Adaptive Immunity Against the L1 Protein of the Human Papilloma Virus: HPV L1 Cross-Reactive $CD4^+$ T Cells Allow Immune Responses Across Type Specific Borders.

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…to my family

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1 Introduction

1.1 The human papillomavirus

1.1.1 Classification and genetic variability

Human papillomaviruses (HPV) belong to the taxonomic family of papillomaviridae (PV). These non-enveloped DNA viruses contain a circular genome of about 8 kDa. The PV infect a wide range of hosts, ranging from mammals to birds and reptiles. Individual virus types are very restricted concerning the site of infection and their host. With the sole exceptions of bovine papilloma virus 1 and 2, which can also infect horses, an individual PV type can only infect one species(Campo, 2002; Kanodia et al., 2008).Because most is known about the human papillomavirus the following text will focus on this human pathogen and will not include the information about PVs infecting members of the animal kingdom.

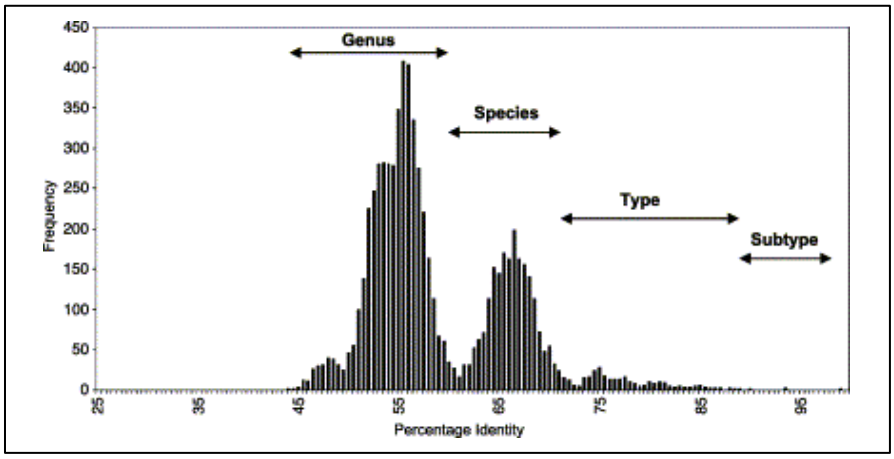

Figure 1-1. Frequency distribution of pairwise identity percentages from nucleotide sequence comparison of the L1 ORFs of 118 papillomavirus types(de Villiers et al., 2004). The 96 HPV types and 22 animal papilloma viruses were compared pairwise for the identity of their L1 ORF. The identity percentage is shown on the x axis. The frequency of HPV-type pairs that reach the indicated identity is shown on the y axis.

HPV are a group of genetically variable viruses. These different viruses are grouped into geni, species, types and subtypes according to sequence differences in the open reading frame (ORF) of the L1 gene. The L1 gene is used to classify new PV isolates because it is the most conserved gene within the whole PV`s genome (Bernard et al., 2010; de Villiers et al., 2004). As can be seen in a sequence comparison of the L1 ORF of 118 different papilloma viruses (Figure 1-1), there are four different levels of organization.

HPV types of different geni have a sequence agreement in their L1 ORF of less than 60%. When the whole genome of two different viruses of different geni is compared, only 23 to 43% of sequence identity is observed. All HPV belong to one of five different geni (Figure 1-2). The Alpha PV is the best characterized genus, with most of the over 130 HPV types belonging to this group. The viruses of this genus infect the mucosa or the skin of humans or primates. Medical data of the Alpha PV allows an additional subdivision of the genus into high-risk and low-risk types. High-risk types are involved in the malignant transformation of human keratinocytes, while low-risk types are not.

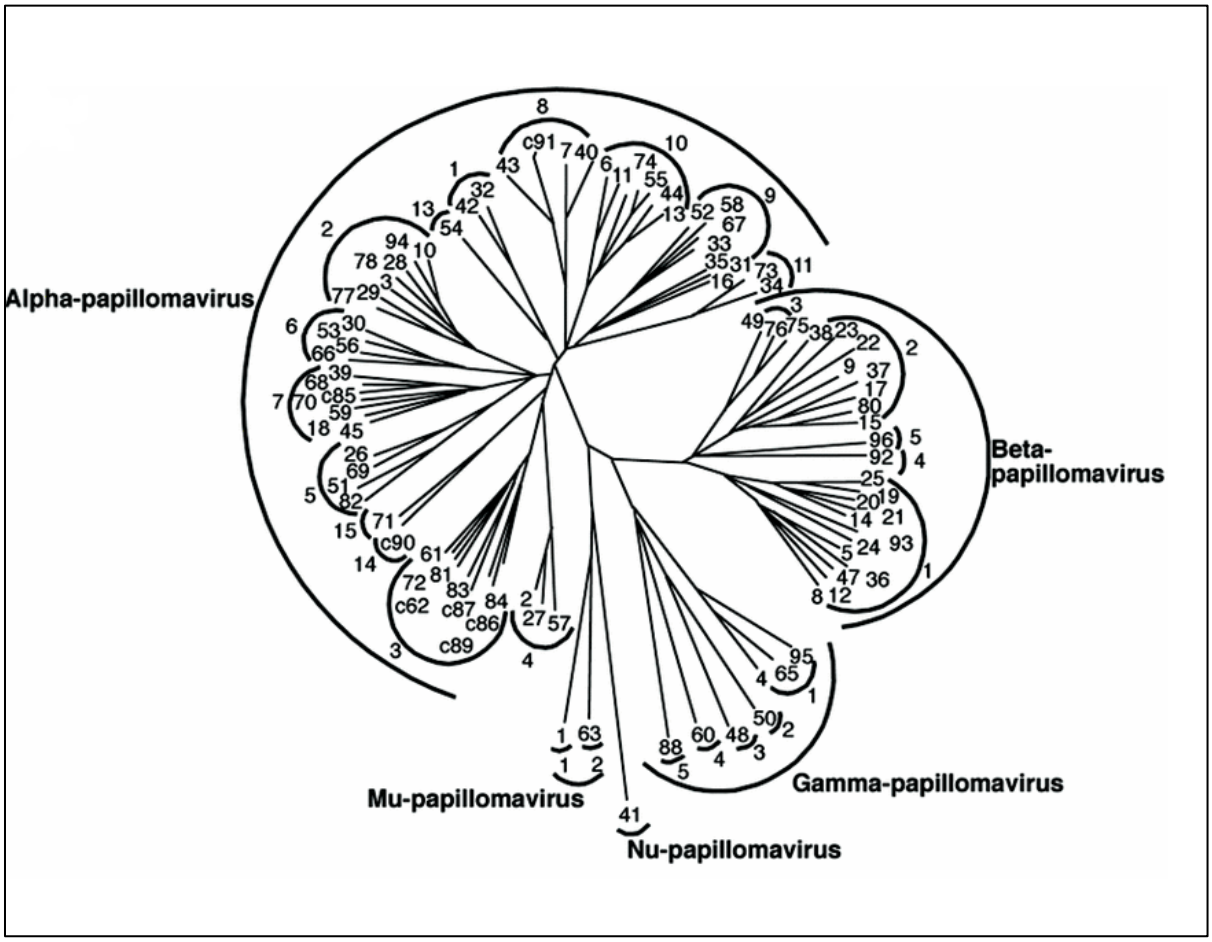

Figure 1-2. Phylogenetic tree of the different HPV types(Doorbar, 2006). The numbers of the branches indicate the type. The species is indicated by the inner semicircles. The outer semicircles show the papillomavirus genera.

