Identification of preferentially targeted tumor-associated antigens in melanoma patients via mRNA stimulation of CD8+ blood lymphocytes

DISSERTATION

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SUMMARY

Until now, therapeutic vaccination of cancer patients has mainly relied on rather few T cell epitopes processed from structurally normal shared tumor antigens and presented by frequent HLA alleles. So far the design of these studies has not addressed the individuality of tumor-host interactions, which are not only determined by the antigenic tumor phenotype or the natural HLA polymorphism, but also by the individual T cell repertoire. The procedure described herein was developed to identify the preferential targets of the individual repertoire from a panel of known shared tumor-associated antigens.

Lymphocytes were isolated from the peripheral blood of cancer patients or healthy donors and stimulated twice with autologous mRNA-transfected FastDC (*Dauer et al., J Immunol. 170:4069, 2003*). FastDC were generated from blood monocytes and separately transfected via lipofection with in vitro transcribed mRNAs encoding the panel antigens. Responder lymphocytes were tested on day 12 in a 20-hour IFN-γ ELISPOT assay for recognition of 293T cells co-transfected pairwise with plasmids encoding the stimulation antigens and the respective individual's HLA class I alleles. In a first step, stimulation parameters were optimized for the detection of anti-HCMV pp65 responses. A maximum amplification of pp65-specific CD8+ T cell responses was obtained at a rather low IL-2 concentration (25 IU/ml) and at a minimum APC-to-effector ratio of 1:10. Addition of IL-4, IL-7 or IL-15 did not substantially improve the stimulatory potential.

The test was applied to the human melanoma models D05 and MZ2, in both of which multiple T cell-defined antigens had previously been identified by expression screening. Blood lymphocytes were stimulated in parallel with autologous tumor cells and with mRNA-transfected FastDC. In D05, T cell reactivities against three out of eleven epitopes induced by stimulation with tumor cells were also found after stimulation with mRNA-transfected FastDC. Two further T cell target epitopes were identified with mRNA but not with tumor cell stimulation. In MZ2, T cell responses against five distinct epitopes were detected on day 12 after stimulation with mRNA transfectants. The same responses were detectable after stimulation with tumor cells only on day 32. mRNA stimulations against 21 tumor-associated antigens in addition to HCMV pp65 were performed in four healthy individuals. In all cases, CD8+ T cells against HCMV pp65 could be expanded. Among tumor-associated antigens, only reactivity against Melan-A/MART-1 in association with HLA-A*0201 was detectable in one of the donors.

The vaccination of patients with targets *a priori* known to be recognized by their T cell repertoire may help to improve the outcome of therapeutic vaccination.

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ABBREVIATIONS

A_{260}	Optical absorbance at 260 nm
A_{280}	Optical absorbance at 280 nm
A_{320}	Optical absorbance at 320 nm
AD	Aqua destillata (see dH_2O)
AEC	3-amino-9-ethylcarbazole
APC	Antigen presenting cell
BSA	Bovine serum albumin
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DEPC	Diethyl pyrocarbonat
dH_2O	Distilled H_2O
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
EGFP	Enhanced green fluorescent protein
ELISPOT	Enzyme-linked immunospot assay
EP	Electroporation
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FastDC	Fast generated dendritic cells
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GM-CSF	Granulocyte-monocyte colony-stimulating factor
HCMV	Human Cytomegalovirus
HLA	Human Leukocyte Antigen
IFN	Interferon
IL	Interleukine
IU	International Unit
IVT	In vitro transcription
IVT-mRNA	In vitro transcribed mRNA
Kb	Kilobase

LB	Lysogeny broth
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA
MLTC	Mixed Lymphocytes Tumor cells Culture
MVA	Modified Vaccinia Ankara
NF	Nucleofection™
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral Blood Mononuclear Cells
PGE-2	Prostaglandin E2
PI	Propidium iodide
rATP	Riboadenosine tri-phosphate
rCTP	Ribocytosine tri-phosphate
rGTP	Riboguanosine tri-phosphate
RNA	Ribonucleic acid
rNTP	Ribonucleotide tri-phosphate
rpm	round per minute
RT-PCR	Reverse-transcription PCR
rUTP	Ribouridine tri-phosphate
SSC	Side scatter
TBE	Tris-borate-EDTA
TM	TransMessenger™
TNF	Tumor necrosis factor
Tris	tris(hydroxymethyl)-aminomethane
UV	Ultraviolet

1 INTRODUCTION

1.1 Melanoma

Melanoma is a malignant tumor of melanocytes, the pigment cells of the skin. Its incidence has more than tripled in the white population in the United Sates over the past 20 years, placing it as the most common fatal malignancy among young adults (NCHS/SEER 2008; Jemal, 2008; Parkin, 2001). The major cause of melanoma is the intermittent and massive exposition to UV light (Elwood, 1997; Gilchrest, 1999). This has emerged from correlations between the UV exposition and the geographic incidence of melanoma in Caucasians (Bulliard, 1994; Devesa, 1999). Indeed, the disease affects per year 6 of 10^5 male individuals in North America versus 38 of 10^5 male individuals in Australia and New Zealand (Parkin, 2005). Predominantly affected are Caucasians with type I and II skins characteristic of fair complexions, whereas Africans or Asians are rarely concerned and are affected with an incidence of 1,1-1,7 for 10^5 per year (Ries, 2008; Cormier, 2006). Other genetic predisposition factors include albinism, defects in DNA-repair genes, such as Xeroderma Pigmentosum, and familial melanoma (Rivers, 1996, Chaudru, 2004). The two most common types of melanoma are the “Superficial Spreading Melanoma” (“SSM”) which represents 65% of the melanoma cases, and the “Nodular Melanoma”, which accounts for 15% of cases (Clark, 1969; McGovern 1973). The nodular form of melanoma is particularly aggressive, as it tends to metastasize very rapidly within a few months from diagnosis. The SSM is characterized by a more delayed progression as it can evolve over years before metastasizing (Clark, 1986). Although most cases of melanoma affect the skin, 10% are found on mucous surfaces (mouth, genitals), in the eye, the ears, in the central nervous system or in lymph nodes.

With epidermal Langerhans cells and Merkel cells, melanocytes represent the 10% of cells, which live in symbiosis with the predominant keratinocytes of the epidermis (Slominski, 1993). Melanocytes are dendritic-shaped cells producing melanin, a dark pigment, that absorbs all wavelengths of light and binds free radicals (Sulaimon, 2003). They are derived from the neural crest during embryogenesis and migrate into the epidermis and hair follicles to reside in the basal lamina of the epidermis (Bennett, 1993; Hirobe, 1994). Melanin is produced in special organelles called melanosomes, which are transferred to neighbouring keratinocytes via dendrites (**Figure 1.1A**). Both the biosynthesis and the maturation of melanin occur in melanosomes, where the polymerisation of the amino acid tyrosin is catalysed by the enzyme tyrosinase (Moellmann, 1988; Nordlund, 1998; Jimbow, 2000) (**Figure 1.1B**). The structural proteins involved in the formation of melanosomes or in the transformation of tyrosine to melanin are called the Melanogenesis-related proteins (MRPs)

and their main representatives are shown in **Table 1.1** (Slominski, 2002). The products of the melanogenesis are the two forms of melanin, namely the black eumelanin, which is predominant in Africans, and the reddish to yellow pheomelanin, which is predominant in Caucasians (Prota, 1992). The distribution of melanosoms in the cytoplasm of keratinocytes will affect the dispersion and absorption of light (Thong, 2003).

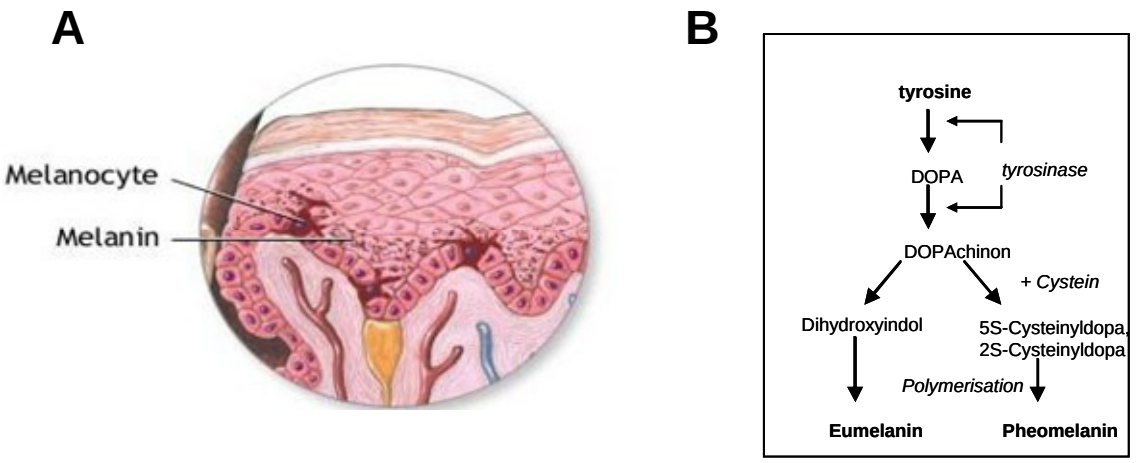

Figure 1.1. **Melanocytes and melanogenesis**. **A**. Melanocytes are dendritic-shaped cells located on the basal lamina of the epidermis and supplying neighbouring keratinocytes with melanin. **B**. The synthesis pathway of melanin is based on the oxydation of tyrosine into intermediates like Dihydroxyphenylalanin (DOPA) or DOPAchinon by the enzyme tyrosinase. DOPAchinon is the common precursor of the two forms of melanin eumelanin and pheomelanin (adapted from Fritsch, 2004).

Table 1.1. **Melanogenesis-related proteins (MRPs)**. The principal MRPs involved in the biosynthesis of melanin are indicated with their function and loci of coding genes (adapted from Slominski, 2002).

Protein	Function	Locus	Chromosome
Tyrosinase	Oxidation of tyrosine, DOPA, DHI	TYR	11q14-q21
TRP-1	DHICA oxidase, TYR stabilisation	TYRP1	9p23
TRP-2	Dopachrome tautomerase	DCT	13q31-q32
Gp100/PMEL17	DHICA polymerisation/stabilizer	SILV	12q13-q14
Melan-A/MART-1	Melanosomal protein	MART-1?	9?
P protein	Regulator of melanosome pH	OCA2	15q11.2-q12
MITF	Transcriptional regulator	MITF	3p12.3-p14.1
MSH receptor	G protein-coupled receptor	MC1R	16q24.3

1.2 Antigens recognized on melanoma by autologous T cells

The observation that the immune system may control the progression of tumors in certain patients dates from the 19th century (Coley, 1893; Gresser, 1987). Later, this gave

rise to the concept of “immunosurveillance” (Burnet, 1967; Thomas, 1959). Melanoma is considered to be immunogenic. Although it is difficult to estimate their frequency, spontaneous regressions do occur, and are often associated with vitiligo (McGovern, 1975; Nordlund, 1983; Baldo, 1992). This is due to the attack and lysis of malignant and surrounding normal melanocytes by cytotoxic T lymphocytes (CTL) (Parmiani, 1993). From 1991 on melanoma antigens were discovered, that can be recognized by autologous T cells (van der Bruggen, 1991; Traversari, 1992; Boel, 1995). These antigens are in part specific for the individual tumor, but some are shared, i.e. they can be found in tumors of different individuals. These antigens are candidate targets for immunotherapy. The following classification is based on the expression pattern of tumor antigens and distinguishes four major groups (Cancer Immunity \ peptide database).

1.2.1 Mutated antigens

Mutated antigens result from point mutations in genes that are often ubiquitously expressed. The mutations are mostly unique to the tumor of an individual patient. Accordingly, they are also referred to as “unique antigens”. Some of these mutations may be implicated in the tumoral transformation. An example is $CDK4^{R24C}$, a mutated form of the Cyclin-Dependent Kinase 4 with oncogenic functions, that was identified as a tumor antigen in an individual melanoma patient (Wölfel, 1995), and in familial melanoma (Zuo, 1996). Until now, very few antigens encoded by regions of genes that are very frequently mutated in cancer cells, like ras or p53, have been found. One exception is B-RAF, a member of the mitogen-activated protein (MAP) kinase cascade, for which the same activating mutation is found in more than 60% of melanomas (Davies, 2002). A peptide encoded by the mutated region was identified as target of CD4+ T cells (Sharkey, 2004). Most strictly tumor-specific mutated antigens may play an important role in the natural anti-tumor immune response of individual patients. But they are still difficult to identify with a reasonable effort and, therefore, can not be reliably targeted by immunotherapy so far.

1.2.2 Shared antigens

In contrast to rare mutated antigens, shared antigens are present on many independent tumors. They can be further divided into three groups according to their expression profile in tumors and in other tissues.

1.2.2.1 Shared tumor-specific antigens

Shared tumor-specific antigens are peptides encoded by "cancer-germline" (CG) genes, that are expressed in many tumors, but not in normal tissues. The prototype of the CG genes is MAGE-A1, a member of the *melanoma antigen*-encoding (*MAGE)* gene family

(van der Bruggen, 1991; De Plaen, 1994; Chomez, 2001; Van der Bruggen, 1994). The only normal cells, in which significant expression of such genes has been detected are placental trophoblasts and testicular germ cells. Because these cells supposedly do not express MHC class I molecules (Haas, 1988), gene expression should not result in expression of the antigenic peptides and such antigens are therefore considered as tumor-specific. The expression of *MAGE* genes in tumors appears to be triggered by the demethylation of their promoters, as a consequence of the widespread demethylation process occuring in many tumors (De Smet, 1996). Other important CG gene families are the *BAGE* (Boel, 1995), *GAGE* (Van den Eynde, 1995; De Backer, 1999), *NY-ESO-1/LAGE-2* (Wang, 1998; Jager, 1998) and *SSX* (Ayyoub, 2002) families.

1.2.2.2 Differentiation antigens

In contrast to shared tumor-specific antigens, differentiation antigens are also expressed in the parental tissue of the respective malignancy. In the case of melanoma, these are melanosomal proteins such as tyrosinase (Brichard, 1993; Wölfel, 1994), Melan-A/MART-1 (Kawakami, 1994a; Coulie, 1994), gp100/PMEL17 (Kawakami, 1994b), tyrosinase-related proteins (TRP)-1 (Wang, 1996a) and TRP-2 (Wang, 1996b). Antigens of this group are structurally intact self-proteins and T cell reactivity against these antigens is most likely controlled by tolerance mechanisms (see 1.3.3.2). Furthermore, they are not tumor-specific, and their use as targets for cancer immunotherapy may result in autoimmunity towards the corresponding normal tissue (Goldberg, 1998; Phan, 2003). Other examples are the carcinoembryonic antigen (CEA), an oncofetal protein expressed in normal colon epithelium and in most intestinal carcinomas (Tsang, 1995), or prostate-specific antigen (PSA) expressed in the prostate and prostate carcinomas (Correale, 1997).

1.2.2.3 Overexpressed antigens

Overexpressed antigens are antigens that are expressed in a wide variety of normal tissues and overexpressed in tumors. The gene PRAME, that was identified as coding for a melanoma antigen, is expressed at a high level in almost all melanomas and in many other tumors (Ikeda, 1997; Kessler, 2001). Survivin, an anti-apoptotic protein of the inhibitor of apoptosis protein (IAP) family, was reported to be overexpressed in tumors, and has also been shown to be a target of T cells (Schmitz, 2000; Schmidt, 2003). Telomerase has been suggested as a good target for immunotherapy, as its presence is essential for the proliferation of tumor cells. Nevertheless, results concerning its potential as tumor antigen are controversial (Vonderheide, 1999; Parkhurst, 2004). Overexpressed antigens are shared between many tumors, but they are not tumor-specific, and the risk of vaccinating with these antigens may be associated with severe side effects.

1.3 Processing and presentation of tumor antigens

T cells recognize cell-bound antigens in association with molecules encoded by the Major Histocompatibility Complex (MHC), which is called MHC restriction (Zinkernagel, 1974). In humans, this complex is known as the Human Leukocyte Antigen (HLA) system. While CD8+ T cells recognize their antigens in association with MHC class I molecules, CD4+ T cells recognize their antigens in association with MHC class II molecules (Bjorkman, 1987; Rötzschke, 1990; Germain, 1994). Antigens are not presented by MHC molecules as intact proteins, but as processed peptides fitting in the peptide-binding groove of the respective MHC molecule (Townsend, 1986; Rötzschke, 1990; Garrett, 1989). MHC class II molecules are preferentially expressed in professional antigen-presenting cells (APC) like macrophages, B cells or dendritic cells, whereas MHC class I molecules are ubiquitously expressed. MHC class I molecules preferentially bind peptides of 8-10 amino acids with an hydrophobic or basic C terminus (Falk, 1991), whereas MHC class II molecules bind longer peptides (13-17 amino acids) (Chicz, 1992; Stern, 1994).

1.3.1 Antigens presented by MHC class I molecules

Antigens presented by MHC class I molecules are derived from proteins synthesized intracellulary and processed via the MHC class I pathway (Lehner, 1996; Koopmann, 1997) (**Figure 1.2**). Intracellular proteins are processed, at least in part, by an organelle known as proteasome (Baumeister, 1998; Schubert, 2000). Proteasomes are multicatalytic proteinase complexes ubiquitously expressed and devoted to preferentially degrade poly-ubiquitinated misfolded, aged or no-longer-needed proteins in an ATP-dependent way (Kloetzel, 2001). After cleavage of a given protein, the proteasome releases precursors of antigenic peptides with the correct C-termini. Cleavage at the N-termini of the peptide precursors is, however, less precise and necessitates further trimming by peptidases in the cytosol or in the endoplasmic reticulum (ER) to the preferred length of 8-10 amino acids (Falk, 2002; Serwold, 2002; Hammer, 2006). Peptides generated by the proteasome are then transported into the ER where MHC class I molecules are synthesized (**Figure 1.2**). This transport process is carried out by two members of the “ABC” superfamily of transporters, the Transporters-associated-with-Antigen-Processing TAP-1 and TAP-2 (Spies, 1990; Shepherd, 1993; Suh, 1994). Within the ER, newly synthesized MHC I molecules bind ß$_2$ microglobulin (ß$_2$m) and are stabilized by several chaperon proteins like calnexin, calreticulin and tapasin (Sadasivan, 1996; Suh, 1996; Ortmann, 1997). Peptides entering the ER bind to MHC class I molecules if they fit their respective binding groove (Neefjes, 1993; Grandea, 2000). Only a minority of peptides will actually bind to one of the available MHC molecules. Peptide-binding stabilizes MHC molecules and induces a conformational change, which releases the peptide-MHC complexes from the chaperon complex. The MHC I-peptide complexes are then transported

to the cell surface via the Golgi complex (Pamer 1998; van Endert, 1999). MHC class I molecules lacking bound peptide are instable, they dissociate and are degraded intracellularly (Fahnestock, 1992; Reich, 1997). It is noteworthy that IFN-γ induces the replacement of the normal proteasome by the immunoproteasome and the synthesis of the proteasome activator PA28 (Gaczynska, 1993; Hisamatsu, 1996). Both alter the proteolytic properties of the proteasome complex end enhance proteasomal function in antigen presentation (Toes, 2001; van Hall, 2000).

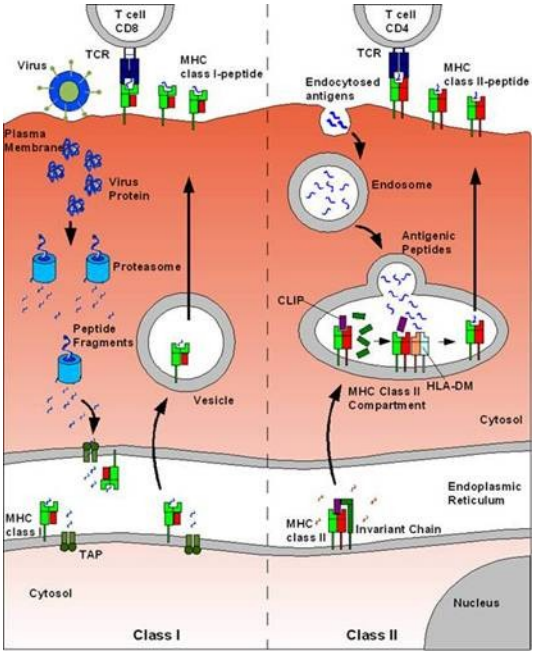

Figure 1.2. **MHC processing and presentation pathways**. Represented are the MHC class I (left) and MHC class II (right) processing pathways.

1.3.2 Antigens presented by MHC class II molecules

The MHC class II pathway processes exogenous antigens that are internalized via endocytosis (Neefjes, 1990; Watts, 1997) (**Figure 1.2**). Antigen processing occurs in the early endosome, where trimming by proteases (like cathepsins) occurs in an acidic environment (Watts, 2001; Stern, 2006). In the ER, MHC class II molecules are found complexed to a chaperon called the invariant chain (Ii) (Cresswell, 1992). This protein prevents loading of the MHC molecule with a peptide and contains sequences that enable it to exit the ER and to be directed towards the endosome via the Golgi complex (Chapman, 1998). In the acidic endosome, Ii becomes degraded by cathepsins, until a minimal fragment called CLIP (class-II-associated invariant-chain peptide) is left, still protecting the peptide-binding groove of the MHC II molecule (Wolf, 1995; Riese, 1998). Peptide and MHC II molecule come in contact in the MIIC (MHC-II compartment), where the peptide displaces CLIP from the MHC molecule to form the MHC-II-antigen complex (**Figure 1.2**), a reaction catalysed by HLA-DM molecules (Denzin, 1995; Kropshofer, 1997). The binding of a peptide

to the MHC molecule is irreversible. When the complexes have reached the cell surface, they remain stable until the MHC molecules become degraded. This is also the case for binding of peptides to MHC class I molecules (Fahnestock, 1992; Reich, 1997).

1.3.3 Antigen recognition by T cells

The peptides presented by MHC molecules are recognized via the T cell receptors (TCR) on T cells (Matsui, 1991; Weber, 1992; Hennecke, 2001). An individual's capability to respond to a given antigen is determined by the individual HLA phenotype, the individual T cell repertoire comprising the pool of available TCRs, and the mechanisms of central and peripheral tolerance protecting the individual against autoimmunity.

1.3.3.1 HLA polymorphism

HLA alleles are highly polymorphic. For HLA class I molecules, this polymorphism is clustered mainly in three regions in and around the peptide binding groove (Little, 1999; Hildebrand, 2002). The structure of this groove determines which peptides can bind (Garrett, 1989). Residues at certain positions of the peptide define the binding capacity and are therefore called anchor residues (Falk, 1991; Deres, 1993; Falk, 1993). Thus, the spectra of peptides presented by HLA molecules to T cells might largely differ among individuals according to their HLA phenotype.

1.3.3.2 T cell repertoire

TCRs are heterodimeric molecules comprising an α chain and a β chain linked by a disulphide bond (Bjorkman, 1997). αβ TCRs are present on more than 95% of peripheral T cells. Subpopulations of T cells in the thymus and in epithelial tissues express γ and δ chains, forming the γδ TCR (Davis, 1998; Hayday, 2000). Like for immunoglobulins, the diversity of the TCR chains is generated by recombinations between V, D, and J gene segments (Muljo, 2000; Rodewald, 1998; Roth, 2000). This process is controlled at least in part by the two recombination activating genes RAG-1 and RAG-2 (Fugmann, 2000). TCR gene recombination might give rise to $2{,}9 \times 10^{20}$ possible TCRs in mice (Hunkapiller, 1989). But only a few of them will be expressed by the T cells that develop during the lifetime. A comprehensive response to foreign antigens is made possible by the crossreactivity of TCRs, such that one T cell potentially interacts with 10^6 different MHC-peptide complexes (Mason, 1998). The TCRs actually expressed on T cells make up for an individual's T cell repertoire.

1.3.3.3 Mechanisms of tolerance

As seen above, the immune system randomly generates a vast diversity of T cell receptors. Some of these might be self-reactive. Developing T cells are therefore subjected to mechanisms of central tolerance in the thymus (Bevan, 1997). T cells expressing receptors with high binding affinity for intrathymic self antigens will be deleted, a process known as negative selection (Kyewski, 2004). In contrast, low-affinity self-reactive T cells and T cells with receptors specific for antigens that are not expressed in the thymus, mature and join the peripheral T cell pool (positive selection) (Hogquist, 2001; Kyewski, 2006; Gallegos, 2006). Other mechanisms of tolerance induction act in the periphery. Autoreactive T cells escaping central tolerance induction can be deleted or rendered anergic, either by a suppressive microenvironment (see below) or by the absence of costimulatory molecules (B7-1, B7-2) on antigen presenting cells (Sakaguchi, 2000; Salomon, 2001; Lohr, 2006; Walker, 2002).

1.4 The tumor-immune escape

The identification of tumor antigens recognized by T lymphocytes in melanoma and in other tumor patients has definitively proven that antitumoral T cell responses spontaneously evolve in these patients. One could have expected that these T cell responses lead to tumor destruction. Although they do exist, spontaneous complete remissions in melanoma and other malignant diseases are rare. Nevertheless, approximatively 5% of cases with metastatic disease show a complete remission of the primary tumor (Avril, 1992; Baldo, 1992; Shai, 1994; Emanuel, 2008). But in most of cases the tumor will progress, indicating that the immune system is overwhelmed at some point. Since a few years, it has become clear that the tumor is able to circumvent the immune attack or to render immune effector cells anergic, i.e. prevent them from exerting their function (reviewed in Rabinovich, 2007).

1.4.1 Antigen and HLA loss

One mechanism of tumor escape is the selection of tumor cell variants that no longer present the antigens that are the targets of effector cells (Restifo, 1993; Ferrone, 1995), which is described by the concept of “immunoediting” (Coulie, 1999; Marincola, 2000; Dunn, 2004). This may be due to a loss or downregulation of HLA I expression (Garrido, 1997; Jäger, 1997; Seliger, 1996), e.g. due to mutations in the $ß_2$m gene (Wang, 1993), to loss of a whole HLA haplotype, or to a loss of a single HLA allele (Koopman, 2000; Marincola, 2000; Garcia-Lora, 2003; Kageshita, 2005). Defects of the antigen processing machinery can in principle also be involved, including mutations and down-regulation of TAP (Gabathuler, 1994; Seliger, 1996), down-regulation of tapasin (Seliger, 2001), and components of the

immunoproteasome (Rivoltini, 2002). At last, loss of antigen presentation may also come from a loss of antigen expression (Spiotto, 2004; Singh, 2007).

1.4.2 Tumor immune barriers

Antigen loss can not be regarded as the only, and perhaps not even as the most important cause of failure of the immune system to fight cancer. Instead, it became clear that lymphocytes are anergized and paralyzed in the microenvironment of the tumor (reviewed in Gajewski, 2007). Tumor cells or their stroma anergize the potential responder T cells directly or indirectly via the recruitment of suppressive T cells.

1.4.2.1 Immune suppressive effects directly induced by the tumor

Many tumors seem to be deficient in expression of B7-1 and B7-2, which are important costimulatory factors for full T cell activation (Denfeld, 1995; Allison, 1994). Stimulation through the T cell antigen receptor without B7 costimulation has been shown to induce a hyporesponsive state of T cells termed anergy (Fields, 1996; Schwartz, 2003). Another negative regulatory pathway is the engagement of the inhibitory receptor PD-1 on T cells by PD-L1 expressed by tumor cells. PD-1 is a receptor expressed by activated T cells, which mediates inhibition of T cell activation (Freeman, 2000). The vast majority of melanomas and other tumors express PD-L1 either constitutively or in response to IFN-γ exposure (Dong, 2003). Metabolic dysregulation represents an other potential escape mechanism. The tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) is expressed in a subset of metastatic melanoma tumor sites. Although expression by tumor cells themselves has been reported (Uyttenhove, 2003), in some settings IDO seems to be expressed by infiltrating dendritic-like cells and endothelial cells (Munn, 2004). IDO is expressed in placenta where it induces immunologic tolerance at the maternal/fetal interface (Munn, 1998). Through tryptophan depletion, its activity has been shown to lead to T cell dysfunction and also apoptosis (Fallarino, 2002; Uyttenhove, 2003). In renal carcinoma patients, production of arginase by a subset of granulocytes has been observed, resulting in a low arginine level in the tumor microenvironment and, again, depressed T cell functions (Zea, 2005; Rodriguez, 2004). Human melanoma cells also secrete galectin-1, which inhibits T cells (Rubinstein, 2004). Additional negative regulatory mechanisms directly induced by the tumor or the stromal cells include the secretion of immunosuppressive factors like transforming growth factor-β (Gorelik, 2001), and interleukin-10 (Wittke, 1999), or the expression of the inhibitory ligand B7-H4 (Kryczek, 2006). Also, the tumor or the tumor endothelium may fail to produce specific chemokines essential for the homing of effector T cells to the tumor sites (Zhang, 2005; Vianello, 2006; Buckanovitch, 2008).

1.4.2.2 Indirect immune suppressive effects

CD4+CD25+ regulatory T cells (Tregs) were identified by Sakaguchi et al. as a naturally occurring CD4+ T cell subset suppressing T cell responses in vivo and crucial for maintaining T cell tolerance to self-antigens (Sakaguchi, 2000). They differentiate in the thymus under the influence of the transcription factor forkhead box P3 (Foxp3) (Sakaguchi, 2005; Fontenot, 2005) and exert their inhibitory effect on the CD4+CD25- T cell populations (Dieckmann, 2001; Levings, 2001; Jonuleit, 2001). Tumors are thought to promote the expansion, recruitment, and activation of Tregs, which inhibit effector T cells in the tumor microenvironment (Zou, 2006). In addition to naturally occurring Tregs, other regulatory cell populations may also contribute to local immune suppression, like IL-10-producing regulatory T cells (Tr1) (O'Garra, 2004; Taams, 2006), CD4+ NKT cells which suppress CTL-effector functions by the secretion of IL-13 and TGF-β (Terabe, 2000) or tumor infiltrating γδ T cells (Peng, 2007).

1.5 Antigen-specific immunotherapy of melanoma

Early observations have shown that T cells may be the major effector cells in anti-tumor immunity (Maryanski, 1982). More special attention has been paid on CD8+ T cells, used as main effectors in the identification of antigens on tumor cells. The discovery of the first tumor-associated antigens definitively proved their role in the recognition and control of tumors (Van der Bruggen, 1991). Correspondingly, the aim of most immunotherapy strategies against cancer has been to boost or elicit potent anti-tumor CD8+ T cell responses. The identification of the nature of tumor antigens provided the targets for the design of antigen-specific cancer vaccines as immunotherapy of cancer.

Such vaccines differ from the classic concept of preventive vaccination in two essential aspects. First, they are intended to help in the treatment of established malignancy. Second, as mentioned, they aim to elicit T cell responses rather than antibodies which are commonly the favoured effectors to be induced by preventive vaccines in infectious diseases. Those vaccines can be divided into vaccines consisting of whole tumor cells or preparations derived thereof, and vaccines based on defined antigens provided in different formats such as synthetic peptides, naked DNA or recombinant viruses, or whole proteins.

1.5.1 Cellular vaccines

Cellular vaccines rely on the use of whole autologous or allogeneic tumor cells or tumor cell lysates for vaccination (Jocham, 2004; Mitchell, 2007). Tumor cells have also been modified by gene transfer to express cytokines, such as IL-2 and GM-CSF, that may enhance their overall immunogenicity (Dranoff, 1993; Hege, 2006; Soiffer, 1998; Salgia, 2003; Nemunaitis, 2004). Alternatively, tumor lysates have been loaded onto DC (Stift, 2003;

Geiger, 2001; Höltl, 2002; O'Rourke, 2003). Phase III clinical trials of cell-based cancer vaccines, however, could not demonstrate so far a clear survival benefit for vaccinated patients (Wallack, 1998; Dillman, 2007; Sondak, 2003).

1.5.2 Vaccination with defined antigens

The other approach to vaccination is to choose well-defined antigens. Several advantages are associated with it. First, the vaccine formulations and the vaccine production can be easily standardized. Second, immune responses against the vaccine can be precisely monitored with standard assays (see chapter 1.6).

Until now, cancer patients with metastatic disease, with a large majority of melanoma patients, have been preferentially vaccinated with cancer-germline or differentiation antigens, which are expressed on a high proportion of different tumors (see **Table 1.2**; Rosenberg, 2004). Many different formats have been used.

Early vaccinations with defined antigens were performed in metastatic melanoma with a peptide derived from the MAGE-A3 antigen (Marchand, 1995), which is expressed in 74% of metastatic melanomas (**Table 1.2**). The first modality consisting of vaccinating with the peptide without adjuvant (Marchand, 1999; Coulie, 2001) induced T cell responses in only few patients. To increase the immunogenicity of antigens, these have been combined with immunological adjuvants, including IL-2, IL-12 (Gajewski, 2001; Cebon, 2003), incomplete Freund's adjuvant (IFA) (Pittet, 2001; Cormier, 1997), GM-CSF (Chianese-Bullock, 2005) or ISCOMATRIX, a saponin-based adjuvant (Davis, 2004). Injection of CpG oligodeoxynucleotides, mimicking signals from innate immunity, increased the rate and the intensity of CD8+ T cell responses after vaccination with a peptide derived from the Melan-A/MART-1 antigen (Speiser, 2005).

Alternatively, recombinant viruses like Vaccinia-PSA (Edler, 2000; Gulley, 2002), Vaccinia-CEA (Conry, 1999), MVA-Tyr (Meyer, 2005) or Avipox-CEA (Von Mehren, 2000; Marshall, 1999) containing the antigen-encoding sequence have been used. One has also tried to co-express the costimulatory molecules B7-H1 and B7-H2 with the antigen (Hörig, 2000; Oertli, 2002), but globally, the response rates achieved with these modalities have been low (Rosenberg, 2004).

Another modality is to use autologous dendritic cells (DC) as adjuvant, as these are known to be the most potent antigen presenting cells (APC) in vivo and essential for priming of adaptive T cell responses (Banchereau, 1998; Jung, 2002). Monocyte- or CD34+ progenitor-derived mature DC pulsed with one (Thurner, 1999; Paczesny, 2004) or several peptides (Nestle, 1998; Banchereau, 2001; Schuler-Thurner, 2002) have been used. Responses observed when peptide pulsed DC were used seemed to be stronger than with peptide alone, and response rates were higher (Rosenberg, 2004).

Finally, the possibility of providing help to the CD8+ T cell response by vaccinating with antigens recognized by CD4+ T cells has been explored. This has been done by injection of the recombinant proteins MAGE-A3 or NY-ESO-1 with adjuvant (Marchand, 2003; Kruit, 2005, Davis, 2004). Proteins delivered in this way are processed preferentially by the MHC-II pathway of APC and stimulate antigen specific CD4+ T cells. A few trials have tested the use of a mix of HLA-I and II peptides to induce both CD4+ and CD8+ T cell responses (Phan, 2003; Knutson, 2005). However, results do not show a major advantage of these combination.

The outcomes of these vaccination strategies have been variable, and in most cases comparisons are rendered difficult by the fact that small number of patients have been included in the studies. The responses to a vaccine have been in first line measured by the extent of the T cell responses induced against the vaccine. Generally, vaccine-specific T cell responses have been induced in large proportions of vaccinated patients (reviewed in Boon, 2006). Nevertheless, the correlation with clinical responses is more hazardous, as only 5%-10% of vaccinated patients have shown an objective clinical response (Rosenberg, 2004; Boon, 2006). This still remains above the frequency of spontaneous regressions, indicating a clear benefit of immunotherapy.

The reasons for the until now limited success of immunotherapy are still incompletely understood (Steinman, 2004). A fist reason may be linked with the fact that most patients enrolled in these studies displayed advanced stages of the disease, although responses are more often observed with localized disease and in early stages (Rosenberg, 2004). Second, as already evoked, mainly antigens from the cancer-germline and differentiation families have been used, although these, as autoantigens, may be subjected to tolerance mechanisms and not be representative of most anti-tumor reactivity (Anichini, 1996; Lennerz, 2005) (see chapter 1.3.3.3). Also, it is now obvious that the escape mechanisms and suppressive environment induced by the tumor are likely to be a major factor in the induction of immunologic tolerance (Anichini, 1999; Rabinovich, 2007; Zou, 2006) (see chapter 1.4). In this line, strategies aiming at depleting Tregs previous to vaccination may increase the immunogenicity of the vaccine (Jones, 2002; Curtin, 2008). At last, the individuality of the tumor-host interactions have been largely ignored. These interactions are not only determined by the individual tumor phenotype or the individual HLA phenotype, but also by the ability of the individual T cell repertoire to respond to a particular antigen (see chapter 1.3.3.2).

Table 1.2. **Antigenic peptides used to vaccinate melanoma patients**. Adapted from Boon, 2006.

Antigen	Frequency of expression in metastatic melanoma	Presenting HLA	Peptide sequence	Position in the protein
MAGE-A1	46	A2 A3	KVLEYVIKV SLFRAVITK	278-286 96-104
MAGE-A3	74	A1 DP4	EVDPIGHLY KKLLTQHFVQENYLEY	168-176 243-258
MAGE-A4	28	A2	GVYDGREHTV	230-239
MAGE-A10	47	A2	GLYDGMEHL	254-262
MAGE-A12	62	Cw7	VRIGHLYIL	170-178
MAGE-C2	59	A2	ALKDVEERV	336-344
NY-ESO-1	28	A2	SLLMWITQ(A,I,L,V)	157-165 (C165A,I,L,V)
Tyrosinase	>90	A2	YMDGTMSQV	369-377
Melan-A/MART-1	>90	A2	ELAGIGILTV	26-35 (A27L)
gp100	>90	A2	IMDQVPFSV	209-217 (T210M)
GNT-V	50	A2	VLPDVFIRC	(intron)

1.6 Methods to quantify antigen-specific T cell responses

Quantitative assays to measure antigen-specific T cell responses were developed when it became possible to target defined antigens. They are indispensable to evaluate responses to a vaccine and are based on the phenotypic and functional analysis of T cells at a single-cell level. Two main methodologies are involved. Some assays detect single T cell receptors (TCR) due to their ability to bind a given antigen. Others detect activated T cells, because they secrete Th1 and Th2 cytokines upon antigen contact (Mosmann, 1989).

1.6.1 Fluorescent HLA/peptide tetramers

Tetramers are soluble complexes of recombinant biotinylated HLA molecules folded in the presence of an antigenic peptide. These complexes are bound to fluorescent avidin, which can bind four biotin moieties, thus forming tetramers of HLA-peptides complexes. T cells expressing TCR specific for the peptide in association with its restricting HLA molecule will bind the tetramers, regardless of their functional characteristics. The lymphocytes can then be quantified and sorted with flow cytometry while staying alive (Altman, 1996; McMichael, 1998). Tetramers do not representent a functional tool evaluating the effector functions of selected populations. But further functional assays can be performed on still viable antigen-specific populations, such as TCR usage, functional status and determination

of TCR avidity for its antigen (Valmori, 1999; Valmori, 2000). These may be important parameters to assess the efficacy of a vaccine. With most HLA class I tetramers, the lower detection limit of the test is ca. 3 x 10^{-4} CD8+ T cells (Speiser, 2004; Comin-Anduix, 2006). Tetramers of HLA class II molecules have proven much more difficult to produce, probably because HLA class II molecules are less stable in solution (Novak, 1999). In combination with sorting and cloning, frequencies as low as 10^{-6} of CD4+ T cells have been measured (Zhang, 2005).

