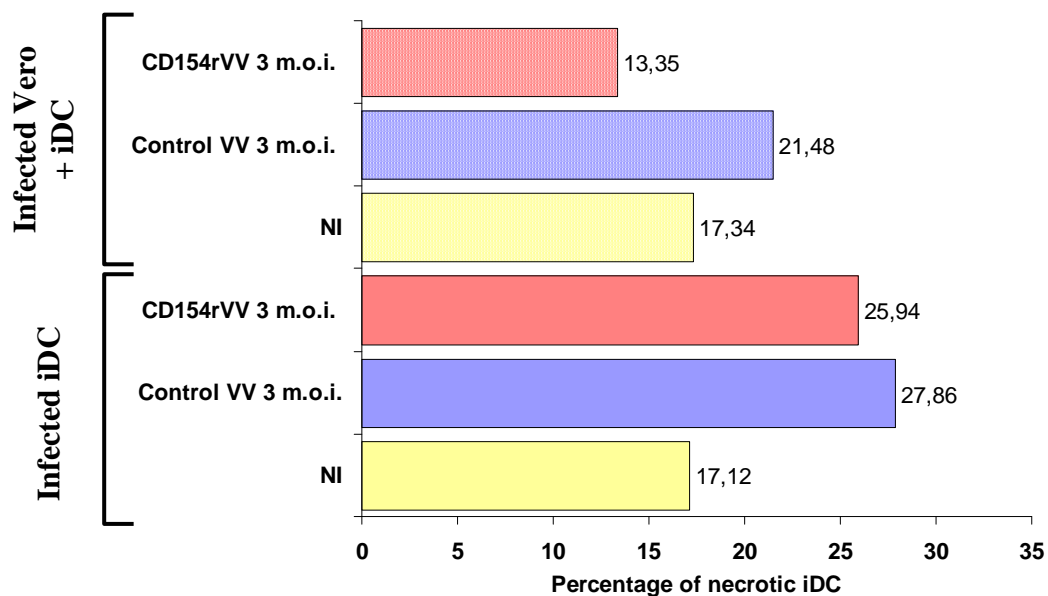
Figure 31. Inhibition of iDC cell death when co-cultured with infected fibroblasts.
6.5×10^5 iDC cells were either directly infected with the replication-incompetent (PLUV) CD154rVV or Control virus at 3 m.o.i., or non-infected and co-cultured with 1×10^5 infected Vero cells. Dead cells were stained with PI and percentages of dead cells were evaluated after overnight culture by flow cytometry.

In conclusion, in our conditions, even if repeated, APC apoptosis inhibition by CD154 properties seems really weak, and not really significant. One explanation could be that apoptotic events begin immediatly within minutes after infection. Indeed, as shown in figure 32, some iDC seem to die already 1 hour after infection, as compared to non infected iDC. On the other hand, the virus requires some hours to express proteins : CD154 ligand was detectable at the surface of PLUV CD154rVV infected cells only after 3 hours infection. Events induced by CD154/CD40 pathways may be simply later in time than apoptotic events, and subsequently too late to have a real significant beneficial effects on infected cells.

A.
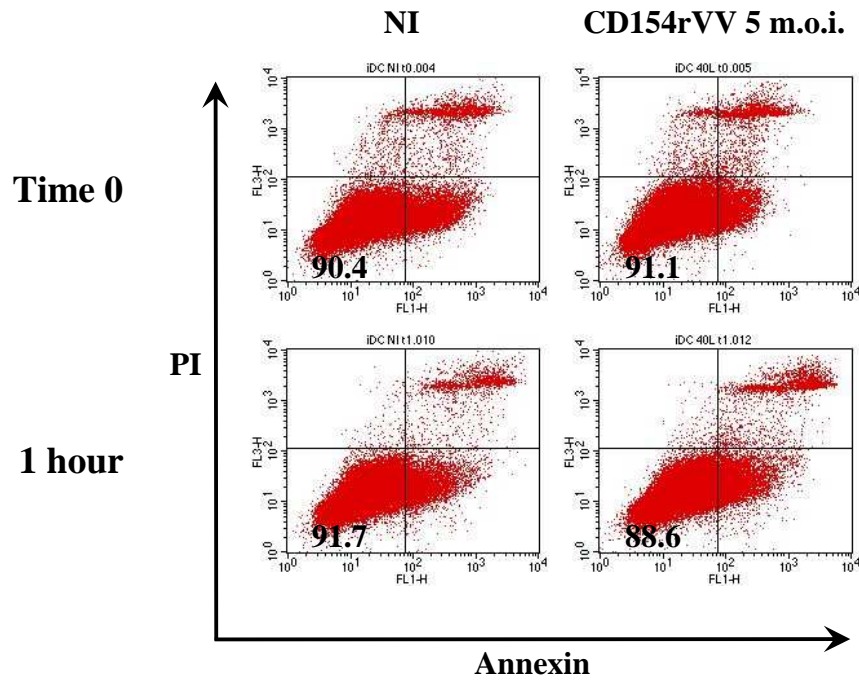
B.
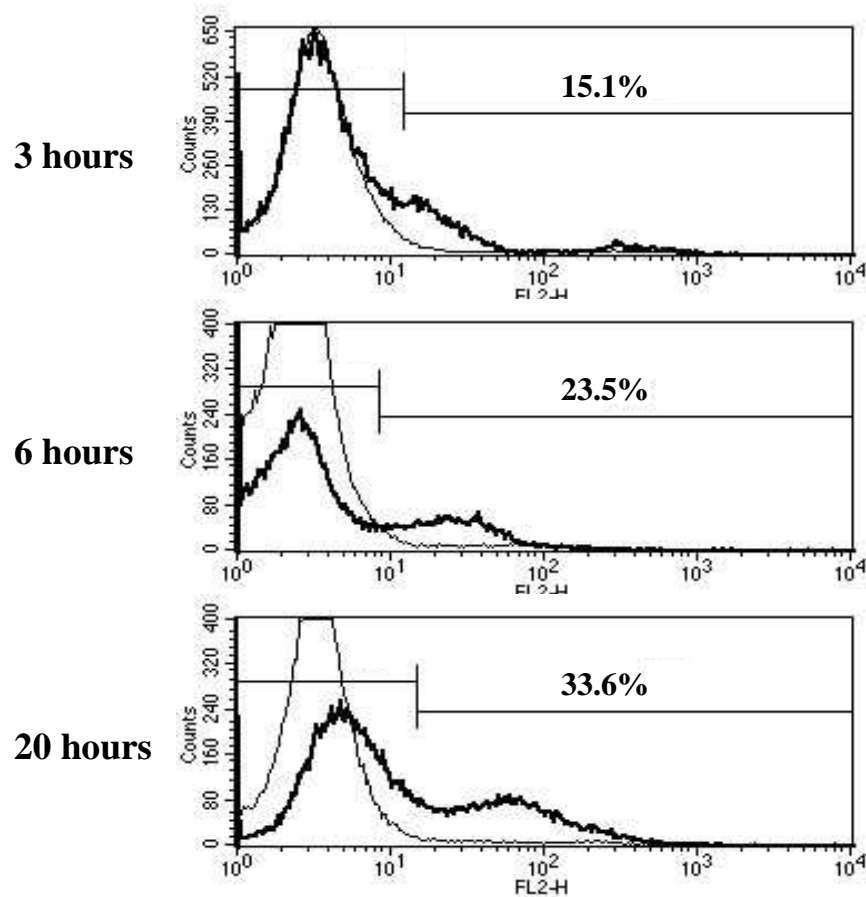
Figure 32. CD154rVV infected iDC: kinetic of CD154 expression on cell surface and apoptosis beginning.
5×10^5 human iDC from a healthy donor were infected with the replication-incompetent CD154rVV (PLUV) at m.o.i. 5. Non infected cells (NI) were used as negative control. **(A.)** Cells were stained with PI/Annexin V and the percentages of cells still alive were evaluated by flow cytometry. **(B.)** Non infected cells (thin line) and CD154rVV infected cells (**thick line**) were stained with PE-labeled anti-CD154 mAbs, washed, fixed with paraformaldehyde 1%, and subsequently analyzed by flow cytometry.

3.3. Induction of cytokines genes expression in APC

3.3.1. Cytokines genes expression induction in directly infected monocytes

The effects of CD154 expression in CD154rVV infected cells on CD40+ antigen presenting cells were then investigated in detail. Indeed, CD40 ligation by its receptor induces in APC the expression of cytokines and surface molecules favoring T helper 1 responses.

IL-12 is a heterodimeric cytokine composed of two different disulfide-linked subunits, p35 and p40 (Kobayashi et al., 1989). Whereas the *IL-12p35* subunit is constitutively expressed in most cell types and post-translationally regulated (D'Andrea et al., 1992), *IL-12p40* expression, is controlled at transcriptional level (Ma et al., 1996) and its up-regulation can be used as marker of CD154/CD40 activation (Cella et al., 1996).

In the experiment shown in figure 33, peripheral blood CD14+ cells from healthy donors, were infected at 10 m.o.i. with CD154rVV or Control VV, and incubated in complete medium for 24 hours. Total cellular RNA was reverse transcribed and cytokines gene expression was quantified by real-time quantitative PCR (qRT-PCR).

As shown in figure 33A, IL-12p40 transcription in monocytes was exclusively detected following CD154rVV infection. Furthermore, as shown in figure 33B and C, expression of GM-CSF and TNF-α genes was indeed also observed following infection with control virus (WT) but at significantly lower levels as compared to those observed upon CD154rVV infection.

A.

IL-12p40

B.

GM-CSF

C.

TNF-α
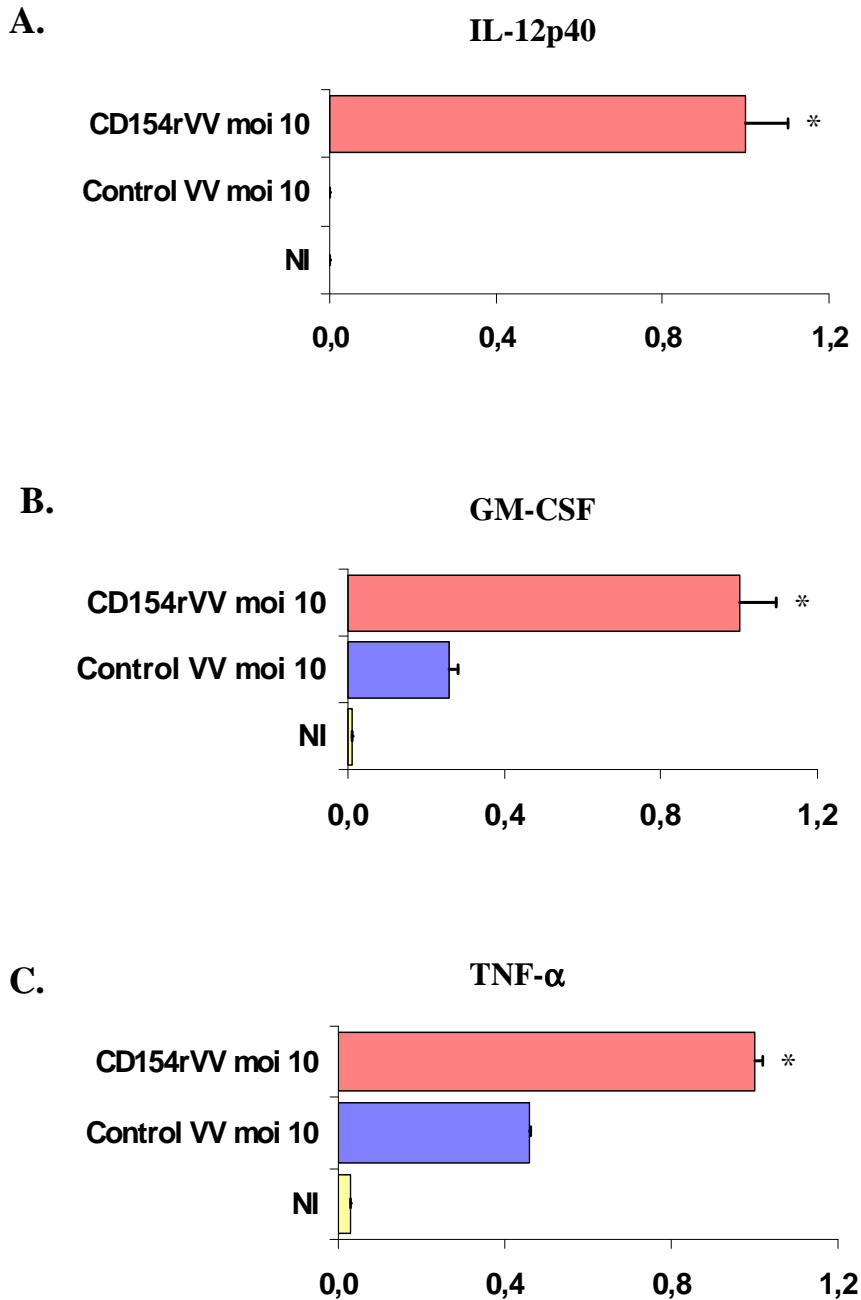
Figure 33. Induction of cytokine genes expression in infected CD14+ cells.
2×10^6 CD14+ cells were infected with the replication-incompetent (PLUV) CD154rVV or control virus at 10 m.o.i. and cultured overnight. Non infected cells (NI) were used as negative control. IL-12p40 **(A.)**, GM-CSF **(B.)** and TNF-α **(C.)** gene expression was analyzed by quantitative real-time PCR and data were expressed as ratio to CD154rVV infected samples. Symbols (∗) indicate values significantly (p< 0.05) different as compared to control VV infected cultures.

IL-6 is a pro-inflammatory cytokine induced during infection (Reviewed in (Hopkins, 2003)). CD154 is known to induce IL-6 secretion by monocytes and dendritic cells (Sugiura et al., 2000; Wesa et al., 2002). This increase in IL-6 secretion may promote IgG secretion accompanied by antibody isotype switching (Urashima et al., 1996). In addition, IL-6 has been shown to render pathogen-specific T cells refractory to the suppressive activity of CD4+ CD25+ T regulatory cells (Pasare et al., 2003). We hypothesized that the effect of CD154rVV on CTL activation could also benefit from an increase in IL-6 induction.

However, as shown in figure 34, probably due to the already strong effect of the virus itself, no significant differences were obtained between CD154rVV and Control virus regarding IL-6 gene expression induction, neither in infected CD14+ or PBMC, nor in CD4+ or CD8+ cells cultured with autologous infected CD14+ cells. Similarly, no differences were observed in infected CD14+ cells cultured alone or with autologous CD14-, CD4+, CD8+ or CD14-CD4-CD8- cells (data not shown). Nevertheless, this demonstrates that inactivated Vaccinia virus by itself can strongly induce IL-6, therefore having a potent effect by itself on T cells. In addition, IL-6 is known to induce *in vivo* migration of human primary T cells but only in the presence of extracellular matrix (Weissenbach et al., 2004), which can also be an obvious advantage for vaccination purpose. Nevertheless, IL-6 also directly favors the polarization of naïve CD4+ T cells to T helper 2 cells (Diehl et al., 2002a; Diehl et al., 2002b), which could hinder T helper 1 effects induced by the virus.
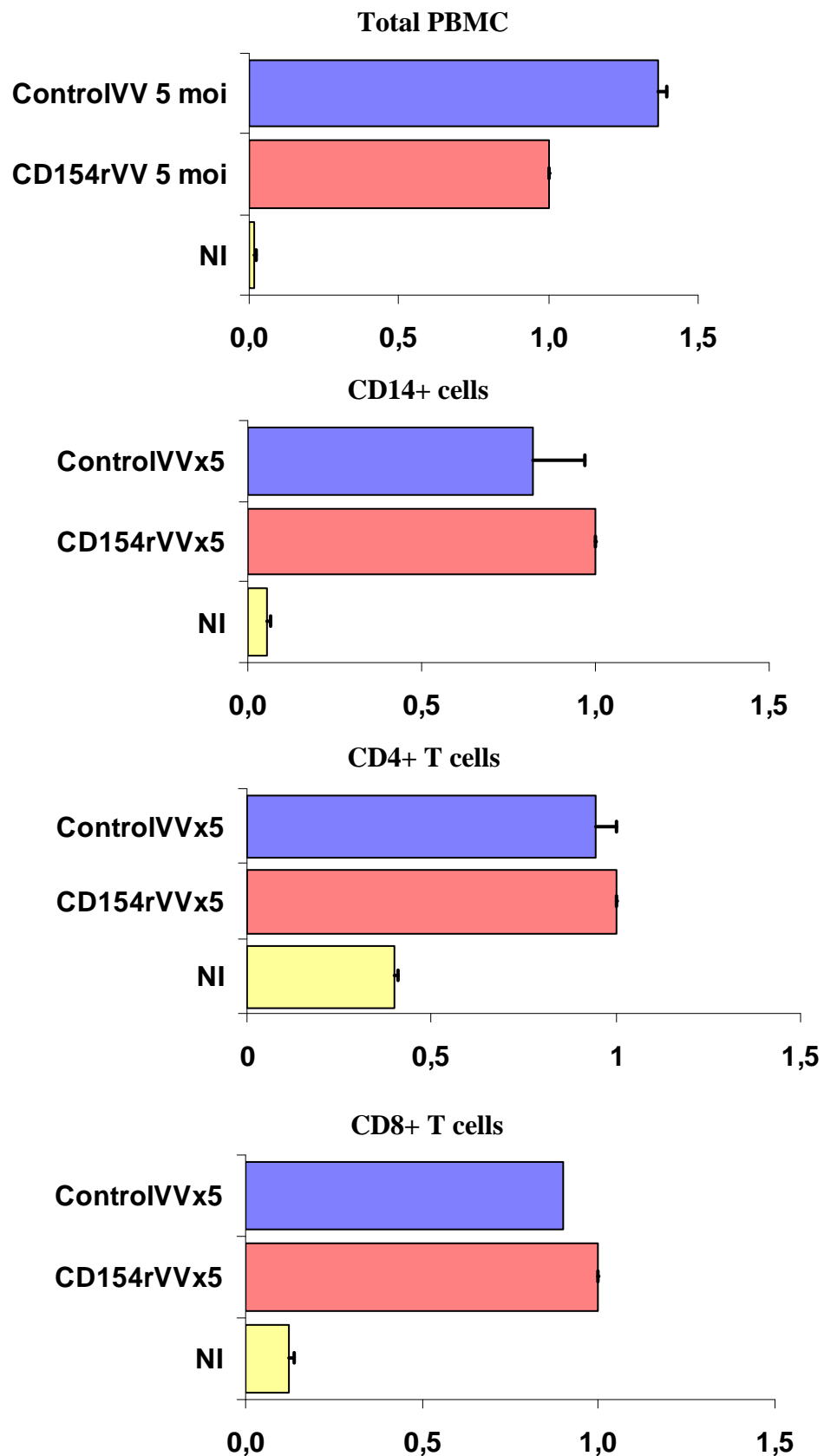
Figure 34. Induction of IL-6 gene expression.
4×10^6 CD14+ cells were infected with the replication-incompetent (PLUV) CD154rVV or control virus at 5 m.o.i. and cultured with 10×10^6 autologous CD14- cells. Non-infected cells (NI) were used as negative control. CD14+, CD4+ and CD8+ cells were sorted after 36 hours co-culture using microbeads technology, and IL-6 gene expression was analyzed by quantitative real-time PCR. Data were expressed as ratio to CD154rVV infected samples.

3.3.2. Cytokines genes expression in directly infected monocytes derived iDC

Dendritic cells (DC) are the most potent type of APCs and are responsible for the initiation of immune responses. Located in peripheral tissues and in lymphoid organs, DCs are only suited to detect and capture pathogens. Upon maturation, especially through TLR signaling (Akira et al., 2001), DC are upregulating MHC and costimulatory molecules and expressing pro-inflammatory cytokines. As a result, DC acquire the ability to prime naïve T cells (Cella et al., 1997). For these reasons, we were interested in evaluating the impact of CD154rVV on DC, especially immature DC.

Monocyte derived iDC from healthy donors were infected with CD154rVV or with control virus at different m.o.i.. Untreated iDC were used as additional control. As shown in figure 35, IL-12p40 and GM-CSF gene expression were induced by CD154rVV infection but not by Control virus. In addition, TNF-α and IL-15 gene expression was found to be significantly higher in iDC infected with CD154rVV as compared to control virus.
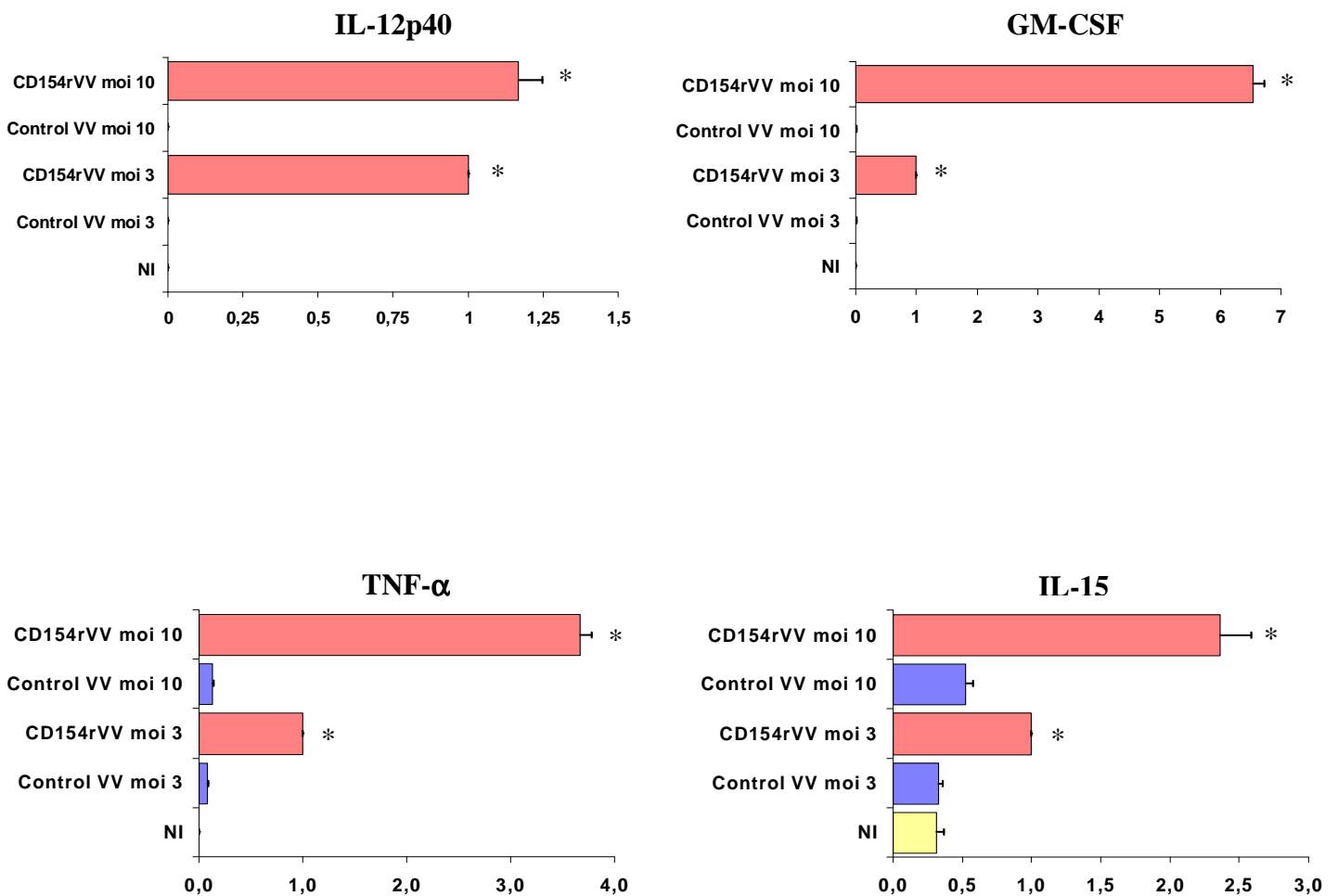
Figure 35. Induction of cytokine genes expression in CD14+ derived iDC.
2×10^6 CD14+ derived iDC were infected with the replication-incompetent (PLUV) CD154rVV or control virus at 3 or 10 m.o.i., washed and cultured in complete medium supplemented with 10 % FCS. Cells were harvested 24 hours after infection. Gene expression was analyzed by quantitative real-time PCR and data were expressed as ratio to the CD154rVV 3 m.o.i. sample. Symbols (∗) indicate values significantly (p< 0.05) different as compared to control VV infected cultures.