A species unites several closely related HPV types into a subgroup that shares between 60 and 70% of sequence identity in the L1 ORF. The members of a species are thought to have similar characteristics. For example, all members of the species 1 are low-risk types infecting the oral or genital mucosa.

The next level in organization of the PV classification is its type. The original serotype, meaning a PV isolate that is recognized by a specific antibody, has been replaced by a genetically more sophisticated definition. To be acknowledged as a new PV type the sequence of the L1 ORF must differ by more than 10% from all known HPV types. All variations that do not fall under this definition are defined as subtypes (2-10% difference in the L1 ORF) or variants (below 2% of such difference). PV, being a stable DNA virus, does not diversify quickly. Mutations or recombinations are rare events that occur as infrequently as in the host's genome, suggesting a coevolution between virus and host (Bernard et al., 1994; Chan et al., 1997). The same types could be isolated several times in different clinical studies at different places. It is noteworthy that a single type was present in only 10-100 different genomic variants (de Villiers et al., 2004). The letter that sometimes accompanies the type description (e.g. HPV6a) refers to such genetic variants. The lettering originated at a time when labs tried to differentiate between different HPV types by digestion. The genomic digestion fingerprints differentiate HPV 6a and HPV 6b, but the underlying genomic difference is only marginal.

1.1.2 Structure

The HPV viron has of a symmetric icosahedral protein coat that is made out of two viral proteins: L1, the major capsid protein, and L2, the minor capsid protein. The viral capsid is approximately 500 Å in diameter. It consists of 72 L1 pentamers, which interact with at least 12 L2 proteins. These capsomeres are organized in a T = 7 icosahedral lattices (Chen et al., 2000) and contain a double-stranded DNA with a length of approximately 8 kDa (Conway and Meyers, 2009). An electron and a cryoelectron micrograph of papilloma virus' capsid are depicted in Figure 1-3.

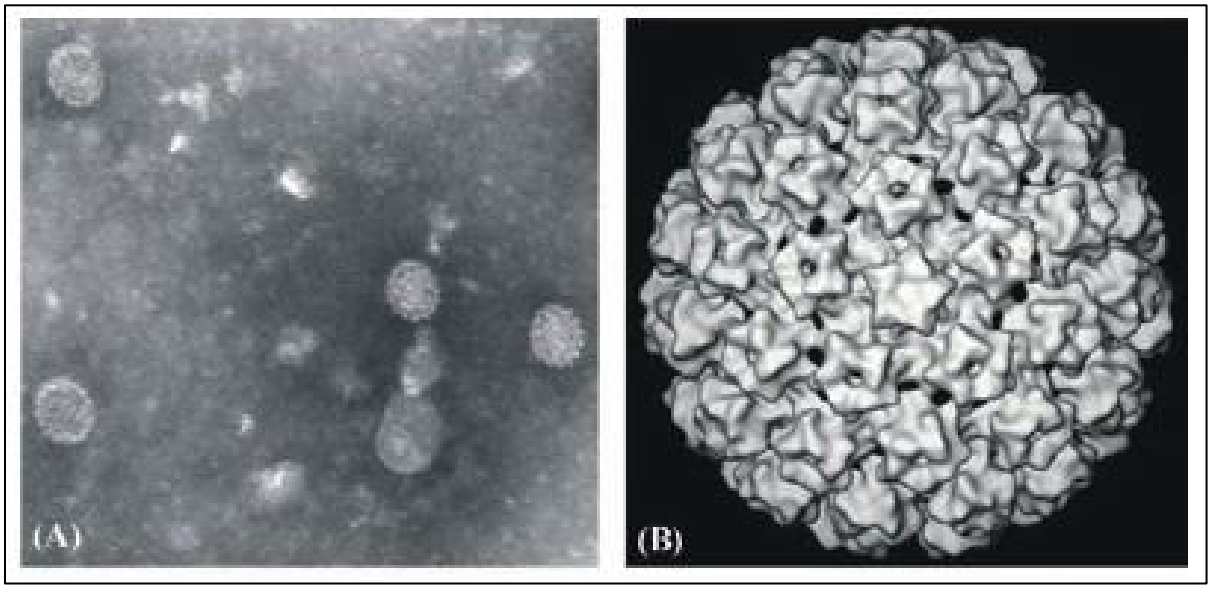

Figure 1-3. Papillomavirus capsid structure: (A) Electron micrograph of native HPV11 virions (B) cryoelectron micrograph of a Bovine papillomavirus 1 virion (taken from www.hpv-curing.com).

1.1.3 Genome and geneproducts

1.1.3.1 Genomic organization

The genetic organization of the well characterized HPV16 genome is depicted in Figure 1-4. It can be divided into three regions that are separated by polyadenylation sites: The early region, the late region and the long control region (LCR).

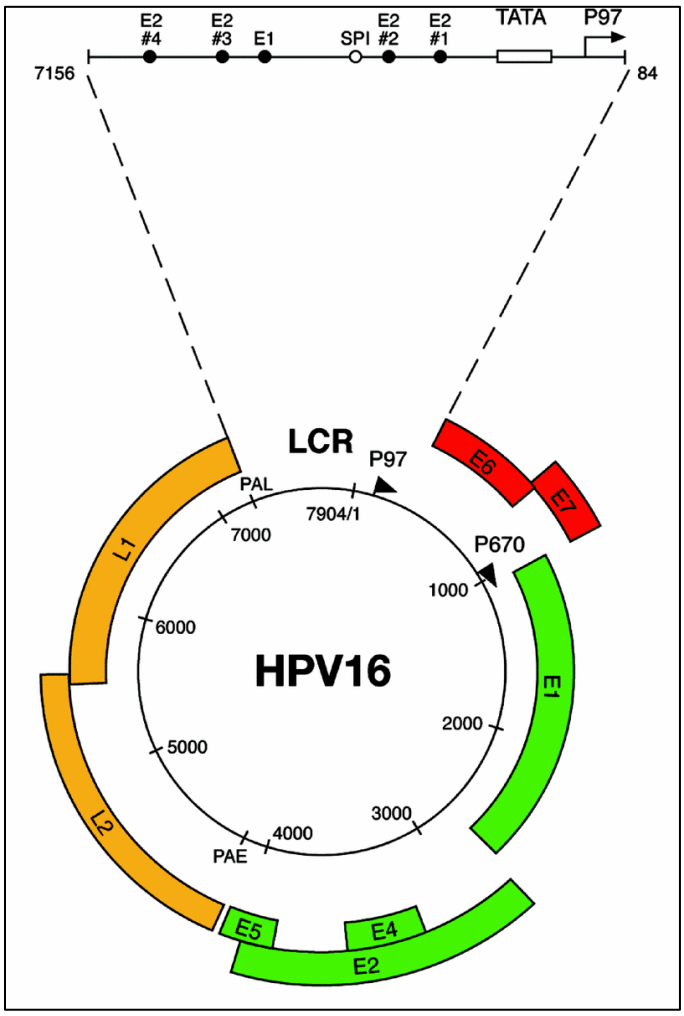

Figure 1-4. HPV16 genome (Doorbar, 2006). The 7904 bp genome is displayed as a black circle with the ORFs' positions illustrated in red, green and yellow. The two promotors P97 and P670 are activated at different differentiation states of the infected keratinocyte. A change in the polyadenylation site from PAE to PAL induces L1 and L2 transcription.