1.6.2 Enzyme Linked Immunospot (ELISPOT) assay

The Enzyme Linked Immunospot (ELISPOT) assay was developed in the late 80s, mostly for the detection of IFN-γ spot formation (Czerkinsky, 1988). Antibodies are used to detect the cytokine produced by individual T cells after a short restimulation period (6h-48h) with the antigen (**Figure 1.3**). The test has been combined with computer-assisted image analysis to standardize the readout procedure (Herr, 1997). The sensitivity of the assay is about 3-5 x 10^{-4} CD8+ T cells. This method has also been applied to the detection of CD4+ T cells using the antigen-triggered secretion of IFN-γ, IL-2 or IL-4. In this context, sensitivities of 3 x 10^{-4} of CD4+ T cells can be reached (Schuler-Thurner, 2002).

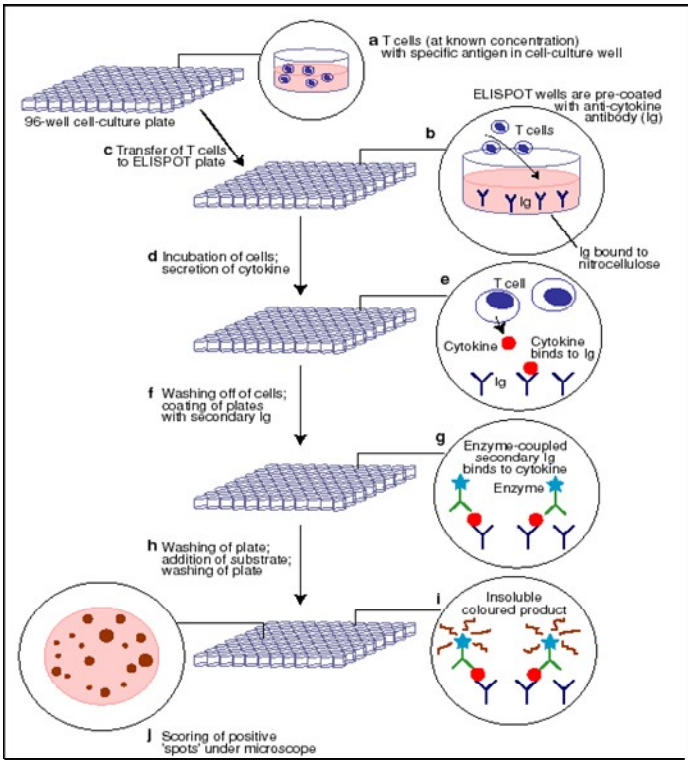

Figure 1.3. **Principle of the ELISPOT assay**. After contact with antigen, T cells release the cytokine which is captured by anti-cytokine antibodies on the ELISPOT plate. Enzyme-coupled secondary antibodies bind another epitope of the cytokine. After addition of a chromogenic enzyme substrate, chromatographic reaction occurs, which produces visible cytokine spots. From Expert Reviews in Molecular Medicine (accession number: fig003jhc).

1.6.3 Intracellular cytokine assay

The intracellular cytokine assay evaluates the number of cells producing IFN-γ upon contact with the antigen using flow cytometry (Andersson, 1988; Desombere, 2004). During the last 4-18h of stimulation, brefeldin is added to block the export of the cytokine, which then accumulates in the cells. The lymphocytes are fixed, permeabilized and stained with an antibody against the cytokine. The fraction of positive cells is evaluated by flow cytometry. The sensitivity of this assay is similar to that of ELISPOT assays. As with the ELISPOT assay, the lymphocytes are lost during the assay, preventing any further assessment of their specificity or phenotype.

1.6.4 Cytokine secretion assay

Further functional characterization or expansion of cytokine-secreting cells requires isolation methods that leave cells viable. Like the intracellular cytokine assay, the cytokine secretion assay is based on the selection of cells producing IFN-γ or another cytokine upon antigen contact. But in the latter method, cells are encapsulated in a permeable antibody-capture matrix which retains the secreted cytokine and do not alter the viability or functionality of cells (Weaver, 1997; Turcanu, 2001). Antibody-stained cells are then sorted with flow cytometry or magnetic-based separation. The sensitivity of the assay is ca. 10^{-3}.

1.7 The rationale and requirements of a procedure identifying the preferential targets of the individual T cell repertoire

As already described, the ability of the immune system to mount effective T cell responses against a specific antigen may be influenced by individual parameters. First, the particular HLA molecules expressed by an individual define in part the antigens against which T cells responses may be mounted, as only a selection of peptides can be presented by the individual's HLA molecules (Garrett, 1989; Falk, 1991). This is important to consider especially when defined peptides are used to vaccinate patients. Second, the ability to respond to a given antigen depends on the individual T cell receptor repertoire (Mason, 1998). Finally, mechanisms of central and peripheral tolerance also influence the response (Kyewski, 2006). In cancer patients, other variables in relation with the tumor, like its antigenic expression profile, the escape mechanisms or the suppression strategies used by the tumor to evade the immune system, may further individualize the ability to respond efficiently to an antigen. The variability of these parameters among individuals make the outcome of vaccination strategies in tumor patients largely unpredictable.

So far, almost all vaccination studies have been restricted to frequently expressed antigens presented by frequent HLA class I alleles (**Table 1.2**). The individuality of tumor-

host interactions was largely ignored and was only exceptionally considered (Tanaka, 2003). For above listed reasons, the improvement of therapeutic vaccination strategies might critically depend on the knowledge of preferential targets of the individual anti-tumor T cell repertoire.

The aim of this project was to develop a procedure that would allow to identify in vitro preferred shared target antigens, against which the immune systems of individual patients can mount readily detectable T cell responses. This procedure should include all potential epitopes that can be presented by the individual patient's HLA class I molecules. Furthermore, it should be applicable even when autologous tumor cells or cell-lines are not available for stimulation in Mixed Lymphocyte Tumor cell Cultures (MLTC). In the following, we show why in vitro stimulation is necessary, and describe briefly the procedure.

1.7.1 Short-term in vitro stimulation

Frequencies of precursor T cells recognizing shared antigens in normal donors vary strongly. For MAGE-encoded antigens, this frequency appears to be around 4×10^{-7} of CD8+ T cells (Chaux, 1998). In tumor patients, a spontaneous amplification may occur through stimulation of responders by the tumor, a process known as "in vivo priming" (Marincola, 1996). Following this first amplification, blood frequencies of 2×10^{-6} to 4×10^{-5} CD8+ T cells have been found in melanoma patients against MAGE-C2 (Germeau, 2005). Others have reported frequencies reaching 4×10^{-4} CD8+ T cells against NY-ESO-1 (Valmori, 2000B). In many patients, frequency of CTL precursors against Melan-A is 20-fold higher than in healthy individuals (Marincola, 1996; Romero, 2002). Other differentiation antigens appear to rarely stimulate spontaneous CTL responses. Even after a substantial amplification of antigen-specific T cells by the tumor, their frequency in peripheral blood are often below the lower detection limits of quantitative assays. Thus, an amplification step is necessary to be able to detect these responses with current methods. Herein, antigen-specific CD8+ T cells present in the peripheral blood of tumor patients were amplified with a short-term in vitro stimulation.

1.7.2 The stimulation procedure

As shown in **Figure 1.4**, unmanipulated, ex vivo CD8+ T cells isolated from patients' peripheral blood are stimulated in vitro. Stimulators are antigen presenting cells (APC) loaded with a panel of shared antigens comprising shared tumor-specific and differentiation antigens. The antigen-specific T cells potentially amplified with the stimulation are detected in an ELISPOT assay. Several requirements influenced our choice concerning the nature of these APC, the format of the antigen to load APC with, and the readout procedure. These are described further.

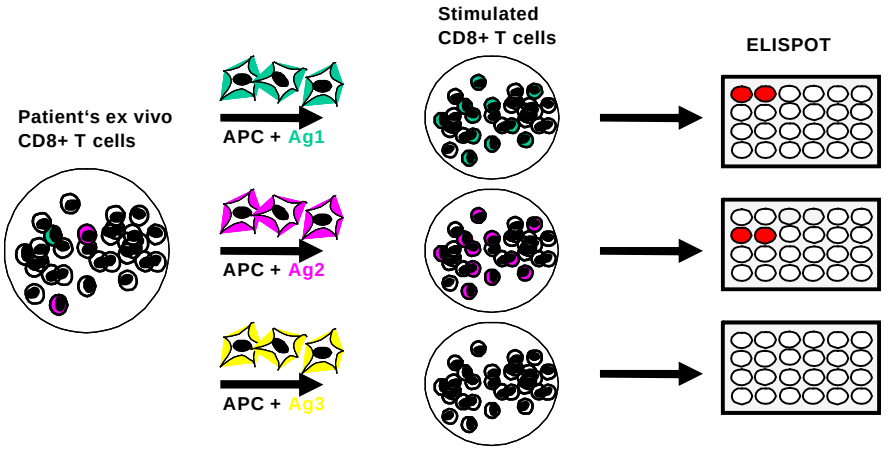

Figure 1.4. **Principle of the stimulation procedure**. Ex vivo CD8+ T cells isolated from patients' peripheral blood contain antigen-specific precursors at low frequency. These precursors are amplified with a short-term in vitro stimulation, where stimulators are antigen presenting cells (APC) loaded with a panel of shared antigens (Ag). The antigen-specific CD8+ T cells potentially amplified by the stimulation procedure and present at a frequency above the detection limit can now be detected in an ELISPOT assay. Here, stimulations with Ag1 and Ag2 elicit the amplification of CD8+ T cell responses against these antigens, which can be detected in the ELISPOT assay. Stimulation with Ag3 amplifies no specific response, and thus induces no signal when stimulated cells are tested against this antigen in the ELISPOT assay.

1.7.3 Antigen format

The antigen format should include all potential known and unknown epitopes of a given antigen that can be presented by the individual patients´ HLA class I molecules. mRNA appeared to be a promising candidate format, and has already been used in vaccination trials (reviewed in Gilboa, 2004). Because the mRNA-encoded antigens are expressed in the cytoplasm of the APC, the antigens are given access to the HLA-I pathway and presented to CD8+ T cells. mRNA loading of DC was first described in 1996 (Boczkowski, 1996). Since then, transfection efficiency has been considerably increased with electroporation (Strobel, 2000; van Tendeloo, 2001). The use of mRNA avoids the transfer of potentially highly immunogenic vectors which may mask low-frequency T cell responses (Kuball, 2002; Meyer, 2005) and proved to be as efficient as peptide pulsing in immune monitoring (Britten, 2004).

1.7.4 Antigen Presenting Cells (APC)

Most of known antigens recognized by patients' T cells have been identified with tumor-reactive T cells generated via in vitro stimulation with available autologous tumor cells or cell-lines (van der Bruggen, 1991; Wölfel, 1995; Lennerz, 2005). Autologous tumor cells have the major advantage to bear all HLA molecules of the patient and can, therefore, present all potential epitopes derived from tumor-associated antigens, provided that the tumor cells' processing and presenting machinery is intact. But tumor cells or tumor cell-lines are available only in a minority of patients. As a remplacement as autologous APC, dendritic

cells (DC) were chosen herein. DC have been shown to be highly potent APC (Banchereau, 1998; Jung, 2002). A decisive aspect was that these cells can be rapidly and efficiently produced in vitro from the patient's monocytes, giving rise to potent monocytes-derived FastDC (Dauer, 2003).

A general concern in T cell monitoring is to avoid the use of the same APC and antigen format for stimulation and for readout. This would increase the risk of expanding T cells against contaminants or artefacts. For this reason, the readout assay was designed to rely, instead on mRNA-transfected DC, on 293T or K562 cells co-transfected with plasmids containing the respective antigen- and HLA-I-encoding cDNA (Lennerz, 2005; Britten, 2002).

1.7.5 Readout assay

The ELISPOT technology permits to achieve detection thresholds in the same order as those achieved with tetramers (see chapter 1.6.2). This is of importance, because frequencies of antigen-specific CD8+ T cells may remain low even after stimulation, especially for those antigens against which precursors can not be detected ex vivo (Lurquin, 2005; Boon 2006). In addition to its sensitivity, the ELISPOT technology is highly versatile with respect to APC and antigen formats. Thus, the aforementioned requirements of applying all potential restricting HLA alleles could easily be achieved with this type of assay.

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Laboratory instruments

Instrument	Description	Provider
Analytical balance	Precisa	PAG Oerlikan AG, Zürich
Bacteria incubator	Heraeus B6200	Heraeus, Hanau
Balance	Sartorius	Sartorius, Göttingen
Centrifuge	Centrifuge 5415 R Megafuge 3.0R	Eppendorf, Hamburg Heraeus, Hanau
CO_2 incubator	CB210	Binder, Tuttlingen
Deionisated water installation	Milli-Q plus	Millipore, Eschborn
Drying hood	UT 6420	Heraeus, Hanau
Electrophoresis chamber	Easy-Cast™ Minigel Systems	AGS, Heidelberg
Electrophoresis power supply	Gene Power Supply GPS 200/400	Pharmacia, Freiburg
Electroporator	Gene Pulser II	BioRad, Munich
ELISPOT analysis software	KS ELISPOT V4.4.35	Carl Zeiss, Jena
Flow cytometer	FACS CANTO	Becton Dickinson, Heidelberg
Flow cytometry analysis software	Expo 32 V1.0	Beckman Coulter, Krefeld
Fluorescence microscope	Olympus-IX71	Olympus, Hamburg
Gel analysis software	BioDocAnalyse	Biometra, Göttingen
Hemacytometer	Fuchs-Rosenthal	Marienfeld, Lauda-Königshofen
Ice generator	UBE50/35	Ziegra, Isernhagen
Laminar flow clean bench	Herasafe K518	Kendro, Langenselbold
Magnetic hotplate stirrer	IKAMAG REC-G	Janke & Hunkel, Staufen
Microscope	Model TMS	Nikon, Japan
Microwave oven	MWS 2819	Bauknecht, Neunkirchen
Multicanal pipets	25-200 µl, 5-50 µl	Dunn Labortechnik, Asbach
Nitrogen tank	XLC 1370	MVE Europe, Solingen

Nucleofector		Amaxa, Köln
PCR thermocycler	Trio-Thermoblock	Biometra, Göttingen
pH-meter	Digital CG 837	Schott, Mainz
Photometer	Ultrospec 3000	Pharmacia, Freiburg
Pipets adjustable 20, 200, 1000 µl 10 µl	Pipetman	Gilson, Bad Camberg Eppendorf, Wesseling-Berzdorf
Pipetus	Pipetboy	IBS Integra biosciences, Fernwald
Refrigerator, freezer	Bosch Economic Ökosuper Sikafrost comfort	Robert Bosch GmbH, Stuttgart Liebherr, Ochsenhausen Siemens, Berlin
Shaking incubator	Certomat® R/H	Braun, Melsungen
Sterilisator	KSG sterilisator	KSG, Olching
Thermomixer	Thermomixer Comfort	Eppendorf, Hamburg
UV illuminator	BioDoc Analyze Transilluminator	Biometra, Göttingen
Vortexer	VF2	Janke und Kunkel, Staufen
Waterbad	Type 1013	GFL, Burgwedel

2.1.2 Chemicals

Acetic acid	Roth, Karlsruhe
3-Amino-9Ethyl-Carbazol (AEC) tablets	Sigma, Deisenhofen
Agarose	Serva Electrophoresis GmbH, Heidelberg
Agarose, ultrapure	Gibco BRL, Karlsruhe
Albumin, bovine	Sigma, Deisenhofen
Bromophenolblue	Merck, Darmstadt
Chloroform	Riedel-de Haen, Seelze
Diethyl Pyrocarbonat (DEPC)	Sigma, Deisenhofen
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
Dimethylformamid	Merck, Darmstadt

Ethanol, 70%	Brüggemann, Heilbronn
Ethanol, 99%	Applichem, Darmstadt
Ethidiumbromid 10 mg/ml	Sigma, Deisenhofen
Ethylendiamin-tetra acetic acid (EDTA)	Merck, Darmstadt
Ficoll	Biochrom KG, Berlin
Formaldehyd 37%	Merck, Darmstadt
Formamid	Merck, Darmstadt
Glycerol	Sigma, Deisenhofen
Hydrogenperoxid 30%	Roth, Karlsruhe
Isoamylalcohol	Merck, Darmstadt
Isopropanol	Fisher Scientific, Schwerte
Lipofectamine 2000™ Transfection Reagent	Invitrogen, Karlsruhe
m^7G(5')ppp(5')G RNA Capping Analog	Invitrogen, Karlsruhe
Mopholinopropansulfonsäure (MOPS)	Roth, Karlsruhe
Phosphat-buffered saline (PBS)	Biochrom KG, Berlin
Roti® Phenol : Chloroform	Roth, Karlsruhe
Sodiumacetat	Merck, Darmstadt
TBE buffer 10x	Serva Electrophoresis, Heidelberg
TransMessenger ™ Transfection Reagent	QIAGEN, Hilden
Tris-HCl	Sigma, Deisenhofen
Trypanblue	Merck, Darmstadt
ß-Mercaptoethanol	Sigma, Deisenhofen
DNA Blue Run 5x	Hybaid-AGS, Heidelberg

The m^7G(5')ppp(5')G RNA Capping Analog was dissolved in 13,7 µl DEPC-H_2O to give a 100 mM solution, and stored at -20°C.

2.1.3 Buffers and solutions

Acetic acid 0,2 N :

11,3 g acetic acid 96% in 1 l H_2O.

Sodium acetate 0,2 M :

16,4 g sodium acetate in 1 l H_2O.

Sodiumacetat 3 M :

24,6 g Sodiumacetat in 100 ml DEPC-H_2O, pH7

Acetatbuffer (for development of ELISPOT assays) :

4,6 ml acetic acid 0,2 N, 11 ml sodium acetate 0,2 N in 46,9 ml H_2O.

AEC solution (for development of ELISPOT assays) :

1 AEC tablet dissolved in 2,5 ml dimethylformamid, 47,5 ml acetatbuffer. Steril filtrated.

PBS :

95,5 g Instamed PBS pulver in 10 l H_2O.

DEPC-H_2O :

2 ml DEPC in 2 l H_2O. Mixed for 1h and autoclaved.

1x TBE buffer :

100 ml 10 x TBE in 900 ml H_2O.

NaCl 0,15 M :

4,5 g NaCl in 100 ml H_2O.

EDTA 0,5M :

18,6 g EDTA in 100 ml DEPC-H_2O, pH8

10 x MOPS buffer :

41,86 g MOPS, 16,7 ml 3M Sodiumacetat, 10 ml 0,5M EDTA in 1l DEPC-H_2O, pH7

1 x MOPS buffer :

100 ml 10xMOPS in 900 ml DEPC-H_2O

RNA probe buffer :

100 µl deionized Formamid, 70 µl 37% Formaldehyd, 50 µl 10x MOPS, 20 µl Bromophenolblau 1%, 1µl Ethidiumbromid 1% in 259 µl DEPC-H_2O

Chloroform : isoamylalcohol (24:1) :

1 ml isoamylalcohol in 23 ml chloroform

Tris-HCl 1M :

157,6 g Tris-base in 1 l H_2O. pH adjusted at 8,5 with HCl. Autoclaved.

Trypan blue (stock solution) :

2 g Trypan blue in 1 l H_2O.

Trypan blue (staining solution) :

75 ml stock solution, 25 ml NaCl 0,15 M.

Erythrocytes lysis buffer :

8.3 g ammonium chloride, 0.84 g sodium bicarbonate and 29.3 mg EDTA, ad 1l H_2O. pH adjusted at 7.4 with HCl. Sterile filtrated.

MACS buffer :

2 ml EDTA 0,5M, 2,5 g BSA in 500 ml PBS. Sterile filtrated.

FACS buffer (0,1% BSA):

0,5 mg BSA in 500 ml PBS. Sterile filtrated.

FACS fixing buffer :

1,35 ml 37% Formaldehyde in 50 ml FACS buffer.

2.1.4 Material for bacterial culture and molecular biology

2.1.4.1 Plasmids

pcDNA6/V5-HisB.pp65 encoding pp65 of HCMV was kindly provided by Dr. B. Plachter (Institute of Virology, University of Mainz), pcR3.1-uni.MVA#28 by Dr. C. Graf (III. Medizinische Klinik, University of Mainz), pRc/CMV.MC1R by Dr. R. Kiessling (Karolinska Hospital, Stockholm, Sweden) and pcDNA3.1(-).NY-ESO-1 by Dr. U. Sahin (III. Medizinische Klinik, University of Mainz).

All other plasmids encoding tumor antigens were kindly provided by Dr. P. van der Bruggen (LICR, Brussels, Belgium).

Most of the plasmids encoding HLA class I molecules were provided by the group of T. Wölfel, except pcDNA3.1.HLA-Cw*0701, which was kindly provided by Dr. P. van der Bruggen (LICR, Brussels, Belgium) and pcDNA3.1.HLA-Cw*0702, which was kindly provided by Dr H. Wang (Japanese Red Cross, Central Blood Center, Tokyo, Japan). HLA class I molecules which were not yet available were cloned. All inserts were controlled by DNA sequencing.

2.1.4.2 Escherichia coli strains

TOP 10 bacteria (Invitrogen, Karlsruhe) were used to propagate all recombinant plasmids, except pRc/CMV.MC1R, which was propagated in DH10B-T1 electrocompetent cells (Invitrogen).

Genotype of TOP 10:
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.

Genotype of DH10B-T1:
F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G *ton*A.

Bateria aliquots were stored at -80°C.

2.1.4.3 Substances for bacterial culture

Bacto Agar	Difco, Stuttgart
Bacto Tryptone	Difco, Stuttgart
Yeast extract	Difco, Stuttgart
Ampicillin	Sigma, Deisenhofen
S.O.C. Medium	Invitrogen, Karlsruhe

Ampicillin was dissolved in H_2O at 50 mg/ml and stored at -20°C in 10 ml alliquots.

2.1.4.4 Media for bacterial culture and conservation

LB (Luria-Bertani) medium :
10 g Bacto Tryptone, 5 g yeast extract, 10 g NaCl, 1 l H_2O

LB agar :

1 l LB medium, 15 g Bacto Agar

Glycerol stocks :

500 µl bacterial culture (in LB medium), 500 µl 30% glycerin

Media were autoclaved and supplemented with Ampicillin at 100 µg/ml for selection of Ampicillin-resistant bacteria when media temperature reached 50-60°C. They were further stored at 4°C. Glycerol stocks were directly stored at -80°C in cryotubes (Nunc, Wiesbaden).

2.1.4.5 Enzymes

Yeast Poly(A) polymerase	USB, Cleveland, USA
DNAse I	Qiagen, Hilden
RNase H	Qiagen, Hilden
T7 RNA polymerase	Promega, Mannheim
Red Taq polymerase	Sigma, Deisenhofen
Easy A polymerase	Stratagene, Heidelberg
Xba I 12u/µl	Promega, Mannheim
*Hind*III 10u/µl	Promega, Mannheim
*Xho*I 10u/µl	Promega, Mannheim
*EcoR*I 12u/µl	Promega, Mannheim

For all digestion reactions with endonucleases, Bovine Serum Albumin Acetylated (BSA) 10 mg/ml (Promega, Mannheim) was added.

All enzymes were stored at -20°C.

2.1.4.6 Molecular markers

DNA Molecular Weight Marker III	Roche, Mannheim
0.5-10 Kb RNA Ladder	Invitrogen, Karlsruhe

DNA marker was stored at 4°C. RNA marker was stored at -80°C in 3 µl aliquots.

2.1.4.7 Kits

High Pure PCR Product Purification Kit	Roche, Mannheim
RNeasy Mini Kit	QIAGEN, Hilden
QIAPrep Spin Miniprep Kit	QIAGEN, Hilden
Hispeed Plasmid Maxi Kit	QIAGEN, Hilden
Super Script III First-Strand	Invitrogen, Karlsruhe
pcDNA 3.1/V5-His TOPO TA Expression Kit	Invitrogen, Karlsruhe
RiboMAX™ Large Scale RNA Production System T7	Promega, Mannheim

2.1.5 Material for cell culture and cellular assays

2.1.5.1 Cell lines

293 T

293T served as APC in ELISPOT assays after transient transfection with recombinant plasmids encoding antigens of interest. 293T cells derive from human embryonal kidney cells that were immortalized with transforming DNA from human adenovirus type 5 (Graham, 1977). Immortalized 293 cells were then stably transfected with the large T antigen from the SV40 virus, giving rise to 293T cells (Lewis, 1985). These were kindly provided by N. Shastri (The University of California, Berkeley, USA). The expression of the large T antigen enables the episomal replication of circular plasmids carrying the SV40 origin of replication (Gluzman, 1981). This is the case for the vectors pcDNA1, pcDNA3, pcDNA3.1, pRC/CMV and pcR3.1-uni used in this work. 293T cells naturally express the HLA-A*0201, B*0702 and Cw*0701 HLA molecules according to high-resolution HLA genotyping and RT-PCR (personal communication from V. Lennerz).

NIH-3T3-CD40L ($3T3_{CD40L}$)

$3T3_{CD40L}$ are immortalized mouse 3T3 fibroblasts that were stably transfected with the CD40 ligand (Schultze, 1997). This cell line is commonly used to expand CD40-activated B cells in vitro by activating B lymphocytes via the CD40-CD40L interaction (Schultze, 1997; von Bergwelt-Baildon, 2002). $3T3_{CD40L}$ were kindly provided by M. von Bergwelt-Baildon (Klinik I für Innere Medizin, University of Cologne).

K562 transfectants

K562 stably expressing HLA molecules were used as alternative APC in ELISPOT assays. K562 are leukemia cells originally derived from a patient with chronic myelogenous leukemia (CML) (Lozzio, 1975) and lacking HLA class I and II surface expression (Drew, 1977). K562 cells stably transfected with single HLA I alleles have been successfully established as low-background APC in ELISPOT assays (Britten, 2002). Several K562 cell lines stably expressing various HLA molecules have been established. Transfectants that have been used in this work are :

Transfectant	Clone	HLA expression
K562/A*0201	#25	HLA-A*0201 (75-95%)
K562/B*0702	#4	HLA-B*0702 (10-40%)
K562/B*1501	#12	HLA-B*1501 (60-75%)

Patients and melanoma cell lines

In 1982, patient MZ2 developed a malignant melanoma with lymph node and abdominal metastases. Throughout 1982 and 1983, the patient was treated with chemotherapy and suffered from several relapses with abdominal metastases, which were removed by surgery. The cell line MZ2-MEL was derived from one of these metastases in 1982. MZ2-MEL-43 was isolated from surviving cell populations obtained after mutagen treatment of MZ2-MEL-3, a clone derived from MZ2-MEL (Hérin, 1987; Van den Eynde, 1989). Patient remained disease free after multiple injections of irradiated tumor cells between 1983 and 1988. MZ2-MEL-43 was a gift from Dr. B. van den Eynde (LICR, Brussels, Belgium). PBL used for stimulation experiments were isolated from peripheral blood drawn in 1990 and 1991 and frozen in alliquots.

After surgical resection of primary melanoma, patient D05 developed metastases in the lymph nodes, kidney, adrenal gland, mediastinum, pleuropericardium and soft tissue. Cell line D05-GS.M1 was derived from a metastasis of a retroperitoneal lymph node. The patient was regularly vaccinated with autologous DC loaded with irradiated tumor cells between 1997 and 2000 (O'Rourke, 2003). Upon treatment, the patient experienced a durable complete remission. D05-GS.M1 was originally provided by Dr. C. W. Schmidt (Queensland Institute of Medical Research, Brisbane, Australia) and further cloned. Clone D05-GS.M1A#6 (further referred to as D05-MEL) was used in all experiments. PBL used for stimulation experiments were isolated from peripheral blood drawn in 09/2005 and frozen in alliquots.

Cytotoxic T lymhocytes and MLTC

Several autologous mixed lymphocyte-tumor cell cultures (MLTC) or MLTC-derived cytotoxic T lymphocyte (CTL) clones were used as effector cells in recognition assays. All of them were generated from peripheral blood mononuclear cells (PBMC) isolated from the blood of patient D05. Cryopreserved PBMC were thawed at different timepoints, and cocultured with the autologous tumor cell line D05-MEL in MLTC. CTL could be isolated from MLTC3 and MLTC4 under limiting dilution conditions and further expanded. MLTC5 was not cloned and instead used as such in recognition assays. Both MLTC and CTL were weekly stimulated with the tumor cell line exactly as described (Lennerz, 2005). Their tumor specificity and HLA restriction were examined. A series of target antigens could be identified (V. Lennerz, M. Fatho, D. Eberts, unpublished results). MLTC and CTL were cryopreserved four days after the last stimulation (d4) for direct use in ELISPOT assays. The following effector populations were used in this work :

PBMC	MLTC	CTL	Antigen	Peptide residues
01.01	#3	D05-3.2/141	Melan-A/MART-1/HLA-A*0201	26-35
-	#3	D05-3.2/173	MAGE-A3/HLA-Cw*0202	143-151
-	#3	D05-3.2/100	MAGE-A4/HLA-B*2705	145-154
05.98	#4	D05-4/50	NY-ESO-1/HLA-A*0201	157-165
-	#4	D05-4/21	Tyrosinase/HLA-Cw*0501	369-377
-	#4	D05-4/95	CCT6Amut/HLA-B*2705	156-164
09.05	#5		GAGE-1/HLA-B*2705	14-22
			MAGE-A1/HLA-B*4402 and Cw*0501	not determined
			BAGE-1/HLA-B*4402	21-29

2.1.5.2 Healthy donors

PBMC used for optimization of the stimulation procedure were isolated from healthy individuals. HLA type and CMV status were determined according to standard serologic procedures. Blood was drawn at the blood bank of the Mainz University Hospital and provided as a “Buffy coat”.

2.1.5.3 Substances for cell culture

Aqua Dest (AD)	B. Braun, Melsungen
PBS	Gibco BRL, Karlsruhe
RPMI 1640 culture medium	Gibco BRL, Karlsruhe
AIM V® culture medium	Gibco BRL, Karlsruhe

OPTIMEM	Gibco BRL, Karlsruhe
X-VIVO-15	Bio Whittaker, Walkersville, MY
Trypsin-EDTA	Gibco BRL, Karlsruhe
FCS (fetal calf serum)	Biochrom KG, Berlin
HS (human serum)	Healthy donors' blood donations
Geneticin (G418)	Biochrom KG, Berlin
Penicillin-Streptomycin	Gibco BRL, Karlsruhe
Gentamycin	Gentamicin 80SF, Ratiopharm, Ulm
Human transferin	Sigma, Deisenhofen
Human insulin	Insuma Rapid, Aventis, Frankfurt
Cyclosporin A (CsA)	Sandimmun, Novartis, Nürnberg

Penicillin-Streptomycin was stored at 10 mg/ml in 10 ml aliquots at -20°C.
FCS was heat-inactivated at 56°C for 30 min before being alliquoted and frozen at -20°.
HS was gained from the blood of healthy donors and prepared by the blood bank of the Mainz university hospital before heat inactivation at 56°C for 30 min, sterile filtration and storage in aliquots at -80°C.
Transferin was stored at -20°C as pulver stock. Upon dilution in AD at 30 mg/ml, it was sterile filtrated and preserved upon 2 weeks at 4°C. CsA was diluted in PBS to a concentration of 1 mg/ml and stored at 4°C.
All other components were stored at 4°C.

2.1.5.4 Media for cell culture

$RPMI_{complete}$
RPMI 1640 supplemented with 10% (v/v) FCS and 1% (v/v) Penicillin-Streptomycin.

AIM $V_{complete}$
AIM V supplemented with 10% HS.

B cell medium
RPMI 1640 supplemented with 10% HS, 1% Penicillin-Streptomycin, 50 µg/ml human transferin, 5 µg/ml human insulin and 15 µg/ml gentamycin. 0,5 µg/ml CsA and 100 IU/ml rhIL-4 were freshly added before use.

FastDC medium

RPMI 1640 supplemented with 2% human serum.

Classical DC medium

X-VIVO 15 supplemented with 1% human serum.

Freezing medium

FCS supplemented with 10% DMSO.

All media were stored at 4°C.

2.1.5.5 Cytokines

Interleukine-2 (rhIL-2)	Proleukin, Chiron Behring, Marburg
Interleukine-4 (rhIL-4)	Strathmann-Biotech, Hannover
Interleukine-6 (rhIL-6)	R&D Systems, Wiesbaden
Interleukine-7 (rhIL-7)	Strathmann-Biotech, Hannover
Interleukine-15 (rhIL-15)	R&D Systems, Wiesbaden
Interleukine-1ß (rhIL-1ß)	R&D Systems, Wiesbaden
rhGM-CSF (Leukine Sagramostim)	Berlex, Richmond, CA, USA
rhTNF-a	R&D Systems, Wiesbaden
Prostaglandin-E2 (PGE2)	Sigmal Aldrich, Taufkirchen

rhIL-2 was dissolved in AIM $V_{complete}$ to obtain a concentration of 25 IU/µl, rhIL-4 to a concentration of 500 IU/µl, rhIL-6 to a concentration of 1000 IU/µl, rhIL-7 and rhIL-15 to a concentration of 5 ng/µl, rhIL-1ß and rhTNF-a to a concentration of 10 ng/µl.
rhGM-CSF was diluted in AIM $V_{complete}$ to a concentration of 1000 IU/µl and PGE2 was dissolved in 100% ethanol to a concentration of 1 µg/µl.

2.1.5.6 Antibodies

The following conjugated mouse antibodies were used to characterize cells for expression of differentiation or maturation surface markers :

Antibody	**Clone**	**Isotype**	**Provider**
Anti-CD3-Fitc	UCHT1	IgG1	Beckman Coulter, Krefeld
Anti-CD4-PE	13B8.2	IgG1	Beckman Coulter, Krefeld
Anti-CD8-PE	B9.11	IgG1	Beckman Coulter, Krefeld

Anti-CD13	MY7	IgG1	Immunotech/Coulter, Hamburg
Anti-CD14-PE	RM052	IgG2a	Beckman Coulter, Krefeld
Anti-CD19-PE	J4.119	IgG1	Immunotech/Coulter, Hamburg
Anti-CD80-Fitc	MAB104	IgG1	Immunotech/Coulter, Hamburg
Anti-CD83-PE	HB15A	IgG2b	Immunotech/Coulter, Hamburg
Anti-CD86-Fitc	2331 (Fun.1)	IgG1	Pharmingen, Heidelberg
Anti-CCR7-Fitc	FAB197F	IgG2a	R&D Systems, Wiesbaden

In addition, a few mouse monoclonal antibodies purified from hybridoma supernatants and specific for some groups of HLA molecules were used to characterize the surface HLA expression pattern of FastDC or K562 transfectants :

Antibody	**Specificity**	**Isotype**	**Origin**	**Reference**
L243	HLA-DR	IgG2a	ATCC, HB-55	Bridges, 1987
W6/32	HLA-I	IgG2a	(1)	Barnstable, 1978; Parham, 1979
MA2.1	HLA-A2, Bw57, Bw58	IgG1	ATCC, HB-54	McMichael, 1980; Ways, 1986
B1.23.2	HLA-B, C, Aw19	IgG2b	(2)	Lemonnier, 1982; Zinszner, 1990
BB7.1	HLA-B7	IgG1	ATCC, HB-56	Toubert, 1988

(1) kindly provided by Dr P. Parham (Departments of Structural biology and microbiology and Immunology, Stanford University, Stanford, USA)

(2) kindly provided by Dr F.A. Lemonnier (Centre d'immunologie INSERM-CNRS de Marseille Luminy, France).

To enable detection by flow cytometry, primary mouse antibodies were detected with Fitc-conjugated secondary goat anti-mouse-Fab antibodies (Beckman Coulter, Krefeld).

2.1.5.7 Synthetic peptides

The following peptides, defining antigens recognized by CD8+ T lymphocytes, were used in recognition assays. They were synthesized on solid-phase using Fmoc chemistry, purified by reversed-phase high performance liquid chromatography and characterized by mass spectrometry (performed by Dr. J.W. Drijfhout, Department of Immunohematology and Blood Transfusion, University Medical Center, Leiden, The Netherlands).

Antigen	Peptide sequence	HLA restriction	Position in the protein	Reference
CMV/pp65	TPRVTGGGAM	HLA-B*0702	417-426	Wills, 1996
CMV/pp65	NLVPMVATV	HLA-A*0201	495-503	Wills, 1996
MVA/ORF28 (K7R)	SIIDLIDEY	HLA-B*1501	25-33	Meyer, 2007
Melan-A/MART-1	EAAGIGILTV	HLA-A*0201	26-35	Kawakami, 1994
TRP-2	NMVPFFPPV	HLA-A*0201	431-439	Reynolds, 1998

Lyophilised peptides were dissolved in DMSO to a concentration of 40 mg/ml and diluted in PBS to a final concentration of 2 mg/ml. Aliquots were stored at -20°C.

2.2 Methods

2.2.1 Molecular biology

2.2.1.1 Amplification and isolation of plasmid DNA

All recombinant plasmids were propagated in TOP10 bacteria, except plasmid pRc/CMV.MC1R which was propagated in DH10B-T1. Glycerol stocks were systematically generated and stored at 80°C, providing expandable plasmid reserves. For plasmid amplification, 250 ml LB medium supplemented with 100 µg / ml ampicillin were inoculated with 10 µl bacteria from a glycerol stock and bacteria were allowed to grow over night at 37°C in a shaking incubator at 240 rounds per minute (rpm). Alternatively, culture was inoculated with a single colony from an overnight culture on agar plate. In this case, bacteria were picked and transferred into 2 ml LB medium supplemented with 100 µg / ml ampicillin and allowed to grow for 8h at 37°C. 0,5 ml from this preculture were then transferred into 250 ml LB medium supplemented with 100 µg / ml ampicillin, and bacteria were allowed to grow over night. Isolation of plasmid DNA was performed with the Plasmid Maxi Kit (QIAGEN) according to the manufacturer's instructions. DNA was eluted with 500 µl TE buffer, and DNA concentration was determined by spectrophotometric measurement. Yields between 250 and 500 µg of plasmid DNA were commonly obtained from 250 ml overnight cultures. For cloning of HLA molecules and verification of recombinant clones (see chapter 2.2.1.7), plasmid DNA was isolated from 5 ml overnight bacteria cultures using the Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions. DNA yields between 10 and 20 µg were obtained in this case.

2.2.1.2 Generation of in vitro transcribed (IVT)-mRNA

In vitro transcription (IVT) of antigen-encoding sequences was performed from recombinant plasmids carrying the antigens of interest using the T7 Polymerase (Promega). Transcription start was enabled by the presence of the T7 promotor in 5' of the insert on all the plasmids used. Transcription ending was ensured by previously linearizating the plasmid after the insert, producing the adequate template for IVT.

Plasmid linearization

pcDNA6/V5-HisB.pp65 was linearized with *Xho*I, pcR3.1-uni/ORF#28 with *EcoR*I, and pcDNA3.1(-).NY-ESO-1 with *Hind*III. All other plasmids were linearized with *Xba*I (**Table 2.1**).

Table 2.1 : Antigen encoding IVT-mRNAs with the corresponding DNA templates, the enzymes used for linearization and the lengths of the IVT products.

Antigen	Plasmid	Lin. enzyme	Length (kb)
CMV/pp65	pcDNA6/V5-HisB	Xho1	1,9
MVA/ORF#28	pcR3.1-uni	EcoRI	0,6
Tyrosinase	pcDNA3	XbaI	2,4
TRP-2	pcDNA3.1	XbaI	1,7
Melan-A/MART-1	pcDNA3.1	XbaI	0,7
Gp100	pcDNA1	XbaI	2,5
MC1R	pRC/CMV	XbaI	1,8
MAGE-A1	pcDNA1	XbaI	2
MAGE-A3	pcDNA1	XbaI	1,6
MAGE-A4	pcDNA1	XbaI	2
MAGE-A6	pcDNA1	XbaI	1,5
MAGE-A12	pcDNA1	XbaI	1,6
MAGE-C2	pcDNA1	XbaI	2
BAGE	pcDNA1	XbaI	1,2
GAGE-1	pcDNA1	XbaI	0,9
GAGE-4	pcDNA1	XbaI	0,7
NY-ESO-1	pcDNA3.1(-)	HindIII	0,7
PRAME	pcDNA1	XbaI	2
RAB-38	pcDNA3.1	XbaI	0,75
Surv-1	pcDNA3.1	XbaI	0,6
Surv-2	pcDNA3.1	XbaI	0,8
N-WASP^{mut}	pcDNA3.1	XbaI	1,9

Linearization occured for 8h at 37°C in the following reaction mix:

Buffer D (Promega) 10x	10µl
Plasmid	5 µg
Acetylated BSA, 10mg/ml	1µl
Xba I (Promega), 12u/ul	5 µl
AD	ad 100 µl

Digestion products were purified using the High Pure PCR Product Purification Kit (Roche) according to the manufacturer's instructions, and eluted in 25 µl DEPC-H_2O before use as templates for IVT.