3.3.3. Indirect APC stimulation: cytokine genes expression in PBMC after coculture with autologous CD154rVV infected fibroblasts

Intradermal injection of VV is likely to result in infection of fibroblasts. Thus, we were interested in indirect effects of fibroblast infection on uninfected immunocompetent cells.

Primary skin fibroblasts were infected either with CD154rVV, or with WT virus, and co-cultured with autologous total PBMC. Cytokine genes expression was evaluated 48 hours after infection. As shown in figure 36, consistent with results obtained in directly infected APC (see above), IL-12p40 mRNA expression was strongly induced upon CD154rVV infection. Furthermore, background expression of IFN-γ and IL-2 genes in PBMC co-cultured with WT infected fibroblasts, was increased following CD154rVV infection. Thus, CD154 signaling provided by infected bystander cells (here fibroblasts) is sufficient to induce strong APC activation and Th1 cytokine gene expression.
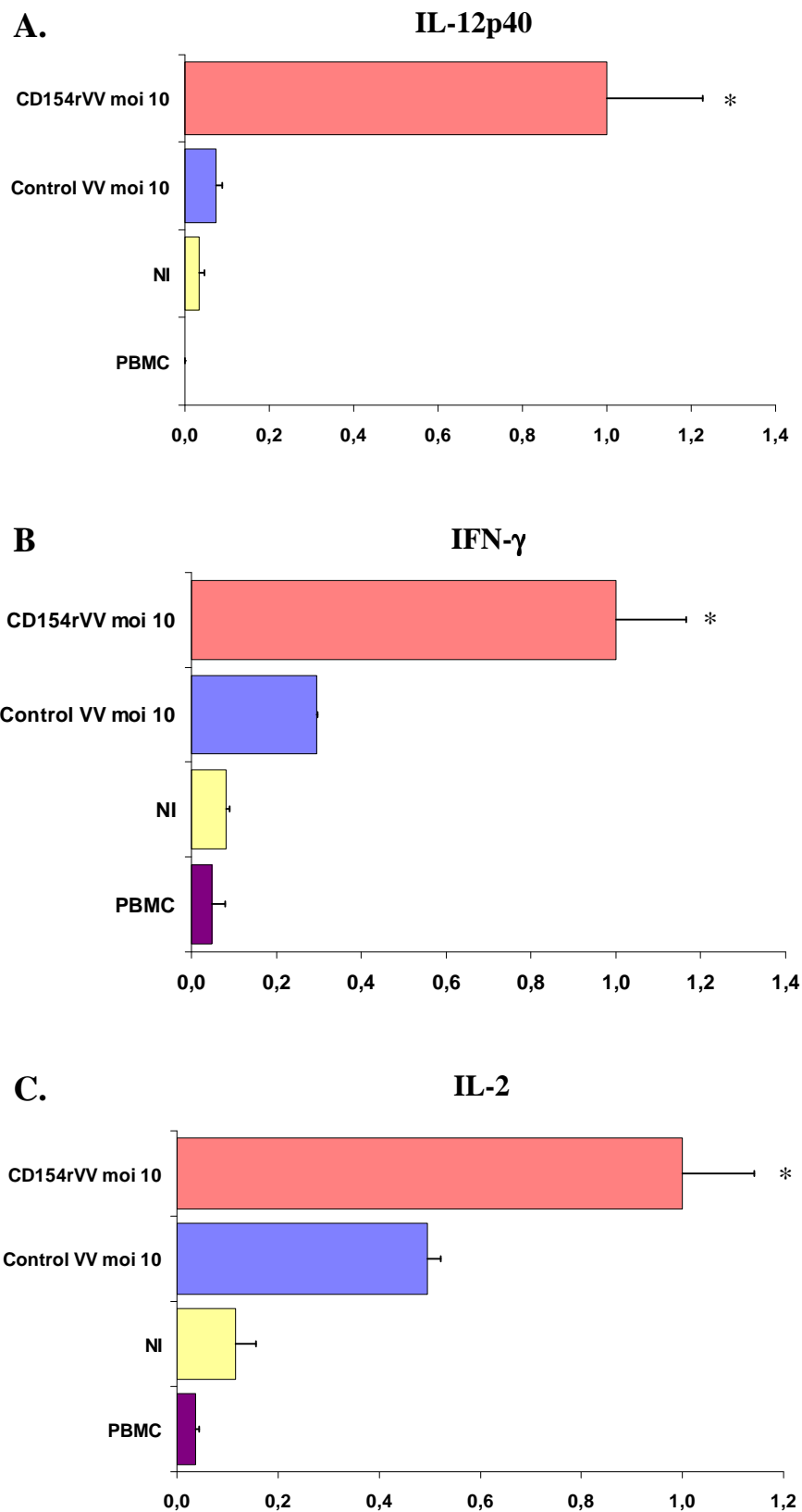
Figure 36. Genes expression in PBMC co-cultured with autologous infected skin fibroblasts.
2.5×10^5 cultured Human Primary Skin fibroblasts were infected with the replication-incompetent (PLUV) CD154rVV or control virus at 10 m.o.i., washed and cocultured for 48 hours with 4×10^6 autologous PBMC. Non infected (NI) skin fibroblasts and PBMC cultured alone were used as controls. Gene expression was analyzed by quantitative real-time PCR and expressed as ratio to CD154rVV infected sample, as reference. IL-12p40 **(A.)**, IFN-γ **(B.)**, IL-2 **(C.)** gene expression. Symbol (∗) indicates values significantly (p< 0.05) different between CD154rVV and control VV infected cultures.

3.4. Blockage of CD154rVV effect by an antagonist anti-CD40 monoclonal antibody

3.4.1. IL-12p40 gene expression inhibition

Taken together, these data suggested strong CD40 triggering by CD154rVV infection on APC. In order to formally confirm whether these effects were indeed due to transgene expression, we performed similar experiments in the presence of an antagonist anti-human CD40 monoclonal antibody (generous gift from M. de Boer, Pan Genetics B.V., the Netherlands). This chimeric antibody was constructed by substituting mouse variable regions on a IgG4 human backbone (mAb ch5D12). This antibody was demonstrated to effectively inhibit a range of APC activities mediated by CD40/CD154 interaction (Laman et al., 2002).

As shown in figure 37A, IL-12p40 gene expression induced in CD154rVV infected CD14+ cells was indeed abolished in the presence of anti-CD40 mAb, whereas it was not affected by the presence of a control antibody, formally demonstrating that effects observed with CD154rVV are due to transgene expression.

3.4.2. IL-12 protein inhibition

These data were also confirmed at the protein level. As shown in figure 37B, the functional IL-12p70 heterodimer was only detected in the supernatant of CD154rVV infected CD14+ cells, but not in the supernatants of control VV infected CD14+ cells. Furthermore, IL-12 secretion induced by CD154rVV was fully prevented by the addition of anti-CD40 monoclonal antibody (mAb ch5D12), but was not affected by a control antibody. These data confirm that CD154rVV is able to induce IL-12p40 gene transcription resulting in IL-12p70 protein production in CD40+ infected cells due to functional ligand expression.
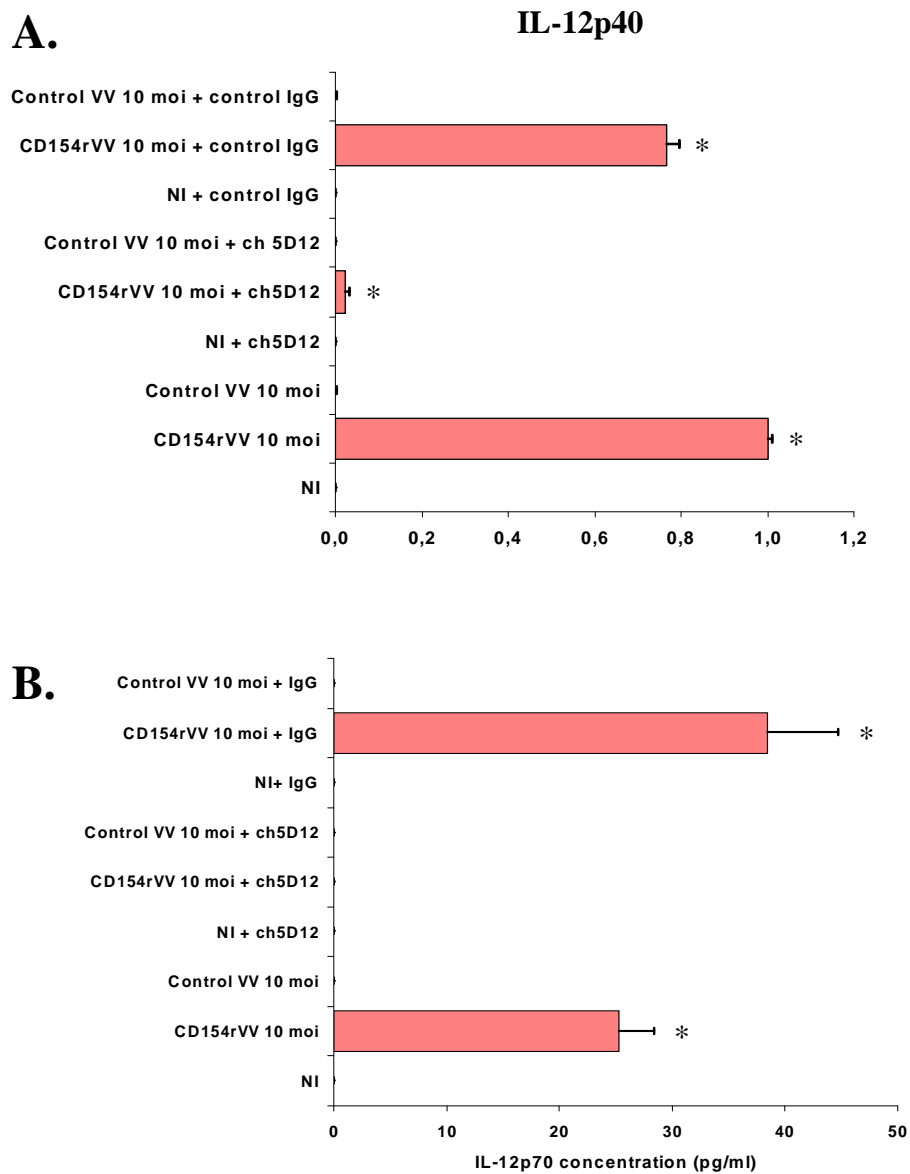
Figure 37. Inhibition of IL-12 expression induction in infected CD14+ cells, by an antagonist anti-CD40 monoclonal antibody.

2 x 10^6 CD14+ cells were infected with the replication-incompetent (PLUV) CD154rVV or control virus at 10 m.o.i. and cultured overnight, in the presence or absence of mAb ch5D12 (10 μg/ml), a chimeric anti-human CD40 monoclonal antibody were the mouse variable regions were placed on a IgG4 human backbone (provided by PanGenetics B.V., Utrecht, The Netherlands), or with an IgG mAb used as control. Non infected cells (NI) were used as negative control. **(A.)** Inhibition of IL-12p40 gene expression induction was analyzed by quantitative real-time PCR and expressed as ratio to a reference sample (infection with CD154rVV at 10 m.o.i. without any mAbs). **(B.)** Inhibition of IL-12p70 secretion was analyzed by ELISA. Symbol (∗) refers to significant (p< 0.05) differences as compared to control VV infected cultures.

3.5. APC activation and iDC maturation

3.5.1. Monocytes activation

3.5.1.1. CD80 and CD86 as activation markers

APC activation by direct infection resulting in CD40 cross ligation, is often characterized by an increased surface expression of costimulatory and MHC molecules. We therefore chose to monitor monocyte activation by following expression of CD80, CD86, and HLA-DR, on the surface of infected CD14+ cells.

Monocytes from healthy donors were infected with CD154rVV or with WT virus as control virus, and effects on surface markers expression were evaluated in comparison to non infected cells. As shown in figure 38, in 1 day infected monocytes, CD86 and CD14 expression are already clearly increased and downregulated respectively, in CD154rVV infected cells as compared to non infected monocytes and cells infected with the control virus. In contrast, CD80 and CD1a remained unmodified at that time point.

 Similar results were observed at 4 days after infection: CD80 and CD86 expression induced by control virus (Mean Fluorescence Intensity or MFI = 15.9 and 681.2 respectively as compared to MFI = 3.1 and 605.7 respectively for NI cells), were enhanced in CD154rVV infected CD14+ cells (MFI = 19.7 and 839.1 respectively). On the other hand, CD14 downregulation induced by control virus (MFI = 589.0 as compared to MFI = 603.9 for NI), was strongly enhanced in CD154rVV infected CD14+ cells (MFI = 346.3). However, CD154rVV infection did not appear to promote differentiation of CD14+ cells into iDC since no CD1a upregulation was observed.

Figure 38. Phenotypic analysis of infected CD14+ cells.
2×10^6 human CD14+ cells from an healthy donor were infected with the replication-incompetent (PLUV) CD154rVV or control virus at 10 m.o.i., washed and cultured for 1 (**A.**) or 4 days (**B.**). Non infected CD14+ cells (NI) were also studied. Cells were stained either with Isotype control antibodies (thin line), or with specific antibodies (**thick line**). Relevant mean channel fluorescence intensities were calculated by subtracting the mean fluorescence corresponding to isotype control values, and are indicated on each panel.

A.
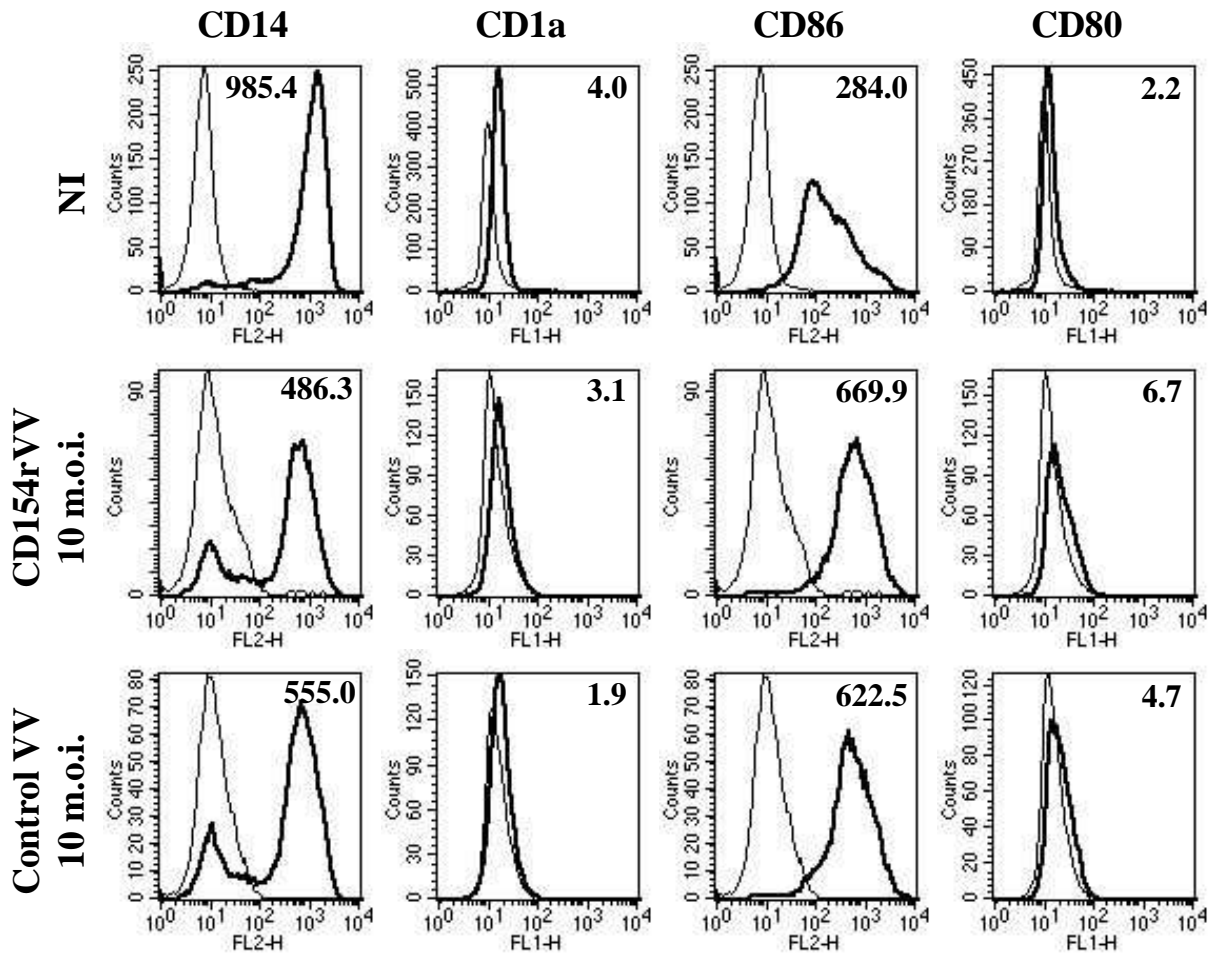
B.
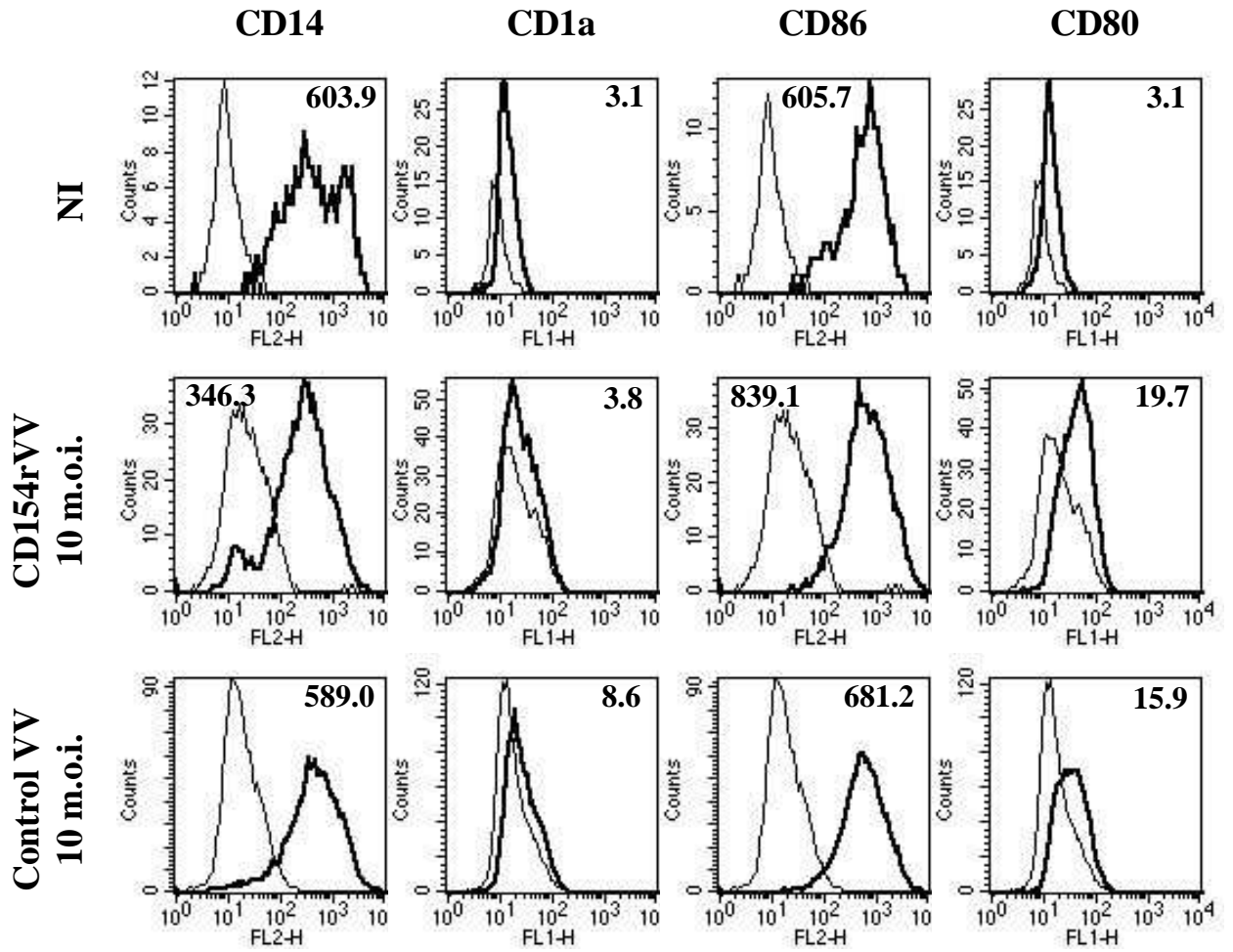
3.5.1.2. HLA-DR as activation marker

Monocyte activation following infection is characterized by MHC molecules up-regulation. Similarly, CD40 ligation on APC is known to trigger ICAM-1 expression. Thus, to further confirm monocyte activation following CD154rVV infection, we also verified MHC and ICAM-1 expression on infected CD14+ cells surface.

Figure 39 displays HLA-DR surface expression on infected monocytes. Infection with PLUV Vaccinia virus induces for one part of the population, already upon 12 hours infection, HLA-DR up-regulation as compared to non infected monocytes. This expression increases over time and concerns almost 100% of the population after 48 hours infection, as opposed to LPS activation which decreases rapidly after 24 hours stimulation. Nevertheless, no differences were observed between the Control virus and the CD154rVV.