Early region: This region uses over 50% of the 5'half of the genome. It encodes the ORFs for the six viral early proteins E1, E2, E4, E5, E6 and E7. These proteins are expressed immediately after viral infection of the cell. The ORF E3 and E8 exist only in very few HPV types. While E3 is never expressed as a protein, the E8 protein is a splice variant of the E1 ORF that functions as a negative regulator of E2 (Lambert et al., 1989).

Late region: The late region is situated downstream of the early region and uses about 40% of the coding region. It encodes the two late proteins: L1, the major capsid protein, and L2, the minor capsid protein.

Long control region: The LCR uses about 850 bp (~10% of the whole genome). Although this region does not encode any proteins, it does contain the origin of replication.

1.1.3.2 The very early regulatory genes E1 and E2

The E1 and E2 proteins are acting together for the replication of the viral DNA. The papilloma E1 protein is a helicase. It separates the complementary DNA strands by using ATP and interacts with the DNA-polymerase alpha-primase for replication (Sun et al., 1998). E1 assembles with the help of E2 at the viral origin of replication into a double hexamer that enables the viral genome replication (Morin et al., 2011). E2 has a transactivation and a DNA binding domain and so serves as the main transcriptional regulator. A whole family of splice variants of E2 exists, expressing not only activation but also repressor functions (Dowhanick et al., 1995).

Recent work suggests the role of E2 in regulating cellular genes involved in apoptosis, proliferation and cell differentiation and, thus, in creating a convenient microenvironment for the proliferating cells to replicate the virus (Hadaschik et al., 2003; Ramirez-Salazar et al., 2011). Furthermore, studies show that E2 connects the viral genome to mitotic chromosomes during cell division to ensure their equal distribution to both daughter cells. This happens either (1) by mediation of the double bromodomain-containing chromatin adaptor Brd4 (McBride et al., 2004) or (2) Brd4 independent by interacting with repeated ribosomal DNA genes in the short arms of acrocentric mitotic chromosomes (Poddar et al., 2009). When the viral genome is integrated into the host chromosome, the expression and function of E2 is lost. This is a common event in HPV infection (Collins et al., 2009), which can increase the E6/E7 oncogene expression, and thereby promote transformation and genetic destabilization (Cricca et al., 2009).

1.1.3.3 The late early regulatory gene E4

The products of the E4 ORF are expressed in the early phase of infection, but only at very low levels. As suggested in 1986 (Doorbar et al., 1986), the E4 gene products play an important role in virus maturation as the expression levels continuously rise. In the final state the E4 gene products can make up to 30% of the cellular protein content of the

infected cell. As the infected cell differentiates, the E4 protein also evolves. Through differences in proteolysis, oligomerization and phosphorylation the E4 protein is progressively changed as the infected keratinocyte migrates towards the outer layers of the skin (Doorbar et al., 1988; Grand et al., 1989). The exact function of the E4 protein still needs to be elucidated. It is known, however that the mutant genomes of the cottontail rabbit papillomavirus that cannot express E4 fail to induce a productive viral life cycle (Peh et al., 2004). E4 interacts with the keratin network of the infected cell and reorganizes its structure (McIntosh et al., 2010; Wang et al., 2004a). It has been speculated that the resulting collapse of the cytoplasmic cytokeratin network facilitates the release of the virions of the infected cells (Doorbar et al., 1991).

1.1.3.4 The small early oncogene E5

The early protein E5 is a small hydrophobic membrane molecule with a length of 83 amino acids (in case of HPV type 16). It is weakly oncogenic and is localized to the endoplasmatic reticulum membrane (Conrad et al., 1993). It colocalizes with BCL-2 (Auvinen et al., 2004) and influences the function of such receptor tyrosine kinases as PDGFR (Petti et al., 1991; Talbert-Slagle and DiMaio, 2009), EGFR (Crusius et al., 1998; Pedroza-Saavedra et al., 2010), ErbB2 (Crusius et al., 1998) and ErbB4 (Chen et al., 2007). It has been shown that E5 stabilizes the EGF receptor and, therefore, upregulates ligand-induced activation (Martin et al., 1989; Rodriguez et al., 2000). E5 also enhances the EGF signal by downregulating the degradation of the receptor. It disrupts the ubiquitin EGFR interaction and, therefore, reduces degradation by the proteasome (Zhang et al., 2005). Additionally, it affects the cell-cell communication (Oelze et al., 1995) and is able to downregulate the expression of major histocompatibility complex class I and II molecules on the surface of the infected cell, thus inhibiting the adaptive immune system (Ashrafi et al., 2006; Zhang et al., 2003).

1.1.3.5 The viral early oncogenes E6 and E7

The viral proteins E6 and E7 are small molecules (E6: 18 kDa; E7: 13 kDa). They are located mainly in the nucleus but are also found to a lesser extent in the cytoplasm (Howie et al., 2009; McLaughlin-Drubin and Munger, 2009). Neither molecule is able to immortalize a cell. Together they complement each other and successfully immortalize a keratinocyte (Hawley-Nelson et al., 1989; Munger et al., 1989).

The viral protein E6 is well known for its interaction with the tumor suppressor p53. P53 is a transcription factor that controls cell proliferation by arresting the transition from G_1 to S phase. High levels of p53 due to stress cause the expression of several genes that lead to cell cycle arrest and induction of apoptosis (Baker et al., 1989). E6 binds to p53 and induces its degradation (Scheffner et al., 1990; Werness et al., 1990). Cytoplasmic E6 also inhibits the transport of p53 to the nucleus and, thereby, achieves low levels of p53 in the nucleus, which inhibits a transition to S phase. Additionally, E6 targets the pro-apoptotic protein BAK for degradation. BAK is expressed at high levels in epithelial cells (Thomas and Banks, 1998). E6 also activates the telomerase to elongate the telomeres, enabling the infected cell to divide without limits (Liu et al., 2009).

The viral E7 protein has no DNA binding domain and no enzymatic activity. Its sole purpose lies in protein binding. The best characterized target of E7 is another tumor suppressor protein family. E7 targets the members of the retinoblastoma (rb) family, namely RB, p107 and p130. In the normal state, RB, which has several phosphorylation sites, is associated with transcription factors such as the E2F family. As the cell undergoes mitosis, RB becomes more and more phosphorylated. Hyperphosphorylated RB is no longer able to bind E2F and, consequently, releases the transcription factors that for their part activate the transcription of genes involved in cell cycle progression (Chen et al., 2009). E7 disrupts this pathway by binding hypo-phosphorylated RB through its LXCXE motif, thereby releasing E2F and uncoupling cell cycle progression from rb-mediated checkpoint control (Cho et al., 2002).

Besides the interaction with the rb family, E7 also associates via a zinc finger-like motif with the histone deacetylase complexes through Mi2ß (Brehm et al., 1999). This inhibition leads to more acetylated lysine residues on the histone proteins and a decreased attraction among the single proteins due to masked charges. The resulting open structure of the chromatin enables better chromatin accessibility for the E2F transcription factors (Longworth and Laimins, 2004). The same zinc finger-like motif is also able to directly interact with AP-1 transcription factors such as c-Jun, JunB, JunD and c-Fos (McMurray et al., 2001). In humans the expression of E6 and E7 alone is not sufficient to induce cancer. This is indicated by the fact that whereas a high proportion of humans is infected at least at one point in their lifetime by a high-risk type HPV, only few will develop cancer (Hildesheim et al., 1994).