IVT reaction

IVT was performed with the RiboMAX™ Large Scale RNA Production System T7 (Promega) according to the manufacturer's instructions. Briefly, linearized DNA templates were incubated for 3h at 37°C in a reaction containing the T7 reaction buffer, the four ribonucleotides and the m^7G(5')ppp(5')G capping analog (Invitrogen) with the T7 RNA polymerase, as follows:

transcription buffer 5x	20 µl
rATP 100 mM	7,5 µl
rCTP 100mM	7,5 µl
rGTP 100 mM	1 µl
rUTP 100mM	7,5 µl
Template	5 µg in 25 µl
Capping analog 100 mM	4 µl
Enzyme Mix	10 µl
DEPC H_2O	ad 100 µl

The m^7G(5')ppp(5')G capping analog is incorporated at the 5' extremity of the RNA and has been shown to improve its stability, as well as the initiation of translation (Shatkin, 1976; Banerjee, 1980). Nevertheless, its use in the IVT reaction may be linked with a reduce in the RNA yields to 20-50% of the yields obtained without capping analog. The ratio of capping analog : GTP was chosen to balance the percentage of capped products with the efficiency of the transcription reaction.

After IVT, DNA template was removed by digestion with 1u DNase I / µg template for 15 minutes at 37°C. RNA was then purified with phenol extraction and precipitated with 100% ethanol, as described below. RNA pellet was resuspended in 100 µl DEPC-H_2O, and quality of IVT product was controlled by electrophoretic analysis. IVT product was typically visualized as a sharp band on the gel, characteristic of RNA molecules of a unique and common length. RNA concentration was determined by spectrophotometry and IVT product stored at -80°C until polyadenylation.

Purification of IVT-mRNA

Reaction volume containing IVT-mRNA was mixed with an equal volume of Phenol-Chloroform (1:1) by vortexing for 1 minute. Proteins were concentrated at the interface by centrifugation for 5 minutes at 14 000 rpm. Upper aqueous phase containing RNA was gently removed, and mixed with an equal volume of Chloroform-Isoamyl alcohol (24:1) by vortexing 1 minute. After centrifugation for 5 minutes at 14 000 rpm, the upper phase containing RNA was gently removed. RNA was precipitated by addition of 0,1 volume of 3M Sodium Acetate and 2,5 volumes of 99% ethanol, and incubation for 5 minutes on ice. After centrifugation for 20 minutes at 14 000 rpm, supernatant was discarded and RNA washed with 0,5 ml 70% ethanol to remove salts. After a last centrifugation step for 10 minutes at 14 000 rpm, supernatant was completely removed, and pellet was air dried for 15 minutes to enable evaporation of residual ethanol. RNA pellet was dissolved in 100 µl DEPC-H_2O by resuspending well with the pipet.

2.2.1.3 Polyadenylation of IVT-mRNA

A poly(A) tail of approximatively 300 As was added to the IVT-mRNA using the Poly(A) polymerase (USB) according to the manufacturer's instructions. Previously, the number of RNA molecules to be polyadenylated was calculated, based on the RNA concentration and the length of the IVT product (Table 2.1) :

$$n_{RNA\ mol}\ (pmol) = [weight\ (pg)] / [MW_{base} \times length_{IVT}\ (base)]$$

with weight (pg) = concentration (µg / µl) x 10^6 x volume (µl),
MW_{base} = molecular weight of an RNA base = 330 g / mol
$length_{IVT}$ = length of the IVT product (base).

The rATP need was calculated to generate a poly(A) tail of approximatively 300 As. As rATP should not be the limiting factor in the reaction mix, it was used in a two-fold excess :

$$rATP\ (pmol) = n_{RNA\ mol}\ (pmol) \times 300 \times 2$$

1 IU of Poly(A) polymerase is defined as the quantity of enzyme adding 1 A to one substrate molecule in 1 minute. Thus, the quantity of Poly(A) polymerase necessary to add 300 As to the RNA substrate in an excess of ATP was calculated as follows :

$$\text{Poly(A) (IU)} = [\, n_{RNA\ mol}\ (\text{pmol}) \times 300] \,/\, \text{incubation time (min)}$$

with an incubation time commonly between 90 and 120 min.

The example of EGFP illustrates the procedure : the IVT product encoding the EGFP protein has a length of approximatively 900 bases. Thus, 100 µg IVT-mRNA correspond to a number of RNA molecules of:

$$n_{RNA\ mol} = [100 \times 10^6\ (\text{pg})] \,/\, [330 \times 900\ (\text{base})] = 337\ \text{pmol}$$

To generate a poly(A) tail of approximatively 300 As, the ATP need is:

$$337\ (\text{pmol}) \times 300 \times 2 = 202 \times 10^3\ \text{pmol}$$

and the need in Poly(A) polymerase is:

$$\text{Poly(A)} = [\, 337\ (\text{pmol}) \times 300] \,/\, 120\ (\text{min}) = 842\ \text{IU}$$

for an incubation time of 120 min.

Accordingly, IVT-mRNA was incubated for 120 min at 37°C in the following reaction mix to generate a poly(A) of approximatively 300 As:

IVT-mRNA	100 µg (in 50 µl)
rATP 10 mM	20 µl
5x Poly(A) buffer	20 µl
Poly(A) pol. 600 IU/ µl	1,4 µl
DEPC-H_2O	ad 100 µl

To polyadenylate smaller amounts of IVT-mRNA, incubation time, rather than amount of Poly(A) polymerase was reduced.

Polyadenylated IVT-mRNA was purified with phenol extraction and precipitated with 100% ethanol as described above. RNA pellet was resuspended in 100 µl DEPC-H_2O, and concentration was evaluated with spectrophotometry. Concentrations of IVT-mRNA-poly(A) between 3 and 8 µg/µl were commonly obtained. Electrophoretic analysis was also performed to control RNA quality, as well as the shift in length of IVT-mRNA after polyadenylation. In contrast to IVT-mRNA, IVT-mRNA-poly(A) appeared sometimes as a light smear centred around a sharp band. This is due to the fact, that the poly(A)s generated by the poly(A) polymerase may slightly differ in size. Nevertheless, the large majority of the

product was always of a definite length, appearing as the sharp band on the gel. After polyadenylation, IVT-mRNA was alliquoted and stored at -80°C.

2.2.1.4 Spectrophotometric quantification of nucleic acids

DNA quantification

Plasmid DNA concentration was determined by measuring the absorbance at 260 nm (A_{260}) with a spectrophotometer (Pharmacia, Freiburg). 1 µl DNA solution was diluted in 79 µl AD in a quartz cuvette, and the following parameters were determined with the measurement:

A_{260} = absorbance at 260 nm
A_{280} = absorbance at 280 nm
A_{320} = absorbance at 320 nm
Ratio = A_{260} / A_{280}

The plasmid DNA concentration was determined as follows :

DNA concentration (µg / ml) = A_{260} x 50 x dilution factor

Where dilution factor = 80 as described above.

DNA quantification was considered as reliable when A_{260} was between 0,1 and 1,0. The ratio of the readings at 260 nm and 280 nm (A_{260} / A_{280}) provides an estimate of DNA purity with respect to contaminants that absorb UV light, such as proteins. DNA solution was considered to be exempt from protein contamination when the ratio was between 1,8 and 2,0.

RNA quantification

Like for quantification of plasmid DNA, RNA concentration was determined by measuring the absorbance at 260 nm (A_{260}) using a RNase-free quartz cuvette exclusively dedicated to RNA measurement. RNA was diluted 1:100 to 1:200 in AD and RNA concentration was caluculated as follows :

RNA concentration (µg / ml) = A_{260} x 40 x dilution factor

With a dilution factor commonly between 100 and 200.

RNA quantification was considered as reliable when A_{260} laid between 0,15 and 1,0.

RNA solution was considered exempt from protein contamination when the ratio was between 1,9 and 2,3.

2.2.1.5 Electrophoretic visualization of nucleic acids

Visualization of DNA

DNA electrophoresis was performed to check length of insert after digestion of recombinant plasmids or to check complete linearization of templates previously to IVT. Indeed, as linearized plasmids are less efficient at migrating through the electrophoresis gel matrix, a shift in length is visible after migration of linearized versus unlinearized plasmids. If linearization was complete, a single DNA band should be visible on the gel after migration of the linearization product.

1% agarose gels were commonly used as matrix, enabling detection of DNA fragments from 0,5 to 10 kb. For the agarose solution, 1 g agarose was dissolved in 100 ml 1 x TBE buffer by heating in a microwave. The solution was enabled to cool to 50-60°C, and supplemented with 0,1 µg / ml ethidium bromide. Ethidium bromide intercalates between DNA bases and enables fluorescent vizualisation of DNA under UV light. Agarose solution was poured onto a gel tray carrying a comb, to a thickness of 3-5 mm. Gel was allowed to polymerize by incubation for 30-40 minutes at room temperature, and the comb was removed. Gel was then put into an electrophoresis tank previously filled with 1x TBE buffer. DNA samples were prepared by mixing 0,25-1 µg DNA with 5 µl DNA probe buffer (Blue Run 5x). DNA samples were loaded into the preformed wells of the gel and allowed to migrate at 10V / cm for 20-30 minutes.

To enable analysis of DNA fragment sizes, 1 µg Molecular weight marker III (Roche) were parallely run on the gel. Vizualisation of DNA bands was performed by illumination of the gel under UV light (254-366 nm) and images were recorded using the BioDocAnalyse System gel documentation system (Biometra, Göttingen).

Visualization of RNA

RNA electrophoresis was performed to check the quality of total RNA extracted from blood cells, or to control quality and length of IVT-mRNA before and after polyadenylation. In the first case, quality was assessed by vizualisation of the 28S and 18S ribosomal bands from rRNA which appeared sharp if RNA was intact. In the second case, as mono-specific RNA was analyzed, a single band was vizualised, which, again, appeared sharp if RNA was not degraded. After enzymatic polyadenylation, formation of polyadenylated products slightly different in size are produced, which result in a slight smeared band on the gel. This should

not be interpreted as a degraded RNA. In all the cases, a shift in length should be clearly apparent when polyadenylated IVT-mRNA is analysed parallely to non-polyadenylated IVT-mRNA.

As matrix, 0,8% agarose denaturing gels were used. Denaturing conditions are important for RNA visualization, as, unlike DNA, RNA has a high degree of secondary structures. These structures are disrupted in the gel by Formaldehyde, making migration of RNA dependent on the charge and not on the secondary structures formed. For the agarose solution, 0,56 g agarose was dissolved in 56 ml DEPC-H_2O by heating in a microwave. The solution was enabled to cool to 50-60°C, and supplemented with :

10 x MOPS	7 ml
37% Formaldehyde	7 ml

This mix was allowed to polymerize in a tray and the gel was put into an electrophoresis tank, previously filled with 1x MOPS.

RNA samples were prepared by mixing 7-10 µg RNA with 25 µl RNA probe buffer (containing Ethidium Bromid), and denaturing at 75 °C for 10 min. After chilling on ice for 5 min and a short centrifugation step to collect drops of evaporated buffer, samples were loaded onto the gel, which was run for 10 min at 6V / cm, followed by 60 min at 8V / cm. Higher voltages may cause trailing and smearing of RNA bands.

3 µg of 10 kb Molecular weight markers (Invitrogen) was parallely run on the gel to appreciate RNA length. Vizualisation of RNA bands was performed as described for DNA analysis.

2.2.1.6 HLA RT-PCR

Patient's HLA type and subtype were determined by specific amplification of HLA-A, -B, und -C alleles from cDNA using Polymerase Chain Reaction (PCR).

Extraction of total RNA

Total RNA was extracted from 5-10 x 10^6 PBMC using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions, and eluted with 30 µl RNase-free water. Concentration was determined by spectrophotometry using a 1:20 dilution, and 1 µg was used for quality check on an RNA gel (see chapter 2.2.1.5). Total RNA was stored at -80 °C until reverse transcription.

Reverse Transcription

cDNA was generated from total RNA by reverse transcription PCR (RT-PCR) using the Super Script III RT Kit (Invitrogen) including Oligo DT primers specifically amplifying mRNA :

Total RNA	5 µg
Oligo dT 50 µM	1 µl
dNTP Mix 10 mM	1 µl
DEPC-H_2O	ad 10 µl

Primer annealing occurred by incubation for 5 minutes at 65°C, and 10 µl cDNA Synthese Mix containing the Super Script III reverse transcriptase (Invitrogen) was added after chilling on ice for 1 minute :

10x buffer	2 µl
25 mM MgCl	4 µl
0,1M DTT	2 µl
RNase Out (40 U/ µl)	1 µl
Super Script III RT (200 U/ µl)	1 µl

Reverse transcription was performed by incubating the reaction mix for 50 min at 50°C, followed by 5 min at 85°C. After chilling on ice for 1 min, RNA template was removed by incubation with 1 µl RNase H (2U/ µl) for 20 min at 37°C.

HLA amplification

HLA-cDNA was amplified with PCR from cDNA using the sense primer 5p2, binding a region common to all HLA sequences, and one of the antisense primers 3pA, 3pB or 3pC, respectively binding either HLA-A, -B or -C alleles (Appendix 2, **Supplementary Table 3**). cDNA was diluted 1:5 in AD and applied to HLA-PCR in the following mix:

10x buffer	5 µl
dNTP Mix 10 mM	1 µl
5p2 Primer 5 µM	0,5 µl
3pA, B or C Primer 5 µM	0,5 µl
Easy A polymerase (5U/ µl)	1 µl
Diluted cDNA	5 µl
AD	ad 50 µl

Parallely, a negative control with 5 µl AD instead of diluted cDNA was run. HLA-PCR was performed with the following program:

1. 95°C,	4 min (denaturation of cDNA)
2. 95°C,	45 sec (denaturation)
3. 55°C,	45 sec (annealing)
4. 70°C,	1 min 30s (elongation)
5. 70°C,	2 min (final elongation)
6. 4°C,	pause

where steps 2 to 4 were repeated 35 times.

The lengths and concentrations of the PCR products obtained were analysed with electrophoresis by applying 8 µl of the PCR products to an 1% agarose gel as described in chapter 2.2.1.5.

HLA sequencing

Before sequencing, HLA-PCR product was purified using the High Pure PCR Purification Kit (Roche) according to the manufacturer's instructions. Purified product was eluted in 50-100 µl elution buffer, according to the concentration appreciated on the electrophoresis gel. Sequencing reaction contained either the sense primer 2S or the antisense primer 4N, both binding at sequences common to all HLA alleles :

5x sequencing buffer	2 µl
Big Dye 3.1	2 µl
2S or 4N primer 10 µM	1 µl
Purified HLA-PCR product	100-200 ng
AD	ad 10 µl

Sequencing reaction was applied to the following PCR program:

1. 95°C,	2 min
2. 95°C,	20 sec
3. 55°C,	4 min
4. 4°C,	pause

where steps 2 to 3 were repeated 30 times.

Electrophoretic analysis and readout of the sequencing product was performed by GENterprise GENOMICS (Mainz), and sequences were edited with Chromas 2.3 (Technelysium Pty Ltd). Analysis of the sequence was performed with programs EditSeq 3.9.10 and SeqMan 3.61 (DNASTAR Inc.) and sequence was run against standard HLA databases (IMGT/HLA database from EMBL-EBI; NCBI-BLAST).

2.2.1.7 HLA cloning

If patient's HLA alleles, determined as described above, were not yet available in expression vectors, they were cloned with the pcDNA3.1/V5-HIS TOPO TA Expression Kit (Invitrogen) as described below.

Transformation

PCR product was cloned into the pcDNA3.1/V5-HIS TOPO vector (Invitrogen) according to the manufacturer's instructions. Briefly, 2 µl HLA-PCR product from the HLA amplification step described in 2.2.1.6 were incubated with 0,5 µl pcDNA3.1/V5-HIS TOPO and 0,5 µl Salt Solution (Invitrogen) for 20 min at room temperature. 2 µl of this mixture were then added to a shot of TOP10 bacteria (Invitrogen) and incubated for 15 min on ice. Transformation of bacteria was performed by incubation for 30 sec in a 42°C water bath. Bacteria were enabled to recover by immediately putting them on ice and supplementing them with 250 µl SOC medium. They were then cultured for 1 hour in a thermomixer at 37°C, 800 rpm. 100-120 µl of the culture were streaked on an agar plate and colonies were enabled to grow overnight at 37°C.

Control of recombinant clones

10-20 single colonies were picked into 150 µl LB medium supplemented with 100 µl/ml ampicillin, and allowed to grow for 4-6 hours in a thermomixer at 37°C. Presence of the plasmid and orientation of the insert were controlled by amplification of the cloned HLA sequence with the primers 2S, specifically binding to the HLA sequences, and BGH reverse, binding the BGH polyadenylation sequence of pcDNA3.1/V5-His TOPO, situated 3' of the multiple cloning site. 50 µl of the bacteria cultures were centrifugated in 0,2 ml PCR tubes at 14 000 rpm for 3 min. Supernatant was discarded and pellet was resuspended in 20 µl AD. 20 µl Master Mix (see below) containing the Red Taq Polymerase (Sigma) was added.

Master Mix for 30 reactions:

Red Taq ready Mix	646 µl

2S primer 5 µM	7 µl
BGH rev. primer 5 µM	7 µl

Reaction mix were applied to the following PCR program:

1. 95°C,	2 min (denaturation)
2. 95°C,	1 min (denaturation)
3. 47°C,	30 sec (annealing)
4. 72°C,	45 sec (elongation)
5. 72°C,	2 min (final elongation)
6. 4°C,	pause

where steps 2 to 4 were repeated 30 times.

15 µl of the PCR products were analyzed by electrophoresis as described in 2.2.1.5 (without previously mixing the probes with DNA probe buffer, as the Red Taq ready Mix already contains a loading dye). Clones for which a band was visualized, i.e. containing an insert in the right orientation, were expanded by transferring the 100 µl precultures left in 5 ml LB medium supplemented with 100 µl/ml ampicillin. Bacteria were allowed to grow over night at 37°C. Plasmids were extracted using the Spin Miniprep Kit (QIAGEN) and submitted to sequencing with 500-600 ng for each reaction, as described in 2.2.1.6. Bacteria carrying the wild-type and complete HLA-encoding sequence were stored as glycerol stocks.

2.2.2 Cell culture and manipulation

2.2.2.1 Cell counting

Cells put in suspension were mixed with an equal volume of trypan blue staining solution. 10-20 µl from the mixture were transferred by capillarity under a glass coverslip previously fixed on the pillars of a Fuchs Rosenthal hemacytometer (Marienfeld). Cells were allowed to settle for 2-3 min and analyzed under light microscopy. Viable cells with an intact plasma membrane exclude the dye and appear yellow and bright under the microscope, in contrast to dead cells, which internalize the dye and become blue stained. Number of viable cells within one big square of the hemacytometer (**Figure 2.1**) was determined, and the cell concentration was calculated as follows:

Number of cells / ml = (number of cells / big square) * dilution * 1000 / 0,2

with dilution = 2 when equal volumes of cell suspension and trypan blue staining solution were mixed.

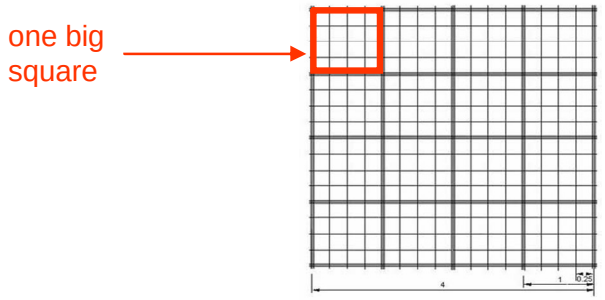

Figure 2.1 : **Fuchs Rosenthal hemacytometer**. Cells were counted within two big squares and the mean value was used to determine the cell concentration. The surface of one big square is 1 mm2, and the depth between the glass coverslip and the chamber floor is 0,2 mm.

2.2.2.2 Passaging of cell lines

To prevent bacterial and fungial contaminations, cells were manipulated under a laminar flow sterile bench, taking standard precautions to maintain a sterile environment (Freshney, 2000). Centrifugation steps were always performed at 1500 rpm for 5 minutes at 20°C, if not otherwise specified. Adherent cells were commonly maintained in T175 cm^2 flasks, and suspension cells in T75 cm^2 flasks (BD Falcon, Heidelberg).

Adherent cells

293T and melanoma cells were maintained in culture in RPMI 1640 supplemented with 10% FCS, penicillin (10 IU/ml) and streptomycin (100 µg/ml) ($RPMI_{complete}$). Cells were subcultured every 3-4 days. 293T cells were detached by incubation for 5 minutes with 5 ml Trypsin-EDTA (Gibco, Karlsruhe) at 37°C after washing with 10 ml PBS. They were resuspended by gently taping and shaking the flask, splitted 1:25 for long-term culture and recultured in 25 ml fresh medium. Three days before transfection, they were replated at 8 x 10^5 cells in a new T175 flask. Melanoma cells were detached with 5 ml PBS/0,05% EDTA after washing with PBS. After 10 minutes incubation at 37°C, cells were resuspended by gently taping and shaking the flask, and reseeded at 1,8 x 10^6 cells in the T175 flask.
$3T3_{CD40L}$ were maintained in $RPMI_{complete}$ supplemented with 200 µg/ml G418 and passaged every 3-4 days. Cells were detached with 5 ml Trypsin-EDTA and plated at 1 x 10^6 cells in a T175 flask. After expansion, cells were irradiated at 1 x 10^4 rad and cryopreserved in small aliquots. Cells were thawed one day before use as B cell stimulators for generation of CD40L-activated B cells.

Suspension cells

K562 transfectants were maintained in $RPMI_{complete}$ supplemented with 1 mg/ml G418. They were subcultured every 3-4 days and adjusted to a cell density of 2 x 10^5 cells/ml. Medium was completely replaced every 3-4 weeks.

CTL clones and MLTC

All CTL clones and MLTC were maintained in 24 well plates (Greiner, Nürtingen) in 2 ml AIM V® medium supplemented with 10% human serum (AIM $V_{complete}$) and 250 IU/ml rhIL-2. Long-term culture was achieved by weekly stimulations with autologous tumor cell lines, as already described (Wölfel, 1995; Lennerz, 2005). Generally, for use as effector cells in ELISPOT assays, aliquots of cells cryopreserved on day 4 after the last stimulation were thawed, and incubated in AIM $V_{complete}$ supplemented with 250 IU/ml rhIL-2 over night before use in the assay.

All cell cultures were kept in a water-saturated atmosphere with 5% CO_2 at 37°C.

2.2.2.3 Cryopreservation and thawing

For cryopreservation, cells were centrifugated, pellet was taken up in 1 ml freezing medium and transferred into cryotubes (Nunc, Wiesbaden). Cells were frozen at -1°C/min in a cryofreezing container (Nalgene, Nuremberg) to -80°C and transferred into liquid nitrogen at -196°C the next day for long term storage. Thawing was performed by holding tubes in a 37°C water-bath until detachment of the cells was visible. Cells were then immediately poured into 10 ml of RPMI 1640, and washed two times before use.

2.2.2.4 PBMC isolation from donors' blood

Donors' blood was obtained as concentrated "buffy coats" from the blood bank of the Mainz university hospital. PBMC were isolated from the "buffy coat" by centrifugation on a Ficoll-Hypaque gradient. Briefly, blood was diluted 1:2 in PBS and distributed on 50 ml LeucoSep tubes (Greiner Bio-One, Frickenhausen) prefilled with 15 ml Ficoll. Cells were centrifugated 30 min at 2100 rpm, Ø brake. PBMC, concentrated as a thick cell layer above the ficoll phase, were gently collected and washed three times with PBS to remove platelets. If pellet still appeared strongly red after the washing steps, erythrocytes were lysed by incubating cells 5 minutes on ice in 5 ml erythrocytes lysis buffer, and PBMC were washed again. Centrifugation after each washing step was performed 10 minutes at 1800 rpm with brake. PBMC were then either directly used for isolation of CD8+ T cells, or frozen in 50-100 x 10^6 cells aliquots.

2.2.2.5 Isolation of CD8+ lymphocytes from PBMC

If possible, PBMC were used immediately after Ficoll density centrifugation for CD8+ T cell isolation to maximize the number of CD8+ T cells obtained. If used after thawing, cells were previously treated with 1 mg/ml DNase I (QIAGEN) directly applied to the pellet for 5 minutes at room temperature, a procedure which has been shown to efficiently eliminate cell clumps. Positive selection of CD8 + T cells was performed with immunomagnetic MACS CD8 MicroBeads on MS separation columns (Miltenyi Biotec, Bergisch Gladbach) following the manufacturer's instructions. In brief, 50-100 Mio PBMC were washed one time in 10 ml MACS buffer and incubated with 150 µl CD8+ beads in 350 µl MACS buffer 15 min at 4°C. Cells were resuspended every 5 minutes. After incubation, 3,5 ml MACS buffer was added, and cells were applied slowly in two steps on the pre-equilibrated separation column. After separation, column was washed three times with 500 µl MACS buffer and removed from the magnetic separator. CD8+ T cells were flushed out with 2ml MACS buffer and washed two times with PBS before use. Resulting cell populations were > 85% CD8-positive according to FACS analysis.

2.2.2.6 Generation of dendritic cells (DC)

Dendritic cells were generated from the CD8-negative fraction resulting from the CD8+ isolation (see chapter 2.2.2.5).

Generation of "classical DC"

Classical DC were generated as already described (Sallusto, 1994; Jonuleit, 1997). Briefly, CD8-negative cells (still containing the monocytic population) were resuspended at 5 x 10^6 cells/ml in X-VIVO-15 medium and were incubated for 60 minutes at 37°C in six-well plates (Costar) at 3 ml/well. Non-adherent cells (peripheral blood lymphocytes, PBL) were gently removed by rinsing the wells three times with PBS prewarmed at 37°C. Adherent cells (monocytes) were cultured in 3 ml of X-VIVO-15 containing 1% human serum (Classical DC medium). On day 1 of culture, 800 U/ml rhGM-CSF and 1000 U/ml rhIL-4 were added. Cells were fed with 1 ml of fresh classical DC medium, 1600 U/ml rhGM-CSF and 1000 U/ml rhIL-4 on day 3 and day 5. Immature DC were harvested on day 7 with cold PBS and replated at 7,5 x 10^5 cells/well in 3 ml fresh classical DC medium. Maturation was achieved by incubation for 48h with 1000 U/ml rhIL-6, 10 ng/ml rhIL-1ß, 1 µg/ml PGE-2 and 10 ng/ml rhTNF-a. Mature, non-adherent classical DC were harvested on day 9 and washed one time with PBS before transfection or freezing in alliquots. Classical DC generated with this protocol downregulated CD14 and upregulated HLA-DR, CD80, CD86 and maturation marker CD83. Yield of DC obtained with this method reached 2-3% of starting PBMC.

Generation of "FastDC"

FastDC were generated as described by Dauer et al. (Dauer, 2003). Briefly, CD8-negative cells were resuspended at 5 x 10^6 cells/ml in RPMI 1640 medium and were incubated for 60 min at 37°C in 6-well plates (Costar) at 3 ml/well. Non-adherent cells (PBL) were gently removed by rinsing the wells 3 times with PBS prewarmed at 37°C. Adherent cells (monocytes) were cultured 24h in 3 ml RPMI containing 2% human serum (FastDC medium) supplemented with 1000 U/ml rhGM-CSF and 500 U/ml rhIL-4. On day 2, rhIL-6, rhTNF-a, PGE-2 and rhIL-1ß were added at concentrations of 1000 U/ml, 10 ng/ml, 1 µg/ml and 10 ng/ml, respectively. On day 3, mature, non-adherent FastDC were harvested and washed one time with PBS before either transfection or freezing in alliquots. Like classical DC, FastDC generated with this protocol downregulated CD14 and upregulated HLA-DR, CD80, CD86 and maturation marker CD83. Yield of DC obtained with this method was higher than with the classical method and reached 5-10% of starting PBMC.

2.2.2.7 Transfection of classical DC or FastDC with IVT-mRNA

Transfection of classical DC or FastDC with IVT-mRNA was performed with the TransMessenger™ Transfection reagent (QIAGEN) using a modified protocol derived from the manufacturer's standard guidelines. For each transfection, 0,8 µg IVT-mRNA and an RNA : TransMessenger™ ratio of 1:8 (w:v) were used. For the formation of the transfection complexes, 100 µl EC-R buffer was mixed with 1,6 µl enhancer-R. 0,8 µg IVT-mRNA was added in a minimum volume of 1 µl, and the mixture was incubated 5 minutes at room temperature to enable compaction of RNA. 6,4 µl TransMessenger™ reagent was added, and transfection complexes were formed by incubating the mixture 20 minutes at room temperature. Transfection complexes were then diluted 1:2 in RPMI 1640.

Parallely, 2 x 10^5 DC were poured into a 15 ml plastic tube (Falcon) and centrifugated. Supernatant was completely discarded and diluted transfection complexes were added directly on the pellet. After resuspension of the pellet, cells were incubated 3 hours at 37°C to enable the transfection process. Following transfection, DC were centrifugated and transfection complexes were removed carefully. Transfected DC were directly used as APC for stimulating antigen-specific CD8+ T cells (see chapter 2.2.2.11) or cryopreserved.

2.2.2.8 Generation of CD40-activated B cells

CD40-activated B cells were generated from PBMC and expanded in the presence of NIH-3T3-CD40L ($3T3_{CD40L}$) via CD40-CD40L activation. Irradiated $3T3_{CD40L}$ were thawed and plated in a 6-well plate at a density of 4 x 10^5 cells / well in 3 ml $RPMI_{complete}$ medium

supplemented with 200 µg/ml G418. On the next day, medium was removed and $3T3_{CD40L}$ were washed carefully with PBS.

PBMC were resuspended at 1 x 10^6 cells / ml in B cell medium supplemented with 100 IU/ml rhIL-4 and 500 ng/ml CsA. 4 ml of this mixture were transferred carefully on $3T3_{CD40L}$ and cells were incubated at 37°C.

After 6 days, cells were resuspended and cell concentration was determined. Cells were fed with fresh B cell medium supplemented with 100 IU/ml rhIL-4 and 500 ng/ml CsA and adjusted to a density of 1 x 10^6 cells / ml. 4 ml of this mixture were replated onto 4 x 10^5 irradiated $3T3_{CD40L}$ that had been plated the day before. Further stimulations by replating B cells onto $3T3_{CD40L}$ at 1 x 10^6 / ml occurred every 3-4 days.

Phenotyping of generated B cells according to CD3, CD13, CD19 expression was regularly performed upon the second week, until achieving a purity of 95% of B cells in the population. A purity of 90% was commonly achieved after 2 weeks.

2.2.2.9 Electroporation of APC with IVT-mRNA

Cells were washed with OPTIMEM and adjusted to the following densities :

- 5-10 x 10^6 cells / ml for FastDC, or
- 10-25 x 10^6 cells / ml for CD40-activated B cells, or
- 25 x 10^6 cells / ml for PBMC.

200 µl of these cell suspensions were transferred into a 0.4 cm-gap electroporation cuvette (BioRad) and placed on ice for 5 minutes. 20 µg IVT-mRNA was added, and cells were immediately pulsed with the Gene Pulser II (BioRad) using the following parameters :

- 350 V, 300 µF for FastDC
- 300 V, 150 µF for CD40-activated B cells
- 350 V, 300 µF for PBMC

Immediately after electroporation, cells were placed on ice for 5 minutes. They were then transferred in prewarmed medium until use as APC for stimulating antigen-specific CD8+ T cells (see chapter 2.2.2.11).

2.2.2.10 Nucleofection™ of FastDC with IVT-mRNA

Cells were washed with PBS and resuspended in DC solution (Amaxa, Cologne) at 10-20 x 10^6 / ml. 100 µl of this cell suspension was mixed with 10 µg IVT-mRNA and directly transferred into a nucleofection cuvette (Amaxa). Nucleofection was performed by running program U-02 of nucleofector. After completion of the program, cells were transferred into a 6 well plate prefilled with 3 ml prewarmed FastDC medium per well, until use as APC for stimulating antigen-specific CD8+ T cells (see chapter 2.2.2.11).

2.2.2.11 Stimulation of lymphocytes with mRNA-transfected APC

Stimulation of CD8+ T cells or unisolated PBL was performed on day 0 in 24 well plates with at least 1,5 x 10^6 effector cells per well, or in 48 well plates with a number of cells between 1 x 10^6 and 1,5 x 10^6 per well. AIM $V_{complete}$ supplemented with 25 IU/ml rhIL-2 (if not otherwise specified) was used as stimulation medium. For the 24 well format, effector cells were resuspended in 1 ml medium. 2 x 10^5 mRNA-transfected FastDC (or alternative APC) were added in 0,5 ml either directly after the transfection procedure with TransMessenger™, or 3h after electroporation or nucleofection™. CD8-negative cells depleted from the adherent population (see chapter 2.2.2.6) were irradiated at 1 x 10^4 rad and used as feeders at 2 x 10^5 / 0,5 ml. For the 48 well format, effector cells were resuspended in 0,5 ml medium and cocultured with 1 x 10^5 mRNA-transfected FastDC in 0,25 ml and 1 x 10^5 irradiated CD8-negative cells in 0,25 ml. Cocultures were incubated at 37°C, and eventually splitted over two wells on day 4 if signs of growth factors depletion were visible (yellow medium). Restimulation occurred on day 7 : effector cells were resuspended in fresh medium at a concentration not exceeding 2,5 x 10^6 cells per well in the 24 well format, and 1,5 x 10^6 cells per well in the 48 well format. mRNA-transfected FastDC and irradiated CD8-negative cells were added in the same concentrations as described above. Commonly, stimulated cells were applied on day 7 + 5 as effector cells in ELISPOT assays.

2.2.3 Analysis

2.2.3.1 IFN-γ ELISPOT assay

Coating

Multiscreen HTS IP plates (Millipore, Bedford, MA, USA) were pretreated with 20 µl 35% ethanol and wasched three times with 150 µl PBS. They were then coated with 10 µg/ml of monoclonal antibodies anti-human IFN-γ (1-D1K; Mabtech AB, Hamburg) in PBS overnight at 4°C. Unbound antibodies were removed by washing three times with 150 µl PBS.

Transcient transfection of 293T cells

293T cells were used as target APC expressing the antigen of interest with the corresponding HLA molecule. To this aim, they were plated in duplicates on the ELISPOT plate at a concentration of 2 x 10^4 cells / well in 120 µl RPMI supplemented with 10% FCS. Plates were incubated for 30 minutes at 37°C. During this step, transfection complexes were build by mixing, for one well:

- 100 ng cDNA encoding the relevant HLA molecule in 5 µl RPMI 1640,

- 300 ng cDNA encoding the relevant antigen in 7 µl RPMI 1640,
- 0,25 µl Lipofectamine 2000™ in 20,75 µl RPMI 1640.

This mixture was incubated for 15 minutes at ambient temperature. After incubation, 30 µl transfection complexes were added onto 293T and the ELISPOT plate was incubated for 24h at 37°C.

Transcient transfection of K562 transfectants

Alternatively, K562 transfectants stably expressing the relevant HLA molecule were used as target APC. Cells were plated in duplicates on the ELISPOT plate at a concentration of 7,5 x 10^4 cells / well in 20 µl RPMI supplemented with 10% FCS. Transfection complexes were formed by mixing, for one well:

- 300 ng cDNA encoding the relevant antigen in 4 µl RPMI 1640 with
- 0,25 µl Lipofectamine 2000™ in 6,75 µl RPMI 1640.

This mixture was incubated for 15 minutes at ambient temperature. After incubation, 10 µl transfection complexes were added onto K562 transfectants and the ELISPOT plate was incubated for 24h at 37°C.

Loading of K562 transfectants with peptide

For presentation of a single epitope, K562 transfectants were loaded with exogenous peptide. On the day of coculture with the effector cells, K562 transfectants were incubated 30 minutes at 37°C with 33 µg/ml peptide in RPMI supplemented with 10% FCS at a concentration of 2,5 x 10^6 cells / ml. After incubation, 30 µl of this mixture were plated per well of the ELISPOT plate.

Addition of effector cells

Stimulated CD8+ T cells or PBL were used as effector cells. They were added to the target APC in 70 µl RPMI 1640 supplemented with 10% FCS at a concentration of 3 x 10^4 cells / well, if not otherwise specified. When 293T were used as APC, 120 µl / well were previously removed from the ELISPOT plate. rhIL-2 was added to effector cells to achieve an endconcentration of 25 IU / ml in the well, if not otherwise specified. Final volume per well was 100 µl. ELISPOT plate was incubated at 37°C for 20h.

Development

Development of cytokine spots produced by effector cells relied on the use of avidin-biotin-peroxidase complexes (ABC method, **Figure 2.2**). Cells were removed by washing the ELISPOT plate six times with PBS/0,05% Tween 20. Captured cytokine was detected by incubation for 2h at 37°C with 60 µl biotinylated monoclonal antibodies anti-human IFN-γ (7-

B6-1; Mabtech AB, Hamburg) at 2 µg/ml in PBS/0,5% BSA. During this time, ABC complexes were prepared by adding one drop of reagent A (Vectastain Elite Kit; Vector Laboratories, Burlingame, USA) and one drop of reagent B (Vectastain Elite Kit; Vector Laboratories, Burlingame, USA) to 10 ml PBS/0,1% Tween 20. Solution was immediately mixed and incubated for 30 min at room temperature in the obscurity. After washing the wells of the ELISPOT plate six times with PBS/0,05% Tween 20, 100 µl ABC complexes were added for 1h at room temperature in the obscurity. Unbound complexes were removed by washing the plate three times with PBS/0,05% Tween 20 and three times with PBS alone. 100 µl AEC solution containing the Peroxidase substrate 3-amino-9-ethyl-carbazole (AEC, Sigma) were added and reaction was allowed to occur for 5-10 minutes before stopping by rinsing the plate ten times under running tap water. Plate was allowed to dry by incubation for 1h in a drying hood at 37°C.

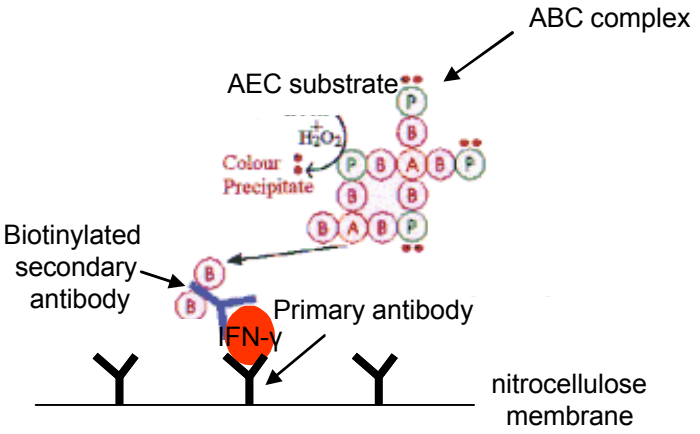

Figure 2.2 : Principle of action of the ABC complex in the development of the ELISPOT assay. Primary antibodies, previously coated on the ELISPOT membrane, catch the IFN-γ molecules secreted by T cells. Secondary antibodies, coupled to biotin (B), recognize and bind to another epitope of the IFN-γ molecules. ABC complexes are formed by one molecule of avidin (A) bound to four molecules of the enzyme peroxydase (P) via biotin moieties. Complexes bind to the biotinylated secondary antibodies, and addition of the peroxydase substrate AEC reveals red cytokine spots in the presence of H_2O_2. Adapted from www.hmds.org.uk.