Indeed, no significant differences were observed between CD154rVV and Control virus regarding MHC class I molecules and ICAM-1 expression, probably because of the already strong effect of the virus itself.
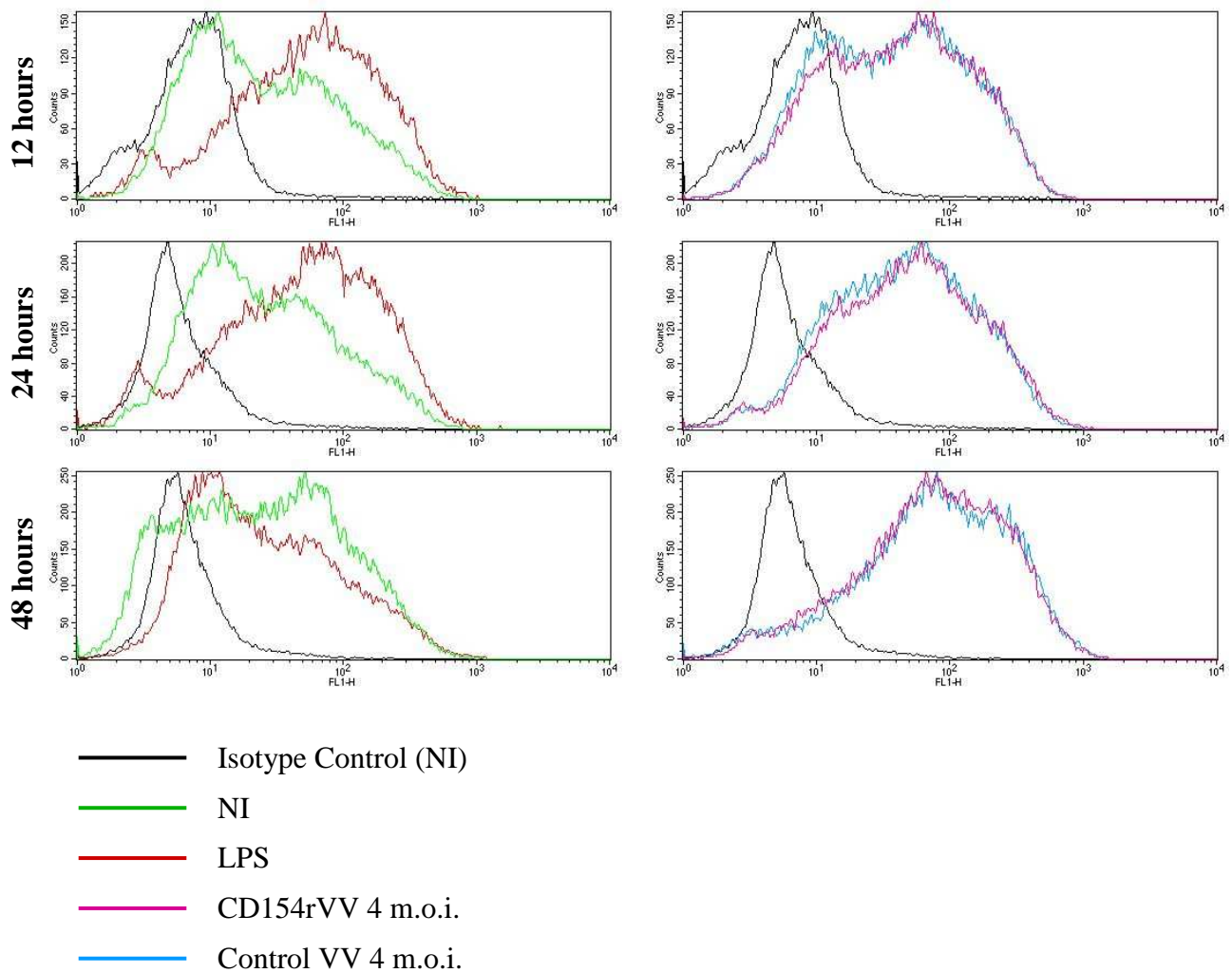
Figure 39. Flow cytometry analysis of the cell surface expression of HLA-DR as activation marker in infected CD14+ cells.

6×10^6 human CD14+ cells from an healthy donor were infected with the replication-incompetent (PLUV) CD154rVV or control VV at 4 m.o.i., washed and cultured for 48 hours. Non infected cells (NI) and cells cultured with LPS (1 μg/ml) were used as negative and positive controls, respectively. Cells were stained at different time points either with Isotype control antibodies, or with FITC-labeled anti-HLA-DR mAbs, washed, fixed with paraformaldehyde 1%, and subsequently analyzed by flow cytometry.

3.5.1.3. Inhibition of activation markers expression by an antagonist anti-CD40 monoclonal antibody

Taken together, these data suggested that CD154rVV was able to induce monocyte activation. As for cytokine gene expression, in order to formally confirm whether these effects were indeed due to transgene expression, we performed similar experiments in the presence of an antagonist anti-human CD40 monoclonal antibody.

As shown in figure 40, CD80 and CD86 surface expression induced in CD154rVV infected CD14+ cells was indeed abolished in the presence of anti-CD40 mAbs, while not affected by the presence of a control antibody (not shown) demonstrating that effects observed with CD154rVV were due to CD40 triggering following transgene expression.
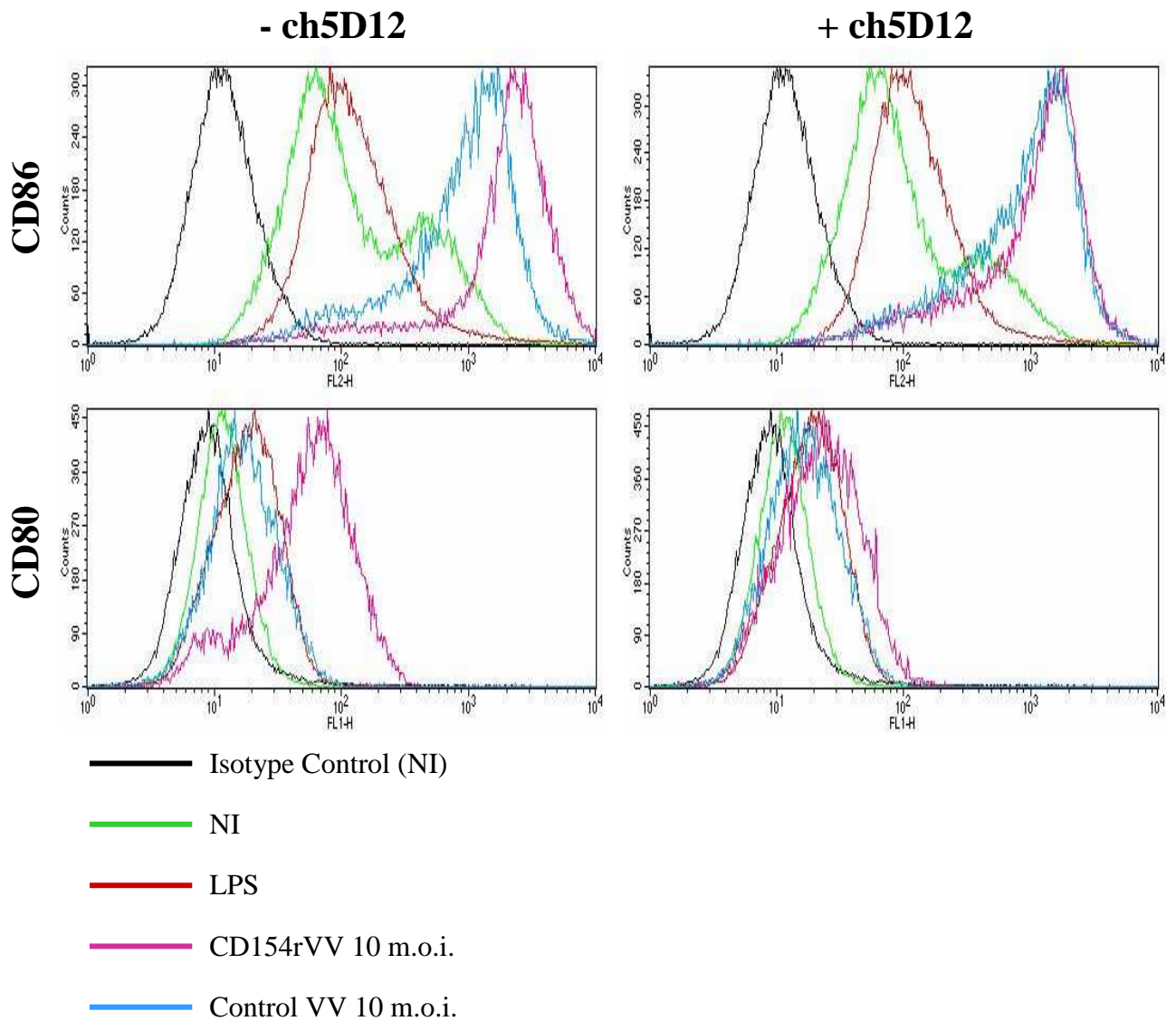
Figure 40. Inhibition of cell surface expression of activation markers in infected CD14+ cells, by an antagonist anti-CD40 monoclonal antibody.

1.5 x 10^6 human CD14+ cells from an healthy donor were infected with the replication-incompetent (PLUV) CD154rVV or control virus at 10 m.o.i., wash and cultured for 4 days. Non infected CD14+ cells (NI) and cells cultured with 1 µg/ml LPS were also studied. Cells were stained with specific antibodies: FITC-labeled anti-CD86 mAbs and PE-labeled anti-CD80 mAbs, washed, fixed with paraformaldehyde 1%, and subsequently analyzed by flow cytometry.

3.5.2. iDC activation and maturation

Maturation of iDC is crucial for the initiation of cellular immune response. Levels of CD86 and CD83 surface expression (Banchereau et al., 1998) were evaluated as markers of activation and maturation of monocyte derived iDC from healthy donors. Since iDC seem to be more sensitive to viral infection than CD14+ cells (see apoptosis results), low viral dose (1 m.o.i.) was chosen to evaluate the impact of CD154rVV on iDC. However, since low viral doses result in lower transgene expression, prolonged culture times (5 days) were necessary prior to phenotypic analysis. In these conditions, low dose CD154rVV infection of iDC resulted in activation and maturation, as indicated by enhanced CD86 and "*de novo*" CD83 expression, as compared to WT infection (Figure 41).
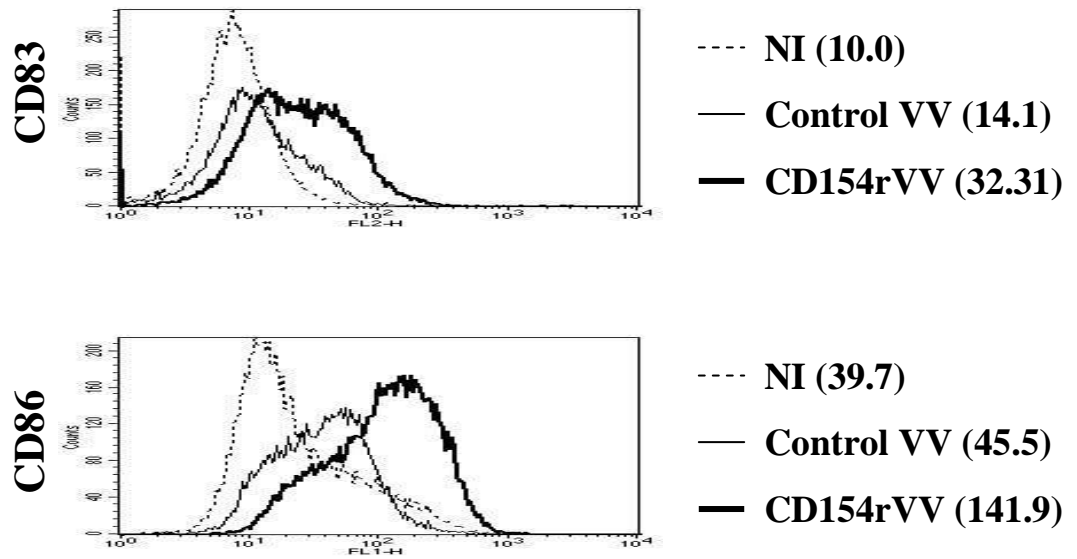
Figure 41. Flow cytometric analysis of the cell surface expression of CD83 and CD86 in infected CD14+ derived iDC.
2 x 10^6 CD14+ derived iDC from an healthy donor were infected with the replication-incompetent (PLUV) CD154rVV or control virus at 1 m.o.i., washed and cultured for 5 days in complete medium supplemented with 10% FCS. Non infected CD14+ cells (NI) were also used as additional control. Cells were harvested and stained with anti-CD83 or anti-CD86 mAbs. Staining of the cells under investigation with isotype control mAb showed identically negative profiles in all cases (data not shown). Mean Fluorescence Intensities are indicated in brackets.

4. Effects of APC activation by CD154rVV on T cell responses

4.1. Induction of T cell response to viral antigen

4.1.1. Cytokine gene expression

The above data prompted us to explore the effects of CD154rVV on antigen presentation to T cells, using Vaccinia virus specific responses as read-out. Peripheral blood CD14+ cells from Vaccinia immunized healthy donors were infected with CD154rVV or Control virus, incubated in complete medium for 24 hours and subsequently co-cultured with autologous CD14- cells.

Total cellular RNA was reverse transcribed and T helper 1 cytokines gene expression were quantified by real-time quantitative PCR (qRT-PCR). As shown in figure 42A, expression of IFN-γ and IL-2 genes readily induced by the control virus, was found to be significantly increased following CD154rVV infection of APC, conspicuously at low moi. Interestingly, the expression of these cytokines genes appeared to be lower in this directly infected APC setting as compared to experiments using "indirect" APC stimulation by infected fibroblasts, thus suggesting that *in vivo*, fibroblasts infected during immunization could have a beneficial effects on T cell responses.
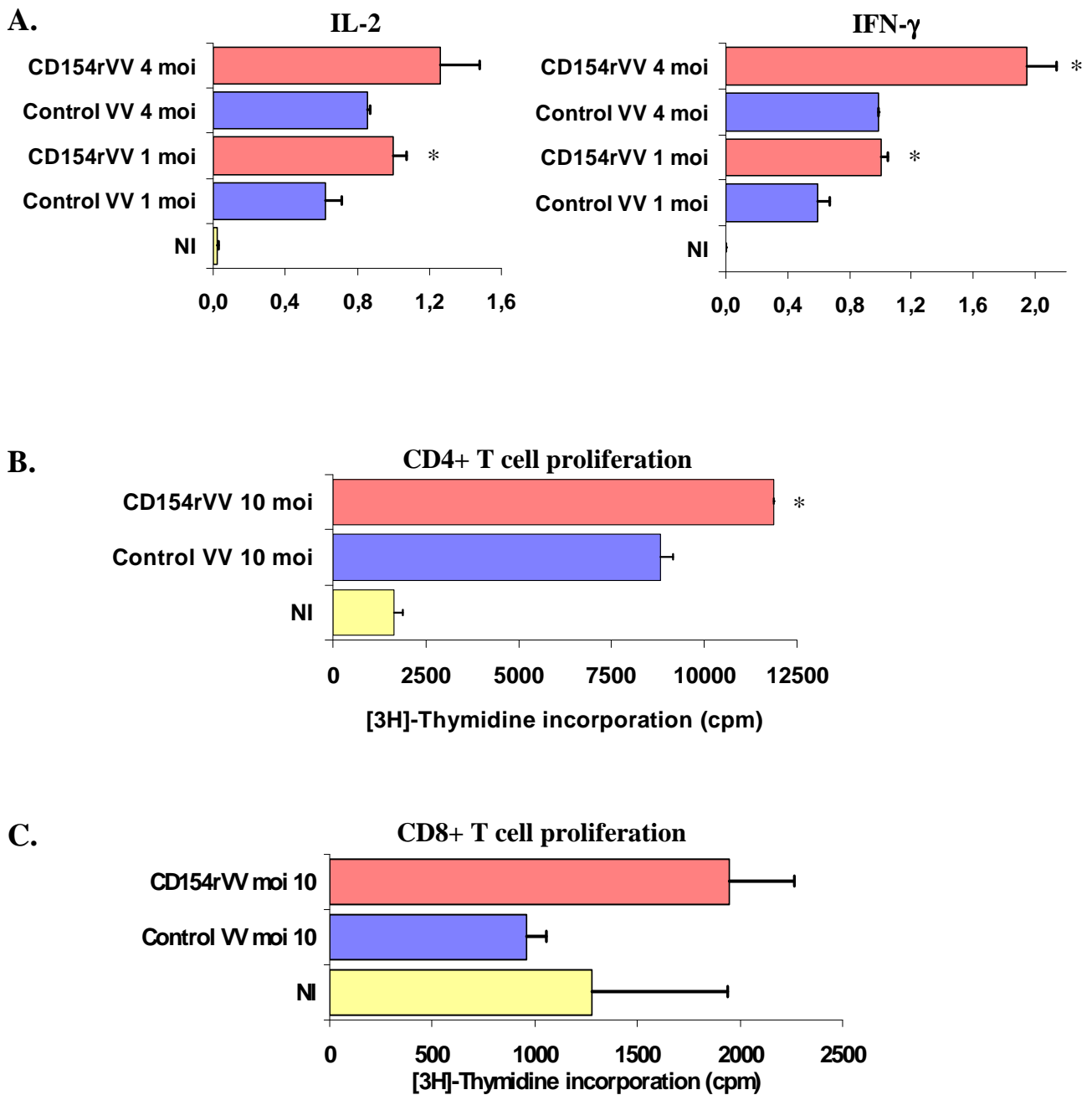
Figure 42. Evaluation of T cell response to viral antigen.
(**A.**) Induction of IL-2 and IFN-γ gene expression in CD14- cells cocultured with autologous infected CD14+ cells. 10^6 CD14+ cells from a vaccinia immune donor were infected with the replication-incompetent (PLUV) CD154rVV or control virus at 1 or 4 m.o.i., washed and co-cultured overnight with 10^6 autologous CD14- cells. Non infected cells (NI) were also used as control. Gene expression was analyzed by quantitative real-time PCR and data were expressed as ratio to those detected upon infection with CD154rVV at 1 moi. Proliferation of purified CD4+ (**B.**) and CD8+ T cells (**C.**) in the presence of autologous infected CD14+ cells. 10^5 CD14+ cells, freshly obtained from a vaccinia immune donor, were infected with the replication-incompetent (PLUV) CD154rVV or control virus at 10 m.o.i., wash and cocultured with autologous T cells (CD4+: 3 x 10^5 , CD8+: 2,5 x 10^5 per well). Co-culture with non infected CD14+ cells (NI) was used as negative control. After 5 days, 0.5 μCi of [methyl ^{3}H]thymidine were added to each well for the following 16 hours of the incubation period. Error bars represent the standard deviation of the mean cpm of triplicate cultures. Symbol (∗) indicates values significantly ($p < 0.05$) different between CD154rVV and control VV infected cultures.

4.1.2. T cell proliferation

In previous work we have shown that MHC class II Vaccinia virus specific CD4+ T cells from Vaccinia immunized donors respond to VV by active proliferation (Marti et al., 1997). Thus, effects of CD154rVV infected APC on CD4+ T cell proliferation were explored using the virus itself as antigen in freshly isolated cells from vaccinated healthy donors. As shown in figure 42B, CD4+ T cell proliferation was significantly enhanced upon APC infection with CD154rVV as compared to Control virus. Even if the differences are not statistically significant, we observed in several experiments that CD8+ T cell proliferation seemed marginaly enhanced upon APC infection with CD154rVV as compared to control virus (figure 42C).

These results were confirmed using CFSE staining as method for proliferation quantification. Monocytes from a Vaccinia immunized healthy donor were infected either with CD154rVV or with the Control virus at 4 m.o.i.. Non infected (NI) and PHA (1 µg/ml) stimulated cultures were used as negative and positive control, respectively. Monocytes were co-cultured for 5 days with CFSE stained autologous CD14- cells. CD4+ T cell proliferation was evaluated by FACS staining. A shown in figure 43, PHA induced strong CD4+ T cells proliferation as opposed to the marginal proliferation induced with non infected CD14+. Interestingly, once again, CD4+ T cell proliferation was manifestly enhanced upon APC infection with CD154rVV as compared to Control virus.
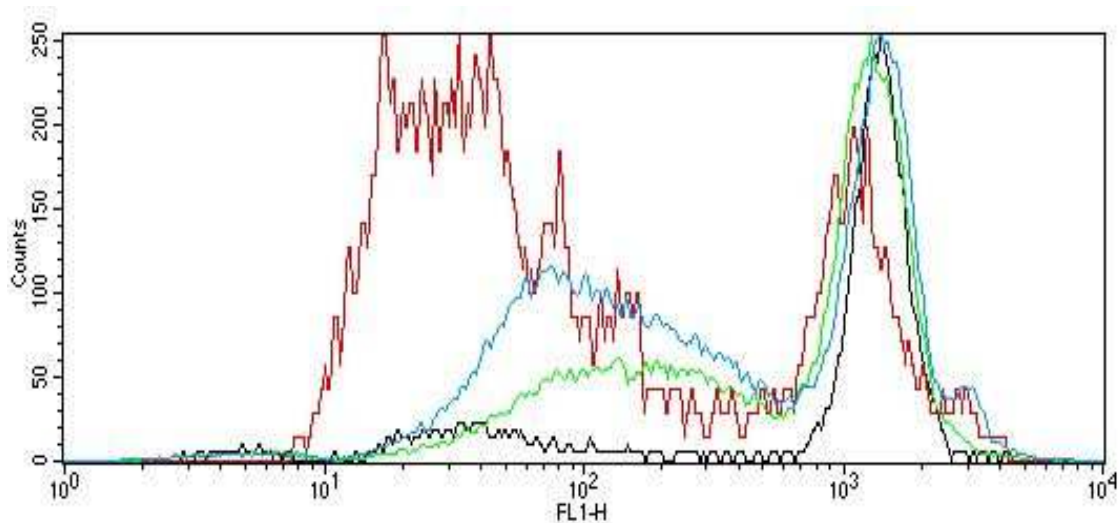
Figure 43. Confirmation of CD4+ T cell proliferation response to antigen.
Proliferation of CD4+ cells in the presence of autologous infected CD14+ cells was evaluated by FACS staining. $2x10^6$ CD14+ cells, freshly isolated from a vaccinia immune donor, were infected with the replication-incompetent (PLUV) CD154rVV (—) or control virus (—) at 4 m.o.i., wash and co-cultured with $2x10^6$ autologous CD14- cells. Co-culture with non-infected CD14+ cells (—) and co-culture with PHA (1µg/ml) (—) were used as negative and positive control, respectively. Proliferation was evaluated after 5 days culture by staining with PE-labeled CD4 and CFSE and flow cytometry analyze.

4.2. CD154rVV promotes APC capacity to prime antigen-specific CTL response

In previous work, we have shown that rVV encoding co-stimulatory molecules provide powerful adjuvance to the induction of specific cytotoxic T-cell responses, with particular reference to tumor associated antigens (Zajac et al., 1998; (Zajac et al., 2003). The capacity of CD154rVV infected APC to enhance specific MART-1/Melan-A specific CTL stimulation *in vitro* was then tested. Monocytes derived iDC from healthy HLA-A0201+ donors were infected with CD154rVV or WT as control, and mixed with autologous CD8+ T cells in the presence of MART-1/Melan-A$_{27-35}$ melanoma associated epitope provided as soluble peptide.