1.1.3.6 The viral late genes L1 and L2

The genes L1 and L2 encode for the two late capsid proteins L1 and L2. L1, the major capsid protein, is around 60 kDa in size and is the major component of the viral capsid. The minor capsid protein L2 is around 50 kDa in size. Both proteins are produced very late in the viral infection cycle, with the L2 expression preceding the expression of L1 (Florin et al., 2002). While L1 readily self-assembles into a virus like particle (VLP), L2 does contact the L1 pentamer to bind and stabilize the viral DNA with its N-terminus (Zhou et al., 1994). Only the amino acids 61-123 of L2 from bovine papilloma virus (BPV) are exposed to be recognized by antibodies, while the biggest part of the protein is buried deep inside the capsid and is not accessible to antibodies (Liu et al., 1997).The variability of L1 is concentrated in five external loop regions. Their positions in the HPV16 L1 sequence are: BC loop (AA76-95), DE loop (136-179), EF loop (AA186-215), FG loop (AA288-317) and the HI loop (AA374-386). The originally published positions were adapted from Chen et al. in order to compensate for the different L1 HPV16 sequences used in this work (see appendix) and in the original data (Chen et al., 2000; Lowe et al., 2008). The loops cover the surface of the capsid. Even among very closely related types, a great diversity can be observed.

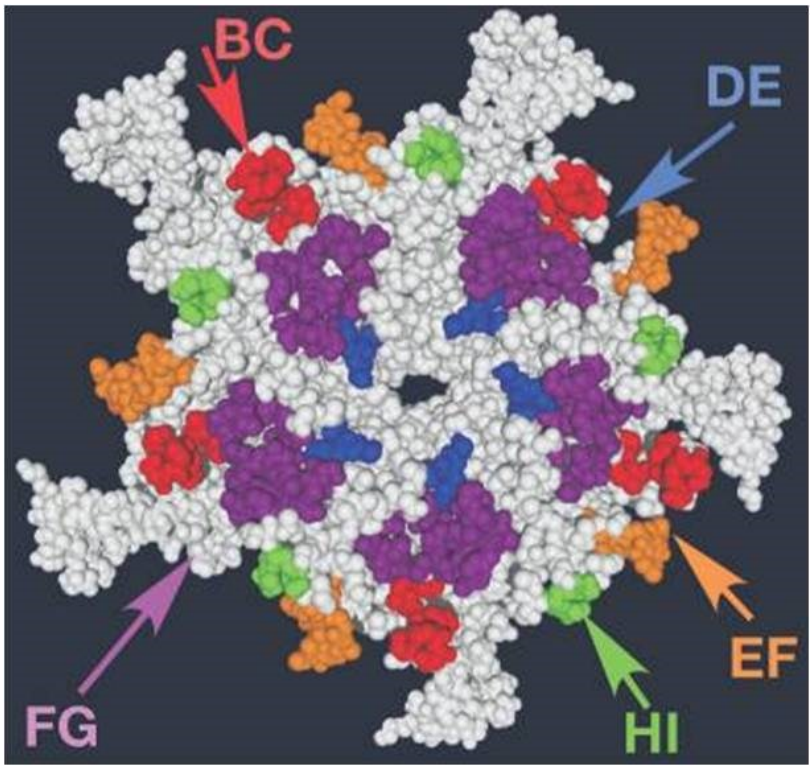

Figure 1-5. HPV L1 pentamer. The arrows indicate the positions of the variability loops. The amino acids that differ between HPV types 6 and 11 are highlighted in color (red: BC-loop, blue: DE-loop, yellow: EF-loop, violet: FG-loop, green: HI-loop).Taken from (Orozco et al., 2005).

As an example, Figure 1-5 shows the differences between the very closely related HPV types 6 and 11. The positions of differing amino acids are highlighted in color. Most antibodies are directed against these regions (Orozco et al., 2005). It can be assumed that the strong variability within these loops is caused by the evolutionary pressure of the immunesystem. This pressure is also clearly illustrated in Figure 1-6. The variable positions are exclusively located on the outside of the capsid and not on the inside. Here the structures are exposed to and can be recognized by antibodies. A strong variability within the especially exposed loops tends to prevent antibodies from recognizing different HPV types, making it difficult for them to cross-react.

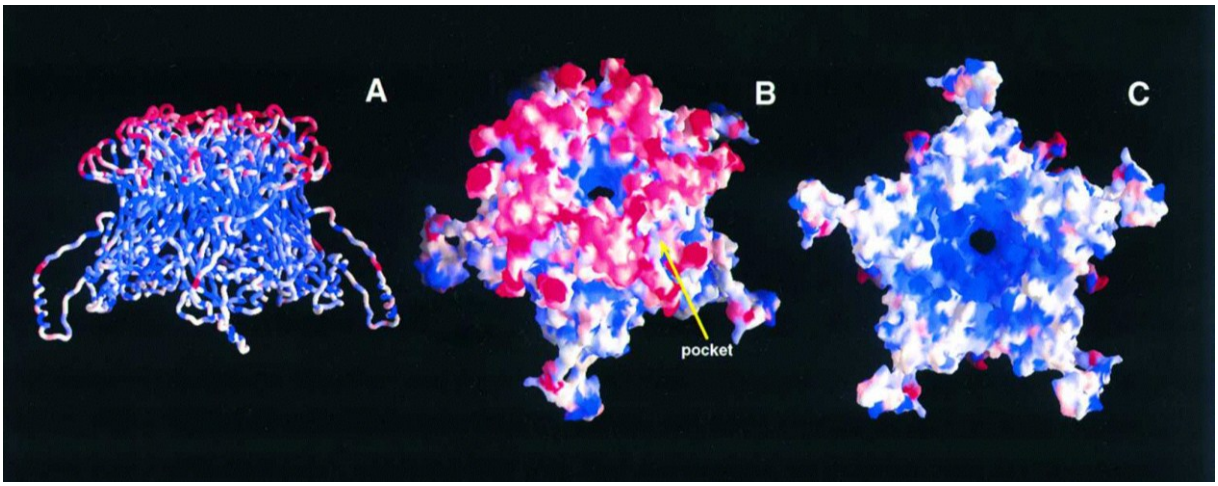

Figure 1-6. Sequence variations in L1 pentamers and their positions. L1 sequences of 49 HPV types were compared. The differences are displayed in a 3D model of the L1 pentamer. Highly variable positions are colored red; fully conserved are blue: (A) side view of L1 pentamer: the upper side of the picture corresponds to the outer side of the virion (B) outer surface and (C) inner surface(Chen et al., 2000).

1.1.4 Papilloma virus infection

1.1.4.1 The human epidermis

To understand HPV, one has to understand the human skin and the differentiation that each keratinocyte undergoes on its journey to the outer epithelium of the skin.