Analysis

For spot evaluation, the nitrocellulose membrane was stripped from the ELISPOT plate on an adhesive foil (Tape MATA09600, Millipore). The foil was then attached to the motor stage of an incident light microscope (Leitz DME, Wetzlar). Spot numbers were determined with the use of a computer-assisted video image analyser equipped with software KS ELISPOT 4.4.35, as already described (Herr, 1997).

2.2.3.2 Ex vivo assay

"Ex vivo" refers to the state of cells that are unmanipulated upon their isolation. Blood PBMC isolated by Ficoll density centrifugation were mostly used as effector cells for the ex vivo assay. PBMC were applied to a previously coated ELISPOT plate at 5 x 10^5 cells/well in 50 µl AIM $V_{complete}$. Antigenic peptide was added in 50 µl AIM $V_{complete}$ to an end concentration of 10 µg/ml. Alternatively, 1 x 10^5 isolated CD8+ T cells were used as effectors in 50 µl AIM $V_{complete.}$ In this case, 7,5 x 10^4 K562 transfectants previously loaded with the peptide (20 µg/ml) were used as APC and added in 50 µl AIM $V_{complete.}$ ELISPOT plate was incubated for 20h at 37°C and developed as described in chapter 2.2.3.1.

2.2.3.3 Flow cytometric analysis

Cells were distributed into polypropylene tubes (BD Biosciences) at 2 x 10^5 cells / tube and washed with PBS. For direct analysis of intracellular EGFP expression, cell pellet was resuspended into 0,5 ml FACS buffer (PBS/0,01% BSA) for parallel evaluation of viability with Propidium Iodide, or into 0,5 ml FACS fixing buffer (PBS/0,01% BSA/1% Formaldehyd) otherwise. EGFP fluorescence was measured in the FITC canal of the CANTO™ flow cytometer. Untransfected cells served as negative control. For surface staining, cells were incubated for 15 min at 4°C with monoclonal antibodies against CD surface markers or the corresponding isotype-matched control antibodies as negative controls. For indirect staining, cells were washed in PBS and incubated with secondary FITC-conjugated goat anti-mouse IgG $F(ab)_2$ antibodies for 15 min at 4°C. After staining, cells were washed in PBS and resuspended in 0,5 ml FACS buffer or FACS fixing buffer. Fluorescence measurement was performed in the FITC or PE canal. For each sample, a minimum of 1 x 10^4 cells were analysed.

For assessment of viability, Propidium Iodide (PI) was added at an end concentration of 1 µg/ml immediately before the measurement. PI fluorescence was measured in the PI canal. Cells which excluded PI were considered as viable and taken into consideration for further analysis.

Data analysis was performed with the EXPO™ V1.0 software (Beckman Coulter). Cell debris were eliminated from the analysis using a gate on forward and side light scatter.

2.2.3.4 Fluorescence microscopy

24h after Transfection with EGFP-IVT-mRNA, 2-5 x 10^5 FastDC were washed in PBS and cell pelett was resuspended in 5 µl Fluorescent Mounting Medium (Dakocytomation) supplemented with Hoechst 33432 (Hoechst), specifically staining the cell nucleus. Cells were placed on a microscope glass slide and images were taken with an Olympus-IX71

inverted microscope (Olympus, Hamburg) with a 20x/0.4 NA objective lens. TILLvisION software (TILL Photonics, Gräfeling) was used for image acquisition.

3 RESULTS

3.1 Antigen format and transfection of Antigen Presenting Cells (APC)

The first part of this work consisted in establishing the nature of the most appropriate stimulators to amplify antigen-specific lymphocytes in a short-term stimulation assay, still fulfilling the requirements described in the Introduction (see chapter 1.7). In this chapter, the antigen format, as well as the chosen APC, and their mode of transfection are adressed.

3.1.1 In vitro-transcribed mRNA (IVT-mRNA) as antigen format

For most non-dividing cells, including dendritic cells, DNA transfection using non-viral transfer methods has been poorly efficient (Van Tendeloo, 1998). In contrast, high and reproducible efficiencies have been obtained with mRNA (Van Tendeloo, 2001; Britten, 2004; Schaft, 2005). This essential characteristic, together with other RNA-inherent advantages described in the Introduction (Chapter 1.7.3) motivated our choice of mRNA as antigen format for the transfection of APC.

mRNAs were generated from a panel of plasmids carrying the cDNAs of 20 tumor-associated antigens. The plasmids, also carrying the T7 promotor from the T7 bacteriophage, were linearized with the appropriate restriction enzyme (Material and Methods, Table 2.1) and used as templates for In Vitro Transcription (IVT) with the T7 RNA polymerase (Material and Methods, Chapter 2.2.1.2).

3.1.1.1 Polyadenylation of IVT-mRNA enhances CTL recognition

Most eukaryotic mRNAs carry a 60-300 poly(A) tail, which is known to play an important role for mRNA stability (Brawerman, 1981; Drummond, 1985; Galili, 1988). We assessed the impact of a poly(A) tail added to IVT-mRNA encoding Melan-A/MART-1 on the recognition of RNA transfectants by CTL clone D05-CTL3.2/141 directed against Melan-A/MART-1_{26-35} in association with HLA-A*0201 (Material and Methods, Chapter 2.1.5.1). Melan-A-IVT-mRNA was enzymatically polyadenylated to generate a poly(A) tail of ca. 300 bases (**Figure 3.1A**). K562/A*0201 cells, stably transfected with HLA-A*0201, were then transfected with polyadenylated or non-polyadenylated Melan-A-IVT-mRNA and tested for recognition by D05-CTL3.2/141 in an IFN-γ ELISPOT assay. As negative controls, the CTL clone was tested alone or with mock-transfected K562/A*0201 cells. The spot number induced by the CTL clone with mock-transfected K562/A*0201 cells was not significantly higher than the background spot production of the CTL clone alone, and too times inferior to the number of spots induced by K562/A*0201 cells transfected with non-polyadenylated Melan-A-IVT-mRNA (**Figure 3.1B**). Strikingly, D05-CTL3.2/141 produced at least three times

more spots when K562/A*0201 cells had been transfected with polyadenylated as compared with non-polyadenylated Melan-A-IVT-mRNA (**Figure 3.1B**). This confirmed that polyadenylation of IVT-mRNA prior to transfection strongly enhances the recognition of transfectants by antigen-specific T cells, which is presumably due to improved RNA stability and translation efficiency (Sachs, 1990; Jackson, 1990). Therefore, IVT-mRNAs were polyadenylated after IVT for all further experiments shown herein (Material and Methods, Chapter 2.2.1.3). Polyadenylated IVT-mRNAs generated from the plasmid panel are shown in **Figure 3.2**. After polyadenylation, shifts of 100 to 300 bases in length, corresponding to the poly(A) length, were regularly observed (see also Table 2.1 in Material and Methods).

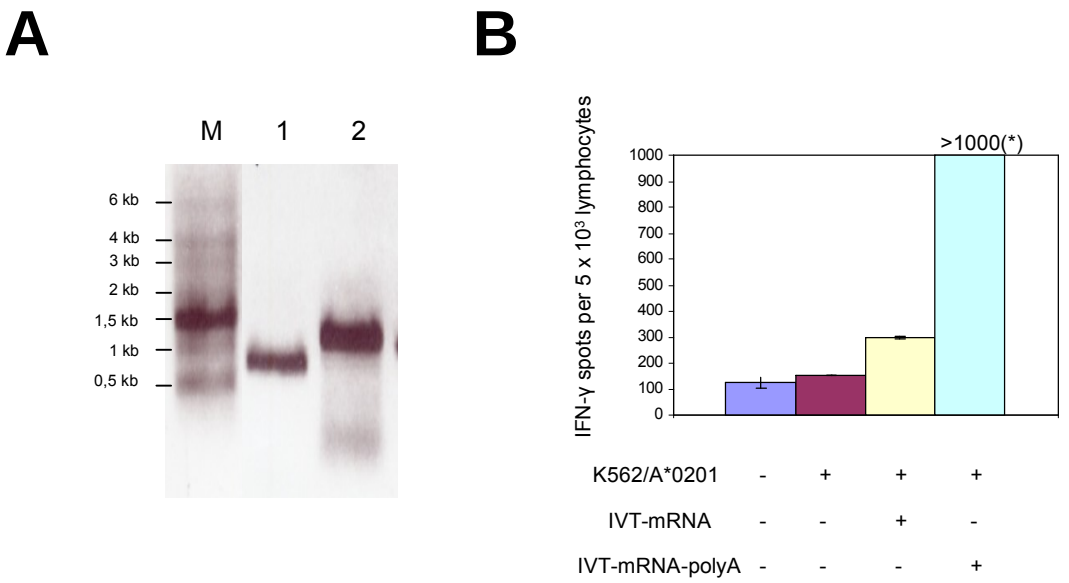

Figure 3.1. **Polyadenylation of IVT-mRNA**. **A.** Polyadenylating Melan-A-IVT-mRNA induces a clear shift in length as seen on a denaturing RNA gel. M: RNA ladder (3 µg), lane 1: non-polyadenylated Melan-A-IVT-mRNA (830 ng), lane 2: enzymatically polyadenylated Melan-A-IVT-mRNA (830 ng). The increase in intensity of the band after polyadenylation is due to the increase in the lengh of the RNA. **B**. Polyadenylation of IVT-mRNA enhances CTL recognition. K562/A*0201 transfectants stably expressing the restriction element HLA-A*0201 were mock transfected, transfected with non-polyadenylated Melan-A-IVT-mRNA (IVT-mRNA) or polyadenylated Melan-A-IVT-mRNA (IVT-mRNA-polyA) using the TransMessenger™ transfection reagent. 3h after transfection, transfected K562/A*0201 (4×10^4 / well) were tested for recognition by CTL clone D05-3.2/141 (5×10^3 / well) in a 20h IFN-γ ELISPOT assay in the presence of IL-2 (250 IU/ml). (*): not enumerable.

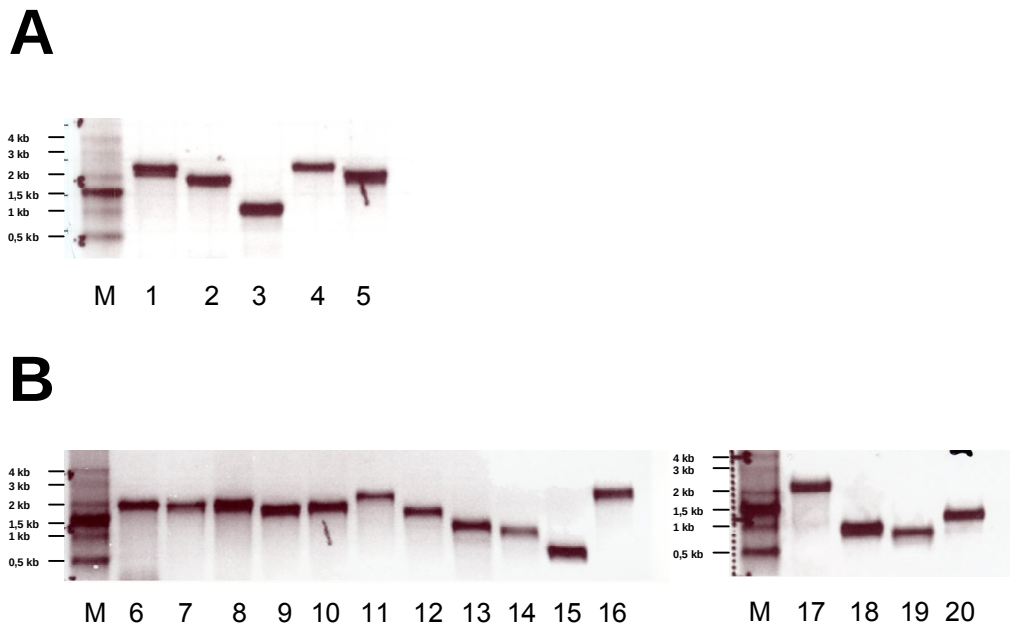

Figure 3.2. **Polyadenylated IVT-mRNAs generated from the plasmid panel**. Quality and length of polyadenylated IVT-mRNAs were controlled on a denaturing RNA gel. **A**. Differentiation antigens. 1: Tyrosinase, 2: TRP-2, 3: Melan-A/MART-1, 4: gp100, 5: MC1R. M: RNA ladder. **B**. Shared tumor-specific, mutated and overexpressed antigens. 6: MAGE-A1, 7: MAGE-A3, 8: MAGE-A4, 9: MAGE-A6, 10: MAGE-A12, 11: MAGE-C2, 12: BAGE-1, 13: GAGE-1, 14: GAGE-4, 15: NY-ESO-1/LAGE-2, 16: N-WASP^{mut}, 17: PRAME, 18: RAB38/NY-MEL-1, 19: Survivin-1, 20: Survivin-2. M: RNA ladder.

3.1.1.2 IVT-mRNA-encoded antigens are recognized by CTL clones or MLTC responders

The functional quality of the generated IVT-mRNAs and their ability to induce recognition by antigen-specific lymphocytes after transfection in APC was tested with MLTC and CTL clones generated from tumor-reactive lymphocytes of patient D05 (Material and Methods, Chapter 2.1.5.1). CTL clones D05-3.2/141, D05-3.2/173 and D05-3.2/100 had been shown to recognize Melan-A/MART1$_{26-35}$ in association with HLA-A*0201, MAGE-A3$_{143-151}$ in association with HLA-Cw*0202, and MAGE-A4$_{145-154}$ in association with HLA-B*2705, respectively. CTL clones D05-4/50, D05-4/21 and D05-4/95 recognized NY-ESO-1/LAGE-2$_{157-165}$ in association with HLA-A*0201 , Tyrosinase$_{369-377}$ in association with HLA-Cw*0501 and CCT6A$^{mut}{}_{156-164}$ in association with HLA-B*2705, respectively. MLTC5 responders recognized GAGE-1$_{14-22}$ in association with HLA-B*2705, MAGE-A1 in association with HLA-B*4402 and HLA-Cw*0501 and BAGE-1$_{21-29}$ in association with HLA-B*4402.

FastDC were generated from D05 monocytes (Material and Methods, Chapter 2.2.2.6) and transfected with individual IVT-mRNAs encoding the tumor antigens mentioned above. Transfected FastDC were then tested for recognition by MLTC5 or the respective CTL clone in an IFN-γ ELISPOT assay. Considering the uncertainty with respect to mRNA and protein stability for individual antigens (Schaft, 2005), we chose to apply transfected

FastDC immediately after the transfection procedure, i.e. ca. 3h after initiation of the transfection, to the ELISPOT assay. As shown in **Figure 3.3**, background spots levels against mock-transfected FastDC were very different among the different effectors. Nevertheless, IVT-RNA-transfected FastDC always induced at least a two-fold increase in the spot production by MLTC5 or the CTL clones when compared with mock-transfected FastDC, except for FastDC transfected with Melan-A- or Tyrosinase-IVT-mRNA. Notably, Melan-A-IVT-mRNA had induced recognition by the same CTL clone when transfected into K562/A*0201 cells (**Figure 3.1**). Anti-Tyrosinase CTL clone D05-4/21 produced a high background level not only against mock-transfected FastDC (**Figure 3.3**) but also spontaneously (not shown). When tested with MLTC5 responders in an independent experiment, Tyrosinase-IVT-mRNA induced recognition after transfection into FastDC (not shown). Taken together, all IVT-mRNAs tested in Figure 3.3 proved to be functional, indicating that the targeted epitopes were processed and efficiently presented at the cell surface after translation of the transfected IVT-mRNA.

In addition, we tested the recognition of FastDC 24h after transfection, thus applying them as APC in the assay one day after the transfection procedure. There was no significant difference in the recognition of transfected FastDC 24h after transfection in comparison to 3h after transfection (not shown). To maximize the timeframe, in which responder lymphocytes may have contact with the antigen, and to ensure that peak of antigenic expression occurs within this timeframe, transfected DC were applied as APC immediately after the transfection procedure in further experiments.

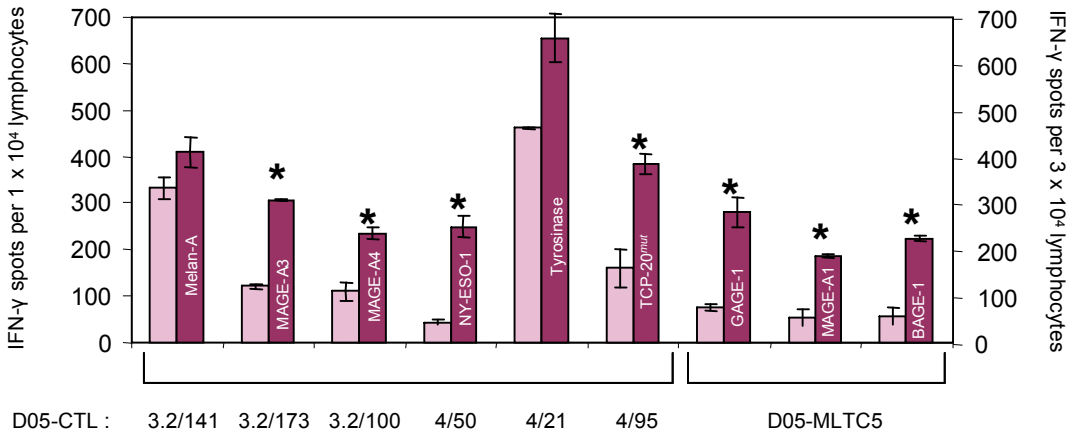

Figure 3.3. **Recognition of IVT-mRNA encoded antigens by D05-CTL clones or MLTC5 responders**. Most IVT-mRNAs are efficiently recognized upon transfection in FastDC. D05-FastDC were mock-transfected or transfected with IVT-mRNA encoding the indicated antigen using the TransMessenger™ transfection reagent. Transfected FastDC (2 x 10^4 / well) were tested 3h after transfection for recognition by MLC5 responders (3 x 10^4 / well) or the relevant CTL clone (each 1 x 10^4 / well) in a 20h IFN-γ ELISPOT assay in the presence of IL-2 (250 IU/ml).* : > 2 x above background (p-value < 0.05).

3.1.2 FastDC as Antigen-Presenting Cells

Dendritic cells (DC) have been shown to be the most potent APC for the priming of adaptive T cell responses in vivo (Banchereau, 1998; Jung, 2002). Among DC originating from pluripotent hemopoietic stem cells, naturally circulating blood DC (myeloid CD11c+ and plasmacytoid CD11c-) are rare, accounting for less than 1% of PBMC. Furthermore, they are difficult to isolate and to maintain in culture (Fong, 2001). Although administration of hemopoietic growth factors like FLT3 ligand or GM-CSF has been shown to increase the frequency of circulating DC in vivo (Pulendran, 2000; Fong, 2001), methods to generate DC in vitro from isolated precursors like CD34+ progenitors or monocytes are now commonly used in experimental or clinical trials (Caux, 1992; Romani, 1994; Fong, 2000; Schuler, 2003). Classical protocols to generate monocyte-derived DC (MoDC) are based on a 5-7 day differentiation step from monocytes to immature DC using GM-CSF and IL-4 (Romani, 1994; Sallusto, 1994), followed by a 2-3 day maturation step with the proinflammatory mediators IL-1ß, IL-6, TNF-a and PGE-2 (Jonuleit, 1997). A more recent protocol based on the same cytokine combinations (Dauer, 2003), described an accelated generation of MoDC which, besides a reduced generation time and cytokine consumption, may resemble more closely the DC development in vivo (Randolph, 1998). In the following, “classical” DC are compared with these “FastDC” with respect to their phenotypic properties and to their ability to stimulate antigen-specific CD8+ T cells.

3.1.2.1 FastDC display the same maturation markers as classical DC

Immature FastDC are generated within 24 hours, and subsequently matured during further 24 hours (Material and Methods, Chapter 2.2.2.6). As a culture period of 5-7 days with GM-CSF and IL-4 is required to induce morphological changes such as increased cell size, development of granules or formation of cytoplasmatic protusions, FastDC are smaller than classical DC as seen under light microscopy and according to forward/side scatter intensity (**Figure 3.4A**). Furthermore, they do not show in light microscopy the typical cytoplasmatic protuberances displayed by classical DC (own observations, not shown). To test, if common maturation and migration markers are upregulated, we generated in parallel classical DC and FastDC from monocytes of a healthy donor, and analyzed expression of some surface molecules by immunofluorescence. Like classical DC, FastDC downregulated monocytic marker CD14, upregulated HLA class II molecules, and typical DC maturation markers like CD80, CD83, CD86 and chemokine receptor CCR7 (**Figure 3.4B**). This was in accordance with the original description of FastDC (Dauer, 2003).

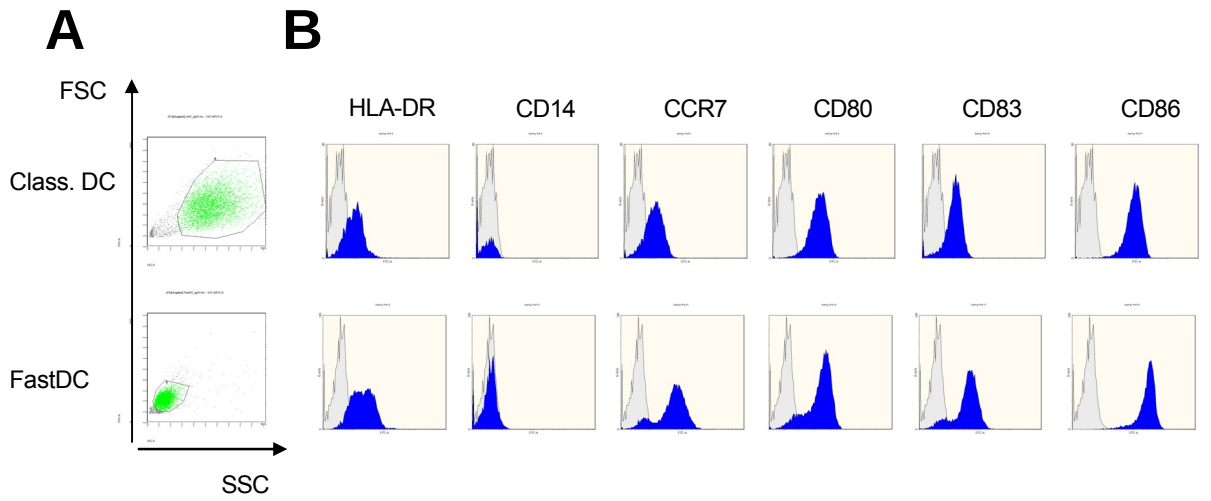

Figure 3.4. Phenotypic analysis of FastDC in comparison with classical DC. FastDC display the same maturation markers as classical DC. Classical DC (top) and FastDC (bottom) were generated from monocytes of healthy donor 109 (Material and Methods, Chapter 2.2.2.6). Classical DC were harvested on day 9, FastDC on day 3. Both populations were cryopreserved after harvesting. Following thawing and washing, DC were stained with FITC- or PE-labeled antibodies directed against surface molecules. **A**. DC morphology according to forward/side scatter measurement in flow cytometry (IgG1-stained populations are shown as representative examples). **B**. Expression of HLA-DR, CD14, CCR7, CD80, CD83 and CD86 in the gated DC populations, as measured with flow cytometry.

3.1.2.2 mRNA-transfected FastDC and classical DC exhibit equivalent stimulatory potentials

FastDC were further compared to classical DC regarding their potency as APC in an in vitro stimulation assay. The phosphoprotein 65 (pp65) of the human cytomegalovirus virus (HCMV) was used as antigen. FastDC and classical DC were generated from monocytes of a CMV seropositive donor and transfected with pp65-IVT-mRNA. Transfected DC were then used as APC to stimulate CD8+ T cells isolated from the peripheral blood of the donor. After one round of stimulation, the expansion of the pp65-specific CD8+ T cells was evaluated in an IFN-γ ELISPOT assay using 293T cells co-transfected with DNA plasmids encoding HLA-B*0702 and pp65. The analysis was restricted to the response restricted by HLA-B*0702, which is known to be a dominant restriction allele for the anti-pp65 response (Lacey, 2003). As negative controls, CD8+ T cells were stimulated against mock-transfected DC.

As shown in **Figure 3.5**, CD8+ T cells stimulated with FastDC transfected with pp65-IVT-mRNA (pp65-transfected FastDC) produced a comparable amount of spots as CD8+ T cells stimulated with classical DC transfected with pp65-IVT-mRNA (pp65-transfected classical DC) (214 versus 181 spots per 1,1 x 10^3 lymphocytes). Background against 293T readout cells was lower after stimulation with pp65-transfected FastDC than after stimulation with pp65-transfected classical DC (14 versus 25 spots). None of the mock-transfected DC could efficiently stimulate pp65-specific CD8+ T cells (**Figure 3.5**). Thus, FastDC could

stimulate pp65-specific CD8+ T cells as efficiently as classical DC did, inducing less background reactivity against 293T readout cells than classical DC.

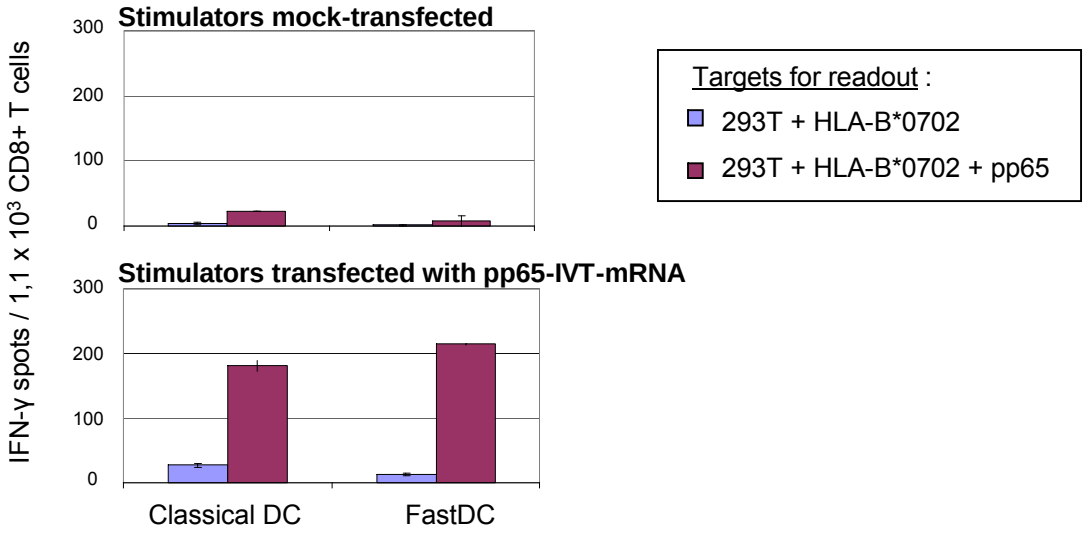

Figure 3.5. **FastDC versus classical DC as APC in an in vitro stimulation assay**. mRNA-transfected classical DC and FastDC exhibit equivalent stimulatory potentials. Classical DC and FastDC were generated from monocytes of CMV seropositive donor 216 (see Material and Methods, Chapter 2.2.2.6). DC were then mock-transfected (upper panel) or transfected with pp65-IVT-mRNA (lower panel) with TransMessenger™, and used as APC to stimulate CD8+ T cells isolated from the peripheral blood of the donor. Amplification of pp65-specific CD8+ T cells was evaluated 5 days after a single stimulation in a 20h IFN-γ ELISPOT assay. Targets were 293T cells (2 x 10^4 / well) co-transfected with plasmids encoding HLA-B*0702 and pp65 using Lipofectamine 2000™.

3.1.2.3 mRNA-transfected FastDC are stronger stimulators than mRNA-transfected CD40-activated B cells or PBMC

The use of Mo-DC is limited by their low precursor frequency in peripheral blood and their lack of expansion in cell culture. Several alternative APC are in principle applicable. CD40-activated B cells can be generated and expanded from small blood volumes and proved to be potent stimulators in vitro after transfection with RNA (Schultze, 1997; von Bergwelt-Baildon, 2002). PBMC directly isolated from peripheral blood have been demonstrated to be potent stimulators of antigen-specific T lymphocytes in vitro (Teufel, 2005; Kreiter, 2007). Following RNA electroporation, mainly the CD14+ subpopulation of PBMC was found to be transfected and thereby served as the true stimulators (C. Graf, personal communication; Kreiter, 2007).

FastDC were compared with CD40-activated B cells and PBMC as APC for stimulation with IVT-mRNA. FastDC and CD40-activated B cells were generated from the peripheral blood of CMV-seropositive donor 065. FastDC, CD40-activated B cells and the donor's PBMC were transfected with pp65-IVT-mRNA by electroporation. In addition, FastDC were transfected with TransMessenger™. These pp65-transfected APC were then used to

stimulate pp65-specific T cells from the donor's CD8+ T cells isolated from peripheral blood. As negative controls, CD8+ T cells were stimulated with mock-transfected APC. Responder lymphocytes were tested for recognition of 293T cells co-transfected with HLA-A*0201 and pp65.

The most efficient amplification of pp65-specific CD8+ T cells was obtained with FastDC electroporated with pp65-IVT-mRNA, directly followed by FastDC transfected with pp65-IVT-mRNA via TransMessenger™ (**Figure 3.6**, lower panel). In order to provide equivalent conditions for all stimulated populations, stimulations with CD40-activated B cells and FastDC were performed at a 1:10 APC-to-CD8+ T cell ratio, whereas stimulations with PBMC, containing approximatively 10% of CD14+ cells, were performed at a 1:1 APC-to-CD8+ T cell ratio. CD40-activated B cells were also applied at a 1:5 ratio. At this ratio, CD40-activated B cells were as efficient as FastDC transfected with TransMessenger™, but inferior to FastDC electroporated with pp65-IVT-mRNA. None of the mock-transfected APC were able to stimulate pp65-specific CD8+ T cells (**Figure 3.6**, upper panel). Of note, no pp65-specific reactivity could be detected among the donor's ex vivo CD8+ T cells in association with HLA-A*0201 in the same experiment (not shown).

Taken together, among mRNA-electroporated APC, FastDC proved to be the most potent stimulators of pp65-specific CD8+ T cells. FastDC transfected with TransMessenger™ were somewhat inferior to electroporated FastDC, but were superior to all non-DC APC with the exception of CD40-activated B cells applied at a 1:5 APC-to-responder T cell ratio. These differences can not only be attributed to the transfection efficiency, as in the same experiment, CD40L-activated B cells were electroporated with EGFP-IVT-mRNA with an efficiency of 32% according to EGFP transgene expression, although FastDC transfected with TransMessenger™ were transfected with an efficiency of 16% (**Figure 3.6**).

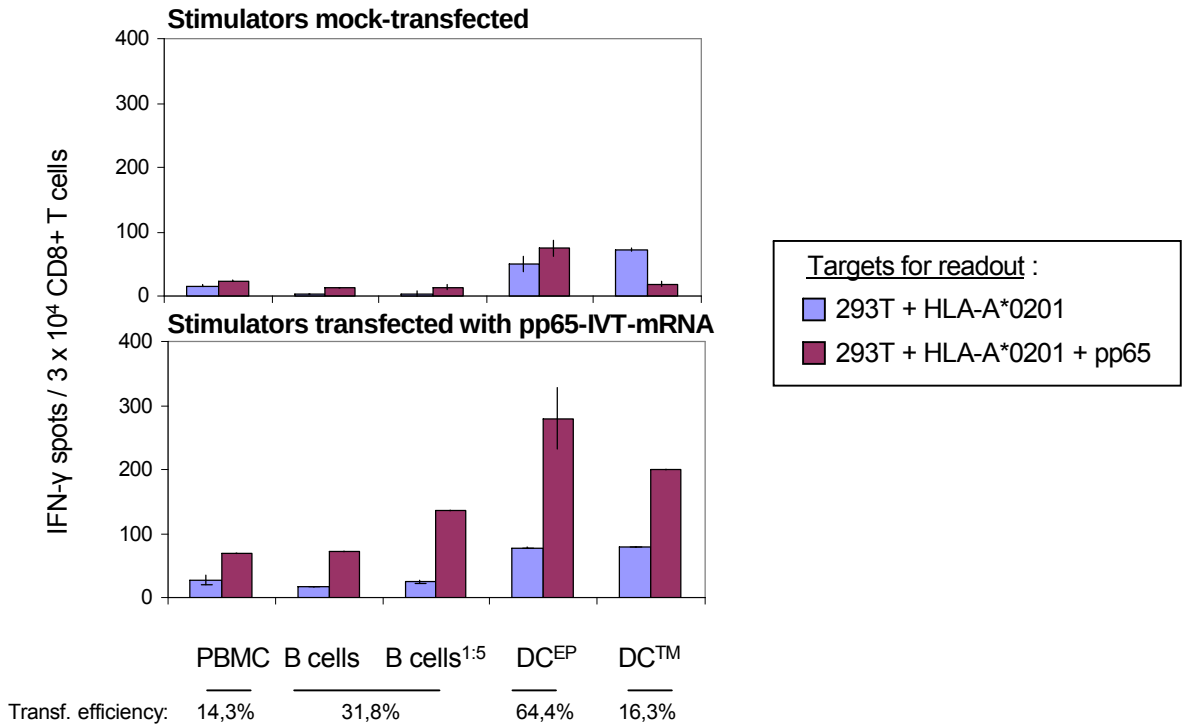

Figure 3.6. **Comparison of different APC for stimulation with IVT-mRNA**. mRNA-transfected FastDC are stronger stimulators than mRNA-transfected CD40-activated B cells or PBMC. FastDC and CD40-activated B cells were generated from the peripheral blood of CMV-seropositive donor 065. FastDC, CD40-activated B cells and the donor's PBMC were electroporated (EP) with pp65-IVT-mRNA (see chapter 2.2.2.9, Material and Methods). In addition, FastDC were also transfected with pp65-IVT-mRNA using TransMessenger™ (TM). pp65-transfected APC were used to stimulate the CD8+ T cells isolated from the donor's peripheral blood (lower panel). As negative controls, CD8+ T cells were stimulated with the respective mock-transfected APC (upper panel). Stimulations were set up at a 1:10 APC-to-CD8+ T cell ratio. CD40-activated B cells were also applied at a 1:5 APC-to-CD8+ T cell ratio. Five days after a single stimulation, responder CD8+ T cells were tested for recognition of 293T cells co-transfected with plasmids encoding HLA-A*0201 and pp65 (2 x 10^4 cells / well) in a 20h IFN-γ ELISPOT assay. Transfection efficiencies (Transf. efficiency) are indicated as the proportion of cells expressing EGFP after transfection with EGFP-IVT-mRNA.

3.1.3 Transfection of FastDC with TransMessenger™

Until now, electroporation has been the method of choice to transfect DC with RNA (Van Tendeloo, 2001; Saeboe-Larssen, 2002; Strobel, 2000). Improvements of electroporation protocols have led to transfection efficiencies reaching 90% in a reproducible way, even in mature DC (Schaft, 2005). Nucleofection™ is another transfection method that proved very efficient for transfecting primary cells (Martinet, 2003) and that combines modified electroporation protocols with cell-type specific transfection solutions. Both methods rely on the physical desintegration of the plasma membrane and are therefore applied to high cell concentrations to maximize the yield of viable cells after transfection (Canatella, 2001). This limits their use for transfecting DC separately with a high number of antigens. Lipofection procedures, in contrast, permit to transfect small cell numbers with no or very limited cell lost. Here, results that were obtained with TransMessenger™, a lipid-based

formulation specially developed for RNA transfection, are shown in comparison with electroporation and Nucleofection™.

3.1.3.1 *Optimal transfection efficiency is obtained with 800 ng RNA and a RNA:TransMessenger™ ratio of 1:8*

Using EGFP expression as a readout, transfection conditions of FastDC with TransMessenger™ were optimized. According to the manufacturer's instructions, RNA amounts varying from 200 ng to 1600 ng and RNA:TransMessenger™ ratios varying from 1:2 to 1:8 were applied for the transfection of FastDC with EGFP-IVT-mRNA. Mock-transfected FastDC were used as a negative control. The best transfection efficiency (22%) was obtained with 800 ng EGFP-IVT-mRNA at a RNA:TransMessenger™ ratio of 1:8, corresponding to 6,4 µl of the reagent for each transfection (**Figure 3.7**). Of note, EGFP expression on a per cell basis did not increase significantly with increasing RNA amounts above 400 ng, as measured by the Mean Fluorescence Intensity (MFI). With the above mentioned parameters, transfection efficiencies ranging from 20 to 40% have been obtained.

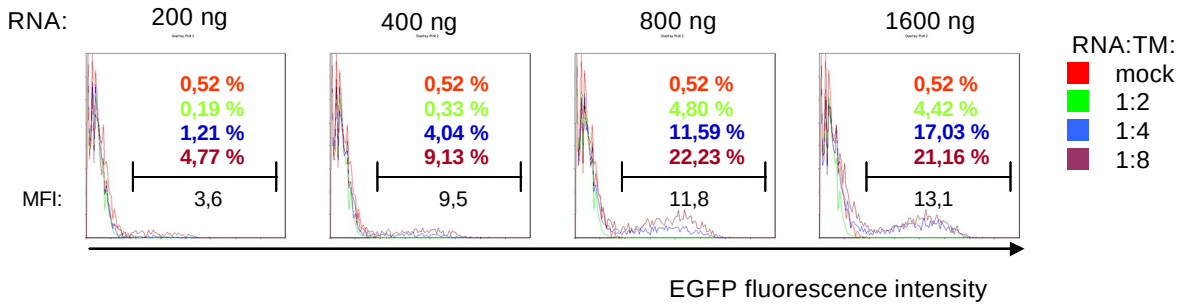

Figure 3.7. **Optimization of FastDC transfection using TransMessenger™ reagent (TM).** Optimal Transfection efficiency is obtained with 800 ng RNA and a RNA:TM ratio of 1:8. FastDC were generated from monocytes of healthy donor 748 and transfected with varying amounts of EGFP-IVT-mRNA, at varying RNA:TM ratios according to the manufacturer's instructions (see chapter 2.2.2.7, Material and Methods). Mock-transfected FastDC served as a negative control. EGFP fluorescence intensity in transfected cells was measured by flow cytometry 24h after transfection. Mean fluorescence intensity (MFI) is indicated for the 1:8 RNA:TM ratio.

3.1.3.2 *Transfection with TransMessenger™ leads to a higher transgene expression on a per cell basis than electroporation*

In parallel to the transfection with TransMessenger™ using the aforementioned parameters, FastDC were also electroporated and transfected with Nucleofection™ with EGFP-IVT-mRNA. EGFP fluorescence was then measured at several timepoints between 3h and 72h after transfection. Mock-transfected cells served as negative controls. As shown in **Figure 3.8**, the transgene was already expressed 3h after transfection with all methods. The percentage of transfectants expressing the transgene and the mean fluorescence intensity (MFI) of transfected cells reached maxima 24h after transfection and then decreased over

time. After 24h, efficiency was at ca. 40% with TransMessenger™, 90% with electroporation and 80% with Nucleofection™. Distribution of cells according to fluorescence intensity showed a transfected population distinct from the untransfected population with TransMessenger™. Furthermore, transfected cells expressed the transgene at very different levels as revealed by the wide distribution of intensities within the transfected population. In contrast, 24h after electroporation or Nucleofection™, most of the cells appeared to be transfected, but expression levels of the transgene were quite homogenous, and lower than with TransMessenger™, as measured by the MFI (**Figure 3.9A** : 31% or 9% with electroporation or Nucleofection™, respectively, versus 100% with TransMessenger™). Correspondingly, with fluorescence microscopy, the transfectants showed medium and low but quite homogenous fluorescence with electroporation and Nucleofection™, respectively (**Figure 3.9B**). In contrast, cells transfected with TransMessenger™ displayed a heterogeneous, for some of them very high fluorescence (**Figure 3.9B**). Thus, using optimized conditions, transfection efficiency of FastDC transfected with TransMessenger™ is lower than with electroporation or Nucleofection™, but the transgene expression in the TransMessenger™-transfected cells is between three and four times higher than with electroporation.