As shown in figure 44A.1, on day 11, after the priming, specific tetramer staining did not detect major differences among the cultures under investigation. In contrast, the enhancing effect of CD154rVV activation on CTL priming became dramatically evident after a first and particularly after a second boost. Indeed, cultures primed with CD154rVV infected APC displayed 13.84% HLA-A0201/MART-1/Melan-A$_{27-35}$ tetramer positive CD8+ T cells, as compared to 2.26% in non-infected conditions, and 1.59% in cultures where APC were infected with control VV (figure 44A.2). The cytotoxic capacity (figure 44B) of these cultures was confirmed by standard chromium release assays.

Similar results were obtained using CD14+ cells as APC (figure 45). Indeed, the cultures infected with CD154rVV displayed 3.80% tetramer positive cells, as compared to 0.44% in non-infected condition, and 0.79% in cultures where APC were infected with control virus.

Same results were obtained by using other peptides (EBV BMLF1, EBV LMP2, CMVpp65) and monocytes as APC (Figure 46): after 7 only days, cultures primed with CD154rVV infected CD14+ cells already displayed usually higher percentage of peptide-specific CTL as compared to infected cultures, or cultures primed with control virus.
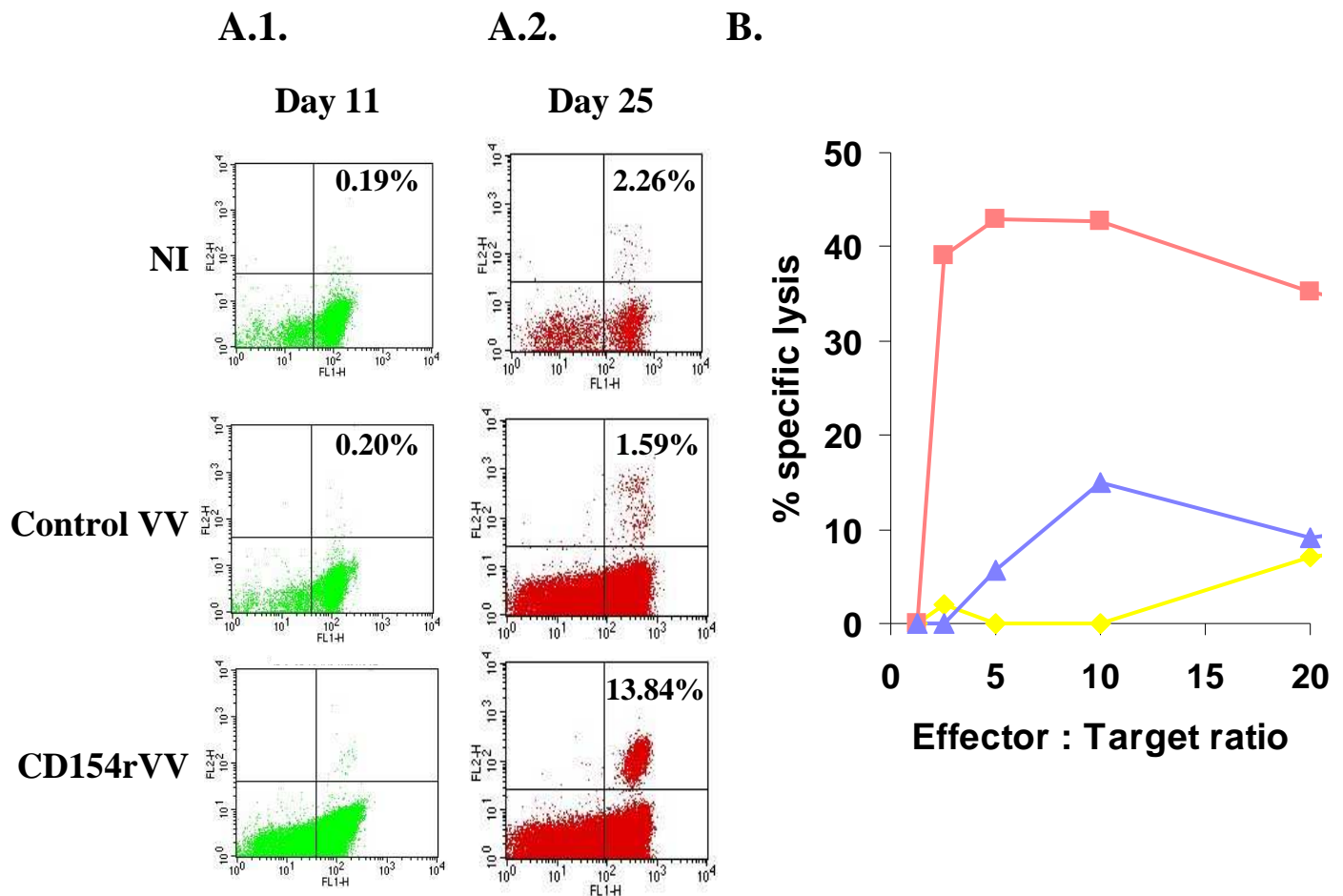
Figure 44. Induction of MART-1/Melan-A$_{27-35}$-specific CD8+ T cells using infected iDC as APC.

2.7×10^5 iDC were infected with the replication-incompetent CD154rVV or control virus (PLUV) at 8 m.o.i., washed and co-cultured with 5×10^5 autologous CD8+ T cells. Non infected (NI) iDC were also used as control. 10 µg per ml of MART-1/Melan-A$_{27-35}$ peptide were added to all cultures. Cells were cultured for 25 days, and regularly supplemented from day 8 on with medium containing a final concentration of 10 U/ml of IL-2. Furthermore, cultures were restimulated on days 12 and 19 using 20 µg/ml peptide-pulsed and irradiated autologous EBV-BL resuspended in complete medium supplemented with 10 U/ml IL-2. **(A.)** Flow cytometric analyses of CD8+ T cells were performed at day 11 and day 25, before the first boost (A.1.) and after the second boost (A.2.) respectively, by staining with FITC-labeled anti-CD8 mAbs and PE-labeled specific MART-1/Melan-A$_{27-35}$-HLA-0201 tetramer. **(B.)** Chromium-release assays were performed, at the indicated effector-to-target ratios, by using as targets, MART-1/Melan-A$_{27-35}$-peptide (20 µg/ml)-pulsed HLA-0201+ EBV-BL. CTL generated upon stimulation with the MART-1/Melan-A$_{27-35}$-peptide alone (◆), or with CD154rVV (■) or with control virus (▲) were tested. Standard deviations, never exceeding 10% of the reported values,are not shown.
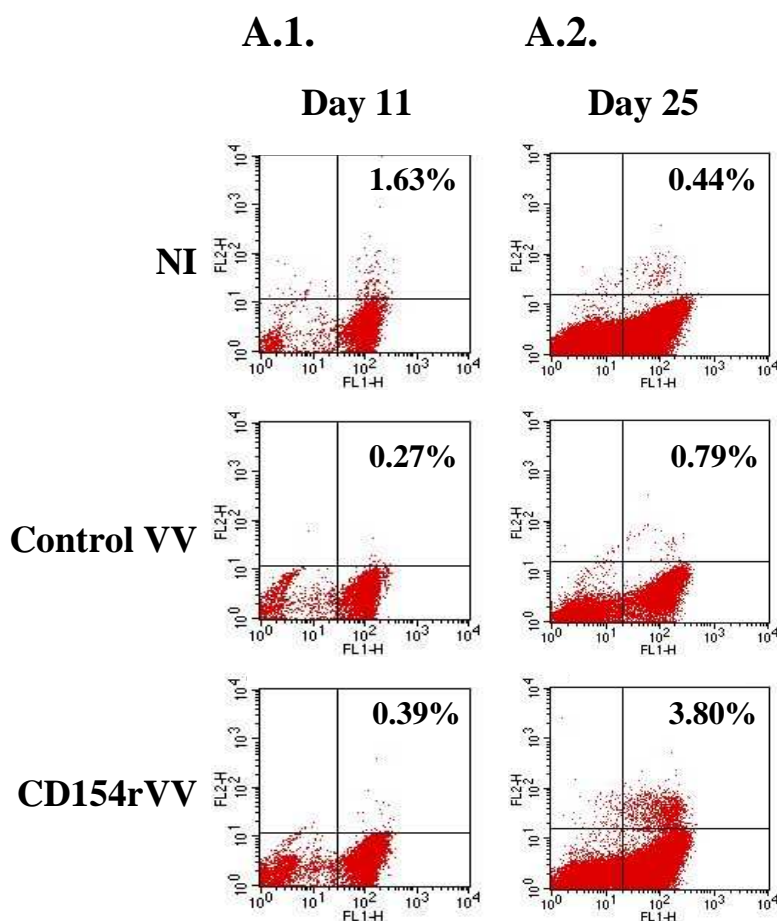
Figure 45. Induction of MART-1/Melan-A$_{27-35}$-specific CD8+ T cells using infected CD14+ cells as APC.

5 x 10^5 CD14+ cells were infected with the replication-incompetent CD154rVV or control virus (PLUV) at 8 m.o.i., washed and co-cultured with 6 x10^5 autologous CD8+ T cells. Non infected (NI) CD14+ cells were also used as control. 10 µg per ml of MART-1/Melan-A$_{27-35}$ peptide were added to all cultures. Cells were cultured for 25 days, and regularly supplemented from day 8 on with medium containing a final concentration of 10 U/ml of IL-2. Furthermore, cultures were restimulated on days 12 and 19 using 20 µg/ml peptide-pulsed and irradiated autologous EBV-BL resuspended in complete medium supplemented with 10 U/ml IL-2. Flow cytometric analyzes of CD8+ T cells were performed at day 11 and day 25, before the first boost **(A.1.)** and after the second boost **(A.2.)** respectively, by staining with FITC-labeled anti-CD8 mAbs and PE-labeled specific MART-1/Melan-A$_{27-35}$-HLA-0201 tetramer.
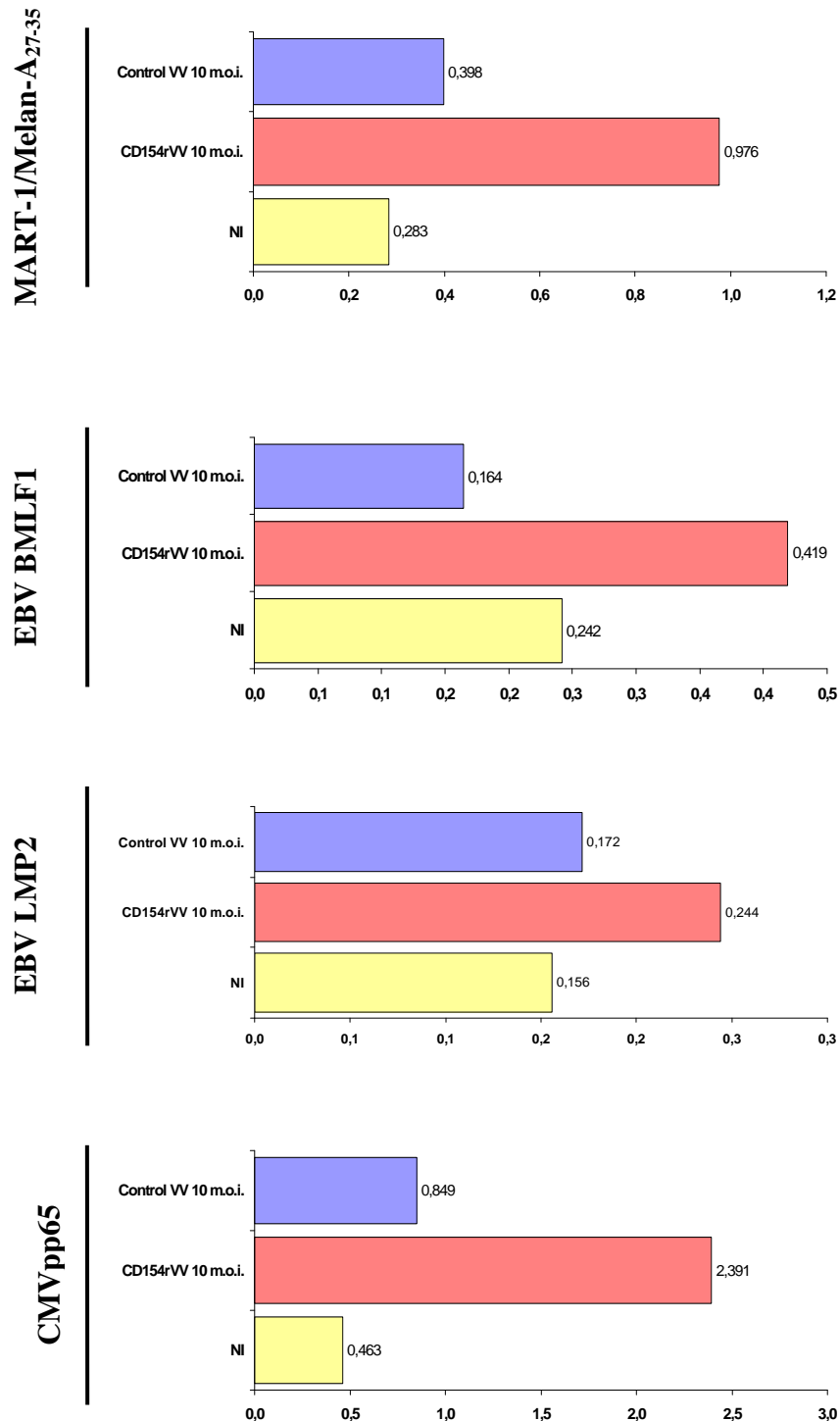
Figure 46. Induction of other antigen-specific CD8+ T cells using infected CD14+ cells as APC.
2 x 10^6 CD14+ cells were infected with the replication-incompetent CD154rVV or Control VV (PLUV) at 10 m.o.i., washed and co-cultured with 2 x 10^6 autologous CD8+ T cells. Non infected (NI) CD14+ cells were also used as control. 10 µg per ml of peptide (EBV BMLF1, EBV LMP2, CMVpp65 or MART-1/Melan-A$_{27-35}$) were added to the cultures. Cells were cultured for 7 days in complete medium without any IL-2 supplementation. Flow cytometry analyzes of CD8+ T cells were performed on day 7, by staining with FITC-labeled anti-CD8 mAbs and PE-labeled peptide specific tetramer.

Discussion

DISCUSSION

Innovative approaches have to be created to cure patients with tumor burden. Even if immunological approaches are actually tested with late stage cancer, they are probably most appropriate for early stages tumor or after resection, with high risk of recurrence. One promising approach seems to be the generation of therapy stimulating specific tumor-directed immune response, combined with non specific immune-modulation. For this purpose, efficient presentation of immunogens to T cells, leading to generation of a large numbers of Tumor Associated Antigen (TAA) specific cytotoxic T cells (CTL), represents a critical issue in cancer immuno-therapy. Nevertheless, even if this method seems promising, the overall clinical success of this experimental approach in cancer patients remains limited, to date. Indeed, paradoxically, T cell activation as detected *ex vivo*, does not always correlate with clinical response (Nielsen et al., 2000). Current approaches obviously need to be improved.

The intrinsic properties of poxvirus, especially Vaccinia virus, render this virus highly interesting as a vaccine vector (Moss, 1996). It is one of the most frequently used in modern cancer immunotherapy. It has been employed as a vector for many tumor antigens like Carcinoembryonic Antigen Epitopes (CEA) (Tsang et al., 1995) or Tyrosinase (Yee et al., 1996). In addition, molecular characterization of HLA restricted epitopes lead to an improved formulation of the vaccine (Celis et al., 1995).

Our group's main focus is the development of an anti-cancer therapeutic approach through the generation of tumor specific cytotoxic T cell responses in melanoma patients. As principal vaccine vector, we are using recombinant Vaccinia virus expressing melanoma tumor associated antigens (gp$_{100}$, MART-1/Melan-A$_{27-35}$, Tyrosinase$_{1-9}$) in combination with co-stimulatory molecules: CD80 and CD86. This vaccine was used in a phase I/II clinical trial (Zajac et al., 2003) and resulted in a substantial immunogenic response.

Nevertheless, even if the specific response to TAA was frequently enhanced after vaccination, these responses were mild and transient.

Therefore, further investigation on vector providing ligands for stimulatory pathways susceptible to increase or at least to maintain vaccine driven TAA specific CD8+ T cell response in cancer patients appears to be a critical issue. As an interesting new immunogenic vaccine, a recombinant Vaccinia virus expressing CD154, known to play a major role in the activation of different help-dependent immune responses, was constructed and evaluated *in vitro*.

Activation of specific T cell responses, especially for tumor antigens, do require more signals than the antigenic peptide restricted by the MHC class I molecule and recognized by the T cell receptor (Matzinger, 2002). In order not to lead to anergy but to T cell activation, this signal must be followed by a second one generated by co-stimulatory molecules, such as CD80 and CD86. This signal can be reinforced by a third signal mediated by soluble factors such as cytokines (for instance IL-12, IL-15), which are usually resulting from activation of "helper mechanisms". Among CD4+ driven help, activation of APC through CD40/CD154 is a major pathway. Indeed, this co-receptor, expressed on activated CD4+ T cells and, upon binding CD40 expressed on APC, has been reported to increase their antigen presentation and immunomodulatory capacities, in particular through the expression of T helper 1 cytokines. Underlying this mechanism, CD40 cross-linking has been shown to bypass the absolute requirement for CD4+ T cells in CTL generation (Bennett et al., 1998; Ribas et al., 2001; Toes et al., 1998). CD40/CD154 interaction controls APC function by upregulating expression of costimulatory ligands, MHC molcules and multiple cytokines (Grewal et al., 1998) such as IL-12 and IL-15 playing important roles in CTL induction and homeostatic

expansion (Banchereau et al., 1994; Bourgeois et al., 2002; Curtsinger et al., 1999; Curtsinger et al., 2003; Ha et al., 1999; Kieper et al., 2001; Kuniyoshi et al., 1999; Maruo et al., 1997; Shu et al., 1995).

Based on our previous experience with recombinant Vaccinia virus and immunomodulation, we hypothezised that a recombinant Vaccinia virus expressing CD154 may reproduce this mechanism and ultimately optimize CTL generation and activation.

CD154 gene was first cloned from human PHA stimulated PBMC. A mutagenesis step was required because the CD154 sequence contained two TTTTTNT sequences, known to be STOP sequence for Vaccinia virus early transcription. The mutated CD154 gene was then inserted in the A56R locus of Wild Type Vaccinia virus (Copenhague strain) genome by homologous recombination.

In the perspective of eventual human application where safe vectors are preferred, the potency of CD154rVV was evaluated in a non replicative form. Replication was inactivated by treatment with psoralen and long wave UV (PLUV), inducing cross-linking in genomic DNA. Because early phase of viral transcription is not affected, the resulting virus is non replicating, thus less cytopathic, but still expresses inserted gene under viral promoters control.

At first, the level of CD154 expression over time was characterized in cells infected with PLUV CD154rVV. Indeed, following transient T cell receptor triggering of CD4+ T cells *in vitro*, CD154 surface expression is peaking 8 hours following activation and is then rapidly down-regulated (Yellin et al., 1994b). In contact with PLUV Vaccinia virus infected monocytes, CD4+ T cells activation, as demonstrated by an increase in CD154 expression, remains detectable up to 36 hours infection. At that time point, CD154 expression, in infected monocytes, was still marked. Thus, CD154 expression seems longer and stronger in CD154rVV infected monocytes as compared to MHC class II activated CD4+ T cells. This observation could already be an advantage of expressing CD154 with a recombinant Vaccinia virus, whereas the longer infected cells are strongly expressing CD154, the more this molecule could induce APC's activation and subsequently enhance T cell activation.

The effects of CD154rVV infection on CD40+ cells endowed with antigen presenting capacity were further investigated. We were able to show that replication incompetent CD154rVV induced a marked cytokine secretion upon APC infection, especially IL-12 and IL-15, which are known to play major roles in CTL induction and in the maintainance of CD8+ T cell memory (Fehniger et al., 2001; Jonuleit et al., 1997; Lu et al., 2002; Wilkinson et al., 1995). Indeed, IL-12p40 transcription in monocytes was exclusively detected in the presence of CD154rVV infected cells. GM-CSF and TNF-α gene expression were induced following APC infection with Control virus but to significantly lower levels as compared to those observed upon CD154rVV infection. Similarly, IL-12p40 and GM-CSF transcription in monocytes derived iDC were as well exclusively detected following CD154rVV infection, and TNF-α gene expression is significantly higher with CD154rVV as compared to the Control virus infection. Interestingly, we also show that IL-15 transcription is significantly higher in iDC infected with CD154rVV as compared to Control virus.

These cytokines inductions in APC upon CD154rVV stimulation are of major interest. Previous study showed that CD40 ligation on APC, and notably on DC, triggers production of high level of IL-12 (Cella et al., 1996), indicating that CD154rVV could notably enhance CD8+ T cells expansion and activation, through to IL-12 properties. For instance, IL-12 is known to play important role during CTL generation (Bourgeois et al., 2002; Kieper et al., 2001; Kuniyoshi et al., 1999). Indeed, IL-12p40 is normally produced by activated CD14+ cells (Reiner et al., 1994; Skeen et al., 1996) and dendritic cells (Cella et al., 1996; Heufler et al., 1996; Macatonia et al., 1995). It may act as a chemotactic molecule for macrophages (Ha et al., 1999) and one of its major role is to enhance CD8+ T cell homeostatic expansion

(Kieper et al., 2001) and activation (Curtsinger et al., 1999; Curtsinger et al., 2003). IL-15 is expressed by DC, monocytes and macrophages (Fehniger et al., 2001; Jonuleit et al., 1997), is a potent chemoattractant for T cells (Wilkinson et al., 1995), and promotes long-term survival of anti-tumor cytotoxic T lymphocytes. One of its crucial role is also to stimulate the proliferation of human memory (CD45+RO+) CD4+ and CD8+ T cells (Kanegane et al., 1996). Due to IL-15 characteristics, activation of CD40/CD154 on APC following infection by CD154rVV, should subsequently also play an important role in the survival of T cells and in the proliferation of tumor specific memory T cells.

Similarly, GM-CSF increased expression following CD154rVV infection is of interest since this cytokine, secreted by monocytes and macrophages, is crucial for APC recrutment and development (Inaba et al., 1992). Our data confirmed a previous study demonstrating that CD40 stimulation with soluble CD154 induces pro-inflammatory responses in CD14+ cells, notably TNF-α (Kiener et al., 1995), which is also known to induce cells death, especially tumor cells (Carswell et al., 1975).

The role of Vaccinia virus expressed CD154 was further confirmed by an antagonist chimeric anti-human CD40 monoclonal antibody (generous gift from M. de Boer, Pan Genetics B.V., Utrecht, The Netherlands). This antibody binds with high affinity (Kd = 2.2×10^{-10}) and blocks the CD40 receptor, effectively demonstrating the specific activity of CD154 in the activation of cells (de Vos et al., 2004).

In this work, we have shown that a replication incompetent CD154rVV is able to induce a marked APC activation, due to CD40 receptor triggering within monocyte or iDC infected populations. Similarly, phenotypic characterization of infected CD14+ derived iDC suggests that inactivated CD154rVV is able to induce iDC maturation which is a crucial event for the initiation of cellular immune response, as verified by CD83 expression (Banchereau et al., 1998). Therefore, CD154rVV was thereby overcoming the intrinsic detrimental effects of WT VV. Indeed, previous studies have demonstrated that WT VV inhibits iDC maturation (Drillien et al., 2000; Engelmayer et al., 1999) whereas CD40 ligation promotes it (Banchereau et al., 1994; Larsson et al., 2001; Straw et al., 2003).