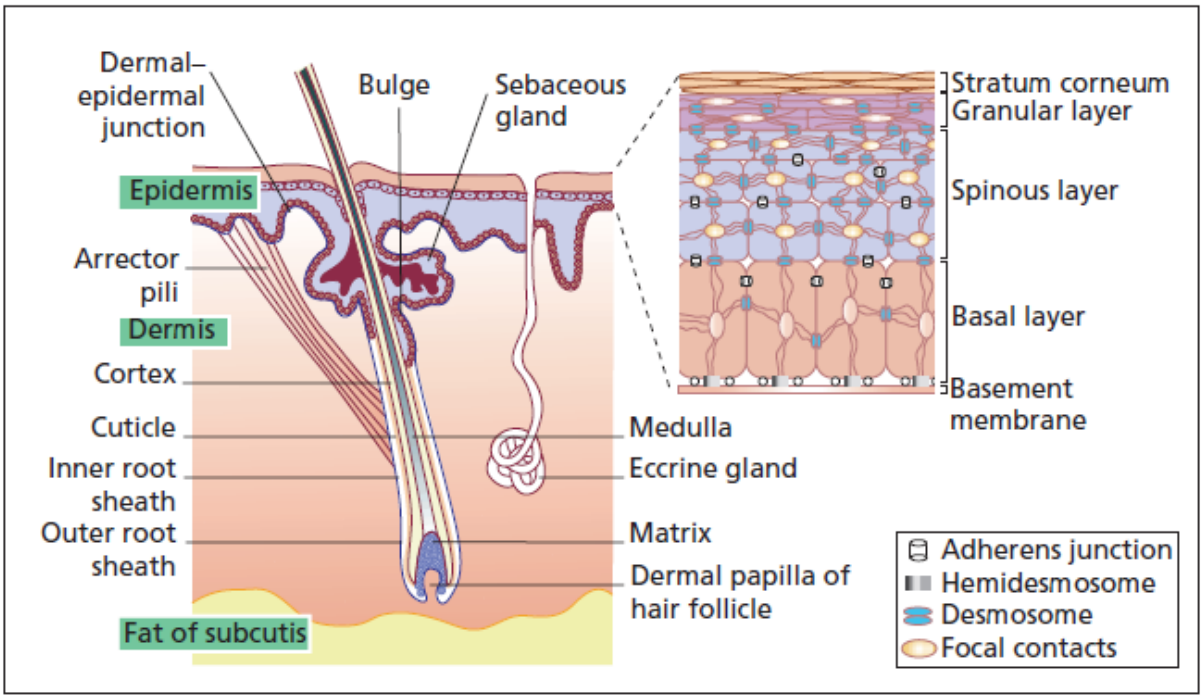

Figure 1-7. Architecture of the human skin (McGrath and Uitto, 2010).

The skin is divided into three different layers that strongly vary in thickness depending on the localization (Figure 1-7). The subcutis consists of a loose connective tissue with many embedded lipocytes. Its main functions are the isolation and the mechanical protection of the underlying tissue. The middle region, the dermis, is the main pillar of the skin's structural integrity. The supporting matrix consists mainly of polysaccharides and the structural proteins, collagen and elastin. Embedded in this matrix are fibroblasts, mast cells and histiocytes. The dermis, where all the appendages of the skin (e.g. sebaceous/perspiratory glands, hair follicle) are located, is very well supplied with blood vessels and enables the nourishment of the adjacent epidermis via diffusion (McGrath and Uitto, 2010).

The epidermis is the outmost layer of the skin. It is a stratified squamous epithelium, which acts as the main barrier of the body against environmental influences. It consists mainly of keratinocytes that are formed in the basal layer and pass through four to five differentiation stages before reaching the outmost layer of the skin as squamous sheets.

The basal/germinal layer (stratum basalum/germinativum) is where the stem cells of the epidermis are located. The stem cells are clustered within this one to two cell thick layer directly contacting the basal membrane. These cells are undifferentiated and are continuously dividing to produce transient amplifying cells that are, themselves capable of another 4-5 cell divisions. The keratinocytes' journey to the surface takes about 40 days. Melanocytes are primarily found in this layer, too.

The spinous layer (stratum spinosum) is adjacent to the basal layer. Here transient amplifying cells are located and are constantly dividing.

The granular layer (stratum granulosum) is characterized by cells storing large amounts of keratohyalin granules for later use and smaller lamellated *Odland bodies* that discharge their lipid content into the intercellular space.

The clear/translucent layer (stratum lucidum) is a transitional layer between granular and cornified states, which can only be observed in palmoplantar skin. Here, the keratinocytes have not yet lost their nucleus.

The cornified layer (stratum corneum) is the outmost layer of the epidermis. The changed, flattened keratinocytes, now called corneocytes have lost their nuclei and all cell organelles. The keratin filaments are aggregated by filaggrin to disulfide cross-linked macrofibres (Lynley and Dale, 1983). Through the slow degradation of the intercellular lipids and the loss of remaining desmosomal intercellular connections, desquamation takes place.

1.1.4.2 Papilloma virus infection cycle

HPV enters the epithelium through micro wounds, i.e. a small scratch that exposes the basement membrane. Here HPV infects primitive transient amplifying keratinocytes in the basal layer of the epidermis. In these cells, HPV uses the DNA replication machinery of the proliferating cells to establish a copy number of 50-100 HPV genomes per cell. At this stage, the expression of all viral genes is very low. The proteins E6 and E7 are only marginally expressed because of the regulation by E2. When a cell division takes place, the viral genome is also amplified by the cellular DNA polymerases to reach a copy number of 50 to 100 in both daughter cells. As one of the daughter cells stops proliferation and undergoes cell differentiation, the expression of E6 and E7 is highly upregulated. HPV is not encoding any replicative enzymes and is, therefore, totally dependent on the replicative machinery of

the infected host cell. The E6 and E7 proteins are activated to escape the HPV specific dilemma. On one side the virus needs the DNA replicating enzymes that are only accessible in proliferating nondifferentiated cells. One the other side, it needs to replicate in differentiated nondividing keratinocytes. This problem is solved by the simultaneous expression of E6 and E7. Their combined force uncouples G1-S phase entry from differentiation (see 1.1.3.5: The viral early oncogenes E6 and E7). It enables the virus to take full advantage of the program of the differentiating keratinocyte, while continuing to use the cellular replicative machinery.

The infection cycle of HPV is illustrated in Figure 1-8. Just as a healthy keratinocyte undergoes several stages on its way to becoming a fully differentiated corneocyte, so does a HPV-infected one.

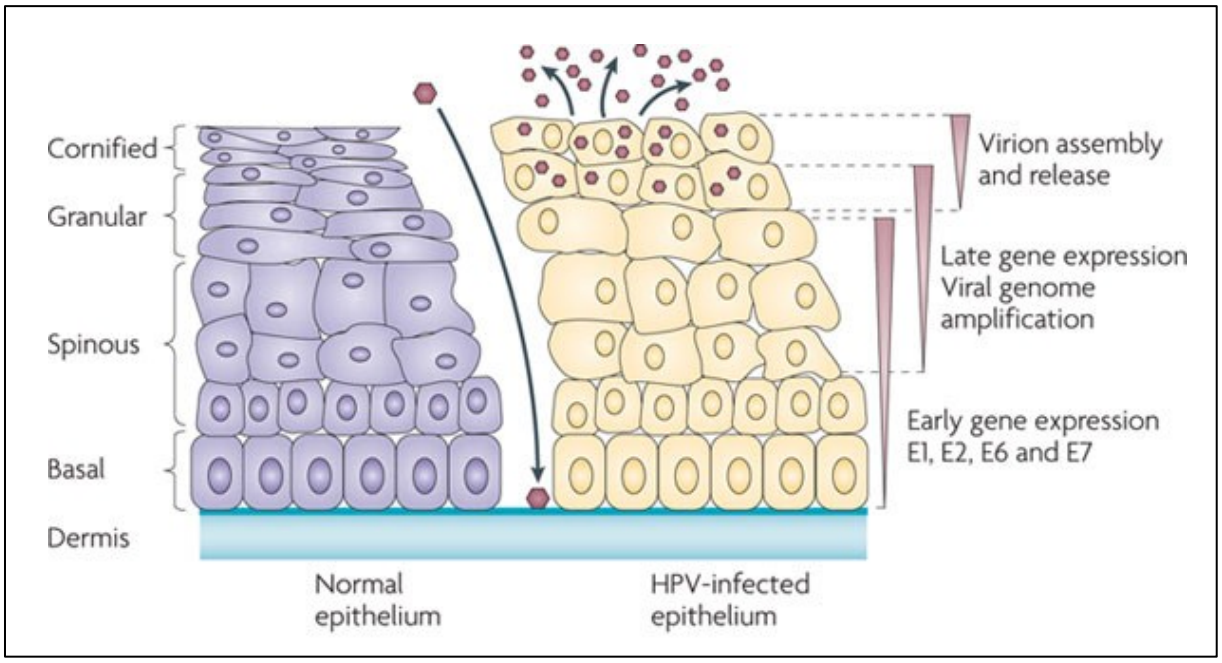

Figure 1-8. Life cycle of HPV taken from (Moody and Laimins, 2010). Normal epithelium is shown on the left and HPV-infected is shown on the right. HPV enters through microwounds exposing the basal layer of the epithelium (middle).