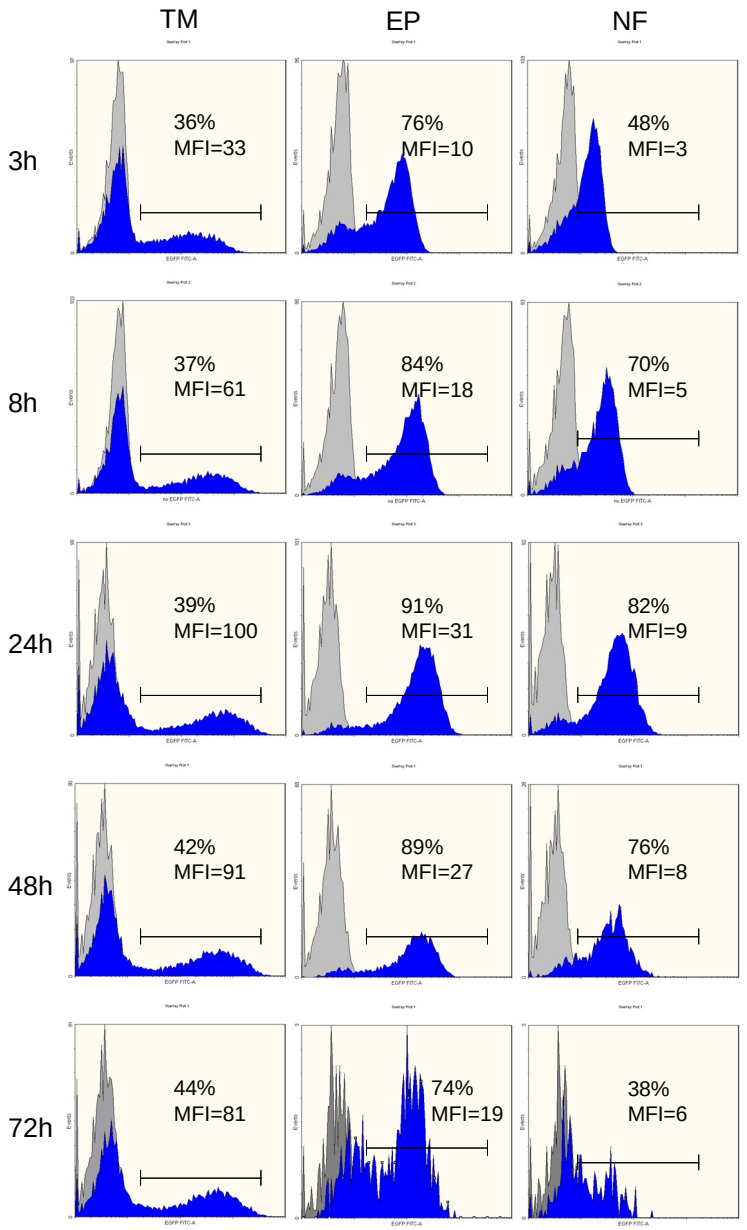

Figure 3.8. **EGFP transfection kinetics after transfection of FastDC with TransMessenger™ (TM), electroporation (EP) or Nucleofection™ (NF).** Electroporation leads to the maximum transfection efficiency after 24h, but maximum mean fluorescence intensity (MFI) is achieved with TransMessenger™. FastDC were generated from monocytes of healthy donor 254 and transfected with EGFP-IVT-mRNA using TM, EP or NF (see Material and Methods, chapter 2.2.2). Mock-transfected cells served as negative controls. EGFP fluorescence was measured in transfected cells 3h, 24h, 48h or 72h after transfection. The percentage of cells expressing EGFP and the MFI of the transfected population are indicated for each timepoint. MFI values were normalized to the maximum value obtained 24h after transfection with TM.

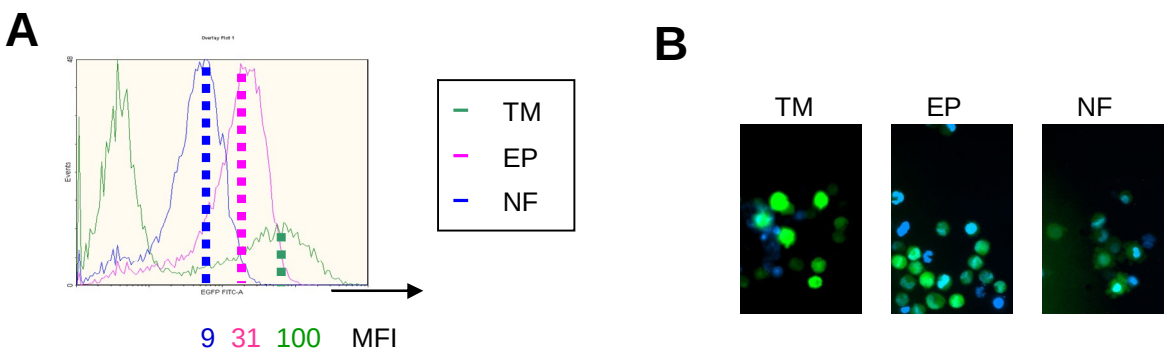

Figure 3.9. **EGFP fluorescence intensity in FastDC 24h after transfection with TransMessenger™ (TM), electroporation (EP) or Nucleofection™ (NF).** Transfection with TransMessenger™ leads to a higher transgene expression on a per cell basis than electroporation or Nucleofection™. **A**. Distributions of EGFP fluorescence intensities 24h after transfection of FastDC with EGFP-IVT-mRNA (same populations as analyzed in Figure 3.8) are shown in an overlay diagram. Mean fluorescence intensities (MFI) of the transfected populations are indicated. MFI values were normalized to the maximum value obtained 24h after transfection with TM. **B.** Representative cells from the same transfected FastDC populations were photographed under fluorescence microscopy. EGFP appears green whereas cell nuclei were stained blue with DAPI.

3.1.3.3 TransMessenger™ transfection kinetics show a high transgene expression in FastDC together with a high viability of transfected cells up to 72h after transfection

As shown in **Figure 3.10A**, the proportion of FastDC remaining in the gate defining morphologically intact FastDC according to Forward scatter/Side scatter measurement decreased down to 14 and 4% 72h after electroporation and Nucleofection™, respectively. In contrast, it remained at 53% with TransMessenger™, which corresponds to the proportion of FastDC within the gate after 24h (not shown). This indicates that the long-term survival of FastDC transfected with TransMessenger™ is high, whereas the physical damages caused by electroporation or Nucleofection™ lead to the loss of most of the cells 72h after transfection. Furthermore, cells transfected with TransMessenger™ and remaining in the gate 72h after transfection were viable according to propidium iodide (PI) exclusion (**Figure 3.10B**), and still efficiently expressed EGFP (**Figure 3.8**). In contrast, the electroporated and nucleofected populations underwent a dramatic loss of viability after 48h and correspondingly lost antigen expression (**Figures 3.10B** and **3.8**). Thus, TransMessenger™ appears considerably less toxic for transfected cells in comparison to electroporation or Nucleofection™ and provides longer duration of transgene expression.

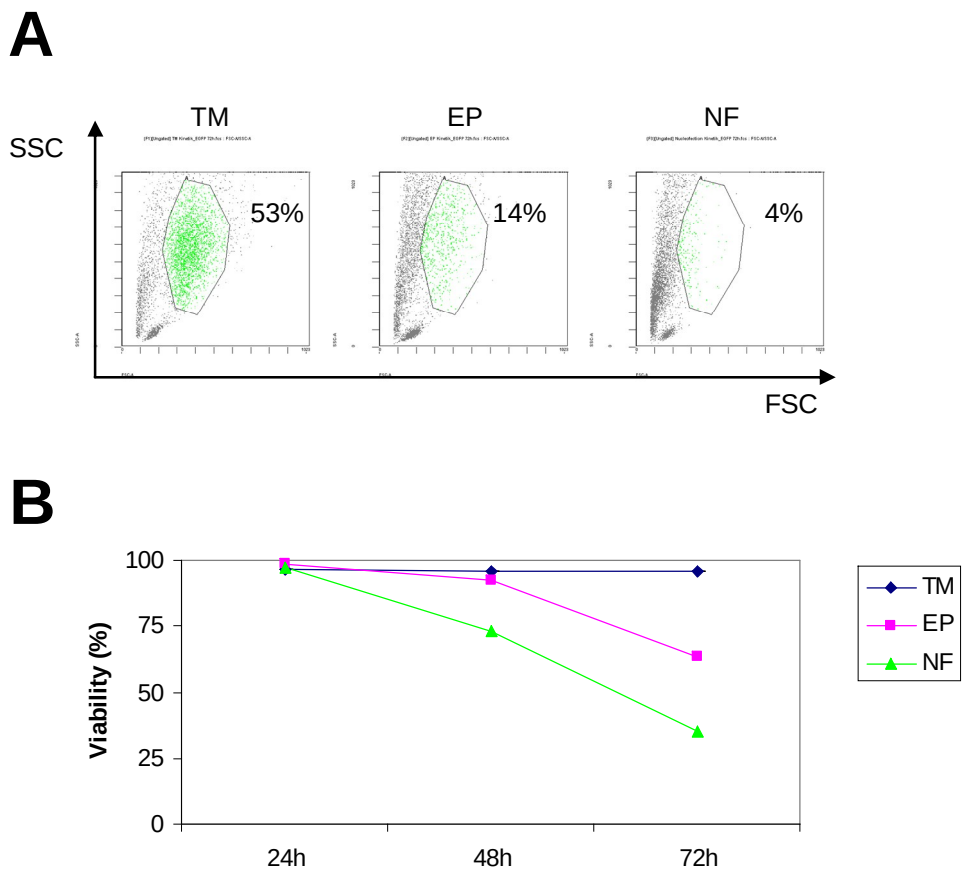

Figure 3.10. Yield and viability of FastDC after transfection with TransMessenger™, electroporation or Nucleofection™. Transfection with TransMessenger™ permits to achieve improved yield and viability after 72h compared to electroporation or Nucleofection™. **A**. Transfected FastDC (same populations as analyzed in Figure 3.8) are shown 72h after transfection with TransMessenger™ (TM), electroporation (EP) or Nucleofection™ (NF) in Forward Scatter (FSC) / Side Scatter (SSC) measurement. 5 x 10^3 cells are shown on each diagram. Percentages of cells remaining in the gate defining morphologically intact FastDC are indicated. **B**. FastDC were stained 24h, 48, or 72h after transfection with propidium iodide (PI, 1 µg/ml) and PI fluorescence of the gated populations was immediately assessed by flow cytometry. Percentages of cells excluding PI, corresponding to viable cells, are indicated.

Conclusion chapter 3.1:

These analyses identify FastDC transfected with IVT-mRNA as potent antigen-presenting cells to stimulate anti-HCMV pp65 CD8+ T cell responses. Transfection of FastDC with TransMessenger™ leads to a higher antigen expression level on a per cell basis than electroporation, a prolonged antigen expression, and a better viability of transfected cells. In the following section, extrinsic stimulation parameters are optimized in viral models.

3.2 Optimization of the stimulation parameters in viral models

Using the transfection conditions described in chapter 3.1, FastDC were transfected with IVT-mRNA using TransMessenger™. To evaluate the capacity of mRNA-transfected FastDC to stimulate antigen-specific CD8+ T cells, we applied them as APC in the Human Cytomegalovirus (CMV) and Modified Vaccinia Ankara (MVA) viral models. In a first step, the influence of the common cytokine-receptor γ-chain (γc)-cytokine family members IL-2, IL-4, IL-7 and IL-15, and the impact of the APC-to-CD8+ T cell ratio on the stimulation of antigen-specific cells were evaluated in the CMV model. In a second step, the stimulation protocol as elaborated in chapters 3.2.1-3.2.3 was applied to the stimulation of virus antigen-specific T cells in a cohort of CMV- or MVA-positive donors.

3.2.1 IL-2 at a concentration of 25 IU/ml leads to the most efficient expansion of pp65-specific CD8+ T cells

As concentrations of IL-2 commonly used to expand T cells in vitro may also contribute to the expansion of non-specific responses, we tested the effects of different IL-2 concentrations on the expansion of pp65-specific CD8+ T cells after stimulation with FastDC transfected with pp65-IVT-mRNA.

FastDC were generated from monocytes of a CMV-seropositive donor and transfected with pp65-IVT-mRNA (Material and Methods, Chapter 2.2.2.7). Transfected FastDC were used as APC to stimulate CD8+ T cells isolated from the donor's PBMC in the presence of IL-2 at different concentrations, or in the absence of IL-2. As further controls, CD8+ T cells were also stimulated with mock-transfected FastDC at the same IL-2 concentrations, or in the absence of IL-2. After one stimulation, expansion of pp65-specific CD8+ T cells restricted by HLA-B*0702 was evaluated in an IFN-γ ELISPOT assay. 293T cells co-transfected with HLA-B*0702 and pp65 were applied as targets.

As shown in **Figure 3.11A**, the overall T cell expansion depended on the presence of IL-2. In the absence of IL-2 the number of viable cells decreased after one stimulation. Analyzing the expansion of pp65-specific CD8+ T cells, it appeared that cells stimulated in the presence of IL-2 at a concentration of 25 IU/ml produced maximum IFN-γ spot amount in response to target cells, whereas cells stimulated with a concentration of 250 IU/ml produced ca. one third less spots (**Figure 3.11B**, lower panel). In the absence of IL-2, stimulated cells produced only ca. one third of maximum spot number. In all the cases, background spot level against 293T was negligeable (< than 20 spots per 1,1 x 10^3 CD8+ T cells). Notably, none of the CD8+ T cell populations stimulated with mock-transfected FastDC produced significant spot levels (**Figure 3.11B**, upper panel).

Thus, although the presence of IL-2 remained essential to drive an efficient expansion of antigen-specific CD8+ T cells, an IL-2 concentration above 25 IU/ml did not improve the stimulatory potential.

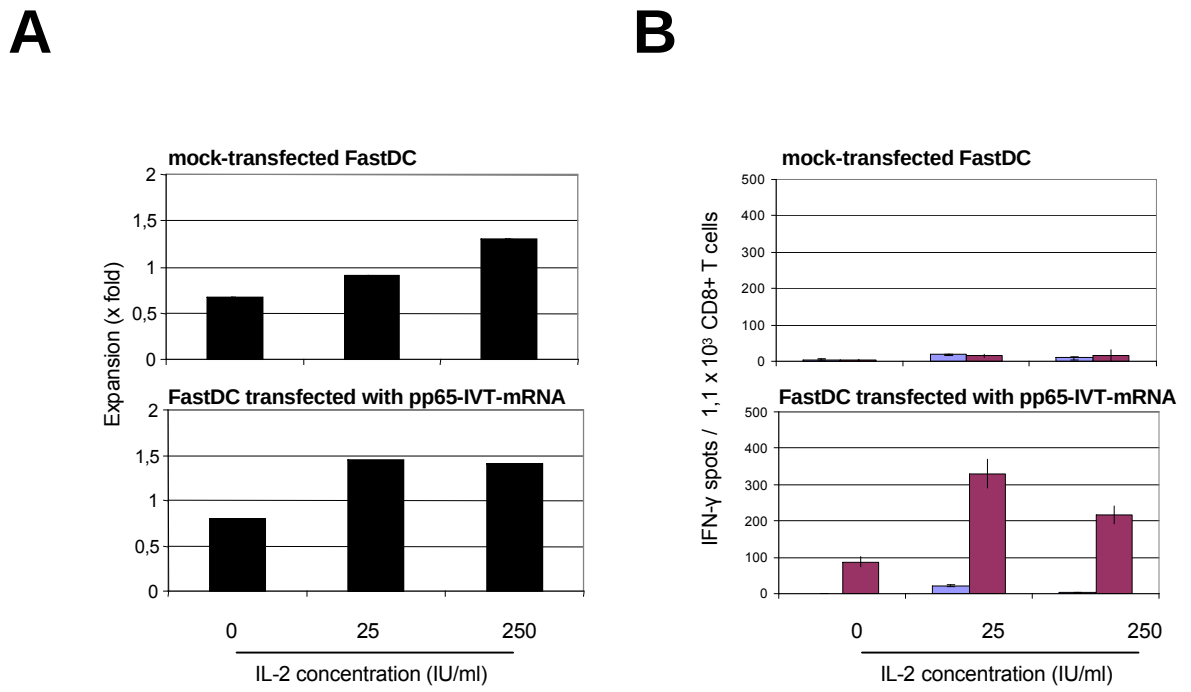

Figure 3.11 : Influence of IL-2 on the stimulation of pp65-reactive CD8+ T cells. IL-2 at a concentration of 25 IU/ml leads to the most efficient expansion of pp65-specific CD8+ T cells. FastDC were generated from monocytes of CMV seropositive donor 744 and transfected with pp65-IVT-mRNA. 2 x 10^6 CD8+ T cells, isolated from the donor's PBMC, were stimulated with 2 x 10^5 pp65-transfected FastDC in the presence of IL-2 at 25 IU/ml, 250 IU/ml, or in the absence of IL-2. **A.** Five days after the stimulation with mock-transfected FastDC (top) or FastDC transfected with pp65-IVT-mRNA (bottom), T cells were stained with trypan blue. Viable cells excluding the dye were counted, and overall cell expansion was calculated relative to the initial T cell number. **B.** Five days after the stimulation with mock-transfected FastDC (top) or FastDC transfected with pp65-IVT-mRNA (bottom), T cell responders (1,1 x 10^3 / well) were tested in a 20h IFN-γ ELISPOT assay for recognition of 293T cells (2 x 10^4 / well) co-transfected with plasmids encoding HLA-B*0702 and pp65 (■), or for recognition of 293T cells transfected with HLA-B*0702 only (■).

3.2.2 The addition of IL-4, IL-7 or IL-15 does not improve the stimulation of pp65-specific CD8+ T cells

Further cytokines of the γc-cytokine family, especially IL-7 and IL-15, have been shown to improve the effects of IL-2 in some settings (Klebanoff, 2004; Schluns, 2003; Judge, 2002). Therefore, the influence of these cytokines on the expansion of antigen-specific CD8+ T cells was analyzed.

CD8+ T cells from another CMV-seropositive donor were stimulated with FastDC transfected with pp65-IVT-mRNA in the presence of IL-2 combined with IL-4, IL-7 and/or IL-15. As negative controls, CD8+ T cells were also stimulated with mock-transfected FastDC. Expansion of antigen-specific CD8+ T cells was evaluated by analyzing the responses

against the $pp65_{495-503}$ peptide presented by HLA-A*0201 in an IFN-γ ELISPOT assay, using K562/A*0201 transfectants pulsed with the peptide as readout cells (see Material and Methods, chapter 2.2.3.1).

All cytokines increased overall T cell expansion, in comparison to stimulation with IL-2 alone (**Figure 3.12A**). The combination of IL-2 with IL-4 and IL-7 led to the strongest overall expansion. But this cytokine combination was clearly inferior to IL-2 alone with respect to the expansion of pp65-specific T cells (**Figure 3.12B**, lower panel). The addition of IL-2 alone led to the strongest expansion of pp65-reactive CD8+ T cells. Addition of IL-4 or IL-7 did not improve this response, whereas combination of both, or addition of IL-15, even reduced it. Background spot levels against readout cells were negligible (<10 spots per 3 x 10^4 lymphocytes). Mock-transfected FastDC were not able to stimulate expansion of pp65-specific cells, except in the presence of all cytokines (**Figure 3.12B**, upper panel). Of note, no reactivity against $pp65_{495-503}$ could be detected among ex vivo CD8+ T cells tested in the same experiment (not shown).

Thus, the most efficient expansion of $pp65_{495-503}$-specific CD8+ T cells was obtained with IL-2 alone, although overall cell proliferation was the lowest. Therefore, only IL-2 was added in further experiments.

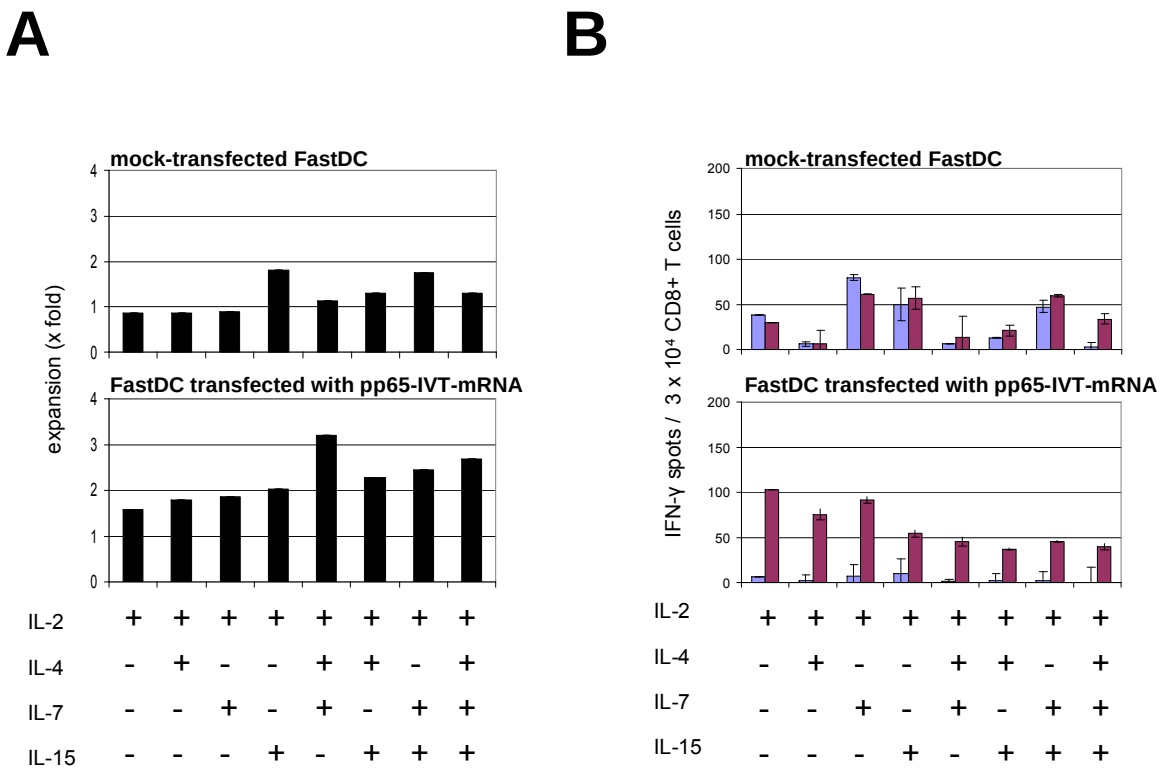

Figure 3.12 : Influence of IL-4, IL-7, IL-15 on the stimulation of pp65-reactive CD8+ T cells. The addition of IL-4, IL-7 or IL-15 does not improve the stimulation of pp65-specific CD8+ T cells. FastDC were generated from monocytes of CMV seropositive donor 811 and transfected with pp65-IVT-mRNA. 1 x 10^6 CD8+ T cells, isolated from the donor's PBMC, were stimulated with 1 x 10^5 pp65-transfected FastDC in the presence of IL-2 (25 IU/ml) and combinations of IL-4 (500 IU/ml), IL-7 (10 ng/ml) and IL-15 (10 ng/ml). **A.** Five days after the stimulation with

mock-transfected FastDC (top) or FastDC transfected with pp65-IVT-mRNA (bottom), T cells were stained with trypan blue. Viable cells excluding the dye were counted, and overall cell expansion was calculated relative to the initial T cell number. **B.** Five days after the stimulation with mock-transfected FastDC (top) or FastDC transfected with pp65-IVT-mRNA (bottom), T cell responders (3 x 10^4 / well) were tested in a 20h IFN-γ ELISPOT assay for recognition of K562/A*0201 transfectants (7,5 x 10^4 / well) stably expressing HLA-A*0201, and pulsed with peptide $pp65_{495-503}$ (■) or unpulsed (□).

3.2.3 Maximum expansion of pp65-specific CD8+ T cells is achieved at a 1:10 APC-to-CD8+ T cell ratio

DC are highly efficient in recruiting peptide-specific T cells and can engage more than ten T cells simultaneously (Bousso, 2003). We therefore investigated the influence of the APC-to-CD8+ T cell ratio on the expansion of pp65-specific CD8+ T cells after stimulation with FastDC transfected with pp65-IVT-mRNA.

CD8+ T cells isolated from the PBMC of a CMV seropositive donor were stimulated with pp65-transfected FastDC at APC-to-CD8+ T cell ratios ranging from 1:40 to 1:2,5. As negative controls, CD8+ T cells were also stimulated with mock-transfected FastDC at the same ratios. Overall expansion of stimulated cells and their reactivity against the $pp65_{417-426}$ peptide presented by HLA-B*0702 were evaluated in an IFN-γ ELISPOT assay, using K562/B*0702 cells pulsed with the peptide as targets.

We noticed only a slight increase in the overall expansion of stimulated cells with increasing APC-to-CD8+ T cell ratios (**Figure 3.13A**). In contrast, an almost linear and relatively strong increase of the pp65-specific reactivity was seen after stimulation at APC-to-CD8+ T cell ratios from 1:40 to 1:10 (**Figure 3.13B**, lower panel). The strongest responses were obtained at ratios between 1:10 and 1:2,5. Background spots levels against readout cells K562/B*0702 were below 10 spots per 1,1 x 10^3 CD8+ T cells. None of the populations stimulated with mock-transfected FastDC could efficiently recognize the antigen, even at the 1:2,5 ratio (**Figure 3.13B**, upper panel). Of note, reactivity of ex vivo CD8+ T cells against the antigen accounted for 11 spots for 1,1 x 10^3 CD8+ T cells (background against K562/B*0702 subtracted, not shown).

Thus, antigen-specific reactivity of stimulated cells increased with increasing APC-to-CD8+ T cell ratios, and a ratio of 1:10 was sufficient to achieve maximum reactivity. This ratio was therefore used in further experiments.

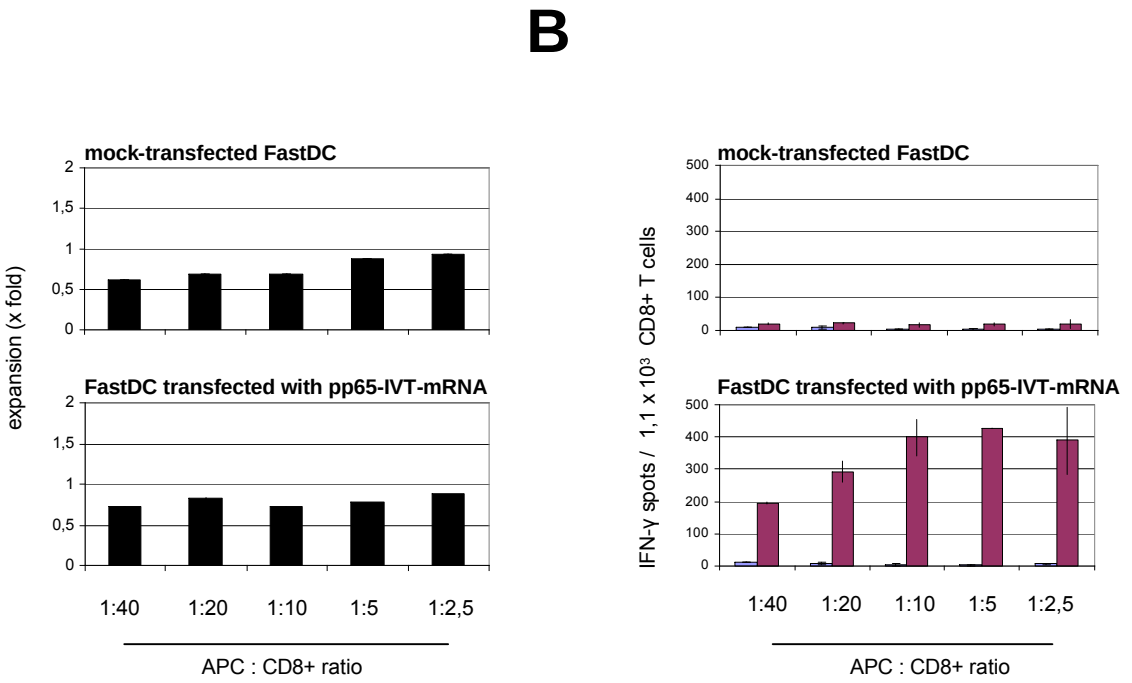

Figure 3.13 : Influence of the APC-to-CD8+ T cell ratio on the expansion of pp65-reactive CD8+ T cells. Maximum expansion of pp65-specific CD8+ T cells is achieved at a minimum APC-to-CD8+ T cell ratio of 1:10. FastDC were generated from monocytes of CMV seropositive donor 514 and transfected with pp65-IVT-mRNA. 1 x 10^6 CD8+ T cells, isolated from the donor's PBMC, were stimulated with pp65-transfected FastDC at APC-to-CD8+ T cell ratios (APC : CD8+ ratio) varying from 1:40 to 1:2,5. **A.** Five days after the stimulation with mock-transfected FastDC (top) or FastDC transfected with pp65-IVT-mRNA (bottom), T cells were stained with trypan blue. Viable cells excluding the dye were counted, and overall cell expansion was calculated relative to the initial T cell number. **B.** Five days after the stimulation with mock-transfected FastDC (top) or FastDC transfected with pp65-IVT-mRNA (bottom), T cell responders were tested in a 20h IFN-γ ELISPOT assay for recognition of K562/B*0702 cells (7,5 x 10^4 / well) stably expressing HLA-B*0702 and pulsed with peptide $pp65_{417-426}$ (■) or unpulsed (■).

3.2.4 Simultaneous stimulation of pp65-specific CD8+ T cells restricted by distinct HLA class I alleles

Multiple peptides can be processed from single proteins and may bind to different HLA class I molecules. Due to the limited amount of APC per effector cell and due to the size limitation for stimulation units of the mRNA-stimulation assay, distinct CD8+ T cell responder populations recognizing distinct peptides of the same protein antigen may compete within a stimulation unit. The following experiments were performed to find out, if the stimulation conditions elaborated in the previous sections would allow to expand T cells with distinct peptide specificities within single stimulation units. We selected three CMV-seropositive donors, who were HLA-A2- and HLA-B7-positive according to standard HLA serotyping. In these donors, distinct anti-pp65 CD8+ T cell responses restricted by HLA-A*0201 and HLA-B*0702, respectively, may coexist (Diamond, 1997; Lacey, 2003). CD8+ T cells from these donors were stimulated with autologous FastDC transfected with pp65-IVT-mRNA, and the

responses against peptides pp65$_{495-503}$, restricted by HLA-A*0201, and pp65$_{417-426}$, restricted by HLA-B*0702, were evaluated in an IFN-γ ELISPOT assay.

In ex-vivo analyses of T cell reactivity against pp65, reactivities against the HLA-B*0702 restricted peptide predominated in all donors (**Figure 3.14**), which is in accordance with the literature (Lacey, 2003). Ex-vivo reactivities against the HLA-A*0201 restricted peptide were very low (< 25 spots for 5 x 10^5 PBMC) but could be stimulated in 2 out of the 3 donors tested (**Figure 3.15B**, upper panel). No response against pp65$_{495-503}$ could be stimulated in donor 514. In all three donors, strong CD8+ T cell responses directed against the immunodominant peptide pp65$_{417-426}$ were obtained after stimulation (**Figure 3.15B**, lower panel). No response could be detected against pp65$_{495-503}$ or pp65$_{417-426}$ in donors 381 and 811 after stimulation with mock-transfected FastDC (**Figure 3.15A**). In donor 514, a response against pp65$_{417-426}$ could be detected after stimulation with mock-transfected FastDC, but this response was 7 times weaker than the response obtained after stimulation with FastDC transfected with pp65-IVT-mRNA.

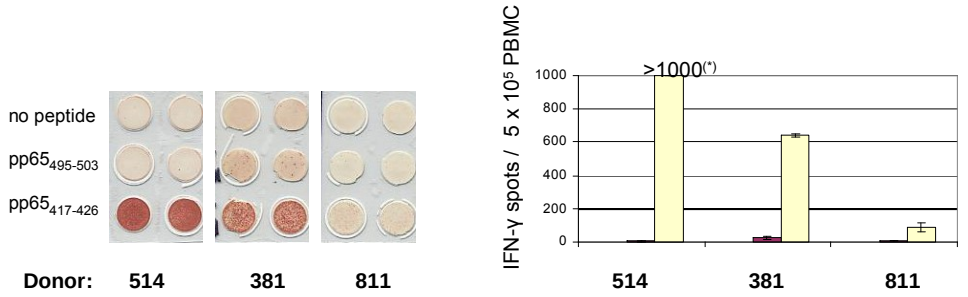

Figure 3.14 : Ex vivo reactivities against pp65 in CMV-seropositive donors. Ex vivo reactivities against the immunodominant peptide pp65$_{417-426}$ are detected at various levels in all donors, while reactivity against pp65$_{495-503}$ can be detected only in donor 381 at a very low level. PBMC (5 x 10^5 / well) from HLA-A2- and HLA-B7-positive as well as CMV-seropositive donors 514, 381 and 811 were tested ex vivo in the absence (■) and in the presence of peptide pp65$_{495-503}$ restricted by HLA-A*0201 (■) and pp65$_{417-426}$ restricted by HLA-B*0702 (■) (10 µg/ml) in a 20h IFN-γ ELISPOT assay. ELISPOT filters (left) as well as result of semi-automated spot evaluation (right) are shown. (*): not enumerable.

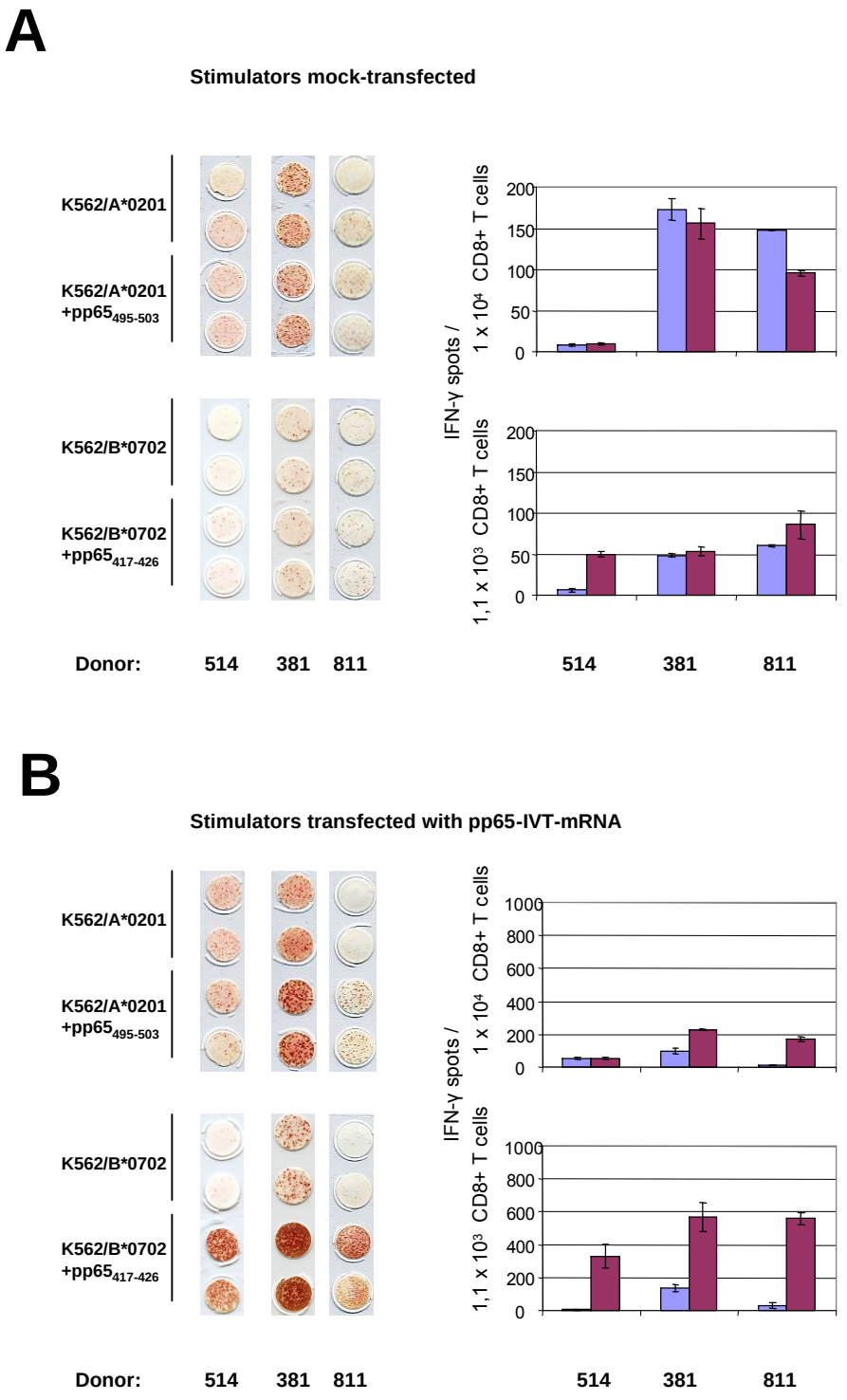

Figure 3.15 : Parallel stimulation of distinct pp65-specific CD8+ T cells responses restricted by HLA-A*0201 or HLA-B*0702. Responses against both epitopes $pp65_{495-503}$ and $pp65_{417-426}$ can be simultaneously stimulated in donors 381 and 811. FastDC were generated from monocytes of CMV-seropositive donors 514, 381 and 811. 1 x 10^6 CD8+ T cells isolated from the donors' PBMC were stimulated with 1 x 10^5 mock-transfected FastDC (**A**) or FastDC transfected with pp65-IVT-mRNA (**B**). Five days after the stimulation, stimulated cells were tested for recognition of peptides $pp65_{495-503}$ (upper panel, 1 x 10^4 / well) and $pp65_{417-426}$ (lower panel, 1,1 x 10^3 / well) in a 20h IFN-γ ELISPOT assay. K562/A*0201 and K562/B*0702 cells alone (□) or pulsed with the relevant peptide (■) (10 µg/ml) were used as target cells (7,5 x 10^4 / well). ELISPOT filters (left) as well as result of semi-automated spot evaluation (right) are shown.

To quantify the expansion of antigen-specific CD8+ T cells after stimulation, stimulation indices were calculated for each response as the fold-increase of spot-producing cells in response to the antigen (**Table 3.1**). In donors 514 and 381, although ex vivo reactivities against $pp65_{417-426}$ were very high (>1,3% and 1,3%, respectively), relatively weak stimulation indexes were achieved for this response (<16 and 21, respectively). In contrast, in donor 811, where the ex vivo reactivity against $pp65_{417-426}$ was ca. 10 times weaker (0,1%), a ca. 18 times higher stimulation index was obtained. Thus, those antigen-specific CD8+ T cells which are hardly detectable ex vivo may be stimulated more efficiently.