As a consequence, these effects on APC are reflected by enhanced functions of T cells: especially CD4+ but also CD8+ T cell proliferation, IL-2 and IFN-γ expression were significantly increased in cultures stimulated with CD154rVV as compared to Control virus. Because IFN-γ activates monocytes, induces expression of MHC class II molecules and promotes directly T- and B- lymphocytes differentiation (Younes et al., 2002), CD154rVV might promote T lymphocytes differentiation and also have an indirect effect on tumor cell killing.

Similar to natural situation of CD154 triggering by helper cells, the elicitation of these effects are not limited to direct immunocompetent cell infection. They can be mediated by bystander infected cells as fibroblasts, a situation likely occurring *in vivo* following intradermal injection. This was demonstrated by increased IL-12p40, IFN-γ and IL-2 gene expression in PBMC co-cultured with autologous human skin fibroblasts infected with CD154rVV as compared to Control VV infection.

Most importantly, our data clearly indicate that CD154rVV provides efficient adjuvance during the induction of CTL specific for MART-1/Melan-A$_{27-35}$ model TAA in the absence of CD4+ T cells, a function exquisitely mediated by APC licensing via CD40/CD154 interaction (Bennett et al., 1997; von Herrath et al., 1996). Indeed, in cultures of infected APC with autologous CD8+ T cells and MART-1/Melan-A$_{27-35}$ soluble peptide, the enhancing effects of CD154rVV activation on CTL priming became dramatically evident after restimulation, as measured with specific tetramer or by cytotoxicity.

Taken together these data indicate that functional CD154 expression from recombinant Vaccinia virus infected cells induces APC activation thereby enhancing their capacity to

generate T cell immune responses. Since triggering of the CD40/CD154 pathway by CD154rVV efficiently mimics key features of activated helper T cells, this recombinant vector, active in non replicating form, may help bypassing their role in CTL induction, thus qualifying as a potentially relevant reagent in the generation of CD8+ T cell responses in cancer immunotherapy.

Similarly, agonistic anti-CD40 mAbs, recombinant CD154 and CD154-transfected cells are already in use for experimental therapy of human cancer. For instance, clinical effects of recombinant CD154 protein were observed in the treatment of solid tumors and non-Hodgkin's lymphoma. In phase I studies, soluble recombinant CD154 was administrated subcutaneously with encouraging anti-tumor activity including a long-term complete remission (Vonderheide et al., 2001; Younes, 2001). Nevertheless, potential risk of systemic inflammation and auto-immune consequences remains a concern for systemic CD154-based experimental therapy. In the same way, an adenoviral vector encoding CD154 was well tolerated, in phase I clinical trials using infusion of transduced autologous leukemia cells (Kipps et al., 2000; Wierda et al., 2000), in which increased number of leukemia-specific T cells as well as reduction in leukemia cell count and lymph node size was demonstrated. However, once again, potential side effects should be carefully evaluated, since patients treated with recombinant adenovirus expressing CD154 transduced leukemia cells commonly experienced Influenza-like symptoms (Kipps et al., 2000). Likewise, efficacy of agonistic CD40-reactive mAb for clinical applications was limited by pro-inflammatory cytokine production by CD40-activated endothelial cells (Singh et al., 2001).

Nevertheless, CD154-based therapy alone or in combination with other therapies may offer an effective and safe strategy for the treatment of human cancers. The promise of CD154 as a tumor therapeutic agent to directly modulate tumor cell growth, and indirectly activate anti-tumor immune response, may just depend on the way of administration or on selective CD154 expression within tumor environment.

Recombinant Vaccinia virus expressing CD154 seems in this regard a good candidate. Based on viral characteristics, PLUV CD154rVV should be safe and could, as demonstrated here, enable bystander cells to activate APC, resulting in enhanced CD8+ T cells priming. Because CD154 is only expressed at the surface of infected cells, side effects of systemic CD154 administration could also be limited. In addition, the use of a viral vector can prolong the expression of the recombinant protein as compared to soluble CD154, which is susceptible to rapid proteolysis as opposed to efficient presentation by MHC molecules loaded through the class I endogenous pathway of antigen processing (Maffei et al., 1997; Townsend et al., 1986; van Endert, 1999).

In order to further characterize the effects of CD154rVV on T cell response, we could analyze the phenotype of the CTL in condition primed with CD154rVV infected APC and peptide. Indeed, CTL can be classified in five distinct classes regarding the expression of CD45RA, CCR7, CD27, CD28 and CD62L (Champagne et al., 2001; Geginat et al., 2003; Tomiyama et al., 2002; Valmori et al., 2002). Antigenic stimulation of CD8+ T cells results in the expansion of naïve and central memory cells mainly (Champagne et al., 2001; Geginat et al., 2003). Similarly, CD154 binding to the CD40 expressed on APC (Moodycliffe et al., 2000; Stout et al., 1996b; Stout et al., 1996a) results in an increase of T helper 1 cytokines such as IL-12 (Maruo et al., 1997) and IL-15 (Wilkinson et al., 1995), playing a major role in memory induction (Bourgeois et al., 2002; Judge et al., 2002; Kanegane et al., 1996; Kieper et al., 2001; Kuniyoshi et al., 1999; Yajima et al., 2002). In the same way, CD154 is known to play roles in maintenance of T cell memory (Borrow et al., 1996; Huster et al., 2004; Koschella et al., 2004; Rogers et al., 2003). Due to these properties, we wanted to

demonstrate *in vitro*, that CD154rVV is able to favor memory CD8+ T cell enhancement. Some preliminary results seem to demonstrate that PLUV CD154rVV is able to effectively enhance antigenic peptide specific CD8+ T cells, with in majority central memory phenotype and in some case effector memory or naïve phenotype.

Finally, it may be of interest to evaluate *in vitro* the potential synergy of CD154 with other co-stimulatory molecules such as CD80/CD86 expressed by recombinant virus. Indeed, recombinant poxvirus expressing immunomodulatory molecules (CD80 and CD86) was demonstrated *in vitro* and *in vivo* to enhance the immunogenic capacity of a recombinant Vaccinia virus encoding different tumor associated antigens (Marti et al., 1997; Zajac et al., 1997). rVV encoding Mart-1/Melan-A$_{27-35}$, Gp$_{100}$ and Tyrosinase$_{1-9}$ Tumor Associated Antigens and with CD80 and CD86 (Oertli et al., 2002; Spagnoli et al., 2002) has been successfully used in a phase I/II clinical trial (Zajac et al., 2003). Nevertheless, even if specific responses to TAA were often induced after vaccination, these responses were limited in time and in intensiy. Properties of CD154rVV demonstrated in this work, may eventually improve the intensity and the duration of these T cell responses.

Bibliography

Reference List

ABI-YOUNES, S., SAUTY, A., MACH, F., SUKHOVA, G.K., LIBBY, P., and LUSTER, A.D. (2000). The stromal cell-derived factor-1 chemokine is a potent platelet agonist highly expressed in atherosclerotic plaques. Circ. Res. **86,** 131-138.

ADEMA, G.J., DE BOER, A.J., VAN 'T, H.R., DENIJN, M., RUITER, D.J., VOGEL, A.M., and FIGDOR, C.G. (1993). Melanocyte lineage-specific antigens recognized by monoclonal antibodies NKI-beteb, HMB-50, and HMB-45 are encoded by a single cDNA. Am. J. Pathol. **143,** 1579-1585.

AKIRA, S., TAKEDA, K., and KAISHO, T. (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. **2,** 675-680.

ALDINUCCI, D., POLETTO, D., NANNI, P., DEGAN, M., RUPOLO, M., PINTO, A., and GATTEI, V. (2002). CD40L induces proliferation, self-renewal, rescue from apoptosis, and production of cytokines by CD40-expressing AML blasts. Exp. Hematol. **30,** 1283-1292.

ALTMAN, J.D., MOSS, P.A., GOULDER, P.J., BAROUCH, D.H., MCHEYZER-WILLIAMS, M.G., BELL, J.I., MCMICHAEL, A.J., and DAVIS, M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science **274,** 94-96.

ANDRE, P., NANNIZZI-ALAIMO, L., PRASAD, S.K., and PHILLIPS, D.R. (2002a). Platelet-derived CD40L: the switch-hitting player of cardiovascular disease. Circulation **106,** 896-899.

ANDRE, P., PRASAD, K.S., DENIS, C.V., HE, M., PAPALIA, J.M., HYNES, R.O., PHILLIPS, D.R., and WAGNER, D.D. (2002b). CD40L stabilizes arterial thrombi by a beta3 integrin--dependent mechanism. Nat. Med. **8,** 247-252.

ANNUNZIATA, C.M., SAFIRAN, Y.J., IRVING, S.G., KASID, U.N., and COSSMAN, J. (2000). Hodgkin disease: pharmacologic intervention of the CD40-NF kappa B pathway by a protease inhibitor. Blood **96,** 2841-2848.

ARMITAGE, R.J., FANSLOW, W.C., STROCKBINE, L., SATO, T.A., CLIFFORD, K.N., MACDUFF, B.M., ANDERSON, D.M., GIMPEL, S.D., DAVIS-SMITH, T., and MALISZEWSKI, C.R. (1992). Molecular and biological characterization of a murine ligand for CD40. Nature **357,** 80-82.

ARMSTRONG, B.K. and KRICKER, A. (1994). Cutaneous melanoma. Cancer Surv. **19-20,** 219-240.

AUKRUST, P., MULLER, F., UELAND, T., BERGET, T., AASER, E., BRUNSVIG, A., SOLUM, N.O., FORFANG, K., FROLAND, S.S., and GULLESTAD, L. (1999). Enhanced levels of soluble and membrane-bound CD40 ligand in patients with unstable angina. Possible reflection of T lymphocyte and platelet involvement in the pathogenesis of acute coronary syndromes. Circulation **100,** 614-620.

AVICE, M.N., DEMEURE, C.E., DELESPESSE, G., RUBIO, M., ARMANT, M., and SARFATI, M. (1998). IL-15 promotes IL-12 production by human monocytes via T cell-dependent contact and may contribute to IL-12-mediated IFN-gamma secretion by CD4+ T cells in the absence of TCR ligation. J. Immunol. **161,** 3408-3415.

BABLANIAN, R., BAXT, B., SONNABEND, J.A., and ESTEBAN, M. (1978). Studies on the mechanisms of vaccinia virus cytopathic effects. II. Early cell rounding is associated with virus polypeptide synthesis. J. Gen. Virol. **39,** 403-413.

BALASA, B., KRAHL, T., PATSTONE, G., LEE, J., TISCH, R., MCDEVITT, H.O., and SARVETNICK, N. (1997). CD40 ligand-CD40 interactions are necessary for the initiation of insulitis and diabetes in nonobese diabetic mice. J. Immunol. **159,** 4620-4627.

BANCHEREAU, J., BAZAN, F., BLANCHARD, D., BRIERE, F., GALIZZI, J.P., VAN KOOTEN, C., LIU, Y.J., ROUSSET, F., and SAELAND, S. (1994). The CD40 antigen and its ligand. Annu. Rev. Immunol. **12,** 881-922.

BANCHEREAU, J. and STEINMAN, R.M. (1998). Dendritic cells and the control of immunity. Nature **392,** 245-252.

BARNHILL, R.L. and MIHM, M.C., JR. (1993). The histopathology of cutaneous malignant melanoma. Semin. Diagn. Pathol. **10,** 47-75.

BARRETT, T.B., SHU, G., and CLARK, E.A. (1991). CD40 signaling activates CD11a/CD18 (LFA-1)-mediated adhesion in B cells. J. Immunol. **146,** 1722-1729.

BENNETT, S.R., CARBONE, F.R., KARAMALIS, F., FLAVELL, R.A., MILLER, J.F., and HEATH, W.R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature **393,** 478-480.

BENNETT, S.R., CARBONE, F.R., KARAMALIS, F., MILLER, J.F., and HEATH, W.R. (1997). Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. J. Exp. Med. **186,** 65-70.

BISHOP, D.T., DEMENAIS, F., GOLDSTEIN, A.M., BERGMAN, W., BISHOP, J.N., BRESSAC-DE PAILLERETS, B., CHOMPRET, A., GHIORZO, P., GRUIS, N., HANSSON, J., HARLAND, M., HAYWARD, N., HOLLAND, E.A., MANN, G.J., MANTELLI, M., NANCARROW, D., PLATZ, A., and TUCKER, M.A. (2002). Geographical variation in the penetrance of CDKN2A mutations for melanoma. J. Natl. Cancer Inst. **94,** 894-903.

BJORCK, P., BANCHEREAU, J., and FLORES-ROMO, L. (1997). CD40 ligation counteracts Fas-induced apoptosis of human dendritic cells. Int. Immunol. **9,** 365-372.

BLANCOU, J., KIENY, M.P., LATHE, R., LECOCQ, J.P., PASTORET, P.P., SOULEBOT, J.P., and DESMETTRE, P. (1986). Oral vaccination of the fox against rabies using a live recombinant vaccinia virus. Nature **322,** 373-375.

BLEHARSKI, J.R., NIAZI, K.R., SIELING, P.A., CHENG, G., and MODLIN, R.L. (2001). Signaling lymphocytic activation molecule is expressed on CD40 ligand-activated dendritic cells and directly augments production of inflammatory cytokines. J. Immunol. **167,** 3174-3181.

BONNET, M.C., TARTAGLIA, J., VERDIER, F., KOURILSKY, P., LINDBERG, A., KLEIN, M., and MOINGEON, P. (2000). Recombinant viruses as a tool for therapeutic vaccination against human cancers. Immunol. Lett. **74,** 11-25.

BORROW, P., TISHON, A., LEE, S., XU, J., GREWAL, I.S., OLDSTONE, M.B., and FLAVELL, R.A. (1996). CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory CD8+ CTL response. J. Exp. Med. **183,** 2129-2142.

BOURGEOIS, C., ROCHA, B., and TANCHOT, C. (2002). A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. Science **297,** 2060-2063.

BRASSEUR, F., MARCHAND, M., VANWIJCK, R., HERIN, M., LETHE, B., CHOMEZ, P., and BOON, T. (1992). Human gene MAGE-1, which codes for a tumor-rejection antigen, is expressed by some breast tumors. Int. J. Cancer **52,** 839-841.

BRINES, R.D. and KLAUS, G.G. (1993). Polyclonal activation of immature B cells by preactivated T cells: the role of IL-4 and CD40 ligand. Int. Immunol. **5,** 1445-1450.

BRIONES, J., TIMMERMAN, J., and LEVY, R. (2002). In vivo antitumor effect of CD40L-transduced tumor cells as a vaccine for B-cell lymphoma. Cancer Res. **62,** 3195-3199.

BROWN, J.P., TWARDZIK, D.R., MARQUARDT, H., and TODARO, G.J. (1985). Vaccinia virus encodes a polypeptide homologous to epidermal growth factor and transforming growth factor. Nature **313,** 491-492.

BRUNNER, K.T., MAUEL, J., CEROTTINI, J.C., and CHAPUIS, B. (1968). Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. Immunology **14,** 181-196.

BULLER, R.M. and PALUMBO, G.J. (1991). Poxvirus pathogenesis. Microbiol. Rev. **55,** 80-122.

CALLARD, R.E., ARMITAGE, R.J., FANSLOW, W.C., and SPRIGGS, M.K. (1993). CD40 ligand and its role in X-linked hyper-IgM syndrome. Immunol. Today **14,** 559-564.

CARSWELL, E.A., OLD, L.J., KASSEL, R.L., GREEN, S., FIORE, N., and WILLIAMSON, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. U. S. A **72,** 3666-3670.

CAUX, C., MASSACRIER, C., VANBERVLIET, B., DUBOIS, B., VAN KOOTEN, C., DURAND, I., and BANCHEREAU, J. (1994). Activation of human dendritic cells through CD40 cross-linking. J. Exp. Med. **180,** 1263-1272.

CELIS, E., SETTE, A., and GREY, H.M. (1995). Epitope selection and development of peptide based vaccines to treat cancer. Semin. Cancer Biol. **6,** 329-336.

CELLA, M., SALLUSTO, F., and LANZAVECCHIA, A. (1997). Origin, maturation and antigen presenting function of dendritic cells. Curr. Opin. Immunol. **9,** 10-16.

CELLA, M., SCHEIDEGGER, D., PALMER-LEHMANN, K., LANE, P., LANZAVECCHIA, A., and ALBER, G. (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J. Exp. Med. **184,** 747-752.

CHAMPAGNE, P., OGG, G.S., KING, A.S., KNABENHANS, C., ELLEFSEN, K., NOBILE, M., APPAY, V., RIZZARDI, G.P., FLEURY, S., LIPP, M., FORSTER, R., ROWLAND-JONES, S., SEKALY, R.P., MCMICHAEL, A.J., and PANTALEO, G. (2001). Skewed maturation of memory HIV-specific CD8 T lymphocytes. Nature **410,** 106-111.

CIPOLLONE, F., MEZZETTI, A., PORRECA, E., DI FEBBO, C., NUTINI, M., FAZIA, M., FALCO, A., CUCCURULLO, F., and DAVI, G. (2002). Association between enhanced soluble CD40L and prothrombotic state in hypercholesterolemia: effects of statin therapy. Circulation **106,** 399-402.

CLODI, K., ASGARI, Z., YOUNES, M., PALMER, J.L., CABANILLAS, F., CARBONE, A., ANDREEFF, M., and YOUNES, A. (2002). Expression of CD40 ligand (CD154) in B and T lymphocytes of Hodgkin disease: potential therapeutic significance. Cancer **94,** 1-5.

CONSTANT, S., SCHWEITZER, N., WEST, J., RANNEY, P., and BOTTOMLY, K. (1995). B lymphocytes can be competent antigen-presenting cells for priming CD4+ T cells to protein antigens in vivo. J. Immunol. **155,** 3734-3741.

COULIE, P.G., BRICHARD, V., VAN PEL, A., WOLFEL, T., SCHNEIDER, J., TRAVERSARI, C., MATTEI, S., DE PLAEN, E., LURQUIN, C., SZIKORA, J.P., and . (1994). A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J. Exp. Med. **180,** 35-42.

CUDMORE, S., RECKMANN, I., GRIFFITHS, G., and WAY, M. (1996). Vaccinia virus: a model system for actin-membrane interactions. J. Cell Sci. **109 (Pt 7),** 1739-1747.

CURTSINGER, J.M., LINS, D.C., and MESCHER, M.F. (2003). Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. J. Exp. Med. **197,** 1141-1151.

CURTSINGER, J.M., SCHMIDT, C.S., MONDINO, A., LINS, D.C., KEDL, R.M., JENKINS, M.K., and MESCHER, M.F. (1999). Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. J. Immunol. **162,** 3256-3262.

D'ANDREA, A., RENGARAJU, M., VALIANTE, N.M., CHEHIMI, J., KUBIN, M., ASTE, M., CHAN, S.H., KOBAYASHI, M., YOUNG, D., NICKBARG, E., and . (1992). Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. J. Exp. Med. **176,** 1387-1398.

DAVISON, A.J. and MOSS, B. (1989a). Structure of vaccinia virus early promoters. J. Mol. Biol. **210,** 749-769.

DAVISON, A.J. and MOSS, B. (1989b). Structure of vaccinia virus late promoters. J. Mol. Biol. **210,** 771-784.

DE VOS, A.F., MELIEF, M.J., VAN RIEL, D., BOON, L., VAN EIJK, M., DE BOER, M., and LAMAN, J.D. (2004). Antagonist anti-human CD40 antibody inhibits germinal center formation in cynomolgus monkeys. Eur. J. Immunol. **34,** 3446-3455.

DENGER, S., JAHN, L., WENDE, P., WATSON, L., GERBER, S.H., KUBLER, W., and KREUZER, J. (1999). Expression of monocyte chemoattractant protein-1 cDNA in vascular smooth muscle cells: induction of the synthetic phenotype: a possible clue to SMC differentiation in the process of atherogenesis. Atherosclerosis **144,** 15-23.

DIEHL, S., CHOW, C.W., WEISS, L., PALMETSHOFER, A., TWARDZIK, T., ROUNDS, L., SERFLING, E., DAVIS, R.J., ANGUITA, J., and RINCON, M. (2002a). Induction of NFATc2 expression by interleukin 6 promotes T helper type 2 differentiation. J. Exp. Med. **196,** 39-49.

DIEHL, S. and RINCON, M. (2002b). The two faces of IL-6 on Th1/Th2 differentiation. Mol. Immunol. **39,** 531-536.

DISANTO, J.P., BONNEFOY, J.Y., GAUCHAT, J.F., FISCHER, A., and DE SAINT, B.G. (1993). CD40 ligand mutations in x-linked immunodeficiency with hyper-IgM. Nature **361,** 541-543.

DRILLIEN, R., SPEHNER, D., BOHBOT, A., and HANAU, D. (2000). Vaccinia virus-related events and phenotypic changes after infection of dendritic cells derived from human monocytes. Virology **268,** 471-481.

DUBOIS, B., MASSACRIER, C., VANBERVLIET, B., FAYETTE, J., BRIERE, F., BANCHEREAU, J., and CAUX, C. (1998). Critical role of IL-12 in dendritic cell-induced differentiation of naive B lymphocytes. J. Immunol. **161,** 2223-2231.

DUDLEY, M.E., WUNDERLICH, J.R., SHELTON, T.E., EVEN, J., and ROSENBERG, S.A. (2003). Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients. J. Immunother. **26,** 332-342.

EARL, P.L., HUGIN, A.W., and MOSS, B. (1990). Removal of cryptic poxvirus transcription termination signals from the human immunodeficiency virus type 1 envelope gene enhances expression and immunogenicity of a recombinant vaccinia virus. J. Virol. **64,** 2448-2451.

ENGELMAYER, J., LARSSON, M., SUBKLEWE, M., CHAHROUDI, A., COX, W.I., STEINMAN, R.M., and BHARDWAJ, N. (1999). Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. J. Immunol. **163,** 6762-6768.

ESCHE, C., GAMBOTTO, A., SATOH, Y., GEREIN, V., ROBBINS, P.D., WATKINS, S.C., LOTZE, M.T., and SHURIN, M.R. (1999). CD154 inhibits tumor-induced apoptosis in dendritic cells and tumor growth. Eur. J. Immunol. **29,** 2148-2155.

EVANS, R.D., KOPF, A.W., LEW, R.A., RIGEL, D.S., BART, R.S., FRIEDMAN, R.J., and RIVERS, J.K. (1988). Risk factors for the development of malignant melanoma--I: Review of case-control studies. J. Dermatol. Surg. Oncol. **14,** 393-408.

FALKNER, F.G. and MOSS, B. (1988). Escherichia coli gpt gene provides dominant selection for vaccinia virus open reading frame expression vectors. J. Virol. **62,** 1849-1854.

FALKNER, F.G. and MOSS, B. (1990). Transient dominant selection of recombinant vaccinia viruses. J. Virol. **64,** 3108-3111.

FEHNIGER, T.A. and CALIGIURI, M.A. (2001). Interleukin 15: biology and relevance to human disease. Blood **97,** 14-32.