In the stratum basalum, the HPV genome exists in the nucleolus of the host cell as low copy number episomes. There is only a marginal expression of the viral early antigens E1, E2, E6 and E7. As differentiation starts and the cell moves to the stratum spinosum, expression of E6 and E7 is strongly upregulated due to the genetic program running in the developing keratinocyte. The cell is forced into the S-phase (Cheng et al., 1995) and the cellular DNA replication machinery is used to amplify the episome number to more than 1000 episomes

per cell. By the time the cell reaches the stratum granulosum, the expression of the viral late proteins is already started and viral particles are already self-assembling. In the stratum granulosum and the stratum corneum this process of virus self-assembly continues as more and more viral particles accumulate in the developing corneocyte. The finally differentiated HPV infected corneocyte is sloughed off by natural desquamation or releases viral particles after being broken by mechanical stress. Virions are passed to another person or to the same individual at second site by skin contact and enter the epithelium through microtrauma. A new viral infection cycle begins.

1.1.4.3 HPV and cancer

It is widely accepted that HPV is the cause of invasive cervical cancer. Harald zur Hausen was awarded the Nobel Prize for Medicine in 2008 for his part in establishing this connection.

First attempts to connect cancer to viral infection date back to the early 1970s. Not only HPV (zur Hausen et al., 1974a) but also herpes viruses (zur Hausen et al., 1974b) and, especially, the Epstein-Barr virus (Wolf et al., 1975) were the points of interest. In 1976 and 1977 Meisels et al. published two studies showing for the first time histological evidence for HPV infection in cervical smears (Meisels and Fortin, 1976; Meisels et al., 1977). The late 1970s were blessed with the development of nucleic-acid hybridization. This technique allowed the "Human wart virus", as it was then called, to be reclassified as not one but many related, not identical types (Gissmann and zur Hausen, 1980; Law et al., 1979; Orth et al., 1977). This raised the questions whether there are special HPV types infecting different tissues and whether some of these are associated with the development of cancer.

In 1983 and 1984 it was proven by isolation and subsequent cloning that cervical cancer cells contain the viral DNA of two distinct HPV types, namely HPV 16 and 18 (Boshart et al., 1984; Durst et al., 1983; Gissmann et al., 1984). After these findings the research into HPV associated cancer exploded. The genomes of HPV 16 and 18 were described in 1985 (Schwarz et al., 1985). The viral proteins E6 and E7 were found in cancer cell lines and biopsies in 1987 (Durst et al., 1987) and were soon thereafter recognized as major factors in the transformation of the host cells (de Villiers et al., 1987; Munger et al., 1989). The first large scale epidemiological studies in 1995 provided convincing evidence that HPV16 and 18 are the main risk factors for the development of cervical cancer.

1.1.4.4 High risk types and cancer burden

Not all HPV types cause cancer. Depending on minor changes in its protein sequences HPV can cause insignificant skin warts (e.g. HPV1, 2, 4), bothersome genital warts (e.g. HPV6 and HPV11) or high-grade squamous intraepithelial neoplasias (e.g. HPV16 and 18) (Handisurya et al., 2009). The HPV types that are known to cause cancer are called high-risk types; the others are called low-risk types. The 15 known high-risk types and the proportion of cervical cancers caused by them are summed up in Figure 1-9 according to data from Smith et al. (Smith et al., 2007).

	Proportion of cervical cancers caused	Cumulative total
HPV16	54·6%	54·6%
HPV18	15·8%	70·4%
HPV33	4·4%	74·8%
HPV45	3·7%	78·5%
HPV31	3·5%	82·0%
HPV58	3·4%	85·4%
HPV52	2·5%	87·9%
HPV35	1·8%	89·7%
HPV59	1·1%	90·8%
HPV56	0·8%	92·2%
HPV51	0·7%	92·9%
HPV39	0·7%	93·6%
HPV73	0·5%	94·1%
HPV68	0·5%	94·6%
HPV82	0·2%	94·8%
No type identified	5·2%	100%

Figure 1-9. The 15 known high-risk HPV types. The first column indicates the proportion of cervical cancer caused by single HPV types. The left column sums up the proportions of the already mentioned types (Schiffman et al., 2007).

More than half of all cervical carcinomas are induced by HPV16 (55%). Adding the 15% caused by HPV18, these two types alone are responsible for over 70% of all cervix carcinomas. The group of HP31, 33, 45 and 58 together are responsible for an additional 15%. The remaining 11 high risk types (HPV35, 39, 51, 52, 56, 59, 68, 73 and 82) account for 10% of the cases. In 5% of the cervical carcinomas no HPV type was identified due to incomplete diagnostics (Smith et al., 2007).

The high risk types not only cause cervical cancer but are also responsible for other malignancies. Cancer of the vulva, vagina, penis and anus are mainly caused by HPV (Parkin and Bray, 2006). There is also growing evidence that persistent infection by a high risk type is a major risk factor in developing head and neck cancer (Leemans et al., 2011). It is thought that over 4% of all cancers worldwide can be attributed to HPV.

In 2002 there were approximately 500 000 cases per year of cervical carcinoma worldwide. These cancers cause around 250 000 deaths per year. This means that 10% of all cancer related deaths in women are caused by cervical carcinoma alone (Parkin et al., 2005). In the developed world, screening programs have proven to be highly effective and led to a strong decrease in cervical cancer related deaths. Due to high costs and the requirement for professional medical training adequate screening is not available in the undeveloped world. Therefore, undeveloped countries suffer the greatest part (>80%) of the cervical cancer burden (Parkin and Bray, 2006). The social and economic burden in these countries is further impacted by the early onset of HPV associated malignancies.

1.1.4.5 Development of cervical cancer

Most HPV infections do not progress to cancer. Two main factors affect the risk that a HPV-infected keratinocyte will proceed from normal infection to cancer.

First and most important is the HPV type. As mentioned, only infection by a high-risk-type HPV can cause cancer (see 1.1.4.4 High risk types and cancer burden). This is mainly because of small but important changes in the viral E5, E6 and E7 genes of the high-risk HPV types. The viral oncogenes E5 and especially E6 and E7 can have a strong impact on the infected cell and can facilitate the acquisition of additional defects that ultimately lead to the induction of cancer (Schiffman et al., 2007). Only the E6 and E7 proteins of high-risk types are known to immortalize human keratinocytes in tissue culture (zur Hausen, 2002). The exact mechanisms are complex, with possibly different binding partners for each single HPV type. This complex binding network is still being investigated for many different HPV types but most attention is being paid to HPV16 as the main cancer causing HPV type. For this HPV type, it is known that the E7 oncoprotein induces centrosome abnormalities that disrupt mitotic fidelity and increasing the risk for a failing of chromosome segregation and therefore aneuploidy (Korzeniewski et al., 2011). Other effects are similar in the whole group of high-risk HPV types. For example, the E7 protein of the high-risk type binds RB with up to 10-fold

higher affinity than low-risk E7 (Heck et al., 1992) and low-risk E6 lacks the ability to strongly bind and degrade p53 (Oh et al., 2004).