In donor 381, the stimulation index obtained for antigen-specific CD8+ T cells directed against $pp65_{495-503}$ was in the same extent as the index obtained for the T cells directed against $pp65_{417-426}$ (18 versus 21). In donor 811, in contrast, this index was ca. 2 times weaker than the one obtained for the response directed against $pp65_{417-426}$ (167 versus 360). This suggests that a competition may exist in some cases between different CD8+ T cell populations directed against distinct epitopes, and that those responses directed against immunodominant epitopes may be preferentially stimulated (see Discussion, chapter 4.3.2.3).

Table 3.1 : Stimulation indices calculated for pp65-specific CD8+ T cells directed against $pp65_{495-503}$ or $pp65_{417-426}$ in association with HLA-A*0201 or HLA-B*0702, respectively. (1) The frequencies of CD8+ T cells specific for peptides $pp65_{495-503}$ (top) and $pp65_{417-426}$ (bottom) among ex vivo PBMC were determined as described in chapter 2.2.3.2 (Material and Methods) (data shown in **Figure 3.14**). (2) Proportion of CD8+ T cells among PBMC was determined by flow cytometry and number of antigen-specific CD8+ T cells reported to the total number of CD8+ T cells per well. CD8+ T cells accounted for 10% of PBMC in donor 381 and 20% in donor 811. It was estimated to 15% in donor 514. (3) CD8+ T cells isolated from PBMC were stimulated with FastDC transfected with pp65-IVT-mRNA. On d5, responder T cells cells were counted and the proportion of antigen-specific CD8+ T cells was determined in a 20h IFN-γ ELISPOT assay (see **Figure 3.15**). The absolute number of antigen-specific cells was calculated relatively to the total amount of remaining CD8+ T cells per well. (4) Stimulation indices were calculated for each donor as the ratio between (3) and (2). nd: not detectable (<15 spots / 3 x 10^4 CD8+ T cells).

$pp65_{495-503}$ -specific CD8+ T cells

Donor	Ex vivo frequency (d0) (1)	Number of antigen-specific CD8+ T cells per well on d0 (2)	Number of antigen-specific CD8+ T cells per well on d5 (3)	Stimulation index (4)
514	nd	nd	nd	-
381	25 / 5 x 10^5 PBMC	500	9 x 10^3	18
811	6 / 5 x 10^5 PBMC	60	1 x 10^4	167

$pp65_{417-426}$ -specific CD8+ T cells

Donor	Ex vivo frequency (d0) (1)	Number of antigen-specific CD8+ T cells per well on d0 (2)	Number of antigen-specific CD8+ T cells per well on d5 (3)	Stimulation index (4)
514	>10^3 / 5 x 10^5 PBMC	>1,3 x 10^4	2,1 x 10^5	<16
381	641 / 5 x 10^5 PBMC	1,3 x 10^4	2,8 x 10^5	21
811	88 / 5 x 10^5 PBMC	880	3,17 x 10^5	360

3.2.5 Stimulation indices of antigen-specific CD8+ T cells in a cohort of CMV-seropositive and MVA-vaccinated donors

The analysis was extended to a cohort of donors who had been identified as CMV-seropositive or who had been vaccinated with Modified Vaccinia Ankara (MVA) previously (Meyer, 2005). In MVA-vaccinated donors, the CD8+ T cell reactivity was shown to be directed at least in part against the ORF 28R (K7R) gene product of MVA in association with HLA-B*1501. The target peptide was identified as $ORF28_{25-33}$ (Meyer, 2007). CD8+ T cells isolated from the donors' PBMC were stimulated with autologous FastDC transfected either with IVT-mRNA encoding the CMV-pp65 antigen or the MVA-ORF 28R antigen. Stimulated

CD8+ T cells were tested in IFN-γ ELISPOT assays for reactivity against readout cells either transfected with the full-length antigen or pulsed with the relevant peptide.

In all of the CMV-seropositive donors tested, pp65-specific CD8+ T cell responses could be efficiently amplified (**Table 3.2**). They were directed against $pp65_{495-503}$ or $pp65_{417-426}$ and were restricted by the HLA alleles HLA-A*0201 or HLA-B*0702, respectively. Stimulation indices were between 30 and 72. No pp65-specific CD8+ T cell response could be stimulated in three out of four CMV-seronegative donors tested. Donor 660 was serologically typed CMV seronegative, but an HLA-A*0201-restricted CD8+ T cell response could be stimulated against peptide $pp65_{495-503}$.

Table 3.2 : Stimulation indices of pp65-specific CD8+ T cells in a cohort of CMV-seropositive and –seronegative (neg) donors. Indices ranging from 30 to 72 are obtained. (1) Ex vivo frequencies were determined using isolated CD8+ T cells (see Material and Methods, chapter 2.2.3.2). CD8+ T cells were tested for recognition of K562 cells (7,5 x 10^4 / well) stably transfected with the relevant HLA I allele and pulsed with the peptide (10 µg/ml) corresponding to the immunodominant antigen (italic), or for recognition of 293T cells (2 x 10^4 / well) co-transfected with plasmids encoding the relevant HLA I allele and pp65 (bold). HLA restriction of the immunodominant antigen is underlined in the HLA I type of each donor. (2) CD8+ T cells were stimulated with FastDC transfected with pp65-IVT-mRNA and tested on d5 for recognition of K562 cells (7,5 x 104 / well) stably transfected with the relevant HLA I allele and pulsed with the peptide (10 µg/ml) corresponding to the immunodominant antigen (italic), or for recognition of 293T cells (2 x 104 / well) co-transfected with plasmids encoding the relevant HLA I allele and pp65 (bold). (3) After stimulation, responder T cells were counted and the overall proliferation index was calculated as the ratio between remaining CD8+ T cells and starting amount of CD8+ T cells per well. (4) Stimulation indices were calculated as follows: (frequency after stimulation)(2) x (overall proliferation index)(3) / (ex vivo frequency)(1). nd: not determined. 1: stimulated twice. 2: not detectable (< 15 for 1 x 10^5 CD8+ T cells in ex vivo tests, or < 15 for 3 x 10^4 responder CD8+ T cells after stimulation).

Donor	HLA class I type	Frequency of antigen-specific CD8+ T cells ex vivo (1)	Frequency of antigen-specific CD8+ T cells on d5 (2)	Overall proliferation index (3)	Stimulation index (4)
906 (neg)[1]	A2, A24, B35, B52	nd	<0,05%[2]	4	-
216	A2, A32, B7, B36	**0,37%** *0,8%*	**29%** *nd*	0,86	**67**
DH (neg)[1]	A2, B15, B45, Cw3	nd	<0,05%[2]	2,29	-
656	A2, A24, B18, B57	**0,1%** *0,1%*	**18%** *nd*	0,4	**72**
660 (neg)[1]	A1, A2, B44, Bw55, Cw3, Cw5	**<0,05%**[2] *<0,05%*[2]	**1,2%** *nd*	2,28	**>55**
694[1]	A2, A26, B45, B62, Cw3, Cw6	**0,2%** *0,2%*	**1,5%** *3,33%*	1,98	*30*
053 (neg)	A2, B7, B27, Cw2, Cw7	nd	<0,05%[2]	1,27	-

Among the two MVA-vaccinated donors tested, strong ORF28-specific CD8+ T cell reactivities could be detected after stimulation with FastDC transfected with ORF28R-IVT-mRNA (**Table 3.3**). Stimulation index was at least 150 in donor FI and 490 in donor DH. No reactivity against the antigen could be stimulated with mock-transfected FastDC (**Figure 3.16B**). Notably, no CD8+ T cell response was detected against ORF_{25-33} ex vivo (**Figure 3.16A**).

Thus, we calculated stimulation indices ranging from 30 to 72 in the CMV viral model, where ex vivo frequencies against the immunodominant antigen were between 0,1 and 0,8%, and indices higher than 150 in the MVA model, where no ex vivo reactivities against the relevant antigen $ORF28_{25-33}$ were detected.

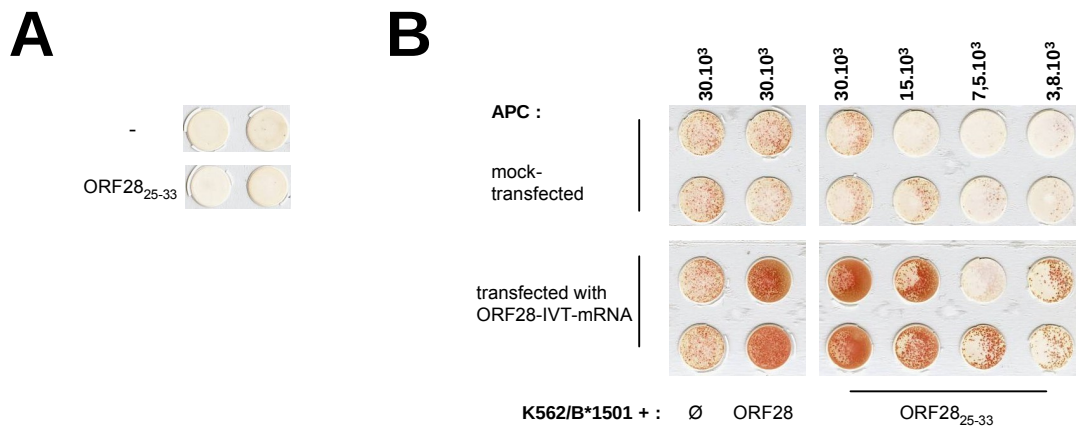

Figure 3.16 : Stimulation of CD8+ T cells with IVT-mRNA encoding the ORF 28R(K7R) gene product in an MVA-vaccinated donor. Strong reactivity against $ORF28_{25-33}$ is found after stimulation. This reactivity can not be detected ex vivo. **A**. PBMC (5 x 10^5 / well) from donor DH were tested ex vivo against peptide $ORF28_{25-33}$ (10 µg/ml). **B.** FastDC were generated from the donor‘s monocytes and transfected with the IVT-mRNA encoding the ORF 28(K7R) gene product. CD8+ T cells, isolated from the donor‘s PBMC, were stimulated on d0 and d7 with FastDC transfected with ORF28-IVT-mRNA. As negative control, CD8+ T cells were also stimulated with mock-transfected FastDC. CD8+ responders were tested on d12 in a 20h IFN-γ ELISPOT assay and titrated from 3 x 10^4 down to 3,8 x 10^3 cells per well. K562/B*1501 cells (7,5 x 10^4 / well), stably expressing the HLA-B*1501 allele and transfected with ORF28-IVT-mRNA using TransMessenger™ or pulsed with peptide $ORF28_{25-33}$ (10 µg/ml) were used as readout cells.

Table 3.3 : Stimulation indices of ORF28-specific CD8+ T cells in MVA-vaccinated donors. Indices of several hundred-fold are obtained. (1) Ex vivo frequencies were determined as described (see Material and Methods, chapter 2.2.3.2). CD8+ T cells were tested for recognition of K562 cells (7,5 x 10^4 / well) stably transfected with HLA*B1501 and pulsed with $ORF28_{25-33}$ (10 µg/ml) (italic) or for recognition of 293T cells (2 x 10^4 / well) co-transfected with plasmids encoding HLA*B1501 and ORF28 (bold). (2) CD8+ T cells were stimulated with FastDC transfected with ORF28-IVT-mRNA and tested on d12 for recognition of K562 cells (7,5 x 10^4 / well) stably transfected with HLA*B1501 and pulsed with $ORF28_{25-33}$ (10 µg/ml) (italic) or for recognition of 293T cells (2 x 10^4 / well) co-transfected with plasmids encoding HLA*B1501 and ORF28 (bold). (3) After stimulation, responder T cells were counted and the overall proliferation index was calculated as the ratio between remaining CD8+ T cells and starting amount of CD8+ T cells per well. (4) Stimulation indices were calculated as follows:

(frequency after stimulation)[(2)] x (overall proliferation index)[(3)] / (ex vivo frequency)[(1)]. nd: not determined. [1]: not detectable (< 15 for 1 x 10^5 CD8+ T cells).

Donor	Frequency of antigen-specific CD8+ T cells ex vivo [(1)]	Frequency of antigen-specific CD8+ T cells on d12 [(2)]	Overall proliferation index [(3)]	Stimulation index [(4)]
DH	**nd** *<0,015% [1]*	**nd** *3,7%*	2	*>490*
FI	**nd** *<0,015% [1]*	**1%** *nd*	2,3	**>150**

Conclusion chapter 3.2:

Extrinsic stimulation parameters like the cytokine concentrations or the APC-to-CD8+ T cell ratio were optimized in the HCMV viral model. Most efficient stimulations of pp65-specific CD8+ T cells with mRNA-transfected FastDC were obtained with IL-2 alone, at a concentration of 25 IU/ml, and at an APC-to-CD8+ T cell ratio of 1:10. With these conditions, up to several hundred-fold increases of CD8+ T cell populations specific for viral antigens CMV/pp65 or MVA/ORF28 were obtained. In the next section, the procedure is applied to stimulate CD8+ T cells specific for tumor-associated antigens.

3.3 Stimulation of CD8+ T cells against tumor antigens

Melan-A/MART-1 represents until now the only tumor antigen for which naïve specific CD8+ T cells can be readily detected in the peripheral blood of a large proportion of HLA-A2 healthy individuals (Pittet, 1999; Romero, 2002). Most of these CD8+ T cell responses are found to be restricted by HLA-A*0201 and directed against Melan-$A_{26(27)-35}$. Thus, this antigen represents a unique candidate to stimulate tumor antigen-specific CD8+ T cells from healthy individuals.

3.3.1 Melan-A/MART-1-specific CD8+ T cells can be stimulated in 5 out of 9 donors

We stimulated CD8+ T cells from 9 HLA-A*0201-positive healthy donors with autologous FastDC transfected with Melan-A-IVT-mRNA. After two stimulations, CD8+ T cells were tested in an IFN-γ ELISPOT assay for recognition of full-length cDNA transfectants or for recognition of APC pulsed with the peptide Melan-A_{26-35}. As negative controls, donors' CD8+ T cells were also stimulated with IVT-mRNA encoding the irrelevant tumor antigen TRP-2. As positive controls, CD8+ T cells of CMV-seropositive donors were stimulated with pp65-IVT-mRNA. Response analysis was restricted to HLA-A*0201-associated epitopes.

As shown in **Figure 3.17A**, Melan-A_{26-35} specific CD8+ T cells could be stimulated in 5 out of 9 donors. Frequencies of spot-producing CD8+ T cells ranged from 0,09% to 0,8% within the stimulated populations. In all the donors, the reactivity was directed against the peptide Melan-A_{26-35} as the responses against K562/A*0201 cells transfected with full-length Melan-A-cDNA were not superior to the responses against K562/A*0201 cells pulsed with the peptide (**Figure 3.17B** for donors 053 and 344). No response against the negative control antigen TRP-2 could be detected after stimulation in any donor. Only 5 out of 8 donors' CD8+ T cells were stimulated with pp65-IVT-mRNA. Anti-pp65 reactivity was seen in all of the CMV-seropositive donors (**Figure 3.17A**).

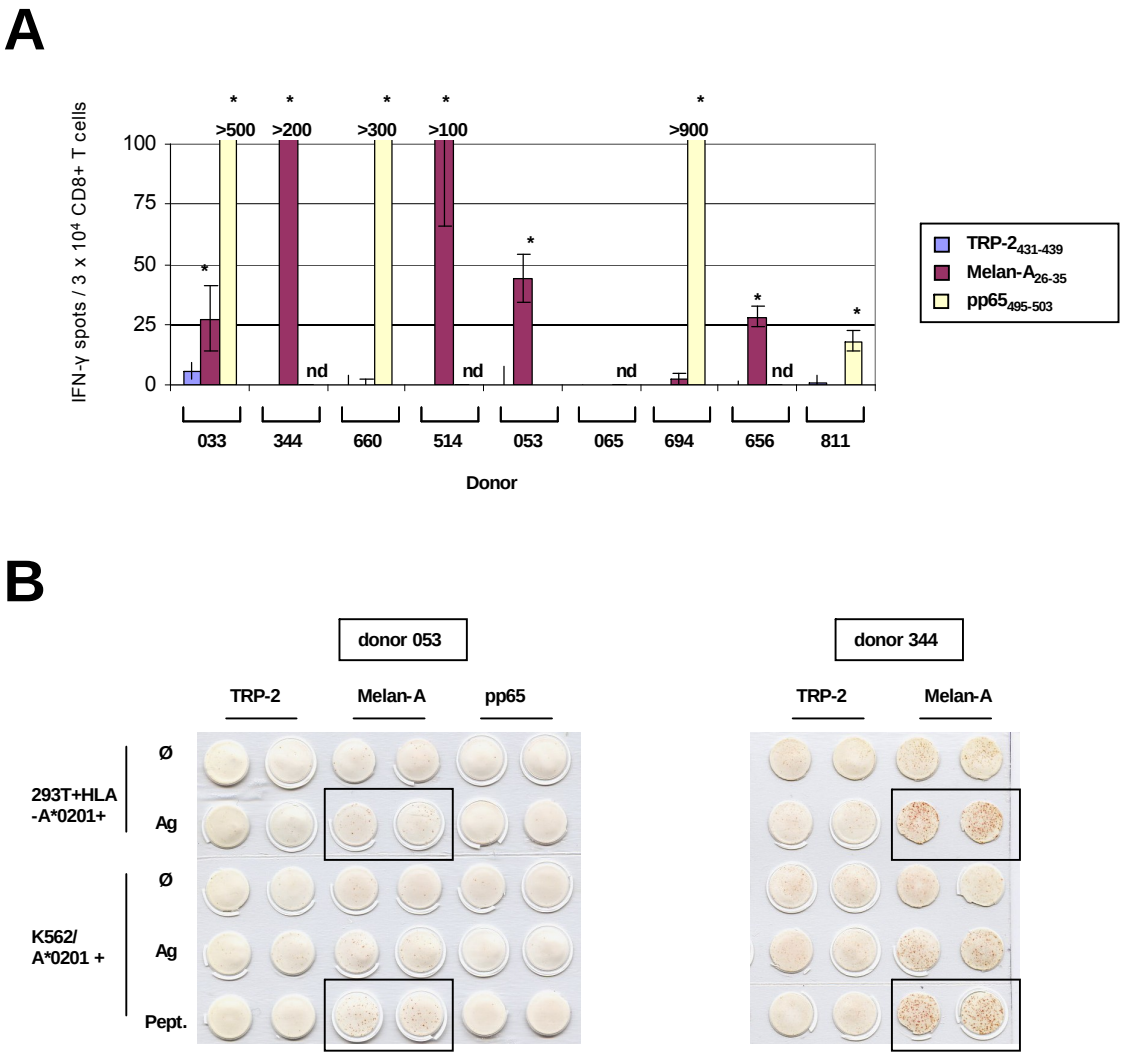

Figure 3.17 : Stimulation of Melan-A-specific CD8+ T cells in HLA-A*0201-positive healthy individuals. Responses against Melan-A_{26-35} are found in 5 out of 9 donors. CD8+ T cells (2 x 10^6) from 9 HLA-A*0201-positive healthy donors were stimulated on d0 and d7 with autologous FastDC (2 x 10^5) transfected with TRP-2-, Melan-A- or pp65-IVT-mRNA. **A**. Responder CD8+ T cells (3 x 10^4 / well) were tested in a 20h IFN-γ ELISPOT assay on d12, for recognition of K562/A*0201 cells (7,5 x 10^4 / well) stably expressing HLA-A*0201 and pulsed with TRP-$2_{431-439}$, Melan-A_{26-35} or $pp65_{495-503}$ (10 µg/ml), respectively. Background activity against K562/A*0201 cells was subtracted. * : > 2x above background (p-value < 0.05). nd: not determined. All donors, with the exception of donor 053, were CMV-seropositive. Donor 053 was stimulated once. **B.** ELISPOT are shown as examples for donors 053 and 344. Responder CD8+ T cells (3 x 10^4 / well) were tested for recognition of 293T cells (2 x 10^4 / well) co-transfected with plasmids encoding HLA-A*0201 and TRP-2, Melan-A or pp65, respectively, and for recognition of K562/A*0201 cells (7,5 x 10^4 / well) transfected with plasmids encoding TRP-2, Melan-A or pp65, respectively, or pulsed with peptides TRP-$2_{431-439}$, Melan-A_{26-35}, or $pp65_{495-503}$, respectively (10 µg/ml).

3.3.2 Melan-A/MART-1 specific CD8+ T cells can not be detected ex vivo

In 4 out of 5 donors, in whom Melan-A-specific reactivity after stimulation was found, CD8+ T cells were tested ex vivo against the peptide Melan-A_{26-35}. No ex vivo-reactivity against this

peptide was seen in any of the donors tested. In contrast, ex vivo-reactivity was seen against the peptide $pp65_{493-503}$ in donors 033 and 656 (**Figure 3.18**).

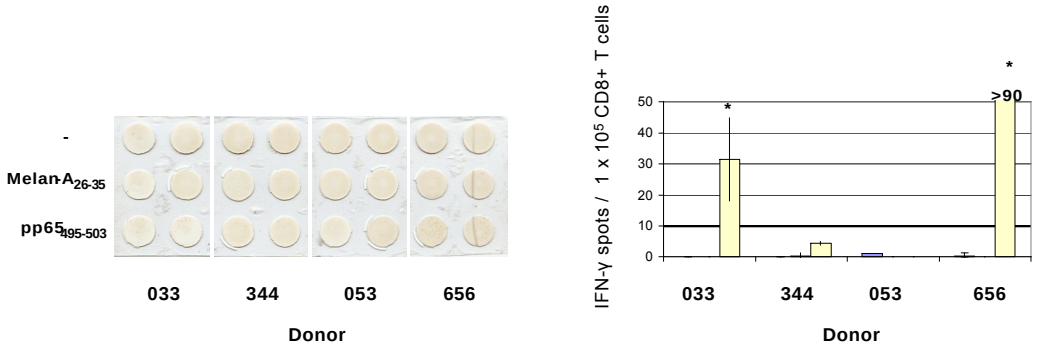

Figure 3.18 : CD8+ T cell ex vivo-reactivity against Melan-A_{26-35} and CMV/$pp65_{495-503}$ in Melan-A-positive healthy donors carrying the HLA-A*0201 allele. No response against Melan-A_{26-35} is detected ex vivo in any of the donors tested. CD8+ T cells (1 x 10^5 / well) were tested ex vivo for recognition of K562/A*0201 cells (7,5 x 10^4 / well) alone (□) or pulsed with peptides Melan-A_{26-35} (■) or $pp65_{495-503}$ (□), respectively (10 µg/ml), in a 20h IFN-γ ELISPOT assay. ELISPOT filters (left) as well as result of semi-automated spot evaluation (right) are shown. All donors, with the exception of donor 053, were CMV-seropositive. * : > 2x above background (p-value < 0.05).

Conclusion chapter 3.3:

CD8+ T cell responses directed against the tumor-associated antigen Melan-A/MART-1 were stimulated with mRNA-transfected FastDC in 5 out of 9 HLA-A*0201-positive healthy donors. Melan-A-specific CD8+ T cells could not be detected in any donor ex vivo. In the next section, the procedure is applied to stimulate anti-tumor CD8+ T cell responses in two melanoma patients.

3.4 Stimulation of tumor-specific CD8+ T cells in melanoma models

Melanoma patients have been shown to develop anti-tumor CD8+ T cell responses in the course of the disease. These responses were found to be directed against melanosomal, cancer-germline or mutated antigens (Boon, 2006). Only some of these reactivities can be detected in ex vivo blood lymphocytes.

In the melanoma model D05, which has been extensively studied in our laboratory, at least 17 different epitopes derived from melanosomal, cancer-germline, and mutated antigens that were presented by different HLA class I molecules, have been identified to date as targets for autologous CD8+ T cells using T cell-based expression screening (**Figure 3.19A**, Lennerz et al., unpublished results). In MZ2, another well characterized melanoma model (Traversari, 1992; Van den Eynde, 1995), one epitope from the melanosomal antigen tyrosinase and 7 epitopes derived from cancer-germline antigens have been identified using expression screening (**Figure 3.19B**).

We generated FastDC from monocytes of patients D05 and MZ2 and transfected them separately with the IVT-mRNAs encoding the respective target antigens. We applied these mRNA-transfected FastDC as stimulators of autologous peripheral blood lymphocytes. In parallel, lymphocytes were stimulated with the autologous tumor cells.

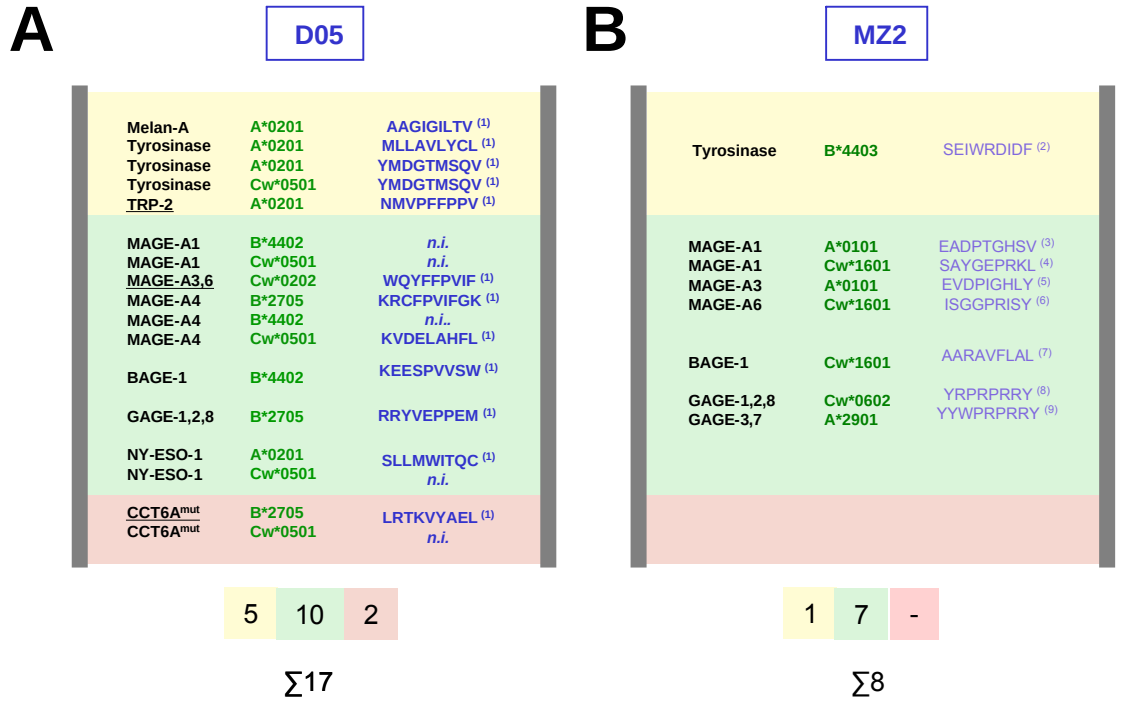

Figure 3.19 : Antigens identified in melanoma models D05 (A) and MZ2 (B) using expression screening procedures. Listed are antigens, their respective presenting HLA I alleles and peptides, if identified, that were shown to be targeted by blood-derived CD8+ T cells on autologous melanoma cells. Antigens are affiliated to their categories (yellow: melanosomal, green: cancer-germline, red: mutated). Reactivities detected ex vivo are underlined. *n.i.*: not identified. (1): Lennerz et al, unpublished results. (2): Brichard, 1996. (3): Traversari, 1992.

(4): Van der Bruggen, 1994B. (5): Gaugler, 1994. (6): Vantomme, 2003. (7): Boel, 1995. (8): Van den Eynde, 1995. (9): De Backer, 1999.

3.4.1 Stimulation of D05-PBL with IVT-mRNA-transfected FastDC uncovers CD8+ T cell responses against 6 tumor antigens

Due to scarce amounts of PBMC available from patient D05, PBL instead of isolated CD8+ T cells were stimulated against a panel of 12 different tumor antigens, 10 of which had been identified with screening procedures (**Figure 3.19A**). As a negative control, PBL were stimulated with mock-transfected FastDC. Responder lymphocytes were tested after two stimulations for recognition of 293T cells separately transfected with plasmids encoding the patient's HLA I alleles and the stimulation antigens.

As shown in **Figure 3.20**, responses against the cancer-germline antigens MAGE-A3, MAGE-A4, MAGE-C2, GAGE-1 and NY-ESO1 could be efficiently stimulated (**Figure 3.20 f-h, j-k**). A particularly strong response was detected after stimulation against the mutated antigen CCT6A^{mut} (**Figure 3.20 l**). No reactivity could be detected against the melanosomal antigens Melan-A/MART-1, Tyrosinase, TRP-2 or gp100 (**Figure 3.20 a-d**).

The responses detected against CCT6A^{mut} and GAGE-1 after stimulation were exclusively restricted by HLA-B*2705 (**Figure 3.20 l, j**). Similarly, the responses against MAGE-A3, NY-ESO-1 and MAGE-A4 were exclusively restricted by HLA-Cw*0202, HLA-Cw*0501 and B*4402, respectively (**Figure 3.20 f, k, g**). MAGE-C2 had not been identified previously as a tumor antigen targeted by autologous CD8+ T cells in D05. Nevertheless we could stimulate a strong response against this protein, which seemed to be restricted by all HLA I alleles applied to the test (**Figure 3.20 h**). Most likely, this response was only restricted by HLA-A*0201, because 293T cells endogenously express HLA-A*0201 (own observations, see Material and Methods, chapter 2.1.5.1). All responder populations stimulated with IVT-mRNA were also tested for recognition of the clonal melanoma cell line D05-MEL (see Material and Methods, chapter 2.1.5.1). While lymphocytes stimulated with TRP-2, MAGE-A3, MAGE-A4, BAGE, NY-ESO1 or CCT6A^{mut} recognized also autologous melanoma cells, cells stimulated with other antigens did not recognize the D05 melanoma cell clone used for the stimulation. Of note, stimulation with mock-transfected FastDC did not uncover any response against the antigens tested, nor did it preserve any reactivity against the tumor cells which was detectable ex vivo (**Figure 3.20 m** and **Figure 3.19A**).

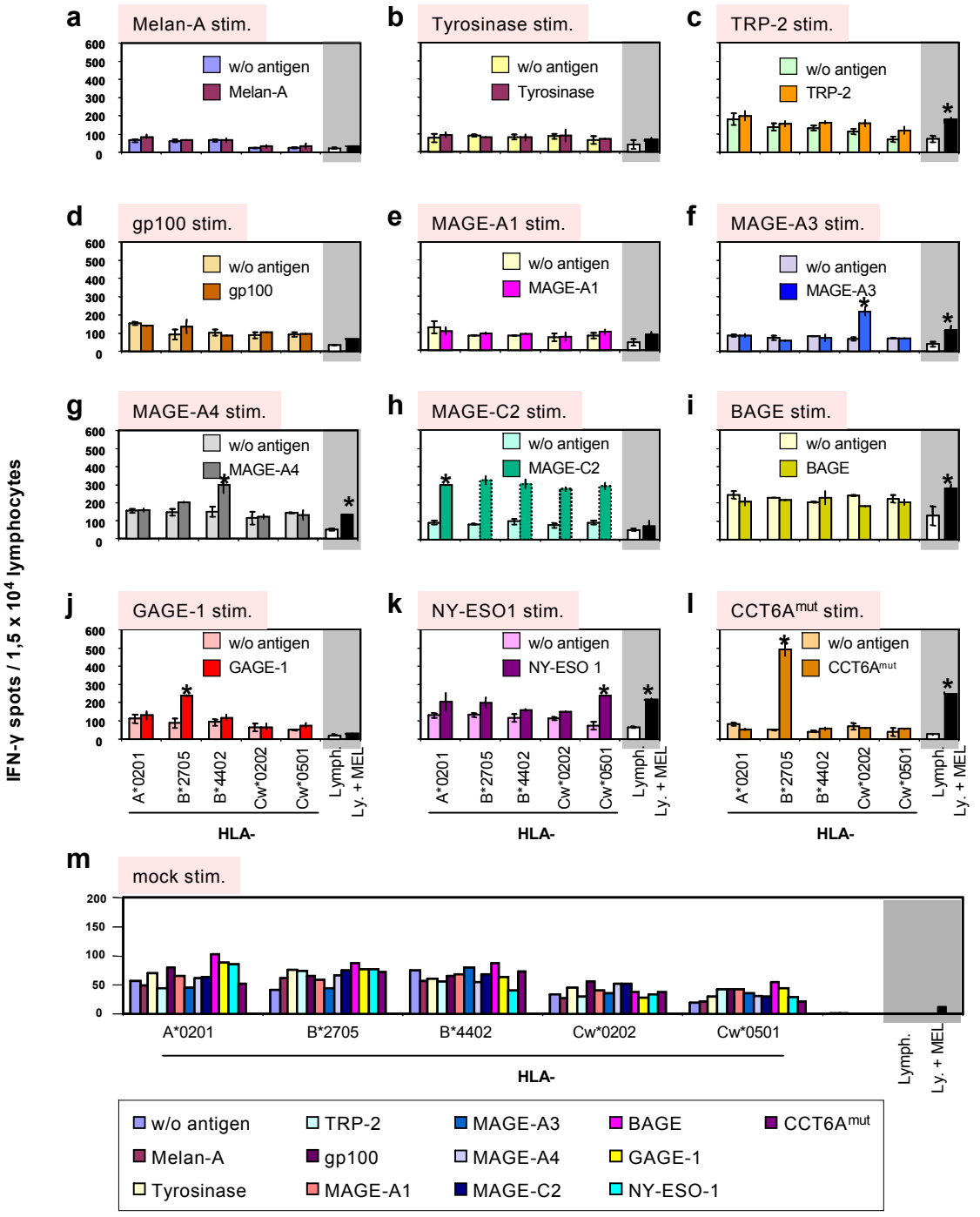

Figure 3.20 : Stimulation of patient D05-PBL with a panel of tumor-associated antigens. Responses against 6 epitopes derived from 6 different antigens are found. FastDC were generated from D05-monocytes and separately transfected with antigen-encoding IVT-mRNAs. D05-PBL (1,15 x 10^6) were stimulated on d0 and d7 with IVT-mRNA-transfected FastDC (1,5 x 10^5) (**a-l**) or mock-transfected FastDC (**m**) and were tested on d12 in a 20h IFN-γ ELISPOT assay. Lymphocyte responders (1,5 x 10^4 / well) were tested either alone (Lymph.), or in the presence of the autologous tumor cell clone D05-MEL (5 x 10^4 / well) (Ly.+MEL), and for recognition of 293T cells (2 x 10^4 / well) co-transfected with plasmid pairs encoding one of the D05 HLA I alleles and the indicated antigen, or for recognition of 293T cells transfected with the respective HLA I allele only (w/o antigen) . * : >2x above background (p-value < 0.05).

3.4.2 Stimulation of D05-PBL with an autologous tumor cell clone uncovers CD8+ T cell responses against 7 tumor antigens

Autologous tumor cells are commonly used in screening procedures for the identification of tumor antigens and, when available, are obviously the most appropriate in vitro stimulators of tumor-specific CD8+ T cell responses that have been primed in vivo. In parallel to the stimulations with IVT-mRNA-transfected FastDC, D05-PBLs were also stimulated with the autologous melanoma cell clone D05-MEL. Like previously, responder lymphocytes were tested after two stimulations for recognition of 293T cells separately transfected with plasmids encoding the patient's HLA I alleles and the panel antigens.

As shown in **Figure 3.21**, 7 of 12 antigens tested were recognized. Among differentiation antigens, responses directed toward Melan-A and Tyrosinase and restricted by HLA-A*0201 and HLA-Cw*0501, respectively, were found. All responses toward MAGE-A1, -A3, -A4, which had been identified with screening procedures, could also be detected after stimulation with the tumor clone, as well as a response directed toward the other cancer-germline antigen NY-ESO-1 and restricted by HLA-A*0201. Both responses toward the mutated antigen CCT6A^{mut} which had been identified with screening procedures were also found after stimulation with the tumor.

Notably, the response against GAGE-1 and restricted by HLA-B*2705 was only found after stimulation with IVT-mRNA-transfected FastDC (**Figure 3.20 j**). This was also the case for the response against NY-ESO-1 and restricted by HLA-Cw*0501, and for the response against MAGE-C2 and restricted by HLA-A*0201 (**Figure 3.20 h**). Neither had the latter response been identified with screening procedures. Further analysis showed that the tumor cell clone used with both methods to stimulate responder lymphocytes had lost MAGE-C2 expression (see Discussion, chapter 4.2.1).

Taken together, 11 of 16 responses detected with screening procedures could also be found after stimulation with the tumor. Out of these 11 responses, only 3 were uncovered with IVT-mRNA-transfected FastDC. Furthermore, these responses were stronger after stimulation with the tumor. Two responses identified with screening procedures were only found after stimulation with IVT-mRNA-transfected FastDC (summarized and further treated in the discussion, chapter 4.2.3; see also Table 4.2) .

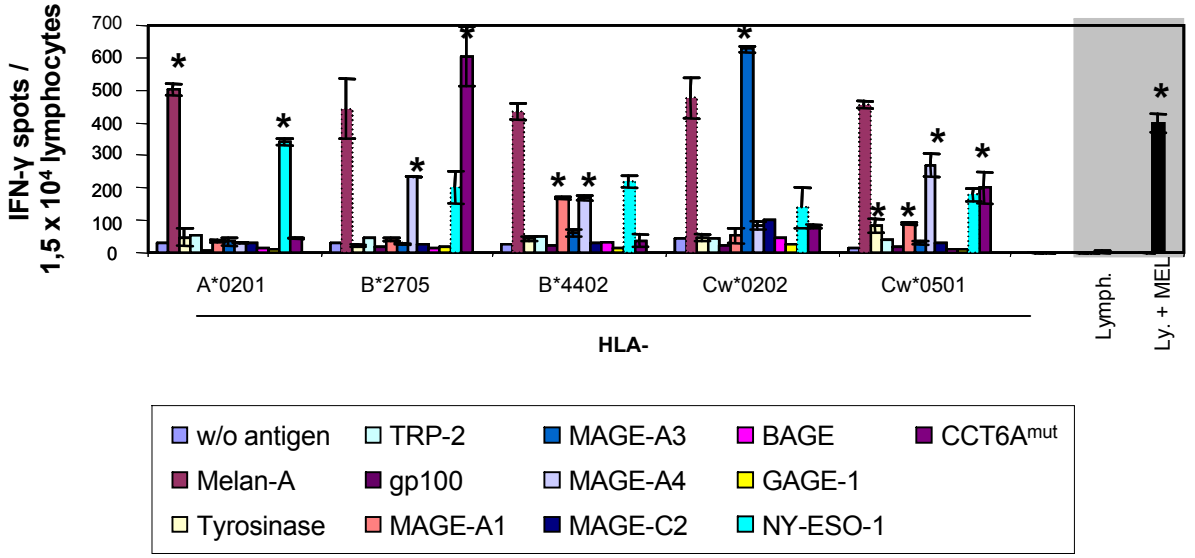

Figure 3.21 : Stimulation of patient D05-PBL with an autologous tumor cell clone. Responses against 11 epitopes derived from 7 different antigens are found. D05-PBL (1,15 x 10^6) were stimulated on d0 and d7 with the autologous melanoma cell clone D05-MEL (2 x 10^5) and tested on d12 in a 20h IFN-γ ELISPOT assay. Lymphocyte responders (1,5 x 10^4 / well) were tested either alone (Lymph.), or in the presence of D05-MEL (5 x 10^4 / well) (Ly.+MEL), and for recognition of 293T cells (2 x 10^4 / well) co-transfected with plasmid pairs encoding one of the D05-HLA I alleles and the indicated antigen, or for recognition of 293T cells transfected with the respective HLA I allele only (w/o antigen). Dot bars indicate the detection of HLA-A*0201 restricted responses in apparent association with other HLA I alleles (see text). * : > 2x above background (p-value < 0.05).