FLEXNER, C., MURPHY, B.R., ROONEY, J.F., WOHLENBERG, C., YUFEROV, V., NOTKINS, A.L., and MOSS, B. (1988). Successful vaccination with a polyvalent live vector despite existing immunity to an expressed antigen. Nature **335,** 259-262.

FLUCKIGER, A.C., DURAND, I., and BANCHEREAU, J. (1994). Interleukin 10 induces apoptotic cell death of B-chronic lymphocytic leukemia cells. J. Exp. Med. **179,** 91-99.

FORD, G.S., BARNHART, B., SHONE, S., and COVEY, L.R. (1999). Regulation of CD154 (CD40 ligand) mRNA stability during T cell activation. J. Immunol. **162,** 4037-4044.

FOY, T.M., LAMAN, J.D., LEDBETTER, J.A., ARUFFO, A., CLAASSEN, E., and NOELLE, R.J. (1994). gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. J. Exp. Med. **180,** 157-163.

FULEIHAN, R., RAMESH, N., LOH, R., JABARA, H., ROSEN, R.S., CHATILA, T., FU, S.M., STAMENKOVIC, I., and GEHA, R.S. (1993). Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM. Proc. Natl. Acad. Sci. U. S. A **90,** 2170-2173.

GARBE, C., BUTTNER, P., WEISS, J., SOYER, H.P., STOCKER, U., KRUGER, S., ROSER, M., WECKBECKER, J., PANIZZON, R., BAHMER, F., and . (1994). Risk factors for developing cutaneous melanoma and criteria for identifying persons at risk: multicenter case-control study of the Central Malignant Melanoma Registry of the German Dermatological Society. J. Invest Dermatol. **102,** 695-699.

GARBE, C. and ORFANOS, C.E. (1992). Epidemiology of malignant melanoma in central Europe: risk factors and prognostic predictors. Results of the Central Malignant Melanoma Registry of the German Dermatological Society. Pigment Cell Res. **Suppl 2,** 285-294.

GARLICHS, C.D., ESKAFI, S., RAAZ, D., SCHMIDT, A., LUDWIG, J., HERRMANN, M., KLINGHAMMER, L., DANIEL, W.G., and SCHMEISSER, A. (2001). Patients with acute coronary syndromes express enhanced CD40 ligand/CD154 on platelets. Heart **86,** 649-655.

GARLICHS, C.D., KOZINA, S., FATEH-MOGHADAM, S., HANDSCHU, R., TOMANDL, B., STUMPF, C., ESKAFI, S., RAAZ, D., SCHMEISSER, A., YILMAZ, A., LUDWIG, J., NEUNDORFER, B., and DANIEL, W.G. (2003). Upregulation of CD40-CD40 ligand (CD154) in patients with acute cerebral ischemia. Stroke **34,** 1412-1418.

GAUCHAT, J.F., AUBRY, J.P., MAZZEI, G., LIFE, P., JOMOTTE, T., ELSON, G., and BONNEFOY, J.Y. (1993). Human CD40-ligand: molecular cloning, cellular distribution and regulation of expression by factors controlling IgE production. FEBS Lett. **315,** 259-266.

GEGINAT, J., LANZAVECCHIA, A., and SALLUSTO, F. (2003). Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. Blood **101,** 4260-4266.

GIULIETTI, A., OVERBERGH, L., VALCKX, D., DECALLONNE, B., BOUILLON, R., and MATHIEU, C. (2001). An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods **25,** 386-401.

GOEBEL, S.J., JOHNSON, G.P., PERKUS, M.E., DAVIS, S.W., WINSLOW, J.P., and PAOLETTI, E. (1990). The complete DNA sequence of vaccinia virus. Virology **179,** 247-263.

GRAF, D., KORTHAUER, U., MAGES, H.W., SENGER, G., and KROCZEK, R.A. (1992). Cloning of TRAP, a ligand for CD40 on human T cells. Eur. J. Immunol. **22,** 3191-3194.

GRAHAM, S., GREEN, C.P., MASON, P.D., and BORYSIEWICZ, L.K. (1991). Human cytotoxic T cell responses to vaccinia virus vaccination. J. Gen. Virol. **72 (Pt 5),** 1183-1186.

GRASSME, H., BOCK, J., KUN, J., and GULBINS, E. (2002). Clustering of CD40 ligand is required to form a functional contact with CD40. J. Biol. Chem. **277,** 30289-30299.

GREWAL, I.S., BORROW, P., PAMER, E.G., OLDSTONE, M.B., and FLAVELL, R.A. (1997). The CD40-CD154 system in anti-infective host defense. Curr. Opin. Immunol. **9,** 491-497.

GREWAL, I.S. and FLAVELL, R.A. (1996a). A central role of CD40 ligand in the regulation of CD4+ T-cell responses. Immunol. Today **17,** 410-414.

GREWAL, I.S. and FLAVELL, R.A. (1996b). The role of CD40 ligand in costimulation and T-cell activation. Immunol. Rev. **153,** 85-106.

GREWAL, I.S. and FLAVELL, R.A. (1998). CD40 and CD154 in cell-mediated immunity. Annu. Rev. Immunol. **16,** 111-135.

GREWAL, I.S., XU, J., and FLAVELL, R.A. (1995). Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. Nature **378,** 617-620.

HA, S.J., LEE, C.H., LEE, S.B., KIM, C.M., JANG, K.L., SHIN, H.S., and SUNG, Y.C. (1999). A novel function of IL-12p40 as a chemotactic molecule for macrophages. J. Immunol. **163,** 2902-2908.

HAMILTON, B.J., GENIN, A., CRON, R.Q., and RIGBY, W.F. (2003). Delineation of a novel pathway that regulates CD154 (CD40 ligand) expression. Mol. Cell Biol. **23,** 510-525.

HANISSIAN, S.H. and GEHA, R.S. (1997). Jak3 is associated with CD40 and is critical for CD40 induction of gene expression in B cells. Immunity. **6,** 379-387.

HANSON, C.V. (1992). Photochemical inactivation of viruses with psoralens: an overview. Blood Cells **18,** 7-25.

HEESCHEN, C., DIMMELER, S., HAMM, C.W., VAN DEN BRAND, M.J., BOERSMA, E., ZEIHER, A.M., and SIMOONS, M.L. (2003). Soluble CD40 ligand in acute coronary syndromes. N. Engl. J. Med. **348,** 1104-1111.

HENDRIKS, R.W., KRAAKMAN, M.E., CRAIG, I.W., ESPANOL, T., and SCHUURMAN, R.K. (1990). Evidence that in X-linked immunodeficiency with hyperimmunoglobulinemia M the intrinsic immunoglobulin heavy chain class switch mechanism is intact. Eur. J. Immunol. **20,** 2603-2608.

HENN, V., SLUPSKY, J.R., GRAFE, M., ANAGNOSTOPOULOS, I., FORSTER, R., MULLER-BERGHAUS, G., and KROCZEK, R.A. (1998). CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature **391,** 591-594.

HEUFLER, C., KOCH, F., STANZL, U., TOPAR, G., WYSOCKA, M., TRINCHIERI, G., ENK, A., STEINMAN, R.M., ROMANI, N., and SCHULER, G. (1996). Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. Eur. J. Immunol. **26,** 659-668.

HILLER, G., JUNGWIRTH, C., and WEBER, K. (1981). Fluorescence microscopical analysis of the life cycle of vaccinia virus in chick embryo fibroblasts. Virus-cytoskeleton interactions. Exp. Cell Res. **132,** 81-87.

HILLER, G., WEBER, K., SCHNEIDER, L., PARAJSZ, C., and JUNGWIRTH, C. (1979). Interaction of assembled progeny pox viruses with the cellular cytoskeleton. Virology **98,** 142-153.

HOLLANDER, G.A., CASTIGLI, E., KULBACKI, R., SU, M., BURAKOFF, S.J., GUTIERREZ-RAMOS, J.C., and GEHA, R.S. (1996). Induction of alloantigen-specific tolerance by B cells from CD40-deficient mice. Proc. Natl. Acad. Sci. U. S. A **93,** 4994-4998.

HOLLENBAUGH, D., GROSMAIRE, L.S., KULLAS, C.D., CHALUPNY, N.J., BRAESCH-ANDERSEN, S., NOELLE, R.J., STAMENKOVIC, I., LEDBETTER, J.A., and ARUFFO, A. (1992). The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity. EMBO J. **11,** 4313-4321.

HOLLENBAUGH, D., MISCHEL-PETTY, N., EDWARDS, C.P., SIMON, J.C., DENFELD, R.W., KIENER, P.A., and ARUFFO, A. (1995). Expression of functional CD40 by vascular endothelial cells. J. Exp. Med. **182,** 33-40.

HOMANN, D., JAHREIS, A., WOLFE, T., HUGHES, A., COON, B., VAN STIPDONK, M.J., PRILLIMAN, K.R., SCHOENBERGER, S.P., and VON HERRATH, M.G. (2002). CD40L blockade prevents autoimmune diabetes by induction of bitypic NK/DC regulatory cells. Immunity. **16,** 403-415.

HOPKINS, S.J. (2003). The pathophysiological role of cytokines. Leg. Med. (Tokyo) **5 Suppl 1,** S45-S57.

HOSTAGER, B.S. and BISHOP, G.A. (2002). Role of TNF receptor-associated factor 2 in the activation of IgM secretion by CD40 and CD120b. J. Immunol. **168,** 3318-3322.

HOSTAGER, B.S., HSING, Y., HARMS, D.E., and BISHOP, G.A. (1996). Different CD40-mediated signaling events require distinct CD40 structural features. J. Immunol. **157,** 1047-1053.

HOWLAND, K.C., AUSUBEL, L.J., LONDON, C.A., and ABBAS, A.K. (2000). The roles of CD28 and CD40 ligand in T cell activation and tolerance. J. Immunol. **164,** 4465-4470.

HRUBY, D.E. (1990). Vaccinia virus vectors: new strategies for producing recombinant vaccines. Clin. Microbiol. Rev. **3,** 153-170.

HUGIN, A.W. and HAUSER, C. (1994). The epidermal growth factor receptor is not a receptor for vaccinia virus. J. Virol. **68,** 8409-8412.

HUSSUSSIAN, C.J., STRUEWING, J.P., GOLDSTEIN, A.M., HIGGINS, P.A., ALLY, D.S., SHEAHAN, M.D., CLARK, W.H., JR., TUCKER, M.A., and DRACOPOLI, N.C. (1994). Germline p16 mutations in familial melanoma. Nat. Genet. **8,** 15-21.

HUSTER, K.M., BUSCH, V., SCHIEMANN, M., LINKEMANN, K., KERKSIEK, K.M., WAGNER, H., and BUSCH, D.H. (2004). Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. Proc. Natl. Acad. Sci. U. S. A **101,** 5610-5615.

INABA, K., INABA, M., ROMANI, N., AYA, H., DEGUCHI, M., IKEHARA, S., MURAMATSU, S., and STEINMAN, R.M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. **176,** 1693-1702.

INUI, S., KAISHO, T., KIKUTANI, H., STAMENKOVIC, I., SEED, B., CLARK, E.A., and KISHIMOTO, T. (1990). Identification of the intracytoplasmic region essential for signal transduction through a B cell activation molecule, CD40. Eur. J. Immunol. **20,** 1747-1753.

JANEWAY, C.A., JR. and BOTTOMLY, K. (1994). Signals and signs for lymphocyte responses. Cell **76,** 275-285.

JOHNSON, P.W., WATT, S.M., BETTS, D.R., DAVIES, D., JORDAN, S., NORTON, A.J., and LISTER, T.A. (1993). Isolated follicular lymphoma cells are resistant to apoptosis and can be grown in vitro in the CD40/stromal cell system. Blood **82,** 1848-1857.

JONULEIT, H., WIEDEMANN, K., MULLER, G., DEGWERT, J., HOPPE, U., KNOP, J., and ENK, A.H. (1997). Induction of IL-15 messenger RNA and protein in human blood-derived dendritic cells: a role for IL-15 in attraction of T cells. J. Immunol. **158,** 2610-2615.

JUDGE, A.D., ZHANG, X., FUJII, H., SURH, C.D., and SPRENT, J. (2002). Interleukin 15 controls both proliferation and survival of a subset of memory-phenotype CD8(+) T cells. J. Exp. Med. **196,** 935-946.

KAMB, A., SHATTUCK-EIDENS, D., EELES, R., LIU, Q., GRUIS, N.A., DING, W., HUSSEY, C., TRAN, T., MIKI, Y., WEAVER-FELDHAUS, J., and . (1994). Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. Nat. Genet. **8,** 23-26.

KAMMULA, U.S., LEE, K.H., RIKER, A.I., WANG, E., OHNMACHT, G.A., ROSENBERG, S.A., and MARINCOLA, F.M. (1999). Functional analysis of antigen-specific T lymphocytes by serial measurement of gene expression in peripheral blood mononuclear cells and tumor specimens. J. Immunol. **163,** 6867-6875.

KANEGANE, H. and TOSATO, G. (1996). Activation of naive and memory T cells by interleukin-15. Blood **88,** 230-235.

KANEKO, Y., HIROSE, S., ABE, M., YAGITA, H., OKUMURA, K., and SHIRAI, T. (1996). CD40-mediated stimulation of B1 and B2 cells: implication in autoantibody production in murine lupus. Eur. J. Immunol. **26,** 3061-3065.

KARMANN, K., HUGHES, C.C., SCHECHNER, J., FANSLOW, W.C., and POBER, J.S. (1995). CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. Proc. Natl. Acad. Sci. U. S. A **92,** 4342-4346.

KARPUSAS, M., HSU, Y.M., WANG, J.H., THOMPSON, J., LEDERMAN, S., CHESS, L., and THOMAS, D. (1995). 2 A crystal structure of an extracellular fragment of human CD40 ligand. Structure. **3,** 1031-1039.

KASKEL, P., SANDER, S., KRON, M., KIND, P., PETER, R.U., and KRAHN, G. (2001). Outdoor activities in childhood: a protective factor for cutaneous melanoma? Results of a case-control study in 271 matched pairs. Br. J. Dermatol. **145,** 602-609.

KIENER, P.A., MORAN-DAVIS, P., RANKIN, B.M., WAHL, A.F., ARUFFO, A., and HOLLENBAUGH, D. (1995). Stimulation of CD40 with purified soluble gp39 induces proinflammatory responses in human monocytes. J. Immunol. **155,** 4917-4925.

KIEPER, W.C., PRLIC, M., SCHMIDT, C.S., MESCHER, M.F., and JAMESON, S.C. (2001). Il-12 enhances CD8 T cell homeostatic expansion. J. Immunol. **166,** 5515-5521.

KIPPS, T.J., CHU, P., and WIERDA, W.G. (2000). Immunogenetic therapy for B-cell malignancies. Semin. Oncol. **27,** 104-109.

KLUIN-NELEMANS, H.C., BEVERSTOCK, G.C., MOLLEVANGER, P., WESSELS, H.W., HOOGENDOORN, E., WILLEMZE, R., and FALKENBURG, J.H. (1994). Proliferation and cytogenetic analysis of hairy cell leukemia upon stimulation via the CD40 antigen. Blood **84,** 3134-3141.

KLUTH, B., HESS, S., ENGELMANN, H., SCHAFNITZEL, S., RIETHMULLER, G., and FEUCHT, H.E. (1997). Endothelial expression of CD40 in renal cell carcinoma. Cancer Res. **57,** 891-899.

KOBAYASHI, M., FITZ, L., RYAN, M., HEWICK, R.M., CLARK, S.C., CHAN, S., LOUDON, R., SHERMAN, F., PERUSSIA, B., and TRINCHIERI, G. (1989). Identification and purification of natural

killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J. Exp. Med. **170,** 827-845.

KOPPI, T.A., TOUGH-BEMENT, T., LEWINSOHN, D.M., LYNCH, D.H., and ALDERSON, M.R. (1997). CD40 ligand inhibits Fas/CD95-mediated apoptosis of human blood-derived dendritic cells. Eur. J. Immunol. **27,** 3161-3165.

KORNBLUTH, R.S., KEE, K., and RICHMAN, D.D. (1998). CD40 ligand (CD154) stimulation of macrophages to produce HIV-1-suppressive beta-chemokines. Proc. Natl. Acad. Sci. U. S. A **95,** 5205-5210.

KORTHAUER, U., GRAF, D., MAGES, H.W., BRIERE, F., PADAYACHEE, M., MALCOLM, S., UGAZIO, A.G., NOTARANGELO, L.D., LEVINSKY, R.J., and KROCZEK, R.A. (1993). Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. Nature **361,** 539-541.

KOSCHELLA, M., VOEHRINGER, D., and PIRCHER, H. (2004). CD40 ligation in vivo induces bystander proliferation of memory phenotype CD8 T cells. J. Immunol. **172,** 4804-4811.

KUNIYOSHI, J.S., KUNIYOSHI, C.J., LIM, A.M., WANG, F.Y., BADE, E.R., LAU, R., THOMAS, E.K., and WEBER, J.S. (1999). Dendritic cell secretion of IL-15 is induced by recombinant huCD40LT and augments the stimulation of antigen-specific cytolytic T cells. Cell Immunol. **193,** 48-58.

KUPFER, A., SWAIN, S.L., and SINGER, S.J. (1987). The specific direct interaction of helper T cells and antigen-presenting B cells. II. Reorientation of the microtubule organizing center and reorganization of the membrane-associated cytoskeleton inside the bound helper T cells. J. Exp. Med. **165,** 1565-1580.

LAMAN, J.D., 'T HART, B.A., BROK, H., MEURS, M., SCHELLEKENS, M.M., KASRAN, A., BOON, L., BAUER, J., BOER, M., and CEUPPENS, J. (2002). Protection of marmoset monkeys against EAE by treatment with a murine antibody blocking CD40 (mu5D12). Eur. J. Immunol. **32,** 2218-2228.

LANE, P., TRAUNECKER, A., HUBELE, S., INUI, S., LANZAVECCHIA, A., and GRAY, D. (1992). Activated human T cells express a ligand for the human B cell-associated antigen CD40 which participates in T cell-dependent activation of B lymphocytes. Eur. J. Immunol. **22,** 2573-2578.

LANGLEY, R.G. and SOBER, A.J. (1997). A clinical review of the evidence for the role of ultraviolet radiation in the etiology of cutaneous melanoma. Cancer Invest **15,** 561-567.

LARSSON, M., FONTENEAU, J.F., SOMERSAN, S., SANDERS, C., BICKHAM, K., THOMAS, E.K., MAHNKE, K., and BHARDWAJ, N. (2001). Efficiency of cross presentation of vaccinia virus-derived antigens by human dendritic cells. Eur. J. Immunol. **31,** 3432-3442.

LATTIME, E.C., LEE, S.S., EISENLOHR, L.C., and MASTRANGELO, M.J. (1996). In situ cytokine gene transfection using vaccinia virus vectors. Semin. Oncol. **23,** 88-100.

LEDERMAN, S., YELLIN, M.J., CLEARY, A.M., PERNIS, A., INGHIRAMI, G., COHN, L.E., COVEY, L.R., LEE, J.J., ROTHMAN, P., and CHESS, L. (1994). T-BAM/CD40-L on helper T lymphocytes augments lymphokine-induced B cell Ig isotype switch recombination and rescues B cells from programmed cell death. J. Immunol. **152,** 2163-2171.

LENSCHOW, D.J., WALUNAS, T.L., and BLUESTONE, J.A. (1996). CD28/B7 system of T cell costimulation. Annu. Rev. Immunol. **14,** 233-258.

LEO, E., WELSH, K., MATSUZAWA, S., ZAPATA, J.M., KITADA, S., MITCHELL, R.S., ELY, K.R., and REED, J.C. (1999). Differential requirements for tumor necrosis factor receptor-associated factor family proteins in CD40-mediated induction of NF-kappaB and Jun N-terminal kinase activation. J. Biol. Chem. **274,** 22414-22422.

LETHE, B., VAN DER, B.P., BRASSEUR, F., and BOON, T. (1997). MAGE-1 expression threshold for the lysis of melanoma cell lines by a specific cytotoxic T lymphocyte. Melanoma Res. **7 Suppl 2,** S83-S88.

LIENENLUKE, B., GERMANN, T., KROCZEK, R.A., and HECKER, M. (2000). CD154 stimulation of interleukin-12 synthesis in human endothelial cells. Eur. J. Immunol. **30,** 2864-2870.

LIU, Y., QURESHI, M., and XIANG, J. (2002). Antitumor immune responses derived from transgenic expression of CD40 ligand in myeloma cells. Cancer Biother. Radiopharm. **17,** 11-18.

LIVAK, K.J. and SCHMITTGEN, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25,** 402-408.

LU, J., GIUNTOLI, R.L., OMIYA, R., KOBAYASHI, H., KENNEDY, R., and CELIS, E. (2002). Interleukin 15 promotes antigen-independent in vitro expansion and long-term survival of antitumor cytotoxic T lymphocytes. Clin. Cancer Res. **8,** 3877-3884.

LUDEWIG, B., GRAF, D., GELDERBLOM, H.R., BECKER, Y., KROCZEK, R.A., and PAULI, G. (1995). Spontaneous apoptosis of dendritic cells is efficiently inhibited by TRAP (CD40-ligand) and TNF-alpha, but strongly enhanced by interleukin-10. Eur. J. Immunol. **25,** 1943-1950.

MA, X., CHOW, J.M., GRI, G., CARRA, G., GEROSA, F., WOLF, S.F., DZIALO, R., and TRINCHIERI, G. (1996). The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. J. Exp. Med. **183,** 147-157.

MACATONIA, S.E., HOSKEN, N.A., LITTON, M., VIEIRA, P., HSIEH, C.S., CULPEPPER, J.A., WYSOCKA, M., TRINCHIERI, G., MURPHY, K.M., and O'GARRA, A. (1995). Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J. Immunol. **154,** 5071-5079.

MACKIE, R.M. (1998). Incidence, risk factors and prevention of melanoma. Eur. J. Cancer **34 Suppl 3,** S3-S6.

MAFFEI, A., PAPADOPOULOS, K., and HARRIS, P.E. (1997). MHC class I antigen processing pathways. Hum. Immunol. **54,** 91-103.

MALEC, M., SODERQVIST, M., SIRSJO, A., MACNAMARA, B., LEWIN, N., SJOBERG, J., BJORKHOLM, M., and PORWIT-MACDONALD, A. (2004). Real-time polymerase chain reaction determination of cytokine mRNA expression profiles in Hodgkin s lymphoma. Haematologica **89,** 679-685.

MARCHES, R., RACILA, E., TUCKER, T.F., PICKER, L., MONGINI, P., HSUEH, R., VITETTA, E.S., SCHEUERMANN, R.H., and UHR, J.W. (1995). Tumour dormancy and cell signalling--III: Role of hypercrosslinking of IgM and CD40 on the induction of cell cycle arrest and apoptosis in B lymphoma cells. Ther. Immunol. **2,** 125-136.

MARTI, W.R., ZAJAC, P., SPAGNOLI, G., HEBERER, M., and OERTLI, D. (1997). Nonreplicating recombinant vaccinia virus encoding human B-7 molecules elicits effective costimulation of naive and memory CD4+ T lymphocytes in vitro. Cell Immunol. **179,** 146-152.

MARTIN, I., JAKOB, M., SCHAFER, D., DICK, W., SPAGNOLI, G., and HEBERER, M. (2001). Quantitative analysis of gene expression in human articular cartilage from normal and osteoarthritic joints. Osteoarthritis. Cartilage. **9,** 112-118.