The second factor is the development of a persistent HPV infection. In order to proceed to precancer, HPV infection must first achieve persistence. The normal HPV infection is transient, with most individuals disposing of the viral infection within 12 to 24 months (Ho et al., 1998; Stanley, 2006). In around 10% of high-risk infections with HPV, the virus persists longer than two years. This long-time persistence is mainly achieved by the HPV type 16 (Londesborough et al., 1996; Wallin et al., 1999) and these infections are strongly associated with a high risk of developing precancer (Bulkmans et al., 2007). How HPV achieves this persistence by hiding from or interacting with the immune system will be explained in 1.4.1.

Although the main risk factor for the progression from persistence to precancer is the type of infecting HPV, not all high risk infections proceed to precancer. Only about 40% of the individuals persistently infected with HPV16 will develop precancer within the next 3-5 years after detection (Castle et al., 2005).

Apart from the risk attributed to HPV infection, there exist other less important risk factors like smoking (International Collaboration of Epidemiological Studies of Cervical Cancer, 2006), the long-term use of oral contraceptives (Smith et al., 2003) and chronic inflammation (Trottier and Franco, 2006) but their effect, however, is only marginal in comparison to the effects of the HPV infection.

In a next step the precancer proceeds to invasive cancer. In unscreened populations the main onset of invasive cervical cancer occurs between the ages of 35 and 55 years (Gustafsson et al., 1997). A timespan of 20 to 40 years from HPV infection to invasive cancer can be concluded from the origin of the malignancy: the sexual transmission of a high risk type of HPV at late adolescence or early adulthood. The incidence and exact timespan for progression from precancer to invasion will remain uncertain because treatment of detected precancer is ethically mandatory in all recent studies. Rough estimates based on older studies assume a time frame of 5-10 years and an incidence rate of around 25% (Chang, 1990; Kinlen and Spriggs, 1978).

1.1.4.6 Medical intervention

With the general introduction of Papanicolaou (Pap) smear screening, deaths from cervical cancer could be reduced by 50-70% (Crosbie and Kitchener, 2006; Sasieni et al., 2003). But this is only true for the well developed countries, where public screening programs are affordable and feasible. The Pap test is a method developed by Georgios Papanicolaou to retrieve cells from the cervical transformation zone and to analyze them for cytological abnormalities (see Figure 1-10). Screening programs recommend Pap tests at 1-5 years intervals for women between the ages of 16 to 65.

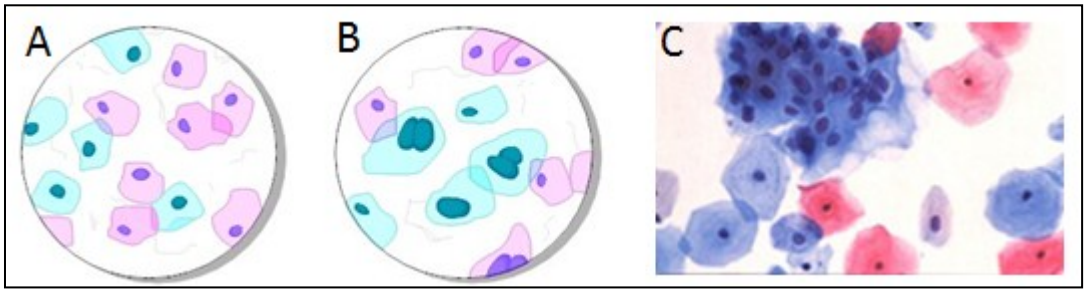

Figure 1-10. Pap test: schematic of (A) normal cells or (B) neoplastic cells (enlarged blue cells with conspicuous nucleus) (taken from www.smartdraw.com). (C) Microscopic picture of a Pap test showing neoplastic cells (taken from www.methodsofhealing.com).

If an abnormal Pap test result is present, colposcopy and HPV testing can be done to triage the equivocal Pap smear. Colposcopy and colposcopically directed biopsies can provide information about the size and histological composition of existing lesions. The lesions are classified into three different grades of cervical intraepithelial neoplasia (CIN):

CIN1: mild dysplasia, confined to the basal third of the epithelium

CIN2: moderate dysplasia, confined to the basal two third of the epithelium

CIN3: severe dysplasia, confined to more than two third of the epithelium

The screening programs, the colposcopically directed biopsies and the CIN system are all controversial (Peto et al., 2004).The sole presence of CIN1 indicates a productive infection by any HPV type, high-risk as well as a low risk (Middleton et al., 2003). These abnormalities are relatively easy to detect, and over 250 000 women are diagnosed with CIN1 in America per year (Kumar and Robbins, 2007). However, most CIN1 spontaneously regress within two years and will never progress to cancer (Bosch et al., 2008). Nonetheless, the diagnosis puts the affected women under permanent pressure and has a serious impact on their lives

(Idestrom et al., 2003). Additionally, CIN2 can spontaneously develop without a CIN1 phase (Agorastos et al., 2005) and CIN2 and CIN3 are more often missed than CIN1 because of the often thinner neoplasia of these lesions (Yang et al., 2008).

To show that the CIN stages do not usually advance to the next higher stage, a newer system was developed. The Bethesda System (Smith, 2002) divides all abnormalities into only two categories, which are called low grade and high grade squamous intraepithelial lesions (LSIL and HSIL). In this system the term LSIL replaces CIN1, and HSIL sums up the groups of CIN2 and CIN3. For diagnostic purposes this system also includes the infecting HPV type and the preparation method of the diagnosed sample. Germany uses yet another classification system: the "Münchener Nomenclatur II". Its system uses the stages I-V to indicate increasing severity and the additional 0 to indicate non-adequately processed and diagnosed smears.

1.2 The immune system

"…the immune system does not care about self and non-self,…its primary force is its need to detect and protect against danger,…" (Matzinger, 1994)

1.3 T cells

1.3.1 The T-cell compartment

T cells are an integral part of the immune system. Together with the B cells they form the adaptive immune system. The difference between the adaptive immune system and the innate immune system lies in the ability to adapt, i.e. to mount a highly specific immune response directed at an explicit need. This strong response is tightly controlled and therefore a specific T or B cell must first be primed by interactions with the innate immune system before it can fulfill its effector function.

T cells can execute three main effector functions and are, accordingly, subdivided into three different subpopulations.

- Cytotoxic T lymphocyte (CTLs): These cells can directly lyse and kill potentially dangerous cells (e.g. virus-infected cells and tumor cells). CTLs can engage target cells with cytolytic granula or activate apoptotic receptors expressed by the target cell.
- Helper cells: T_H cells help and interact with other parts of the immune system to mount an appropriate immune response. They do this by direct cell-cell interaction as well as by releasing cytokines (soluble messenger molecules).
- Regulatory T cells: In order not to pose a threat to the organism itself the immune response needs to be tightly controlled. This control is in part exercised by the regulatory T cells. The immune system is regulated by means of soluble factors and direct cell-cell interaction.