3.4.3 Stimulation of MZ2-CD8+ T cells with IVT-mRNA-transfected FastDC uncovers CD8+ T cell responses against 3 tumor antigens

CD8+ T cells purified from the peripheral blood lymphocytes of patient MZ2 were stimulated twice with autologous FastDC separately transfected with the IVT-mRNAs encoding the 7 antigens previously identified as targets of autologous tumor-reactive T cells with screening procedures (**Figure 3.19B**). As a negative control, CD8+ T cells were also stimulated with mock-transfected FastDC. Like in the D05 melanoma model, responder T cells were tested in a 20h IFN-γ ELISPOT assay for recognition of 293T cells co-transfected with plasmids encoding the patient's HLA I alleles and the candidate antigens, and for recognition of the autologous melanoma cell clone MZ-2-MEL-43 (see Material and Methods, chapter 2.1.5.1).

As shown in **Figure 3.22**, responses against 3 out of 7 known antigens were found. MAGE-A1-stimulated lymphocytes recognized MAGE-A1 in association with HLA-A*0101 and -Cw*1601, GAGE-1-stimulated lymphocytes recognized GAGE-1 with HLA-Cw*0602. Rather strong responses were obtained against BAGE in association with HLA-B*4403 and -Cw*1601 (**Figure 3.22 b**, **f**, and **e**). Of note, the response against BAGE restricted by HLA-B*4403 had not been identified before with screening procedures. None of these responses could be stimulated with mock-transfected FastDC (**Figure 3.22 h**). The autologous tumor cell clone MZ-2-MEL-43 was not or only poorly recognized by the populations stimulated with

GAGE-1 and BAGE. MAGE-A1-stimulated CD8+ T cells recognized the tumor to the same extent as MAGE-A1-plasmid transfectants (**Figure 3.22 f**, **e**, **b**).

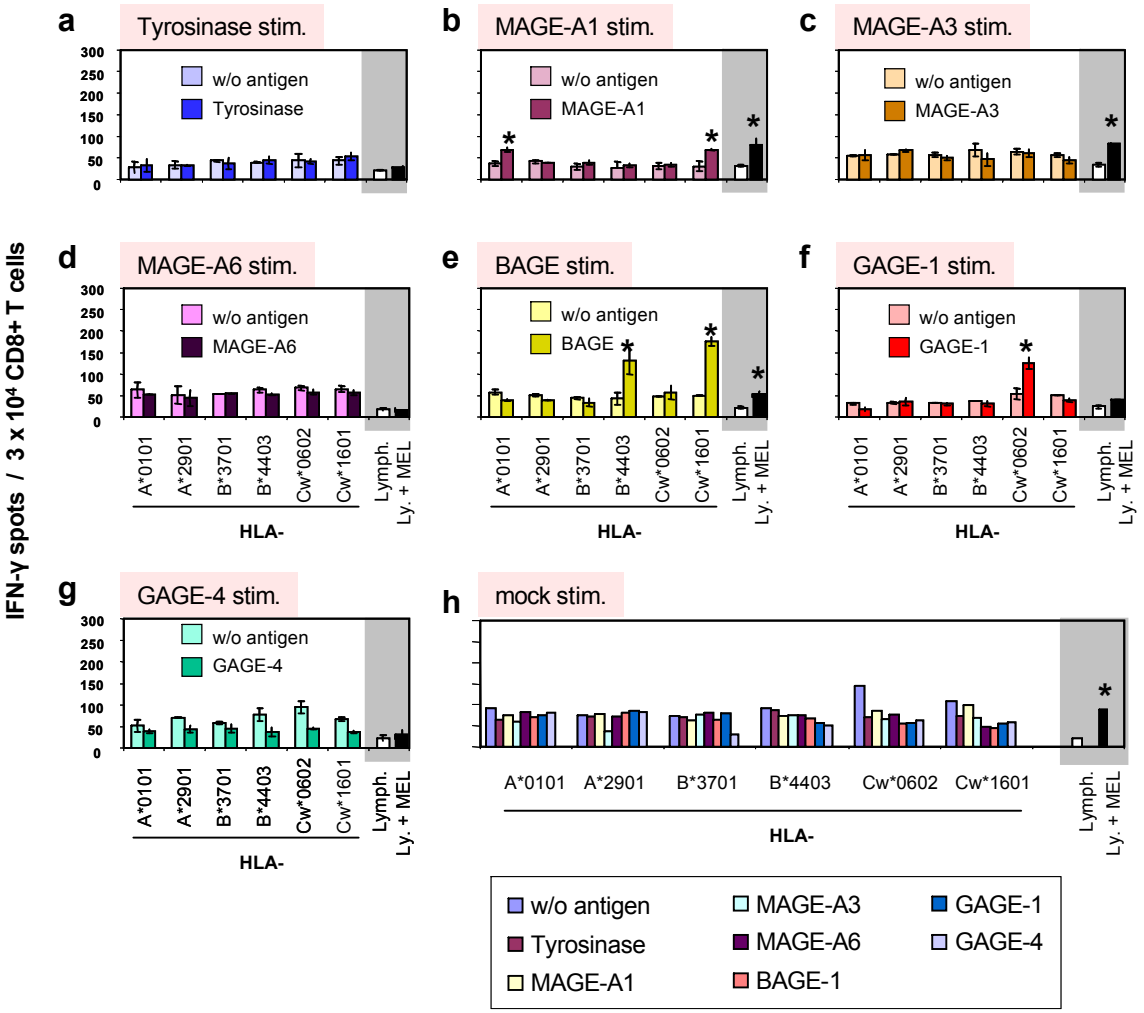

Figure 3.22 : Stimulation of CD8+ T cells from the peripheral blood of patient MZ2 with a panel of tumor-associated antigens. Responses against 5 epitopes derived from 3 different antigens are found. FastDC were generated from MZ2-monocytes and separately transfected with antigen-encoding IVT-mRNA. CD8+ T cells (1,5 x 10^6) isolated from MZ2-PBMC were stimulated on d0 and d7 with IVT-mRNA-transfected FastDC (1,5 x 10^5) (**a-g**) or mock-transfected FastDC (**h**), and were tested on d12 in a 20h IFN-γ ELISPOT assay. Responder T cells (3 x 10^4 cells / well) were tested either alone (Lymph.), or in the presence of the autologous tumor cell clone MZ2-MEL-43 (5 x 10^4 / well) (Ly.+MEL), and for recognition of 293T cells (2 x 10^4 / well) co-transfected with plasmid pairs encoding one of the MZ2-HLA I alleles and the indicated antigen, or for recognition of 293T cells transfected with the respective HLA I allele alone (w/o antigen). * : > 2x above background (p-value < 0.05).

3.4.4 Most of the MZ2-CD8+ T cell responses detected with IVT-mRNA-transfected FastDC on d7+5 are detected with tumor stimulation only on d28+5

In parallel to the stimulations with IVT-mRNA-transfected FastDC, MZ2-CD8+ T cells were stimulated with the autologous melanoma cell clone MZ2-MEL-43 and stimulated CD8+ T cells were tested on d7+5. Among the responses that were detected after stimulation with IVT-mRNA-transfected FastDC on d7+5 (**Figure 3.22**), only the responses against MAGE-A1 in association with HLA-A*0101 and –Cw*1601 could also be detected after two stimulations

with the tumor cells (**Figure 3.23A**). The responses detected against GAGE-4 or MAGE-A6 represent single values and could not be confirmed after further stimulations (**Figure 3.23B**).

CD8+ T cells were further stimulated with autologous tumor cells and responders were tested on d28+5. Targets were 293T cells transfected with pooled HLA I alleles (**Figure 3.23B**). Apart from the responses directed against MAGE-A1, responses against BAGE-1 restricted by HLA-B and C were detected, as well as a response against GAGE-1 restricted by HLA-C. Further analysis of these responses with 293T cells transfected with single HLA I alleles revealed that the responses against MAGE-A1 were restricted by HLA-A*0101 and -Cw*1601, the responses against BAGE-1 were restricted by HLA-B*4403 and -Cw*1601, and the response against GAGE-1 was restricted by HLA-Cw*0602 (not shown).

Thus, the antigens recognized on d28+5 after stimulation with autologous tumor cells were the same that were recognized after stimulation with IVT-mRNA-transfected FastDC on d7+5 (**Figure 3.22**). Though, the intensities of the responses were different. Although the strongest responses after stimulation with IVT-mRNA-transfected FastDC were directed against BAGE in association with HLA-Cw*1601 and HLA-B*4403, stimulation with the tumor cells amplified preferentially the response against MAGE-A1 in association with HLA-Cw*1601 (see Discussion, chapters 4.2.2 and 4.2.3).

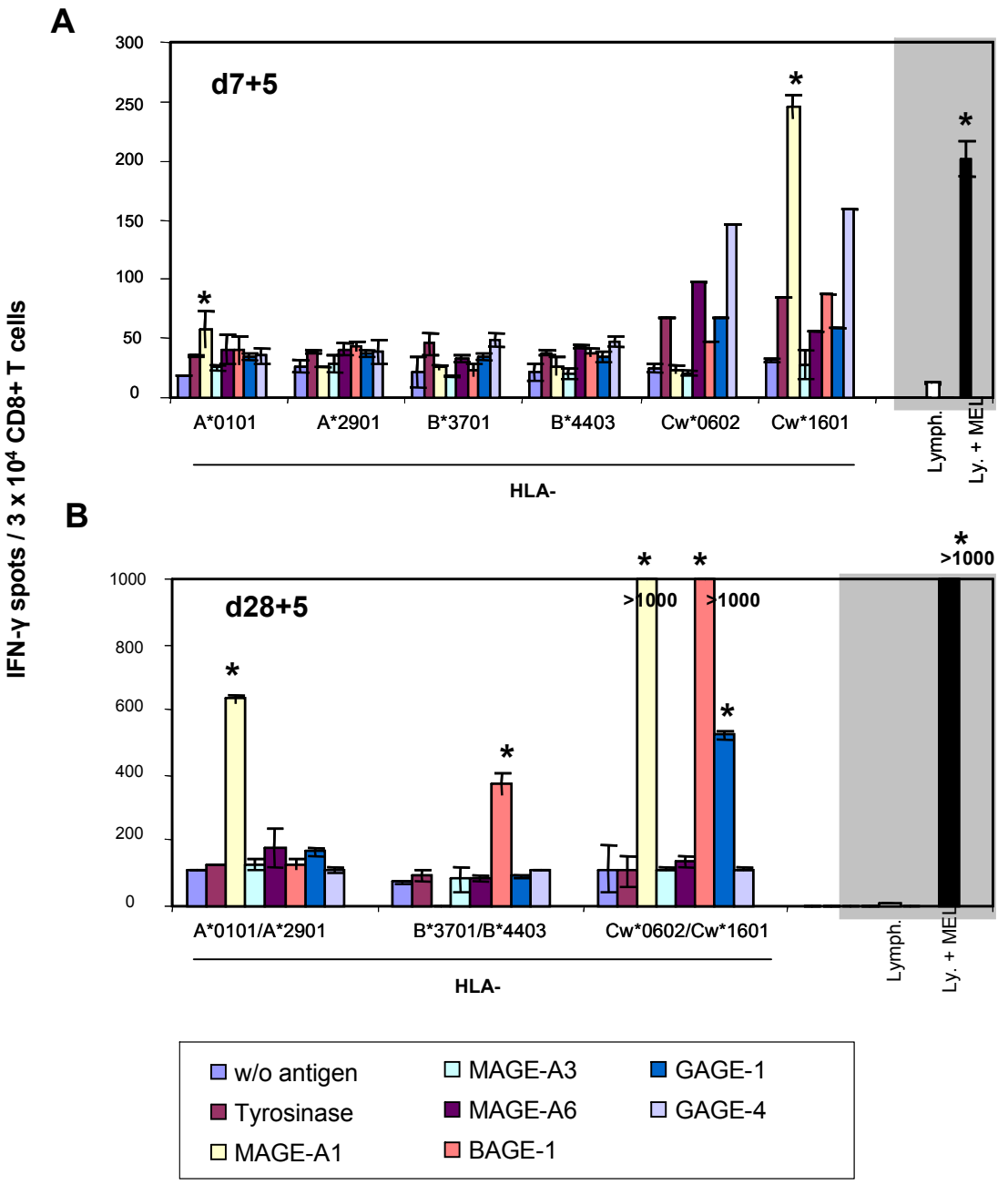

Figure 3.23 : Stimulation of MZ2-CD8+ T cells with an autologous tumor cell clone. Responses against 5 epitopes derived from 3 different antigens are found on day 32, of which only one response is detected on day 12. MZ2-CD8+ T cells (1,5 x 10^6) were stimulated weekly with the autologous melanoma cell clone MZ2-MEL-43 (2 x 10^5) and tested on d7+5 (**A)** and d28+5 (**B**) in a 20h IFN-γ ELISPOT assay. Responder T cells (3 x 10^4 / well) were tested either alone (Lymph.), or in the presence of MZ-2-MEL-43 (5 x 10^4 / well) (Ly.+MEL), and for recognition of 293T cells (2 x 10^4 / well) co-transfected with one (**A**) or two (**B**) of the MZ2-HLA I alleles and the indicated antigen, or for recognition of 293T cells transfected with the respective HLA I allele(s) only (w/o antigen). * : > 2x above background (p-value < 0.05).

3.4.1 Stimulation of MZ2-PBL leads merely to the detection of the strongest responses detected after stimulation of MZ2-CD8+ T cells

To assess the potential of PBL as starting responder population compared to isolated CD8+ T cells, MZ2-PBL were stimulated with autologous FastDC transfected with BAGE- or

MAGE-A1-IVT-mRNA. On day 7+5, responder lymphocytes were mostly (80-90%) CD8+ T cells (not shown). As shown in **Figure 3.24**, the responses directed against MAGE-A1 could not be detected on d7+5. Neither were they detectable after a further stimulation on d14+5 (**Figure 3.24 a**). Only the response against BAGE, restricted by HLA-Cw*1601, was detectable, already on d7+5. This response was greatly enhanced after a further stimulation on d14+5 (**Figure 3.24 b**). No antigen-specific response could be stimulated with autologous mock-transfected FastDC (not shown).

Stimulation of PBL with the autologous tumor cell clone MZ-2-MEL-43 led merely to the detection of the response against MAGE-A1 restricted by HLA-Cw*1601. But this response could not be detected after a further stimulation (**Figure 3.24 c**). Of note, autologous tumor cells were strongly recognized on d14+5. The corresponding responders were obviously directed against antigens other than MAGE-A1 and BAGE.

Thus, stimulation of PBL with IVT-mRNA-transfected FastDC or tumor cells led merely to the recognition of the strongest responses detected after stimulation of isolated CD8+ T cells (**Figures 3.22** and **3.23**). This indicated that the use of CD8+ T cells as starting responder population is superior to the use of PBL.

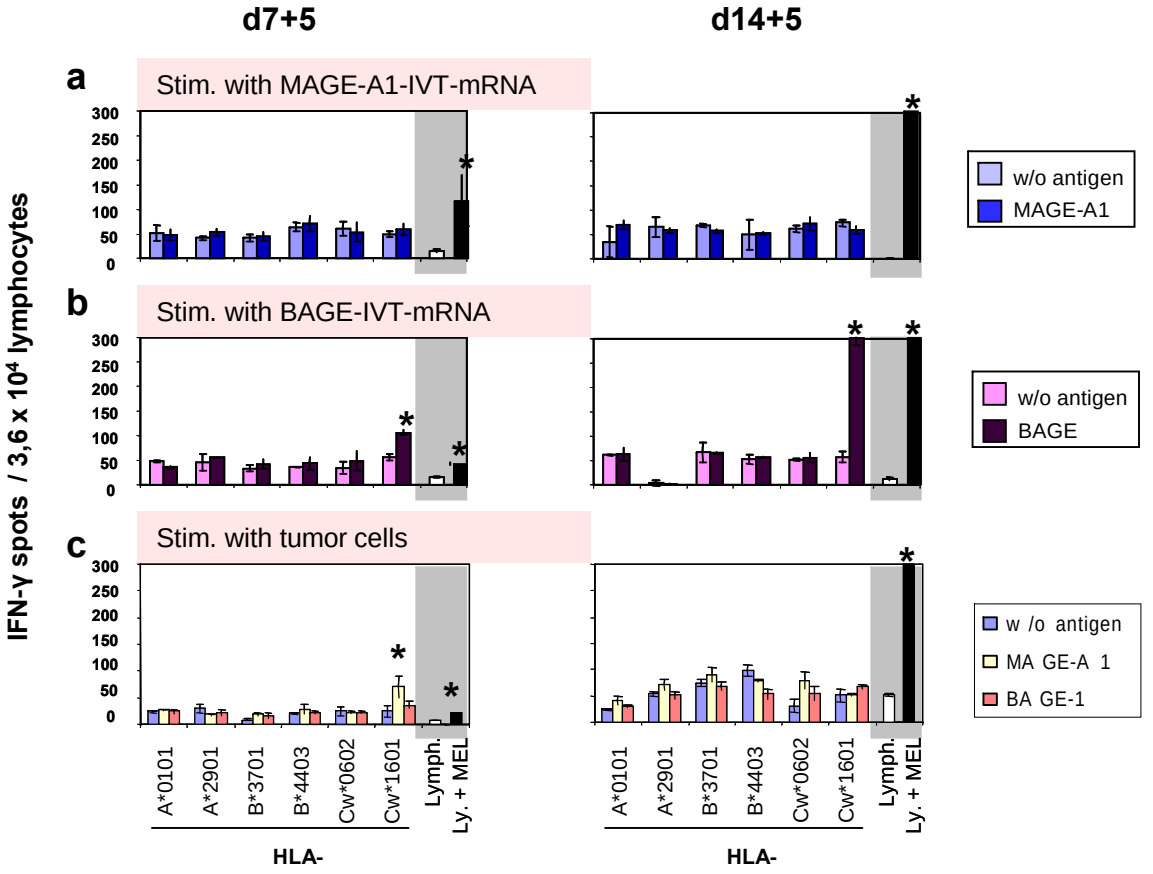

Figure 3.24 : Stimulation of MZ2-PBL. One response against BAGE is found after mRNA-stimulation, while one response against MAGE-A1 is found after stimulation with the tumor cell clone only on day 12. MZ2-PBL (1,5 x 10^6) were stimulated with autologous FastDC (1,5 x 10^5) transfected with MAGE-A1-(**a**) or BAGE-IVT-mRNA (**b**), or with the autologous melanoma cell clone MZ2-MEL-43 (1,5 x 10^5) (**c**). Lymphocyte responders (3,6 x 10^4 / well,

corresponding to 3 x 10^4 CD8+ T cells) were tested on d7+5 and d14+5 alone (Lymph.), or in the presence of MZ2-MEL-43 cells (5 x 10^4 / well) (Ly.+MEL), and for recognition of 293T cells (2 x 10^4 / well) co-transfected with plasmid pairs encoding one of the MZ2-HLA I allele (see x-axis) and the indicated antigen (see boxes), or for recognition of 293T cells transfected with the respective HLA I allele only (w/o antigen). ***** : > 2x above background (p-value < 0.05).

3.4.5 CD8+ T cell responses against tumor antigens detected in patients D05 and MZ2 are not found in healthy donors

To evaluate, if and to what extent CD8+ T cell responses directed against tumor-associated antigens can also be stimulated from PBMC of healthy donors, CD8+ T cells from four CMV-positive donors were stimulated against a panel of antigens including those identified as target antigens in D05 and MZ2. FastDC were generated for each individual donor, transfected with IVT-mRNAs encoding the chosen panel antigens and used to stimulate twice autologous CD8+ T cells. As controls, CD8+ T cells were also stimulated with mock- and with CMV/pp65-transfected FastDC.

As shown in **Figure 3.25**, strong responses against pp65 could be detected after two stimulations in all of the four donors. These responses could also be detected to a lesser extent in three out of four donors, after stimulation with mock-transfected FastDC (**Figure 3.26**). Furthermore, in donor 581, CD8+ T cells exhibited reactivity against Melan-A/MART-1 in association with HLA-A*0201. This response was not stimulated with mock-transfected FastDC (**Figure 3.26**). No response was detected against other melanosomal antigens or against any of the cancer-germline antigens tested.

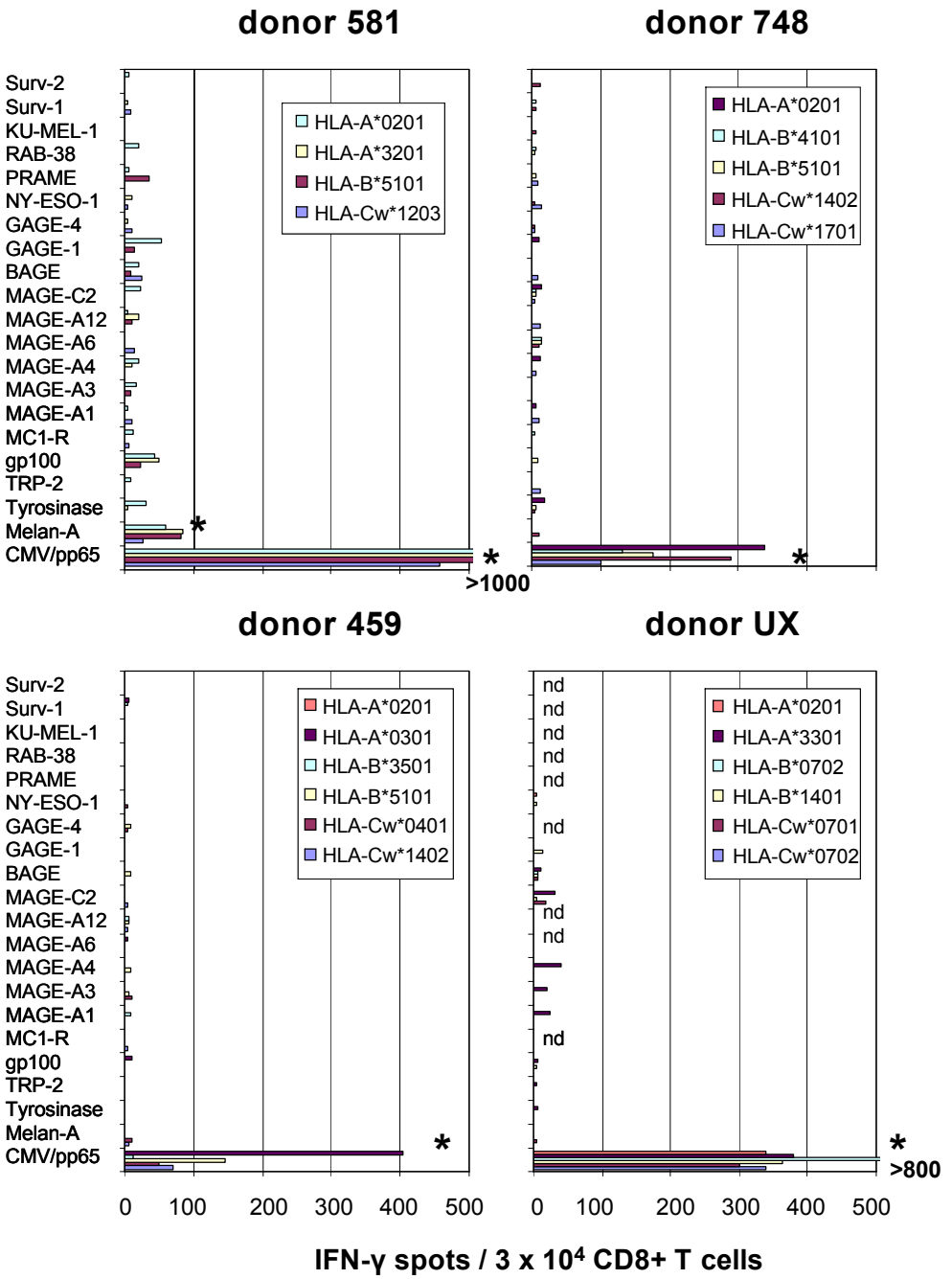

Figure 3.25 : Stimulation of CD8+ T cells from the peripheral blood of healthy donors with a panel of tumor-associated antigens. Among the tumor-associated antigens tested, only responses against Melan-A are found in donor 581. FastDC were generated from the monocytes of four CMV-seropositive donors and were separately transfected with IVT-mRNAs encoding panel antigens. CD8+ T cells (1,5 to 2 x 10^6) isolated from the donors' PBMC were stimulated on d0 and d7 with IVT-mRNA-transfected FastDC (2 x 10^5). CD8+ responders (3 x 10^4 / well) were tested on d12 for recognition of 293T cells (2 x 10^4 / well) co-transfected with plasmid pairs encoding the individual donor's HLA I alleles and the panel antigens in a 20h IFN-γ ELISPOT assay. Background against 293T cells transfected with the respective HLA I allele only was subtracted (background level was below 60 spots / 3 x 10^4 lymphocytes for donors 581 and UX, and below 30 spots / 3 x 10^4 lymphocytes for donors 748 and 459). * : > 2x above background (p-value < 0.05). nd : not determined.

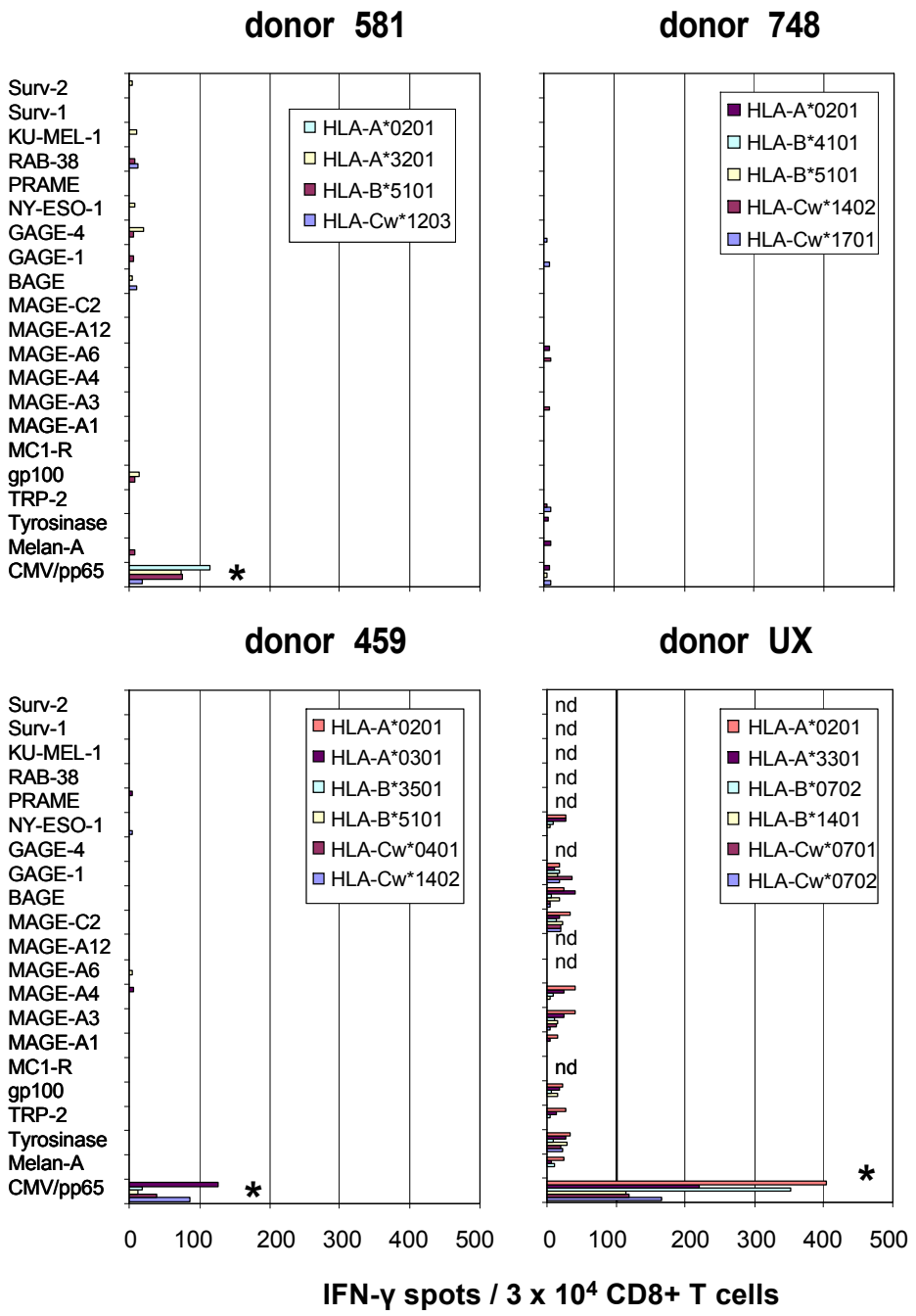

Figure 3.26 : Stimulation of CD8+ T cells from the peripheral blood of healthy donors with mock-transfected FastDC. Only responses against the viral control antigen CMV/pp65 are found in three out of four donors. FastDC were generated from the monocytes of four CMV-seropositive donors. CD8+ T cells (1,5 to 2 x 10^6) isolated from the donors' PBMC were stimulated on d0 and d7 with mock-transfected FastDC (2 x 10^5). CD8+ responders (3 x 10^4 / well) were tested on d12 for recognition of 293T cells (2 x 10^4 / well) co-transfected with plasmid pairs encoding the individual donor's HLA I alleles and the panel antigens in a IFN-γ ELISPOT assay. Background against 293T cells transfected with the respective HLA I allele only was subtracted (background level was below 70 spots / 3 x 10^4 lymphocytes for donors 581 and UX, and below 30 spots / 3 x 10^4 lymphocytes for donors 748 and 459). * : > 2x above background (p-value < 0.05). nd : not determined.

Conclusion chapter 3.4:

Blood lymphocytes from melanoma patients D05 and MZ2 were stimulated in parallel with autologous tumor cells and with FastDC transfected with IVT-mRNAs encoding antigens previously identified as targets of anti-melanoma cytolytic T cells with screening procedures. In D05, T cell reactivities against three out of eleven epitopes induced by stimulation with tumor cells were also found after stimulation with mRNA-transfected FastDC. Two further T cell target epitopes were identified with mRNA, but not with tumor cell stimulation. In MZ2, T cell responses against five distinct epitopes were detected on day 12 after stimulation with mRNA transfectants. The same responses were detectable after stimulation with tumor cells only on day 32. With the exception of Melan-A, no response against the previously targeted antigens could be stimulated in the four HLA-A2-positive healthy donors tested.

4 DISCUSSION

Until now, a large proportion of vaccination studies aiming at boosting cancer patients' T cells against defined tumor antigens rely on the vaccination with antigenic peptides (Parmiani, 2002; Rosenberg, 2004). But, as emphasized in the Introduction, many individual parameters will influence a patient's ability to respond to a given peptide antigen. Parameters varying among individuals include the HLA phenotype, which determines, if peptides can be presented by the individual HLA molecules, or the T cell repertoire, which defines the pool of T cell receptors (TCR) potentially available to recognize peptide-HLA complexes. Other parameters, inherent to individual tumors, such as pre-existing or acquired antigen expression patterns and immune escape mechanisms will influence the ability of antigen-specific T cells induced by the vaccine to effectively attack the tumor. Until now, beside the individual HLA type, the T cell repertoire has been only rarely considered as a selection criterion before vaccinating patients with a specific peptide (Tanaka, 2003). After vaccination with the MAGE-A3 antigen, Lurquin et al. found that the anti-tumor responses in a patient with regressive disease were mostly targeted against MAGE-C2 and gp100 rather than against the vaccination antigen (Lurquin, 2005; Germeau, 2005). Thus, it may be more successful to vaccinate with antigens, which have been identified as targets of the individual T cell repertoire *previous* to vaccination, than with antigens which are not *a priori* targeted by the individual anti-tumor T cell repertoire.

Since the first human tumor antigen recognized by autologous T cells was discovered (Van der Bruggen, 1991), over 250 peptides derived from ca. 70 differentiation, overexpressed or shared antigens, and at least relevant to melanoma, have been identified (http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm). The lists of new antigens and peptides derived thereof are steadily increasing. Considering the low rate of objective clinical responses observed in vaccination trials and the ever growing list of potential candidate antigens available, vaccination of patients should only occur with antigens previously selected among the list of candidates. Selection criteria should include the ability of the patient's T cell repertoire to respond to the antigen.

The goal of the present work was to establish a method, with which antigens against which an individual patient can readily mount a CD8+ T cell response can be determined in vitro, using limited patient blood material and a short-term stimulation protocol. It was important that this protocol did not depend on the availability of autologous tumor material. The analysis was not restricted to specific peptides, but rather involved whole antigens. It was designed to cover the complete HLA class I spectrum of the patient and to potentially

detect responses to unknown peptides presented by rare HLA class I alleles. This is important, as most of the peptides presently known are restricted by frequent HLA alleles.

4.1 Nature of stimulators

The efficient stimulation of antigen-specific CD8+ T cells largely depends on the nature of the antigen-presenting cells (APC) used as stimulators. Among others, their nature and differentiation state should enable:

- their efficient transfection with the whole antigen,
- an efficient co-stimulation of antigen-specific cells, and
- a proteasomal constitution resembling that of tumor cells.

4.1.1 Transfection of DC with TransMessenger™ versus electroporation

In 2001, Van Tendeloo et al. demonstrated that electroporation of DC with clonal mRNA was very efficient, with transfection efficiencies reaching 80-90%, while electroporation with DNA transfected up to 4-5% of cells. Later, it was shown that these transfected DC could efficiently prime and expand antigen-specific T cells in vitro (Saeboe-Larssen, 2002; Kalady, 2002; Tuyaerts, 2003). Since, electroporation with RNA has become a preferred method to load DC with whole antigen (reviewed in Gilboa, 2004). Still, this method is hampered by some drawbacks inherent to the physical nature of the transfection process, which relies on the transient desintegration of the plasma membrane by high intensity electric fields (Neumann, 1982). First, the yield of viable cells after electroporation is low, which can be partially overcome by applying high cell concentrations to electroporation (Canatella, 2001). Commonly 2-10 x 10^6 DC are applied per electroporation attempt. Second, rather high RNA quantities (20-50 µg) are necessary to efficiently transfect such high cell numbers. These aspects clearly limit the use of electroporation for the transfection of DC with a large panel of antigen-coding mRNAs.

The use of mRNA-transfected DC as stimulators in panel testing required an alternative transfection modality. Lipofection technique, which commonly involves much lower starting cell quantitites still maintaining viability, appeared suitable. Among all lipid-based transfection reagents tested, only TransMessenger™ appeared to efficiently transfect DC in a reproducible way. In comparative experiments on DC populations, the transfection efficiencies obtained with electroporation reached 90-95% of transfected cells, whereas efficiencies obtained with TransMessenger™ only reached 20-40%. But the expression level on a per cell basis (measured as the Mean Fluorescence Intensity, MFI) was regularly 2-3 times higher with TransMessenger™ than with electroporation (**Figure 3.9**). In stimulation assays, although electroporated DC revealed as the most efficient stimulators among the different APC used, DC transfected with TransMessenger™ proved to be able to efficiently

stimulate antigen-specific CD8+ T cells (**Figure 3.6**). Interestingly, it has been reported recently that DC transfected with TransMessenger™ induced stronger immune response and protection in an in vivo mouse model of hepatitis than electroporated DC (Yu, 2007). Thus, the proportion of cells expressing the antigen may not be the unique parameter influencing the stimulation of antigen-specific T cells. The antigen expression level on a per cell basis may be equally important (Wherry, 1999). Another criterion which might be of importance in this context is the persistence of the antigen at the surface of APC (Wong, 2004). Although peptide/HLA complexes have been shown to persist beyond extinction of antigen expression (Porgador, 1997), the protein expression duration logically influences the duration of peptide presentation (Princiotta, 2003; Holtkamp, 2006). Whereas DC transfected with TransMessenger™ remained viable for up to 72h after transfection and still efficiently expressed the transgene, the viability of the electroporated population strongly decreased after 48h and the transgene expression was gradually lost (**Figures 3.8** and **3.10**). All in all, with TransMessenger™, the lower transfection efficiency may be counterbalanced by the higher expression levels obtained in these cells, together with the prolonged viability and transgene expression.

Beside these functional considerations, practical advantages made of TransMessenger™ the method of choice to transfect DC with RNA. Although 2-10 x 10^6 DC are currently electroporated in one shot with one antigen-coding mRNA (Van Tendeloo, 2001; Britten, 2004; Schaft, 2005), only 2 x 10^5 DC are transfected using TransMessenger™. This considerably decreases the total need of patients' DC when these have to be transfected separately with a high number of different antigens. This low number of cells per transfection is also linked with reduced RNA amounts needed. Whereas 20-50 µg of RNA are often needed to obtain high transfection efficiencies with electroporation, high RNA amounts do not improve the transfection efficiency with TransMessenger™. 0,4-0,8 µg RNA were needed to transfect 2 x 10^5 DC with maximum efficiency (20-40%), using optimized transfection parameters (**Figure 3.7**). At last, only 10-20% cell loss was observed during transfection with TransMessenger™. In comparison, 2-4 more cells are required to obtain similar numbers of remaining cells after electroporation. Comparative figures for both procedures are given in **Table 4.1** and underline the practicality of the TransMessenger™ technique to transfect patients' DC with a large panel of antigen-coding mRNAs.

Table 4.1. Characteristics of electroporation versus the application of TransMessenger™ for the transfection of FastDC. Transfection with TransMessenger is more applicable than electroporation to transfect patients' FastDC with a large panel of antigen-coding mRNAs.

	Electroporation	**TransMessenger™**
Number of cells per transfection	2-10 x 10^6	0,2-0,4 x 10^5
mRNA amount per transfection	20-50 µg	0,4-0,8 µg
Yield of recovered cells	25-50 %	80-100 %

4.1.2 Maturation of DC before or after transfection

Immature DC have been successfully used as APC in ELISPOT assays (Herr, 1999; Britten, 2004) and have been shown to induce antigen-specific CTL expansion after transfection with antigen-coding mRNA (Strobel, 2000; Ueno, 2004; Saeboe-Larssen, 2002; Tuyaerts, 2003). Nevertheless, they are considered as weakly immunogenic or even tolerogenic, especially in vivo (Steinman, 2003; Dhodapkar, 2002; Roncarolo, 2001). It has been suggested to transfect immature DC and then mature them for stimulation of antigen-specific T cells (Van Tendeloo, 2001; Britten, 2004; Kalady, 2002). But for practical reasons, RNA transfection after maturation is preferable for routine production. It would avoid to harvest immature DC, transfecting them and putting them back into culture again for maturation and final harvest. In addition, immature DC are adherent, which makes cell handling more difficult. At last, during the 24-48h maturation step, antigen presentation may decrease as RNA expression kinetics is highly variable and in some cases transfected mRNA is undetectable already after 6 hours (Schaft, 2005).

One rationale to transfect DC in the immature state is that electroporation of mature monocyte-derived DC (Mo-DC) was reported to be inferior with respect to transfection efficiency and viable DC yield (Van Tendeloo, 2001). But later, it was described that immature and mature DC can be electroporated with equally high yield and transfection efficiency using appropriate parameters (Schaft, 2005). In the same study, DC transfected after rather than before maturation appeared immunologically superior with respect to CTL induction. Using TransMessenger™, higher transfection efficiencies were even obtained in mature than in immature DC (ca. 40% versus 20%).

Furthermore, it is of concern that DC transfected following maturation will only present epitopes generated by the immunoproteasome, but not the standard proteasome-dependent epitopes, as it was described for the Melan-A_{26-35} peptide (Morel, 2000). Blood lymphocytes from 9 HLA-A2 positive healthy donors were stimulated with mature FastDC transfected with Melan-A/MART-1 mRNA after maturation. CD8+ T cell responses against MelanA/HLA-A*0201 were detected in 5 donors (**Figure 3.17 A**). These responses could not be expanded after stimulation with mock-transfected FastDC. This is in accordance with the frequency, at

which anti-Melan-A reactivity was detected among HLA-A2-positive healthy donors by Pittet et al. using the tetramer technology (Pittet, 1999). The responses detected after RNA stimulation exclusively targeted the Melan-A_{26-35} peptide (**Figure 3.17 B**). Although it was not investigated, if and to what extend mature FastDC express the immunoproteasome, these results indicate, that they still produce the Melan-A_{26-35} peptide in quantities sufficient for the stimulation of specific T cells. The shortened maturation step used in the FastDC protocol may alter the proteasome composition less than during the generation of classical mature DC.