MARUO, S., OH-HORA, M., AHN, H.J., ONO, S., WYSOCKA, M., KANEKO, Y., YAGITA, H., OKUMURA, K., KIKUTANI, H., KISHIMOTO, T., KOBAYASHI, M., HAMAOKA, T., TRINCHIERI, G., and FUJIWARA, H. (1997). B cells regulate CD40 ligand-induced IL-12 production in antigen-presenting cells (APC) during T cell/APC interactions. J. Immunol. **158,** 120-126.

MATSUMURA, Y. and ANANTHASWAMY, H.N. (2002). Molecular mechanisms of photocarcinogenesis. Front Biosci. **7,** d765-d783.

MATZINGER, P. (2002). The danger model: a renewed sense of self. Science **296,** 301-305.

MCWHIRTER, S.M., PULLEN, S.S., HOLTON, J.M., CRUTE, J.J., KEHRY, M.R., and ALBER, T. (1999). Crystallographic analysis of CD40 recognition and signaling by human TRAF2. Proc. Natl. Acad. Sci. U. S. A **96,** 8408-8413.

MOODYCLIFFE, A.M., SHREEDHAR, V., ULLRICH, S.E., WALTERSCHEID, J., BUCANA, C., KRIPKE, M.L., and FLORES-ROMO, L. (2000). CD40-CD40 ligand interactions in vivo regulate migration of antigen-bearing dendritic cells from the skin to draining lymph nodes. J. Exp. Med. **191,** 2011-2020.

MOSCA, P.J., HOBEIKA, A.C., CLAY, T.M., NAIR, S.K., THOMAS, E.K., MORSE, M.A., and LYERLY, H.K. (2000). A subset of human monocyte-derived dendritic cells expresses high levels of interleukin-12 in response to combined CD40 ligand and interferon-gamma treatment. Blood **96,** 3499-3504.

MOSS, B. (1996). Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc. Natl. Acad. Sci. U. S. A **93,** 11341-11348.

MOTTONEN, M., ISOMAKI, P., LUUKKAINEN, R., TOIVANEN, P., PUNNONEN, J., and LASSILA, O. (2000). Interleukin-15 up-regulates the expression of CD154 on synovial fluid T cells. Immunology **100,** 238-244.

MULLIGAN, R.C. and BERG, P. (1981). Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. U. S. A **78,** 2072-2076.

MURPHY, W.J., WELNIAK, L., BACK, T., HIXON, J., SUBLESKI, J., SEKI, N., WIGGINTON, J.M., WILSON, S.E., BLAZAR, B.R., MALYGUINE, A.M., SAYERS, T.J., and WILTROUT, R.H. (2003). Synergistic anti-tumor responses after administration of agonistic antibodies to CD40 and IL-2: coordination of dendritic and CD8+ cell responses. J. Immunol. **170,** 2727-2733.

MUSSO, T., CALOSSO, L., ZUCCA, M., MILLESIMO, M., RAVARINO, D., GIOVARELLI, M., MALAVASI, F., PONZI, A.N., PAUS, R., and BULFONE-PAUS, S. (1999). Human monocytes constitutively express membrane-bound, biologically active, and interferon-gamma-upregulated interleukin-15. Blood **93,** 3531-3539.

NANKI, T., HAYASHIDA, K., EL GABALAWY, H.S., SUSON, S., SHI, K., GIRSCHICK, H.J., YAVUZ, S., and LIPSKY, P.E. (2000). Stromal cell-derived factor-1-CXC chemokine receptor 4 interactions play a central role in CD4+ T cell accumulation in rheumatoid arthritis synovium. J. Immunol. **165,** 6590-6598.

NANNIZZI-ALAIMO, L., RUBENSTEIN, M.H., ALVES, V.L., LEONG, G.Y., PHILLIPS, D.R., and GOLD, H.K. (2002). Cardiopulmonary bypass induces release of soluble CD40 ligand. Circulation **105,** 2849-2854.

NI, C.Z., WELSH, K., LEO, E., CHIOU, C.K., WU, H., REED, J.C., and ELY, K.R. (2000). Molecular basis for CD40 signaling mediated by TRAF3. Proc. Natl. Acad. Sci. U. S. A **97,** 10395-10399.

NIELSEN, M.B. and MARINCOLA, F.M. (2000). Melanoma vaccines: the paradox of T cell activation without clinical response. Cancer Chemother. Pharmacol. **46 Suppl,** S62-S66.

NIEMAN, D.C., DAVIS, J.M., BROWN, V.A., HENSON, D.A., DUMKE, C.L., UTTER, A.C., VINCI, D.M., DOWNS, M.F., SMITH, J.C., CARSON, J., BROWN, A., MCANULTY, S.R., and MCANULTY, L.S. (2004). Influence of carbohydrate ingestion on immune changes after 2 h of intensive resistance training. J. Appl. Physiol **96,** 1292-1298.

NOELLE, R.J., ROY, M., SHEPHERD, D.M., STAMENKOVIC, I., LEDBETTER, J.A., and ARUFFO, A. (1992). A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. Proc. Natl. Acad. Sci. U. S. A **89,** 6550-6554.

NOPPEN, C., LEVY, F., BURRI, L., ZAJAC, P., REMMEL, E., SCHAEFER, C., LUSCHER, U., HEBERER, M., and SPAGNOLI, G.C. (2000). Naturally processed and concealed HLA-A2.1-restricted epitopes from tumor-associated antigen tyrosinase-related protein-2. Int. J. Cancer **87,** 241-246.

OERTLI, D., MARTI, W.R., ZAJAC, P., NOPPEN, C., KOCHER, T., PADOVAN, E., ADAMINA, M., SCHUMACHER, R., HARDER, F., HEBERER, M., and SPAGNOLI, G.C. (2002). Rapid induction of specific cytotoxic T lymphocytes against melanoma-associated antigens by a recombinant vaccinia virus vector expressing multiple immunodominant epitopes and costimulatory molecules in vivo. Hum. Gene Ther. **13,** 569-575.

OXENIUS, A., CAMPBELL, K.A., MALISZEWSKI, C.R., KISHIMOTO, T., KIKUTANI, H., HENGARTNER, H., ZINKERNAGEL, R.M., and BACHMANN, M.F. (1996). CD40-CD40 ligand interactions are critical in T-B cooperation but not for other anti-viral CD4+ T cell functions. J. Exp. Med. **183,** 2209-2218.

PAMMER, J., PLETTENBERG, A., WENINGER, W., DILLER, B., MILDNER, M., UTHMAN, A., ISSING, W., STURZL, M., and TSCHACHLER, E. (1996). CD40 antigen is expressed by endothelial cells and tumor cells in Kaposi's sarcoma. Am. J. Pathol. **148,** 1387-1396.

PANICALI, D. and PAOLETTI, E. (1982). Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc. Natl. Acad. Sci. U. S. A **79,** 4927-4931.

PAOLETTI, E. (1996). Applications of pox virus vectors to vaccination: an update. Proc. Natl. Acad. Sci. U. S. A **93,** 11349-11353.

PARISH, C.R. (1999). Fluorescent dyes for lymphocyte migration and proliferation studies. Immunol. Cell Biol. **77,** 499-508.

PASARE, C. and MEDZHITOV, R. (2003). Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science **299,** 1033-1036.

PASTORET, P.P. and BROCHIER, B. (1998). Epidemiology and elimination of rabies in western Europe. Vet. J. **156,** 83-90.

PAULIE, S., EHLIN-HENRIKSSON, B., MELLSTEDT, H., KOHO, H., BEN AISSA, H., and PERLMANN, P. (1985). A p50 surface antigen restricted to human urinary bladder carcinomas and B lymphocytes. Cancer Immunol. Immunother. **20,** 23-28.

PENG, X., REMACLE, J.E., KASRAN, A., HUYLEBROECK, D., and CEUPPENS, J.L. (1998). IL-12 up-regulates CD40 ligand (CD154) expression on human T cells. J. Immunol. **160,** 1166-1172.

PEPLINSKI, G.R., TSUNG, K., and NORTON, J.A. (1998). Vaccinia virus for human gene therapy. Surg. Oncol. Clin. N. Am. **7,** 575-588.

PORTIELJE, J.E., GRATAMA, J.W., VAN OJIK, H.H., STOTER, G., and KRUIT, W.H. (2003). IL-12: a promising adjuvant for cancer vaccination. Cancer Immunol. Immunother. **52,** 133-144.

POUDRIER, J. and OWENS, T. (1994). Co-stimulation by anti-immunoglobulin is required for B cell activation by CD40Llow T cells. Eur. J. Immunol. **24,** 2993-2999.

PULLEN, S.S., LABADIA, M.E., INGRAHAM, R.H., MCWHIRTER, S.M., EVERDEEN, D.S., ALBER, T., CRUTE, J.J., and KEHRY, M.R. (1999). High-affinity interactions of tumor necrosis factor receptor-associated factors (TRAFs) and CD40 require TRAF trimerization and CD40 multimerization. Biochemistry **38,** 10168-10177.

RAMESH, N., FULEIHAN, R., and GEHA, R. (1994). Molecular pathology of X-linked immunoglobulin deficiency with normal or elevated IgM (HIGMX-1). Immunol. Rev. **138,** 87-104.

RANHEIM, E.A. and KIPPS, T.J. (1993). Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. J. Exp. Med. **177,** 925-935.

RANHEIM, E.A. and KIPPS, T.J. (1994). Elevated expression of CD80 (B7/BB1) and other accessory molecules on synovial fluid mononuclear cell subsets in rheumatoid arthritis. Arthritis Rheum. **37,** 1637-1646.

RAYNAL, P. and POLLARD, H.B. (1994). Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. Biochim. Biophys. Acta **1197,** 63-93.

RAZEGHI, P., MUKHOPADHYAY, M., MYERS, T.J., WILLIAMS, J.N., MORAVEC, C.S., FRAZIER, O.H., and TAEGTMEYER, H. (2001). Myocardial tumor necrosis factor-alpha expression does not correlate with clinical indices of heart failure in patients on left ventricular assist device support. Ann. Thorac. Surg. **72,** 2044-2050.

REINER, S.L., ZHENG, S., WANG, Z.E., STOWRING, L., and LOCKSLEY, R.M. (1994). Leishmania promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from CD4+ T cells during initiation of infection. J. Exp. Med. **179,** 447-456.

REISS, J. (1986). Detection of genotoxic properties of mycotoxins with the SOS chromotest. Naturwissenschaften **73,** 677-678.

RIBAS, A., BUTTERFIELD, L.H., AMARNANI, S.N., DISSETTE, V.B., KIM, D., MENG, W.S., MIRANDA, G.A., WANG, H.J., MCBRIDE, W.H., GLASPY, J.A., and ECONOMOU, J.S. (2001). CD40 cross-linking bypasses the absolute requirement for CD4 T cells during immunization with melanoma antigen gene-modified dendritic cells. Cancer Res. **61,** 8787-8793.

RIDGE, J.P., DI ROSA, F., and MATZINGER, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature **393,** 474-478.

RIGBY, W.F., WAUGH, M.G., and HAMILTON, B.J. (1999). Characterization of RNA binding proteins associated with CD40 ligand (CD154) mRNA turnover in human T lymphocytes. J. Immunol. **163,** 4199-4206.

RIVERA, A., CHEN, C.C., RON, N., DOUGHERTY, J.P., and RON, Y. (2001). Role of B cells as antigen-presenting cells in vivo revisited: antigen-specific B cells are essential for T cell expansion in lymph nodes and for systemic T cell responses to low antigen concentrations. Int. Immunol. **13,** 1583-1593.

ROBBINS, P.F., EL GAMIL, M., KAWAKAMI, Y., STEVENS, E., YANNELLI, J.R., and ROSENBERG, S.A. (1994). Recognition of tyrosinase by tumor-infiltrating lymphocytes from a patient responding to immunotherapy. Cancer Res. **54,** 3124-3126.

ROCHESTER, S.C. and TRAKTMAN, P. (1998). Characterization of the single-stranded DNA binding protein encoded by the vaccinia virus I3 gene. J. Virol. **72,** 2917-2926.

ROGERS, N.J., JACKSON, I.M., JORDAN, W.J., LOMBARDI, G., DELIKOURAS, A., and LECHLER, R.I. (2003). CD40 can costimulate human memory T cells and favors IL-10 secretion. Eur. J. Immunol. **33,** 1094-1104.

ROSENBERG, S.A., PACKARD, B.S., AEBERSOLD, P.M., SOLOMON, D., TOPALIAN, S.L., TOY, S.T., SIMON, P., LOTZE, M.T., YANG, J.C., SEIPP, C.A., and . (1988). Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N. Engl. J. Med. **319,** 1676-1680.

ROY, M., ARUFFO, A., LEDBETTER, J., LINSLEY, P., KEHRY, M., and NOELLE, R. (1995). Studies on the interdependence of gp39 and B7 expression and function during antigen-specific immune responses. Eur. J. Immunol. **25,** 596-603.

RUEDL, C., BACHMANN, M.F., and KOPF, M. (2000). The antigen dose determines T helper subset development by regulation of CD40 ligand. Eur. J. Immunol. **30,** 2056-2064.

SCHATTNER, E.J., ELKON, K.B., YOO, D.H., TUMANG, J., KRAMMER, P.H., CROW, M.K., and FRIEDMAN, S.M. (1995). CD40 ligation induces Apo-1/Fas expression on human B lymphocytes and facilitates apoptosis through the Apo-1/Fas pathway. J. Exp. Med. **182,** 1557-1565.

SCHOENBERGER, S.P., TOES, R.E., VAN DER VOORT, E.I., OFFRINGA, R., and MELIEF, C.J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature **393,** 480-483.

SCHONBECK, U. and LIBBY, P. (2001a). CD40 signaling and plaque instability. Circ. Res. **89,** 1092-1103.

SCHONBECK, U. and LIBBY, P. (2001b). The CD40/CD154 receptor/ligand dyad. Cell Mol. Life Sci. **58,** 4-43.

SCHONBECK, U., MACH, F., and LIBBY, P. (2000). CD154 (CD40 ligand). Int. J. Biochem. Cell Biol. **32,** 687-693.

SCHONBECK, U., VARO, N., LIBBY, P., BURING, J., and RIDKER, P.M. (2001c). Soluble CD40L and cardiovascular risk in women. Circulation **104,** 2266-2268.

SEMB, A.G., VAN WISSEN, S., UELAND, T., SMILDE, T., WAEHRE, T., TRIPP, M.D., FROLAND, S.S., KASTELEIN, J.J., GULLESTAD, L., PEDERSEN, T.R., AUKRUST, P., and STALENHOEF, A.F. (2003). Raised serum levels of soluble CD40 ligand in patients with familial hypercholesterolemia: downregulatory effect of statin therapy. J. Am. Coll. Cardiol. **41,** 275-279.

SHAW, G. and KAMEN, R. (1986). A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell **46,** 659-667.

SHU, U., KINIWA, M., WU, C.Y., MALISZEWSKI, C., VEZZIO, N., HAKIMI, J., GATELY, M., and DELESPESSE, G. (1995). Activated T cells induce interleukin-12 production by monocytes via CD40-CD40 ligand interaction. Eur. J. Immunol. **25,** 1125-1128.

SIMONINI, A., MOSCUCCI, M., MULLER, D.W., BATES, E.R., PAGANI, F.D., BURDICK, M.D., and STRIETER, R.M. (2000). IL-8 is an angiogenic factor in human coronary atherectomy tissue. Circulation **101,** 1519-1526.

SIN, J.I., KIM, J.J., ZHANG, D., and WEINER, D.B. (2001). Modulation of cellular responses by plasmid CD40L: CD40L plasmid vectors enhance antigen-specific helper T cell type 1 CD4+ T cell-mediated protective immunity against herpes simplex virus type 2 in vivo. Hum. Gene Ther. **12,** 1091-1102.

SINGH, S.R., CASPER, K., SUMMERS, S., and SWERLICK, R.A. (2001). CD40 expression and function on human dermal microvascular endothelial cells: role in cutaneous inflammation. Clin. Exp. Dermatol. **26,** 434-440.

SKEEN, M.J., MILLER, M.A., SHINNICK, T.M., and ZIEGLER, H.K. (1996). Regulation of murine macrophage IL-12 production. Activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines. J. Immunol. **156,** 1196-1206.

SKOV, S., BONYHADI, M., ODUM, N., and LEDBETTER, J.A. (2000). IL-2 and IL-15 regulate CD154 expression on activated CD4 T cells. J. Immunol. **164,** 3500-3505.

SMITH, G.L., MACKETT, M., and MOSS, B. (1984). Recombinant vaccinia viruses as new live vaccines. Biotechnol. Genet. Eng Rev. **2,** 383-407.

SPAGNOLI, G.C., ZAJAC, P., MARTI, W.R., OERTLI, D., PADOVAN, E., NOPPEN, C., KOCHER, T., ADAMINA, M., and HEBERER, M. (2002). Cytotoxic T-cell induction in metastatic melanoma patients undergoing recombinant vaccinia virus-based immuno-gene therapy. Recent Results Cancer Res. **160,** 195-201.

SRAHNA, M., REMACLE, J.E., ANNAMALAI, K., PYPE, S., HUYLEBROECK, D., BOOGAERTS, M.A., and VANDENBERGHE, P. (2001). NF-kappaB is involved in the regulation of CD154 (CD40 ligand) expression in primary human T cells. Clin. Exp. Immunol. **125,** 229-236.

STOUT, R.D. and SUTTLES, J. (1996a). The many roles of CD40 in cell-mediated inflammatory responses. Immunol. Today **17,** 487-492.

STOUT, R.D., SUTTLES, J., XU, J., GREWAL, I.S., and FLAVELL, R.A. (1996b). Impaired T cell-mediated macrophage activation in CD40 ligand-deficient mice. J. Immunol. **156,** 8-11.

STRAW, A.D., MACDONALD, A.S., DENKERS, E.Y., and PEARCE, E.J. (2003). CD154 plays a central role in regulating dendritic cell activation during infections that induce Th1 or Th2 responses. J. Immunol. **170,** 727-734.

SUGIURA, T., KAWAGUCHI, Y., HARIGAI, M., TAKAGI, K., OHTA, S., FUKASAWA, C., HARA, M., and KAMATANI, N. (2000). Increased CD40 expression on muscle cells of polymyositis and dermatomyositis: role of CD40-CD40 ligand interaction in IL-6, IL-8, IL-15, and monocyte chemoattractant protein-1 production. J. Immunol. **164,** 6593-6600.

TAKAHASHI, T., CHAPMAN, P.B., YANG, S.Y., HARA, I., VIJAYASARADHI, S., and HOUGHTON, A.N. (1995). Reactivity of autologous CD4+ T lymphocytes against human melanoma. Evidence for a shared melanoma antigen presented by HLA-DR15. J. Immunol. **154,** 772-779.

THIENEL, U., LOIKE, J., and YELLIN, M.J. (1999). CD154 (CD40L) induces human endothelial cell chemokine production and migration of leukocyte subsets. Cell Immunol. **198,** 87-95.

TOES, R.E., SCHOENBERGER, S.P., VAN DER VOORT, E.I., OFFRINGA, R., and MELIEF, C.J. (1998). CD40-CD40Ligand interactions and their role in cytotoxic T lymphocyte priming and anti-tumor immunity. Semin. Immunol. **10,** 443-448.

TOMIYAMA, H., MATSUDA, T., and TAKIGUCHI, M. (2002). Differentiation of human CD8(+) T cells from a memory to memory/effector phenotype. J. Immunol. **168,** 5538-5550.

TONE, M., TONE, Y., FAIRCHILD, P.J., WYKES, M., and WALDMANN, H. (2001). Regulation of CD40 function by its isoforms generated through alternative splicing. Proc. Natl. Acad. Sci. U. S. A **98,** 1751-1756.

TONG, A.W., PAPAYOTI, M.H., NETTO, G., ARMSTRONG, D.T., ORDONEZ, G., LAWSON, J.M., and STONE, M.J. (2001). Growth-inhibitory effects of CD40 ligand (CD154) and its endogenous expression in human breast cancer. Clin. Cancer Res. **7,** 691-703.

TOPALIAN, S.L. and ROSENBERG, S.A. (1990). Tumor-infiltrating lymphocytes: evidence for specific immune reactions against growing cancers in mice and humans. Important Adv. Oncol. 19-41.

TOWNSEND, A.R., ROTHBARD, J., GOTCH, F.M., BAHADUR, G., WRAITH, D., and MCMICHAEL, A.J. (1986). The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell **44,** 959-968.

TRAVERSARI, C., VAN DER, B.P., LUESCHER, I.F., LURQUIN, C., CHOMEZ, P., VAN PEL, A., DE PLAEN, E., AMAR-COSTESEC, A., and BOON, T. (1992). A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. J. Exp. Med. **176,** 1453-1457.

TSANG, K.Y., ZAREMBA, S., NIERODA, C.A., ZHU, M.Z., HAMILTON, J.M., and SCHLOM, J. (1995). Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine. J. Natl. Cancer Inst. **87,** 982-990.

TSUBATA, T., WU, J., and HONJO, T. (1993). B-cell apoptosis induced by antigen receptor crosslinking is blocked by a T-cell signal through CD40. Nature **364,** 645-648.

TSUNG, K., YIM, J.H., MARTI, W., BULLER, R.M., and NORTON, J.A. (1996). Gene expression and cytopathic effect of vaccinia virus inactivated by psoralen and long-wave UV light. J. Virol. **70,** 165-171.

URASHIMA, M., CHAUHAN, D., HATZIYANNI, M., OGATA, A., HOLLENBAUGH, D., ARUFFO, A., and ANDERSON, K.C. (1996). CD40 ligand triggers interleukin-6 mediated B cell differentiation. Leuk. Res. **20,** 507-515.

VALENTINE, M.A. and LICCIARDI, K.A. (1992). Rescue from anti-IgM-induced programmed cell death by the B cell surface proteins CD20 and CD40. Eur. J. Immunol. **22,** 3141-3148.

VALMORI, D., SCHEIBENBOGEN, C., DUTOIT, V., NAGORSEN, D., ASEMISSEN, A.M., RUBIO-GODOY, V., RIMOLDI, D., GUILLAUME, P., ROMERO, P., SCHADENDORF, D., LIPP, M., DIETRICH, P.Y., THIEL, E., CEROTTINI, J.C., LIENARD, D., and KEILHOLZ, U. (2002). Circulating Tumor-reactive CD8(+) T cells in melanoma patients contain a CD45RA(+)CCR7(-) effector subset exerting ex vivo tumor-specific cytolytic activity. Cancer Res. **62,** 1743-1750.

VAN ENDERT, P.M. (1999). Genes regulating MHC class I processing of antigen. Curr. Opin. Immunol. **11,** 82-88.

VAN ESSEN, D., KIKUTANI, H., and GRAY, D. (1995). CD40 ligand-transduced co-stimulation of T cells in the development of helper function. Nature **378,** 620-623.

VAN MIERLO, G.J., DEN BOER, A.T., MEDEMA, J.P., VAN DER VOORT, E.I., FRANSEN, M.F., OFFRINGA, R., MELIEF, C.J., and TOES, R.E. (2002). CD40 stimulation leads to effective therapy of CD40(-) tumors through induction of strong systemic cytotoxic T lymphocyte immunity. Proc. Natl. Acad. Sci. U. S. A **99,** 5561-5566.

VARO, N., DE LEMOS, J.A., LIBBY, P., MORROW, D.A., MURPHY, S.A., NUZZO, R., GIBSON, C.M., CANNON, C.P., BRAUNWALD, E., and SCHONBECK, U. (2003a). Soluble CD40L: risk prediction after acute coronary syndromes. Circulation **108,** 1049-1052.

VARO, N., VICENT, D., LIBBY, P., NUZZO, R., CALLE-PASCUAL, A.L., BERNAL, M.R., FERNANDEZ-CRUZ, A., VEVES, A., JAROLIM, P., VARO, J.J., GOLDFINE, A., HORTON, E., and SCHONBECK, U. (2003b). Elevated plasma levels of the atherogenic mediator soluble CD40 ligand in diabetic patients: a novel target of thiazolidinediones. Circulation **107,** 2664-2669.

VON HERRATH, M.G., YOKOYAMA, M., DOCKTER, J., OLDSTONE, M.B., and WHITTON, J.L. (1996). CD4-deficient mice have reduced levels of memory cytotoxic T lymphocytes after immunization and show diminished resistance to subsequent virus challenge. J. Virol. **70,** 1072-1079.

VONDERHEIDE, R.H., DUTCHER, J.P., ANDERSON, J.E., ECKHARDT, S.G., STEPHANS, K.F., RAZVILLAS, B., GARL, S., BUTINE, M.D., PERRY, V.P., ARMITAGE, R.J., GHALIE, R., CARON, D.A., and GRIBBEN, J.G. (2001). Phase I study of recombinant human CD40 ligand in cancer patients. J. Clin. Oncol. **19,** 3280-3287.

VOSE, B.M. and MOORE, M. (1985). Human tumor-infiltrating lymphocytes: a marker of host response. Semin. Hematol. **22,** 27-40.

WEISSENBACH, M., CLAHSEN, T., WEBER, C., SPITZER, D., WIRTH, D., VESTWEBER, D., HEINRICH, P.C., and SCHAPER, F. (2004). Interleukin-6 is a direct mediator of T cell migration. Eur. J. Immunol. **34,** 2895-2906.

WESA, A. and GALY, A. (2002). Increased production of pro-inflammatory cytokines and enhanced T cell responses after activation of human dendritic cells with IL-1 and CD40 ligand. BMC. Immunol. **3,** 14-

WIERDA, W.G., CANTWELL, M.J., WOODS, S.J., RASSENTI, L.Z., PRUSSAK, C.E., and KIPPS, T.J. (2000). CD40-ligand (CD154) gene therapy for chronic lymphocytic leukemia. Blood **96,** 2917-2924.

WILKINSON, P.C. and LIEW, F.Y. (1995). Chemoattraction of human blood T lymphocytes by interleukin-15. J. Exp. Med. **181,** 1255-1259.

WINER, J., JUNG, C.K., SHACKEL, I., and WILLIAMS, P.M. (1999). Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal. Biochem. **270,** 41-49.

WITTEK, R. (1982). Organization and expression of the poxvirus genome. Experientia **38,** 285-297.

WORM, M. and GEHA, R.S. (1994). CD40 ligation induces lymphotoxin alpha gene expression in human B cells. Int. Immunol. **6,** 1883-1890.

XU, Y. and SONG, G. (2004). The role of CD40-CD154 interaction in cell immunoregulation. J. Biomed. Sci. **11,** 426-438.

YAJIMA, T., NISHIMURA, H., ISHIMITSU, R., WATASE, T., BUSCH, D.H., PAMER, E.G., KUWANO, H., and YOSHIKAI, Y. (2002). Overexpression of IL-15 in vivo increases antigen-driven memory CD8+ T cells following a microbe exposure. J. Immunol. **168,** 1198-1203.

YANG, Y. and WILSON, J.M. (1996). CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. Science **273,** 1862-1864.

YAWALKAR, N., KARLEN, S., EGLI, F., BRAND, C.U., GRABER, H.U., PICHLER, W.J., and BRAATHEN, L.R. (2000). Down-regulation of IL-12 by topical corticosteroids in chronic atopic dermatitis. J. Allergy Clin. Immunol. **106,** 941-947.

YEE, C., GILBERT, M.J., RIDDELL, S.R., BRICHARD, V.G., FEFER, A., THOMPSON, J.A., BOON, T., and GREENBERG, P.D. (1996). Isolation of tyrosinase-specific CD8+ and CD4+ T cell clones from the peripheral blood of melanoma patients following in vitro stimulation with recombinant vaccinia virus. J. Immunol. **157,** 4079-4086.

YELLIN, M.J., BRETT, J., BAUM, D., MATSUSHIMA, A., SZABOLCS, M., STERN, D., and CHESS, L. (1995). Functional interactions of T cells with endothelial cells: the role of CD40L-CD40-mediated signals. J. Exp. Med. **182,** 1857-1864.

YELLIN, M.J., SINNING, J., COVEY, L.R., SHERMAN, W., LEE, J.J., GLICKMAN-NIR, E., SIPPEL, K.C., ROGERS, J., CLEARY, A.M., PARKER, M., and . (1994a). T lymphocyte T cell-B cell-activating molecule/CD40-L molecules induce normal B cells or chronic lymphocytic leukemia B cells to express CD80 (B7/BB-1) and enhance their costimulatory activity. J. Immunol. **153,** 666-674.

YELLIN, M.J., SIPPEL, K., INGHIRAMI, G., COVEY, L.R., LEE, J.J., SINNING, J., CLARK, E.A., CHESS, L., and LEDERMAN, S. (1994b). CD40 molecules induce down-modulation and endocytosis of T cell surface T cell-B cell activating molecule/CD40-L. Potential role in regulating helper effector function. J. Immunol. **152,** 598-608.

YOUNES, A. (2001). CD40 ligand therapy of lymphoma patients. J. Clin. Oncol. **19,** 4351-4353.

YOUNES, H.M. and AMSDEN, B.G. (2002). Interferon-gamma therapy: evaluation of routes of administration and delivery systems. J. Pharm. Sci. **91,** 2-17.

YRON, I., WOOD, T.A., JR., SPIESS, P.J., and ROSENBERG, S.A. (1980). In vitro growth of murine T cells. V. The isolation and growth of lymphoid cells infiltrating syngeneic solid tumors. J. Immunol. **125,** 238-245.

YUEN, L. and MOSS, B. (1987). Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. Proc. Natl. Acad. Sci. U. S. A **84,** 6417-6421.

ZAGURY, D., BERNARD, J., CHEYNIER, R., DESPORTES, I., LEONARD, R., FOUCHARD, M., REVEIL, B., ITTELE, D., LURHUMA, Z., MBAYO, K., and . (1988). A group specific anamnestic immune reaction against HIV-1 induced by a candidate vaccine against AIDS. Nature **332,** 728-731.

ZAJAC, P., OERTLI, D., MARTI, W., ADAMINA, M., BOLLI, M., GULLER, U., NOPPEN, C., PADOVAN, E., SCHULTZ-THATER, E., HEBERER, M., and SPAGNOLI, G. (2003). Phase I/II clinical trial of a

nonreplicative vaccinia virus expressing multiple HLA-A0201-restricted tumor-associated epitopes and costimulatory molecules in metastatic melanoma patients. Hum. Gene Ther. **14,** 1497-1510.

ZAJAC, P., OERTLI, D., SPAGNOLI, G.C., NOPPEN, C., SCHAEFER, C., HEBERER, M., and MARTI, W.R. (1997). Generation of tumoricidal cytotoxic T lymphocytes from healthy donors after in vitro stimulation with a replication-incompetent vaccinia virus encoding MART-1/Melan-A 27-35 epitope. Int. J. Cancer **71,** 491-496.

ZAJAC, P., SCHUTZ, A., OERTLI, D., NOPPEN, C., SCHAEFER, C., HEBERER, M., SPAGNOLI, G.C., and MARTI, W.R. (1998). Enhanced generation of cytotoxic T lymphocytes using recombinant vaccinia virus expressing human tumor-associated antigens and B7 costimulatory molecules. Cancer Res. **58,** 4567-4571.

Curriculum vitae
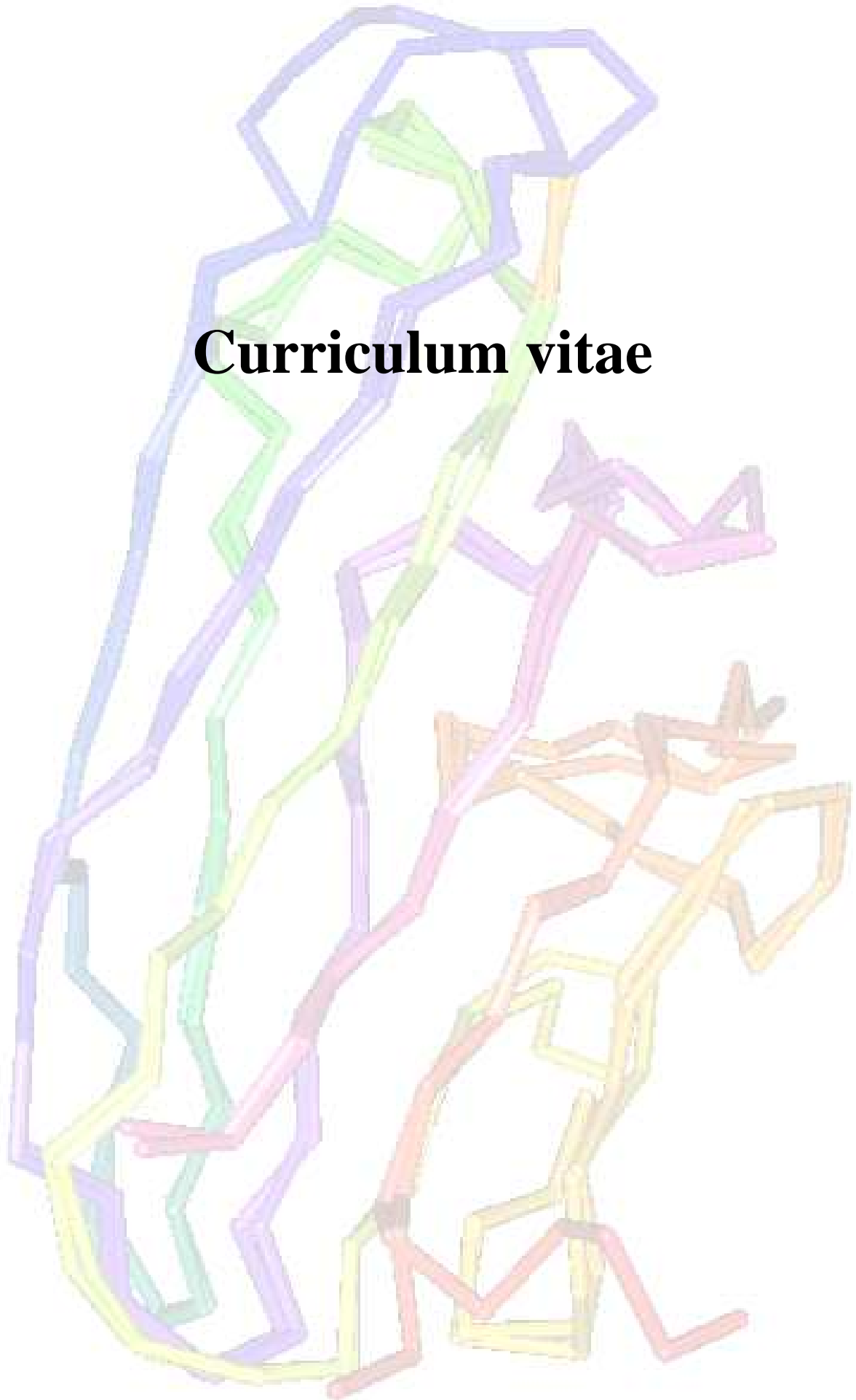
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EDUCATION

2005 Ph.D. in Biology (field of Cancer Immunotherapy) - University of Basel (Switzerland).

2001 Diploma in Pharmacology and Pharmaco-Chemistry (DEA) - University of Strasbourg (France).

1999 Master in Biochemistry, Specialty in Molecular and Cellular Biochemistry, Option Biology of Development (Maîtrise) - University of Strasbourg (France).

1997 Bachelor in Biology, Option Microbiology (Licence) - University of Strasbourg (France).

1996 University Diploma in Biology (DEUG) - University of Strasbourg (France).

1994 Technician Diploma in Biotechnologies (B.T.S.) - L.E.G.T.P. Jean Rostand, Strasbourg (France).

1992 General Certificate of Education in Biology (Baccalauréat série D) - L.E.G.T.P. Jean Rostand, Strasbourg (France).

SCIENTIFIC EXPERIENCE

12.2001 - 12.2005: Institüt für Chirurgische Forschung und Spitalmanagement, Universitätsspital Basel, Basel (Switzerland) - Non-replicating Vaccinia virus expressing CD40 ligand (CD154) : effects on APC capacity to stimulate specific CD4+ and CD8+ T cell responses.

09.2000 - 09.2001: Institut National de la Santé et de la Recherche Médicale, Unité 381 (I.N.S.E.R.M.), Strasbourg (France) - Study of a new powerful type IV phosphodiesterase inhibitor belonging to the 9-benzyladenine family: anti-inflammatory effects in chronic inflammatory intestinal disease such as Crohn disease.

01.1994 - 02.1994: Institut de Biologie Moléculaire des Plantes (C.N.R.S.), Strasbourg (France) - Analyze of an isolated clone of sweet pepper (capsicum): subcloning, sequencing (Sanger method).

06.1993: Laboratoire d'Immunologie et d'Hématologie du C.H.U. de Hautepierre, Strasbourg (France) - Detection and dosage of auto-antibodies by immunoenzymology (E.L.I.S.A.), immuodiffusion (Ouchterlony), and indirect immunofluorescence.

LANGUAGES

French: Mother language.
English: Fluent, good scientific level.
German: Read, spoken, written.

OTHERS

Computer science: Word, Excel, Power Point, Publisher, Internet.

Hobbies: reading, painting and graphic arts, patchwork, embroidery, hiking, swimming.

PUBLICATIONS

- ADAMINA, M., BOLLI, M., ALBO, F., CAVAZZA, A., ZAJAC, P., PADOVAN, E., SCHUMACHER, R., RESCHNER, A., **FEDER-MENGUS, C.**, MARTI, W.R., OERTLI, D., HEBERER, M. and SPAGNOLI, G.C. (2004). Encapsulation into sterically stabilised liposomes enhances the immunogenicity of melanoma-associated Melan-A/MART-1 epitopes. Br.J.Cancer **90**, 263-269.

- BOLLI, M., SCHULTZ-THATER, E., ZAJAC, P., GULLER, U., **FEDER-MENGUS, C.**, SANGUEDOLCE, F., CARAFA, V., TERRACCIANO, L., HUDOLIN, T., SPAGNOLI, G.C. and TORNILLO, L. (2005). NY-ESO-1/LAGE-1 co-expression with MAGE-A cancer-testis antigens: a tissue microarray study. Int.J.Cancer **Vol**, pages.

- VON HOLZEN, U., ADAMINA, M., BOLLI, M., WEBER, W., ZAJAC, P., GROEPER, C., RESCHNER, A., **FEDER-MENGUS, C.**, SCHUMACHER, R., MARTI, W., OERTLI, D., HEBERER, M. and SPAGNOLI, G.C. (2005). Selective responsiveness to common gamma chain cytokines in peripheral blood derived cytotoxic T lymphocytes induced by melan-A/MART-1(27-35) targeted active specific immunotherapy. Int.J.Cancer **115(2)**, 248-255.

- **FEDER-MENGUS, C.**, SCHULTZ-THATER, E., OERTLI, D., MARTI, W., HEBERER, M., SPAGNOLI, G.C. and ZAJAC, P. (2005). Non-replicating recombinant vaccinia virus expressing CD40 ligand enhances APC capacity to stimulate specific CD4+ and CD8+ T cell responses. Hum.Gene Ther. **16(3)**, 348-360.

ORAL PRESENTATIONS

- Departement Klinisch-Biologische Wissenschften der Universität Basel, SchwerPunkt Onkologie 2003, December 3rd, 2002, Landgut Castelen, Kaiseraugst, Switzerland.
  FEDER-MENGUS, C., SCHULTZ-THATER, E., OERTLI, D., MARTI, W., HEBERER, M., SPAGNOLI, G.C. and ZAJAC, P. Co-stimulatory ligands expressed by recombinant Vaccinia virus as tumor vaccine reagent.

- European Cancer Center Basel-Freiburg-Strasbourg, 11th Annual meeting, Clinical and Experimental Oncology, May 14th, 2004, Ungersheim, France.
  FEDER-MENGUS, C., SCHULTZ-THATER, E., OERTLI, D., MARTI, W., HEBERER, M., SPAGNOLI, G.C. and ZAJAC, P. Expression of CD40 ligand (CD154) in recombinant vaccinia virus: effects on APC and CTL priming.

POSTER SESSIONS

- Keystone Symposia, Basic Aspects of Tumor Immunology, February 17th-23th, 2003, Keystone Resort, Keystone, Colorado, USA.
  FEDER-MENGUS, C., SCHULTZ-THATER, E., OERTLI, D., MARTI, W., HEBERER, M., SPAGNOLI, G.C. and ZAJAC, P. Potency of vaccines based on recombinant vaccinia expressing CD40 ligand.

- International Meeting on cancer vaccines, April 19th and 20th, 2004, Istituto Superiore di Sanita, Rome, Italy.

FEDER-MENGUS, C., SCHULTZ-THATER, E., OERTLI, D., MARTI, W., HEBERER, M., SPAGNOLI, G.C. and ZAJAC, P. Expression of CD40 ligand (CD154) in recombinant vaccinia virus: effects on APC and CTL priming.

- Cancer Vaccines 2004, The NEXT Decade-A report from the World, October 4th-6th, 2004, The Manhattan Conference Center at the Millennium Broadway, New York City, NY, USA.
  FEDER-MENGUS, C., SCHULTZ-THATER, E., OERTLI, D., MARTI, W., HEBERER, M., SPAGNOLI, G.C. and ZAJAC, P. Expression of CD40 ligand (CD154) in recombinant vaccinia virus: effects on APC and CTL priming.

- 3rd international Symposium on the Clinical Use of Cellular Products, Cellular Therapy 2005, March 17th and 18th, 2005, Regensburg, Germany.
  FEDER-MENGUS, C., SCHULTZ-THATER, E., OERTLI, D., MARTI, W., HEBERER, M., SPAGNOLI, G.C. and ZAJAC, P. Expression of CD40 ligand (CD154) in recombinant vaccinia virus: effects on APC and CTL priming.

- The Second International Conference on Immunopotentiators in Modern Vaccines 2005, May 18th to 20th, 2005, Malaga, Spain.
  FEDER-MENGUS, C., SCHULTZ-THATER, E., OERTLI, D., MARTI, W., HEBERER, M., SPAGNOLI, G.C. and ZAJAC, P. Vaccine based on recombinant Vaccinia virus expressing CD154: effects on CT L priming.

OTHER COMMUNICATIONS

- Forschungstag 2003, Departemente Chirurgie und Anästhesie, Kantonspital Basel, August 2003, Basel, Switzerland.
  FEDER-MENGUS, C., ZAJAC, P., SCHULTZ-THATER, E., SPAGNOLI, G.C. and HEBERER, M. Potency of tumor vaccines based on recombinant vaccinia expressing CD154 (CD40 ligand).

- Forschungstag 2004, Departemente Chirurgie und Anästhesie, Kantonspital Basel, August 20th 2004, Basel, Switzerland.
  WEBER, W.P., SCHUMACHER, R., RESCHNER, A., **FEDER-MENGUS, C.**, GROEPER, C., SCHULTZ-THATER, E., HEBERER, M. and SPAGNOLI, G.C. IDO: a CTL inhibiting pathway in humans?

- Forschungstag 2004, Departemente Chirurgie und Anästhesie, Kantonspital Basel, August 20th 2004, Basel, Switzerland.
  FEDER-MENGUS, C., SCHULTZ-THATER, E., OERTLI, D., MARTI, W., HEBERER, M., SPAGNOLI, G.C. and ZAJAC, P. Expression of CD40 ligand (CD154) in recombinant vaccinia virus: effects on antigen presenting cells and cytotoxic T lymphocyte priming.

- Forschungstag 2004, Departemente Chirurgie und Anästhesie, Kantonspital Basel, August 20th 2004, Basel, Switzerland.
  VON HOLZEN, U., ADAMINA, M., BOLLI, M., WEBER, W.P., ZAJAC, P., SCHUMACHER, R., GROEPER, C., RESCHNER, A., **FEDER-MENGUS, C.**, MARTI, W., OERTLI, D., HEBERER, M. and SPAGNOLI, G.C. Selective responsiveness to common gamma chain cytokines in peripheral blood derived cytotoxic T lymphocytes induced by Melan-A/MART-1(27-35) targeted active specific immunotherapy.

- Forschungstag 2004, Departemente Chirurgie und Anästhesie, Kantonspital Basel, August 20th 2004, Basel, Switzerland.
  BOLLI, M., ADAMINA, M., SCHULTZ-THATER, E., TORNILLO, L., TERRACCIANO, L., **FEDER-MENGUS, C.**, ZAJAC, P., GULLER, U., OERTLI, D., SPAGNOLI, G.C. and HEBERER, M. NY-ESO-1/LAGE-1 tumor associated antigen expression in clinical samples: a tissue microarray study.

- 3. Gemeinsamer Jahreskongress der Schweizerischen Gesellschaft für Chirurgie und der Schweizerischen Gesellschaft für Thorax-, Herz und Gefässchirurgie (3ème congrès annuel commun de la Société Suisse de Chirurgie et de la Société Suisse de Chirurgie Thoracique et Cardio Vasculaire) , June 8-10th 2005, Zürich, Switzerland.
  WEBER, W.P., ROSENTHAL, R., MISTELI, H., **FEDER-MENGUS, C.**, SPAGNOLI, G.C., OERTLI, D. and HEBERER, M. Tryptophan metabolism as tumor related immunoregulatory mechanism in humans.

- American College of Surgeons Clinical Congress 2005, October 16-20th 2005, San Francisco, CA.
  WEBER, W.P., ROSENTHAL, R., MISTELI, H., **FEDER-MENGUS, C.**, ZAJAC, P., SPAGNOLI, G.C., OERTLI, D. and HEBERER, M. Tryptophan metabolites inhibit common g chain cytokine induced homeostatic proliferation of CD8+ cells.

LECTURES: TEACHER'S LIST

Prof. Max M. Burger
Prof. Giulio Spagnoli
Prof. Christoph Rochlitz
Prof. Nancy Hynes
Prof. Christoph Moroni
Prof. Daniel Oertli
Prof. Antonius G. Rolink
Prof. Gennaro De Libero
Prof. Jean Pieters
Prof. Georg A Holländer
Prof. Kurt Ballmer-Hofer
Prof. Thomas Hohn
Prof. Urs A Meyer
Prof. Markus H. Heim
Prof. Radek C. Skoda
Prof. Paul L. Herrling
PD Barbara C. Biedermann