With respect to the aspects considered above, FastDC were systematically transfected after maturation for all stimulation experiments.

4.1.3 FastDC versus CD40-activated B cells

CD40-activated B cells have been proposed as alternative APC in immunotherapy trials (Schultze, 1997; von Bergwelt-Baildon, 2002; Coughlin, 2004). They can be expanded from small amounts of B cell precursors within a few weeks of stimulation with the CD40 ligand (Schulze, 1997), whereas DC do not expand in culture (Steinman, 2001). Peptide-loaded CD40-activated B cells were reported as effective as DC in the induction of autologous antigen-specific T cell responses against both viral and tumor-associated antigens in vitro (Schultze, 1997; von Bergwelt-Baildon, 2002). In our hands, CD40-activated B cells electroporated with IVT-mRNA were not as efficient as FastDC in stimulating pp65-specific CD8+ T cell responses (**Figure 3.6**). This could not only be explained by differences in the transfection efficiency. While 32% of CD40-activated B cells were successfully transfected in this experiment, only 16% of FastDC transfected with TransMessenger™ expressed the transgene and still revealed to be stronger stimulators.

To compare CD40-activated B cells with FastDC with respect to their antigen-presenting capacity, we transfected both cell types with IVT-mRNA encoding several tumor-associated antigens and tested them for recognition by CTL clones or MLTC responders, respectively. Among 7 antigens that were recognized when transfected into FastDC, only one induced significant recognition of CD40-activated B cells by the T cells applied in this experiment. Beside the lower antigenic expression level at single cell level, CD40-activated B cells may display a distinct proteasome composition than freshly isolated B cells, in relation with their activation stage and their generation time in vitro (Frisan, 2000).

With regard to the lower presentation capacity and stimulatory potential of CD40-activated B cells compared to FastDC, the latter were chosen as standard APC for all stimulation assays.

4.2 Analysis of preferentially targeted antigens in melanoma models D05 and MZ2

Detailed analysis of the anti-tumor T cell repertoire were performed in a number of patients. They suggested a high degree of individuality (Coulie, 1999; Lennerz, 2005). As an example, Germeau et al. found that the spontaneous responses against tumor antigens were dominated by CTL recognizing different epitopes of the MAGE-C2 antigen (Germeau, 2005). No response was observed in this patient against antigens encoded by other cancer-germline genes, even though these were expressed by the tumor at a similar level as MAGE-C2. This clearly supports the notion that, aside from the antigen expression pattern of the individual tumor, the individual T cell repertoire strongly influences the nature of the target antigens, against which patients preferentially respond.

We applied the procedure described herein to two melanoma patients to determine the tumor-associated antigens preferentially targeted by their individual T cell repertoire.

4.2.1 Responses detected in model D05

In the model D05, 17 peptides derived from 10 antigens and presented by different HLA molecules had been identified as targets of the anti-tumor response, using standard screening procedures with previously generated tumor-reactive MLTC responders and CTL clones (**Figure 3.19 A**). We made use of this extensive analysis as a reference to evaluate the stimulation procedure. Bulk PBL were used as starting effector cells and stimulated twice with autologous FastDC separately transfected with the corresponding IVT-mRNAs. Among 16 expected responses against the 10 antigens and restricted by the different HLA molecules of the patient, 5 responses were detected with mRNA-transfected FastDC, whereas 11 responses were detected after stimulation with the tumor cells (**Table 4.2**). Importantly, a response against MAGE-C2 could be efficiently stimulated with mRNA-transfected FastDC, whereas this response was not detected after stimulation with the tumor cells. Neither had this antigen been identified using screening procedures. In fact, it could be confirmed by PCR analysis that the tumor cell line used for the stimulation of PBL and for generation of the CTL clones used to screen the patient's melanoma cDNA bank had lost MAGE-C2 expression (personal communication from P. van der Bruggen).

These results show that, although autologous tumor cells revealed to be more efficient in vitro stimulators in this case, autologous mRNA-transfected FastDC also permitted to stimulate CD8+ T cell responses that were not detected ex vivo.

Table 4.2 : CD8+ T cell responses identified in melanoma model D05 using expression screening, tumor stimulation or mRNA stimulation. Tumor antigens targeted by CD8+ T cells are indicated with the corresponding HLA restriction. 5 out of 16 responses determined with screening procedures (screening) are detected after mRNA stimulation (mRNA stim), versus 11 out of 16 after stimulation with tumor cells (tum stim). PBLs were stimulated twice with mRNA-transfected FastDC or with autologous tumor cells. Responder lymphocytes were tested on d7+5 in a 20h IFN-γ assay. + : ≥ 2x above background. ++ : ≥ 3x above background ($p<0.05$).

D05	screening	tum stim	mRNA stim
Melan-A /A*0201	+	++	-
Tyrosinase /A*0201	+	-	-
Tyrosinase/Cw*0501	+	+	-
TRP-2 /A*0201	+	-	-
MAGE-A1 /B*4402	+	++	-
MAGE-A1 /Cw*0501	+	++	-
MAGE-A3 /Cw*0202	+	++	++
MAGE-A4 /B*2705	+	++	-
MAGE-A4 /B*4402	+	++	+
MAGE-A4 /Cw*0501	+	++	-
MAGE-C2 /A*0201	-	-	++
BAGE-1 /B*4402	+	-	-
GAGE-1 /B*2705	+	-	+
NY-ESO-1 /A*0201	+	++	-
NY-ESO-1/Cw*0501	+	-	++
CCT6A^{mut} /B*2705	+	++	++
CCT6A^{mut}/Cw*0501	+	++	-
∑ 17	16/17	11/16	6/16

4.2.2 Responses detected in model MZ2

In patient MZ2, stimulation of isolated CD8+ T cells with mRNA-transfected FastDC led to the detection of 4 out of 8 responses expected from extensive screening analyses in this model system, whereas only one response was stimulated with the tumor cells, as measured on d7+5 (**Figure 3.19 B** and **Table 4.3**). Furthermore, a response against BAGE-1 in association with HLA-B*4403 was found with mRNA-transfected FastDC but had not been identified before. These five responses could be confirmed with the tumor cells only after 3 further stimulations.

Table 4.3 : CD8+ T cell responses identified in melanoma model MZ2 using expression screening, tumor stimulation or mRNA stimulation. Tumor antigens targeted by CD8+ T cells are indicated with the corresponding HLA restriction. 4 out of 8 responses determined with screening procedures (screening) were detected after mRNA stimulation (mRNA stim) on d7+5. One response was new. All responses were detected after stimulation with tumor cells (tum stim) only on d28+4. PBLs were stimulated twice with mRNA-transfected FastDC or with autologous tumor cells. Responder lymphocytes were tested on d7+5 or d28+4 in a 20h IFN-γ assay. + : ≥ 2x above background. ++ : ≥ 3x above background ($p<0.05$).

MZ2	screening	tum stim (d7+5)	tum stim (d28+4)	mRNA stim (d7+5)
MAGE-A1 /A*0101	+	-	++	+
MAGE-A1 /Cw*1601	+	++	++	+
MAGE-A3 /A*0101	+	-	-	-
MAGE-A6 /Cw*1601	+	-	-	-
BAGE-1 /B*4403	-	-	++	++
BAGE-1 /Cw*1601	+	-	+	++
GAGE-1 /Cw*0602	+	-	+	+
GAGE-4 /A*2901	+	-	-	-
Tyrosinase /B*4403	+	-	-	-
∑ 9	8/9	1/9	5/9	5/9

4.2.3 Stimulation with mRNA-transfected FastDC versus tumor cells

The results obtained in MZ2 suggest that, when sufficient CD8+ precursors can be stimulated, mRNA-transfected FastDC are superior to tumor cells to stimulate antigen-specific responses. One reason might be that mRNA-transfected FastDC at least transciently express higher protein levels of the antigen. The corresponding higher concentration of HLA-peptide complexes at the cell surface might lead to a stronger expansion of antigen-specific cells. In contrast, the concentration of HLA-peptide complexes on the surface of tumor cells might not be sufficient to induce a rapid and strong expansion of the same cells. Instead, further rounds of stimulation might be necessary to progressively expand the precursors to a frequency reached with mRNA-transfected FastDC already after two stimulations (Wherry, 1999). Another reason for this rapid expansion of CD8+ precursors might be the higher expression of co-stimulatory molecules and induction of activating signals by mRNA-transfected FastDC than by the tumor cells.

We wondered why transfected FastDC were not so efficient as tumor cells in expanding antigen-specific CD8+ T cells in model D05. At first, a limited number of FastDC will express the antigen (20-40%) while all tumor cells express it naturally. This might play an important role in this case where PBL instead of isolated CD8+ T cells were stimulated, as

for those few antigen-specific CD8+ T cell among the PBL stimulated with transfected FastDC, the probability to encounter antigen-expressing APC is 2,5 to 5 times lower than for those stimulated with the tumor cells. Furthermore, when PBL instead of CD8+ T cells are stimulated, some CD8+ precursors, which frequency might be below 10^{-6} for some antigens (Boon, 2006), might be excluded from the pool of initial effectors. At last, the presence of CD4+CD25+ regulatory T cells (Tregs) among PBL may inhibit the expansion of antigen-specific CD8+ T cells in vitro (Levings, 2001; Jonuleit, 2001). In the model MZ2, stimulation of PBL led only to the detection of the strongest responses from those detected after stimulation of isolated CD8+ T cells (**Figure 3.24**). This supports the notion that isolated CD8+ T cells are more efficient effectors than bulk PBL for the stimulation of tumor-specific responders in vitro (Coulie, 1992). Another aspect which might influence the efficiency of transfected FastDC to stimulate antigen-specific cells is the shorter duration of antigen expression. Although we measured transgene expression still 72h after transfection of FastDC with IVT-mRNA (**Figure 3.8**), RNA is a labile antigenic format and protein half-lives may considerably vary among different antigens (Schaft, 2005). In contrast, tumor cells stably express the antigen. Nevertheless, strong and transient expression of the antigen may also advantage those T cells which might become anergic or exhausted after persistant contact with the antigen on tumor cells (Klebanoff, 2006). At last, expression of the immunoproteasome by FastDC may have impaired the processing and presentation of some peptides produced by the proteasome of tumor cells and recognized by T cells (see chapter 4.1.2).

4.3 Nature of responders

The results discussed above show that the responder populations definitely depend on the nature of the stimulators. In the following, we discuss more in depth about the nature of those responders that may be stimulated with our stimulation conditions.

4.3.1 Different in vitro stimulation conditions may favour expansion of distinct CTL clones

Despite the fact that mRNA-transfected FastDC could efficiently stimulate antigen-specific CD8+ T cell responses, a significant proportion of responses expected from screening analyses were not detected. To explain this apparent discrepancy, one should consider the different approaches underlying the two methods. Screening procedures in both models were performed with MLTC responders and CTL clones via long-term stimulation of PBL with autologous tumor cells. This method largely selects for CTL clones able to expand with the applied culture conditions at high IL-2 concentrations. In contrast, the short-term stimulation with mRNA-transfected FastDC preferentially expands those precursors which

rapidly proliferate in the presence of the respective antigen and at rather low IL-2 concentration. Although it would be an extensive and, for our goal, dispensable work to analyse which populations are preferentially stimulated under these conditions, it becomes largely apparent that different CD8+ populations might be preferentially stimulated and expanded with one or the other method. Thus, it is not surprising that CTL clones which might have been selected under long term cloning conditions might not expand under short-term stimulation with mRNA-transfected FastDC. In the other way round, the response toward BAGE-1 and restricted by HLA-B*4403 in MZ2 had not been detected with screening methods. The response toward MAGE-C2 in D05 was also only discovered with stimulation using mRNA-transfected FastDC but, as discussed above, this was attributed to a loss of antigen expression by the tumor clone. Furthermore, it has to be noticed that, as the studies of D05 and MZ2 were limited to antigens previously identified with screening procedures, we may have failed to notice responses against other antigens.

4.3.2 Ex vivo frequencies of antigen-specific cells and number of stimulations

4.3.2.1 Spontaneous responses and frequencies of antigen-specific T cells

Although T cell frequencies against the Melan-A/MART-1 antigen as high as 0,1% of blood CD8+ T cells have been documented in some advanced cases of melanoma, this might be an exception, due to the high number of anti-Melan-A T cells naturally occurring in HLA-A2-positive healthy persons (Romero, 2002; Dutoit, 2002). For most of the spontaneous responses that can be detected in advanced melanoma, precursor frequencies are much lower, still after a first amplification which might have been induced by the tumor. Detailled analysis of the blood of one melanoma patient before vaccination revealed that 4×10^{-4} of CD8+ T cells were directed against a NY-ESO-1 peptide (Valmori, 2000B). In another patient, 2×10^{-6} to 4×10^{-5} blood CD8+ T cells recognized MAGE-C2 (Germeau, 2005), which is 10-100 times higher than the background level of T cells of 4×10^{-7} against MAGE-encoded antigens reported in normal donors (Chaux, 1998). Some spontaneous responses against MAGE-A3 (Lonchay, 2004; Hanagiri, 2006) have been documented in a very small proportion of metastatic melanoma patients, with frequencies probably in the order of 10^{-7} (Lurquin, 2005). Differentiation antigens other than Melan-A appear to induce spontaneous CTL responses much less often. In the model analyzed by Germeau et al., CTL precursors directed against gp100 could be detected before vaccination at frequencies below 7×10^{-7} (Germeau, 2005; Lurquin, 2005).

4.3.2.2 Rationale to amplify antigen-specific T cells

We assume that in cases of advanced melanoma, spontaneous responses against antigens expressed by the tumor do exist at levels differing among antigens and among patients, but lead to T cell frequencies still below the detection limits available with current detection methods. The very low frequencies mentioned above could only be determined using labor intensive methods based on mixed lymphocyte-peptide microcultures performed by stimulating tetramer-positive cells under limiting dilution conditions. Such an approach involves long term stimulation of lymphocytes and analysis of a high number of CTL clones. Frequencies that can still be detected among ex vivo CD8+ T cells using tetramer or ELISPOT technology are rather in the order of 10^{-4} (Letsch, 2000; Lonchay, 2004). In other cases where the disease might not have yet spread from the initial tumor and invaded peripheral lymph nodes, the first round of amplification of rare naïve antigen-specific T cells by the tumor might not have yet occurred. The results obtained with Melan-A in normal donors indicate that these naïve cells can be in principle amplified under the stimulation conditions, as reactivities toward Melan-A/MART-1 could be stimulated in normal donors which have been shown to bear exclusively Melan-A precursors with a naïve $CCR7^{+}CD45RA^{hi}$ phenotype (Pittet, 1999; Romero, 2002). Nevertheless, this definition of the naïve phenotype has been recently challenged, as some memory cells have been shown to re-express CCR7 (Schwendemann, 2005) or CD45RA (Carrasco, 2006). On the other hand, we assume that apart the case of Melan-A/MART-1, the starting frequencies of other antigen-specific naïve precursors might be too low (probably below 10^{-7}) to be expanded to such an extent that they can be detected after two stimulations. Otherwise, we would have probably found reactivities against other tumor antigens in the four normal persons we analyzed (**Figure 3.25**). In all the cases, it appears necessary to submit those precursors to in vitro stimulation to either prime naïve cells that have not yet be activated by the tumor and further expand them or/and to expand those experienced responders to frequencies that become detectable with available methods. Beside this requirement to increase starting T cell frequencies, in vitro stimulation has also an additional role. Resting memory and naïve CD8+ T cells, when tested ex vivo, would not be able to secret detectable amounts of IFN-γ during the 20h of antigen contact applied in the ELISPOT assay. In vitro stimulation enables them to acquire or re-acquire effector functions which can be further detected in the assay five days later.

4.3.2.3 Extent of amplifications obtained with mRNA-stimulation

Although CTL precursors have been seen to multiply by several hundredfold during in vitro restimulation with peptide and optimal cytokine environment (Lonchay, 2004; Lurquin, 2005), considerable differences may exist in the proliferation capacity between different

precursors and their differentiation stage. In the case of tumor-specific T cells, memory cells that have been chronically exposed to antigen in vivo may not as easily expand as naïve cells, due to an "exhausted" phenotype (Klebanoff, 2006). Furthermore, a selection of cells by the culture conditions might exist (see chapter 4.3.1). Although we did not analyze the proliferation of antigen-specific cells at single cell level during the stimulation, we obtained up to several hundred-fold increases in the antigen-specific populations after stimulation of pp65 or MVA/ORF#28 specific cells (**Tables 3.1 and 3.3**). Peggs et al. reported expansions of up to 40-fold in CD8+ T cell populations directed toward a single peptide-MHC combination after stimulation with CMV antigen pulsed autologous DC (Peggs, 2002). In the case of Melan-A/MART-1, we assume that, for some patients, the amplification was higher than 100, as we could not detect any reactivity ex vivo in an ELISPOT assay (**Figure 3.18**). Indeed, although the precursor frequency was below 10^{-4} CD8+ T cells, in accordance with the reported frequency of 5×10^{-5} in normal donors (Romero, 2002), frequencies of antigen-specific CD8+ T cells ranged from 0,04% to 0,8% after two stimulations (**Figure 3.17**). This was also the case for most of the reactivities we found in the melanoma models MZ2 and D05 and which had not been detected ex vivo (**Figures 3.19, 3.20** and **3.22**). According to our previous considerations, we do not exclude that these strong amplifications might be exclusively due to the expansion of one or very few CTL clones selected on their differentiation stage and proliferation capacities by the culture conditions. The results obtained in HLA-A*0201 and B*0702 CMV seropositive donors showed that an additional competition may exist between CTLs targeting different epitopes of a same antigen. CD8+ T cells targeting the immunodominant epitope $pp65_{417\text{-}426}$ restricted by HLA-B*0702 (Lacey, 2003) were expanded more efficiently than those targeting the $pp65_{495\text{-}503}$ epitope restricted by HLA-A*0201 (**Table 3.1**). A similar effect had already been reported for the immunodominant epitope of ovalbumin in a transgenic mouse model (Kedl, 2000). This competition might be due to the recognition and lysis of the APC preferentially by the $pp65_{417\text{-}426}$-specific CTLs, predominant in the culture. Thus, APC become depleted at the expense of scarce $pp65_{495\text{-}503}$ specific CTLs. Alternatively, at APC level, the immunodominant epitope might be processed more efficiently than other epitopes from the same antigen, and the higher peptide concentration at the cell surface might induce a more effective proliferation of specific T cells. Nevertheless, the competition between different responder lymphocytes and the selection of CTLs targeting particular epitopes among a stimulated population should not impair our capacity to detect reactivities against a given antigen.

4.3.2.4 Amplification of T cells targeting viral versus tumor antigens

Although a single stimulation was mostly sufficient to obtain strong amplifications of antigen-specific cells in viral models, as in those case memory cells might be rapidly

expanded after antigen re-challenge (Wherry, 2003), the situation might be different with tumor antigens. On the one hand, as already evoked, precursors might still not have been primed in non-advanced melanoma and still have a naïve phenotype. Their frequency is correspondingly very low (see chapter 4.3.2.2), and their capacity to respond rapidly to the antigen limited. On the other hand, antigen-specific cells with a memory phenotype might be already "exhausted" due to a chronic stimulation by the tumor (Klebanoff, 2006). Considering these aspects, stimulations with tumor antigens were systematically performed twice. Further rounds of stimulation may not be advantageous, as some reactivities among CD8+ T cells may fluctuate, even disappear in the course of long term cultures (Lennerz, 2005). Correspondingly, a response against MAGE-A1, which could be detected in MZ2 after 2 stimulations with tumor cells, was not detected anymore after a further round of stimulation (**Figure 3.24 c**). This might be due to different expansion capacities among clones. Furthermore, CD8+ T cells don't expand indefinitely and, at one point, may rather become anergic, or undergo activation-induced cell death (Moskophidis, 1993; Zajac, 1998; Gallimore, 1998; Voehringer, 2001). T cell activation is indeed accompanied by the induction of CTLA-4 one day later (Korman, 2006). Its ligation to CD80 or CD86 on the APC leads to cell cycle arrest in activated lymphocytes and to the delivery of inhibitory signals to T cells. It has also been shown recently that in conditions of chronic exposure to antigen, PD-1 engagement on activated T cells by CD80 can severely inhibit T lymphocytes function (Sharpe, 2007). Still, it is not clear if, or to which extent these inhibitory signals are involved in vitro.

4.3.3 Antigen dependency and influence of cytokines

As negative controls, stimulations with mock-transfected FastDC in the presence of IL-2 (25 IU/ml) were always performed in parallel to stimulations with antigens. None of the populations stimulated with mock-transfected FastDC displayed reactivity against any of the tumor antigens tested. This indicated that the proliferation of antigen-specific CD8+ T cells was antigen-dependent, and that the sole presence of IL-2 was not sufficient to promote their expansion. But, in three CMV-seropositive donors, pp65-specific responses were stimulated with mock-transfected FastDC (**Figure 3.26**). During an acute infection or reactivation of CMV, the virus usually disseminates to virtually every organ in the host. Monocytes are thought to constitute potential vehicles to host organs (Waldman, 1995; Sinclair, 1996; Smith, 2004) although they are abortively infected and do not express pp65 (Grefte, 1994). Nevertheless, it was shown that transendothelial migration of monocytes induces their differentiation into macrophages which are productive for viral replication (Smith, 2004). In this context, differentiation of monocytes into FastDC may also lead to cells permissive for viral replication. Thus, FastDC may have in this case naturally expressed pp65 and

processed it via the HLA class I pathway. A second explanation would be that a subset of antigen-independent memory T cells was stimulated (Kaech, 2002; Wherry, 2003; Kaech, 2003). These antigen-independent memory CD8+ T cells have been shown not to develop during chronic viral infection (Wherry, 2004), a condition often compared to the tumor-bearing state with respect to memory T cell differentiation (Klenerman, 2005; Klebanoff, 2006). This would explain why we did not detect any reactivity toward the tumor antigens tested among the mock-stimulated populations generated from patients' lymphocytes (**Figures 3.20 m and 3.22 h**).

Interestingly, we could also stimulate an antigen-specific response toward pp65 with mock-transfected FastDC when IL-4, IL-7 and IL-15 were simultaneously present, but not when only one of these cytokines was absent. Under these conditions, stimulation was as efficient as with mRNA-transfected FastDC (**Figure 3.12**). As discussed above, these stimulation conditions most probably promoted the expansion of antigen-independent memory T cells, the homeostatic proliferation of which is driven by IL-7 and IL-15 (Kaech, 2002; Wherry, 2003; Kaech, 2003). Unexpectedly, this effect required the presence of IL-4, although IL-4 has been shown to promote survival and canonical differentiation of CD8+ T cells activated in vitro (Vella, 1998; Seder, 1992). Of note, as not only pp65-specific memory T cells, but also other memory T cells might have been stimulated, a smaller proportion of pp65-specific cells was present after stimulation compared to the stimulation with antigen and IL-2 only, which had led to the maximum expansion of pp65-specific cells (**Figure 3.12**). As some memory T cell clones are found to have extensively proliferated in vivo after a CMV infection and can be detected at high frequencies in the blood of seropositive donors (Weekes, 1999; Peggs, 2002), CMV-specific memory T cells may nevertheless constitute an important part of the whole memory pool.

None of the above mentioned cytokines improved the expansion of pp65-specific cells compared to IL-2 alone after stimulation with pp65-transfected FastDC (**Figure 3.12**). Concerning IL-15, this was quite surprising, because it is required to support homeostatic turnover and maintenance of memory CD8+ T cells (Zhang, 1998; Schluns, 2002), but this effect has already been reported (Jackson, 2004). In contrast, IL-7 is necessary for optimal memory generation following infection, but is dispensable for CD8+ T cell activation and expansion (Schluns, 2000). Furthermore, IL-7 is required for survival and homeostatic proliferation of naïve T cells (Tan, 2001). Thus, its absence may not strongly impair the proliferation of pp65-specific T cells which are most probably of the memory phenotype in CMV seropositive donors (Weekes, 1999). Otherwise, we should have been able to prime pp65-specific responses in CMV negative donors, which was not the case (**Table 3.2**). In contrast to IL-4, IL-7, or IL-15, IL-2 proved to be both sufficient and necessary to efficiently stimulate CD8+ T cells in the presence of their specific antigen (**Figure 3.11**). This is in line

with the fact that IL-2 drives the clonal expansion of T cells after TCR ligation of peptide-MHC complexes (reviewed in Schluns, 2003; Ma, 2006). Interestingly, high IL-2 concentrations did not improve expansion of antigen-specific cells (**Figure 3.11**). This might be explained by the ability of IL-2 to also induce death of memory CD8+ T cells after clonal expansion (Lenardo, 1991; Refaeli, 1998; Ku, 2000).

4.4 Implications for vaccination strategies

The stimulation procedure described herein should permit to identify tumor antigens, against which the individual T cell repertoire of tumor patients may preferentially respond. One direct consequence of this analysis would be the vaccination of tumor patients with one or several of these preferentially recognized antigens. At this point, it has to be noted that the T cell populations that expand in vitro depend largely on the stimulation conditions (see chapter 4.2.3), including at first place the nature of the stimulators used. A fortiori, more profound differences may arise between populations that become stimulated in vitro with mRNA-transfected FastDC and CTL that become activated in vivo upon vaccination with the antigen.

4.4.1 The populations stimulated in vitro depend largely on the nature of the stimulators

While stimulation of MZ2-CD8+ T cells with mRNA-transfected FastDC disclosed responses against 3 different antigens, with the strongest responses directed against BAGE, stimulation with the tumor cells amplified only one of the responses against MAGE-A1 on d7+5 (**Table 4.3**). This could be explained by the different expression levels of a single antigen by transfected FastDC or by the tumor cells respectively (see chapter 4.2.3). FastDC transfected with a single antigen potentially express the transgene at a relative high level (**Figure 3.9**), whereas tumor cells may naturally express it at a much lower level. Furthermore, tumor cells also express other antigens, which may induce the proliferation of competitive antigen-specific T cells. Thus mRNA-transfected FastDC, which present a high concentration of HLA-peptide complexes at their surface, may recruit a larger panel of peptide-specific TCR, including those of low avidity, which may not, or to a lower extent, recognize a tumor cell presenting relatively lower concentrations of HLA-peptide complexes. This might explain why, occasionally, CD8+ T cells stimulated with mRNA-transfected FastDC recognized the stimulation antigen on readout 293T cells transfected with the antigen and the respective presenting HLA allele, but not the autologous tumor line (**Figures 3.20 j, 3.22 e, f**). This would also explain why most of the responses detected in MZ2 after stimulation with mRNA-transfected FastDC were detected with the tumor only after 3 further stimulations (see chapter 4.2.3). Tumor cells, on their side, may preferentially stimulate those

CTL with a higher avidity that are less dependent on co-stimulatory signals. Nevertheless, this clearly shows that different CTL specificities may be expanded in vitro depending on the stimulators used.

4.4.2 In vivo versus in vitro responders

A fortiori, in vitro responders may not match with responders expanding in vivo after vaccination. First, different responders may be recruited upon vaccination as a consequence of the application route, the dose of antigen and the nature of the adjuvants eventually applied. Secondly, independently from the mode of vaccination, different responders have been shown to proliferate in response to a tumor cell-line in vitro, compared to the in vivo situation (Ferradini, 1993; Mackensen, 1993; Zorn, 1999). Further, responders that have proliferated in the blood upon vaccination may be rendered ineffective in the tumor environment, although showing full effector functions in vitro (Gattinoni, 2005). This shows that, according to the vaccination modality, the priming conditions and the environment encountered by the vaccine responders, different subsets of cells might be induced to proliferate in vivo compared to the in vitro situation even when the same stimulatory antigens would be used.

Thus, one may argue that the antigens identified in vitro as preferential target antigens may not be relevant as vaccine candidates. In fact, the in vitro stimulation of CD8+ T cell populations specifically recognizing a tumor antigen only indicates that the T cell repertoire of the patient is able to respond to the antigen, as one or several CTL clones can recognize an HLA-peptide complex with sufficient affinity to be efficiently expanded and detected after two stimulations from a very limited number of lymphocytes. The statement that should be deduced, is that the patient can (but does not have to) mount an immune response to the antigen. We assume that some precursors may be refractory to our stimulation conditions. In this case, we may fail to notice the responses to their target antigens. But a response to a stimulation antigen unequivocally indicates that the antigen can be targeted by the patient's immune system. Furthermore, the goals of an in vitro procedure aiming at identifying preferentially targeted antigens, and of a vaccination with these antigens are fundamentally different. For the in vitro stimulation, it is crucial to recruit a large panel of antigen-specific T cells, which will expand enough to be efficiently detected after a short time. Some of these responders may be of low avidity and not even recognize autologous tumor cells. In contrast, the goal of a vaccination strategy might not be to induce large amounts of anti-vaccine T cells, but rather one or very few CTL with high avidity, which will be able to attack the tumor (see below).

4.4.3 Naïve versus memory responders

A few years ago, it was postulated by Boon and colleagues, that the anti-vaccine responders may not be the effector cells that lead to tumor regression in patients showing objective clinical responses to vaccinations (Lonchay, 2004; Boon, 2006). Instead, the anti-vaccine responders are proposed to be some rare highly effective naïve precursors, which are able to migrate to the tumor and resist the local immunosuppressive environment long enough to attack some tumor cells and focally reverse the immunosuppressive environment surrounding the tumor. This would provide conditions that enable a much larger number of antitumor T cells to be stimulated and to proliferate, leading to the destruction of the bulk of the tumor cells and to tumor regression. This hypothesis, although it emerged from the analysis of a very few patients with regressive disease (Lonchay, 2004; Germeau, 2005; Lurquin, 2005), implies that the outcome of a vaccination may depend more on the functional properties than on the number of those vaccine responders which then act as activators of further anti-tumor T cells.

As chronic exposure to the tumor may have led to the formation of “exhausted” or “corrupted” memory T cells (reviewed in Klebanoff, 2006), it may be more effective to target naïve cells still able to be strongly activated and perform the local reversion of the immunosuppressive environment described above.

Although we could detect responses against Melan-A/MART-1, an antigen which has been shown to be exclusively targeted by CD8+ T cells of the naïve phenotype in vivo (Pittet, 1999) (**Figure 3.17**), we do not think that this can commonly be achieved with other antigens, as the frequency of those naïve antigen-specific T cells might be too low to enable their detection after two stimulations (see chapter 4.3.2.2). This is supported by the fact that no response could be stimulated against other tumor antigens than Melan-A in normal persons (**Figure 3.25**). In contrast, antigen-experienced CD8+ T cells that have already proliferated in vivo will be found at higher frequency in the peripheral blood of patients, thus increasing the probability to stimulate them in vitro. These are the cells that were most probably stimulated in the CMV model, where reactivities could be already detected ex vivo (**Tables 3.1** and **3.2**). In tumor models, the higher incidence of antigen-experienced cells may be counterbalanced by a state of exhaustion and senescence which makes these cells less effective at proliferating in vivo. Nevertheless, this effect may be partially reversed in vitro (Gattinoni, 2005), as we could efficiently stimulate responses in both melanoma models D05 and MZ2 (**Tables 4.2** and **4.3**). Thus, although we do not exclude the possibility to stimulate naïve precursors, we assume that most of the responses detected against tumor antigens after stimulation are attributed to antigen-experienced cells that had already proliferated in vivo. This may constitute one limitation of this strategy, as most of the antigens that are found in

vitro to be preferentially targeted by the individual T cell repertoire may essentially be targeted by “corrupted” or “exhausted” memory cells in vivo. Nevertheless, as evoked above, the goal of a vaccination strategy might not be the induction of large numbers of anti-vaccine T cells, but rather of one or a few CTL of high avidity which will be able to induce an anti-tumor response. In this context, it may be sufficient to recruit one or very few naïve anti-vaccine CTL clones upon vaccination to obtain a clinical response.

4.5 Further improvements

Possible reasons were proposed to explain why some responses that could be stimulated with tumor cells in model D05 were not detected using mRNA-transfected FastDC (see chapter 4.2.3). They included the use of PBL instead of CD8+ T cells in the case of D05 on the one hand, and a limited transfection efficiency of FastDC on the other hand. Thus, the major modifications that would further increase the efficiency of the procedure rely on the improvement of the transfection efficiency of APC, and a reduction in the total CD8+ T cell need to enable their systematic use as starting effectors. In a lower extent, modifications of the stimulation environment by addition of new cytokines are also considered.

4.5.1 Modification of the transcript to enhance the antigen expression level

Several technical modifications of the antigen format have been described to improve the density and duration of peptide/MHC complexes at the surface of transfected APC. Such modifications include the use of an anti-reverse cap analog to enhance RNA stability and translational efficiency, or the addition of 3’ UTRs of the human ß-globin gene to the transcript which improves the protein level and persistence (Holtkamp, 2006). Addition of Ubiquitin to the translated product to force proteasomal targeting has also been shown to improve cell-surface presentation of Melan-A/MART-1 (Rasmussen, 2004). Such approaches to improve the antigen expression level have to be considered with caution, as this may not necessarily lead to an enhanced proliferation of antigen-specific cells. It has been demonstrated in vitro (Wherry, 1999) and in animal models that T cells are induced to die by AICD following high-intensity TCR signaling (Mixter, 1999), and increasing antigen concentration in DC/T cell cocultures results in a reduction of T cell proliferation (Peggs, 2001).

4.5.2 Improvement of the transfection efficiency

More than the expression level at single cell level, it might be more advantageous to improve the transfection efficiency of FastDC in a comparable extent to the values currently obtained with electroporation. Indeed, the non-transfected population may lead to the stimulation of antigen-unspecific CD8+ T cells, which may compete with and impede the

expansion of antigen-specific cells. Nevertheless, among the lipofection-based transfection methods tested herein, only TransMessenger™ induced transgene expression in a significant proportion of cells (see chapter 4.1.1). Physical transfection methods often permit to achieve higher efficiencies, but the drawbacks in relation with the high starting cells amount and the low yields of viable cells after transfection impede their use in the present procedure.

4.5.3 Integration of recently described cytokines

Although activation and proliferation of CD8+ T cells is in principle triggered by TCR signalling ("signal 1") and co-stimulatory signals mediated by CD28 ("signal 2"), cytokines ("signal 3") constitute an essential parameter in the full activation of T cells (Curtsinger, 2003A). A growing list of cytokines have been reported to play an important role in CD8+ T cell expansion and survival (see chapter 4.3.3). We already tested the effects of IL-4, IL-7, IL-15 without noticing any benefit to IL-2 alone. Still, it would be worthwhile to test some cytokines like IL-12 or IL-21, which positive effects have been recently described. IL-12 can promote the development of effector functions or survival (Curtsinger, 2003A; Curtsinger, 2003B), whereas IL-21 has been shown to augment expansion and anti-tumor function of cytotoxic T cells in association with IL-7 (Liu, 2007).

4.5.4 Co-stimulation of CD8+ T cells targeting peptides from distinct protein-antigens

Stimulating less than 1 x 10^6 starting CD8+ T cells with one antigen-mRNA may lead to miss precursors occurring at frequencies below 10^{-6}. As the number of potential tumor antigens is constantly increasing, the total number of CD8+ T cells available from one patient to screen all these antigens may constitute the main limiting factor of the procedure. An alternative to reducing the amount of starting CD8+ T cells per stimulation unit would be to pool FastDC separately transfected with different antigen-mRNAs, and co-stimulate CD8+ T cells with the corresponding antigen specificities.

It has been already mentioned, that a competition may exist between different CD8+ T cell populations targeting several peptides derived from one protein-antigen and presented by the same APC, due to the depletion of APC (see chapter 4.3.2.3). In the case of FastDC simultaneously transfected with different antigen-mRNAs, such a competition may occur between CD8+ T cell populations targeting peptides derived from distinct protein-antigens. Although a competition between CD8+ T cells targeting different peptides from one protein-antigen may not hinder the detection of a response against the antigen, the competition between T cells targeting distinct protein-antigens may hide responses against some of the antigens. In contrast, if APC are separately transfected with the different antigen-mRNAs and further pooled to stimulate CD8+ T cells, CD8+ T cell populations targeting different protein-

antigens should not recognize the same APC. Thus, a competition due to the depletion of APC could be avoided. Schaft at al. reported the efficient stimulation of distinct CTL clones by DC simultaneously transfected with up to three antigens in a 16h IFN-γ ELISPOT assay (Schaft, 2005), but the long term stimulatory potential of those transfected DC was only assessed for CD8+ T cells targeting one of the three antigens. Recently, Javorovic et al. observed a decrease of 30% in the stimulatory capacity of DC simultaneously transfected with 3 antigen-mRNAs compared to DC transfected with a single antigen, and a decrease of 60% in the stimulatory capacity after transfection with 6 antigen-mRNAs, due to a diminished presentation of individual epitopes (Javorovic, 2008). In all the cases, it appears necessary to transfect DC separately to avoid both a possible competition by APC depletion and a diminished presentation of individual epitopes.

It would be worthwhile to investigate, if such a co-stimulation leads to the simultaneous expansion of CD8+ T cells targeting the different protein-antigens, or if a competition still exists, due to the selection of specific CTL clones selected by the culture conditions and/or deprivation of growth factors. If it comes out not to be the case, or if such a competition does not fully hinder the detection of the antigen-specific populations, the total CD8+ T cells need could be significantly reduced for one patient, permitting to screen a larger panel of antigens, and correspondingly increasing the probability to find preferential vaccine candidates.

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APPENDIX 1

Supplementary table 1: donors used in this study with their HLA class I phenotype

Donor	HLA-
906	A2, A24, B35, B52
216	A2, A32, B7, B36
DH	A2, B15, B45, Cw3
744	A2, A3, B7, B13
656	A2, A24, B18, B57
514	A2, B7, B51, Cw7
065	A2, A73, B36, Bw60
811	A2, A25, B7, B18, Cw7
381	A2, A23, B7, B49, Cw7
660	A1, A2, B44, Bw55, Cw3, Cw5
694	A2, A26, B45, B62, Cw3, Cw6
053	A2, B7, B27, Cw2, Cw7
033	A2, A24, B7, B44
344	A1, A2, B7, B57
581	A*0201, A*3201, B*5101, Cw*1203
748	A*0201, B*4101, B*5101, Cw*1402, Cw*1701
459	A*0201, A*0301, B*3501, B*5101, Cw*0401, Cw*1402
UX	A*0201, A*3301, B*0702, B*1401, Cw*0701, Cw*0702

Supplementary table 2: patients used in this study with their HLA class I phenotype

Patient	HLA-
D05	A*0201, B*2705, B*4402, Cw*0202, Cw*0501
MZ2	A*0101, A*2901, B*3701, B*4403, Cw*0602, Cw*1601

APPENDIX 2

Supplementary table 3: HLA primers used in this study with their orientation and sequence

Primer	orientation	sequence (5‘- 3‘)
HLA-5p2	sense	GGGCGAATTCGGACTCAGAATCTCCCCAGACGCCGAG
HLA-3pA	antisense	CCGCGTCGACTTGGGGAGGGAGCACAGGTCAGCGTGGGAAG
HLA-3pB	antisense	GGGCGTCGACTGGGGAGGAAACACAGGTCAGCATGGGAAC
HLA-3pC	antisense	GGGCGTCGACCTGCATCTCAGTCCCACACAGGC
HLA-2S	sense	AGGGGCCGGAGTATTGGGAC
HLA-4N	antisense	GCCAGGTCAGTGTGATCTCCGC