1.3.2 The T-cell receptor

All T cells carry several thousand copies of a single receptor that enables them to recognize their target structure. This receptor is called the T-cell receptor (TCR). The TCR recognizes a complex of a major histocompatibility complex (MHC) molecule and a small protein fragment (Townsend et al., 1986; Zinkernagel and Doherty, 1974).

The TCR heterodimer consists of two transmembrane proteins (Figure 1-11). The α chain has a mass of 50 kDa and the β chain weighs about 39 kDa (Garboczi et al., 1996). There is also a γδ-TCR consisting of a γ and a δ chain. T cells carrying this γδ receptor are yet another subgroup. This introduction will be confined to the main group of αß T cells.

The α and the ß chain both consist of a short cytoplasmic domain, a transmembrane helix and two immunoglobulin (Ig) like domains (Moretta et al., 1996). Both chains are connected via a disulfide bridge. The distal Ig-like domain, also called variable domain (V-domain) includes three (in the case of the α chain) or 4 (in the case of the ß chain) hypervariable loops, the so called complementary determining regions (CDR). These almost unique CDRs determine about the specificity of the TCR. Since both chains join together and all CDRs are part of the receptor binding site, all CDRs acting together govern the specificity of the individual T cell (Garcia et al., 1999).

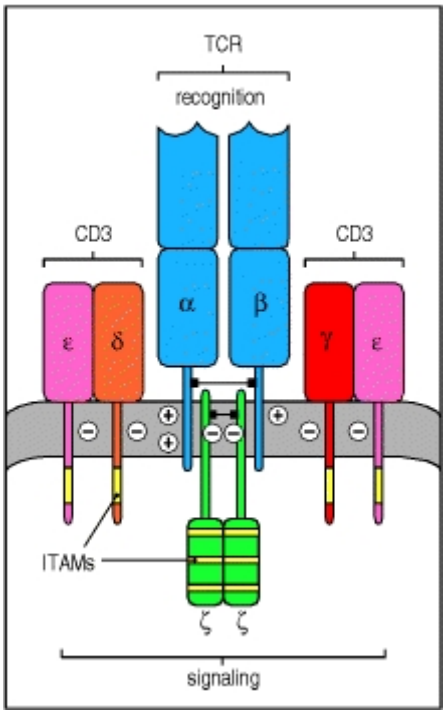

Figure 1-11.T-cell receptor (TCR) complex adapted from (Janeway, 2001). The complex is made up of the TCR, two CD3 molecules and a homodimer of ζ-chains. CD3 and the ζ-chain contain ITAMs (yellow segment) that enable signal transduction into the cell.

While a T cell matures, several mechanisms ensure the almost limitless variability of the TCR. The TCR recognition site is encoded as a sequence of different elements that are recombined in a process called somatic recombination (Hayday et al., 1985). In the α chain sequence there are 70-80 V (variable) and 61 J (joining) segments. The genetic variability of the ß chain is encoded in the 52 V, 13 J and 2 D (diversity) genes. In the recombination process one V and one J segment are combined with the constant domain to form an α-chain. To form a ß chain one V, one D and one J segment are joined with the constant domain (Rowen et al., 1996). The recombination is mediated by RAG1/RAG2 and this joining process produces additional diversity by randomly adding several nucleotides to the emerging TCR sequence (Shinkai et al., 1992). This junctional diversity increases the total theoretical TCR diversity to an estimated 10^{18} unique TCRs. This predicted diversity is so enormous that it cannot be totally utilized by one living organism (Sepulveda et al., 2010).

The α and the ß chain have only very short cytoplasmic tails that are not able to pass information to the cytoplasm. To enable signal transduction into the cell, the TCR is associated with the CD3 complex (Figure 1-11). The CD3 complex consists of six trans-membrane proteins, each containing one or more immunereceptor tyrosine-based activation motives (ITAMs) that are important for intracellular signaling. In particular, the

TCR complex consists of the TCR (one α and one ß chain) associated with two γ, two ε and 2 ζ chains.

1.3.3 The TCR coreceptor

Mature T cells carry one of two possible coreceptors, CD4 or CD8. The engagement of this coreceptor enables effective TCR signaling. The coreceptor binds to conserved regions of the HLA molecule and increases the affinity of the TCR/HLA complex upon binding.

CD4 is the coreceptor of the helper and the majority of regulatory T cells. It is a monomer consisting of four Ig-like domains (D1-D4) and a short cytoplasmic tail interacting with the signaling kinase lck (Campbell et al., 1995). The D1 domain binds the $ß_2$-domain of the MHC class II molecule (Cammarota et al., 1992).

CD8 is the coreceptor of the CTLs. In its most frequent form it is an αß chain heterodimer, the αα homodimer being less abundant. Each chain has one Ig-like domain and a transmembrane domain tying the proteins to the cell membrane (Leahy, 1995). The α chain extends a short tail into the cytoplasm that interacts with lck (Campbell et al., 1995). On the extracellular site the α chain binds to the $α_3$ domain of the MHC class I molecule (Gao et al., 1997).

1.3.4 The MHC molecules

The major histocompatibility complex (MHC) originally referred to a gene cluster encoding genes directly responsible for the rejection of transplanted organs (Stimpfling, 1971). Closer investigation revealed that this gene cluster orchestrates many genes important for the immune system, among others TNF and classical and non-classical MHC molecules (Maenaka and Jones, 1999). In humans, the corresponding region is located on chromosome 6 and is called the Human Leukocyte Antigen (HLA).

MHC or HLA also refer to molecules that present short protein fragments of various origins, so called epitopes to the TCR. There are two main types of classical MHC molecules that differ in size and origin of the presented peptide as well as the T-cell population they address.

- MHC class I: The MHC class I molecule is formed by a 45 kDa heavy chain and a small 12 kDa $ß_2$ microglobulin. This complex, which is anchored in the cell membrane via a trans-membrane domain, is present on the surface of all nucleated cells. In the closed peptide binding cleft, which is formed by the heavy chain, a short peptide of cytosolic origin with a length of 8-10 amino acids is presented (Gromme and Neefjes, 2002; Rammensee, 1995). MHC class I/peptide complexes are recognized by $CD8^+$ CTLs. The three classical HLA class I molecules are HLA-A, HLA-B and HLA-C
- MHC class II: These molecules are found in high amounts only on specialized cells of the immune system. These so called "antigen presenting cells" (APCs), such as dendritic cells (DCs), macrophages and B cells, can process extracellular proteins and present the resulting fragments in the open peptide binding cleft formed by the α and ß chain together. Because of the open form of the binding cleft, the presented peptides are not as firmly restricted in size as the peptides presented by MHC class I. HLA class II epitopes extend between 9 and 25 amino acids (Rammensee, 1995; Villadangos, 2001). MHC class II/peptide complexes are recognized by $CD4^+$ T cells. The three classical HLA class II molecules are called HLA-DR, HLA-DP and HLA-DQ.

MHC molecules can present a great variety of peptides. It is estimated that a somatic cell carrying between 10^5 and 10^6 MHC molecules presents around 10^4 different peptides. This variability of presentation is based on three major factors.

- The peptide binding properties of the MHC molecules are degenerated. The MHC molecule of a certain allele not only binds a predetermined set of peptides but also demonstrates a general affinity towards a specific peptide motive.
- Polygenicity: For the classical HLA molecules, there exist several genes encoding different molecules with different peptide preferences. E.g. for the HLA class I molecules there are the three different genes HLA-A, HLA-B and HLA-C. For class II, the variability is further increased by the fact that different α chain variants can combine with different ß chains.
- Polymorphism: Within the human species there are several hundred different variant alleles of the HLA genes, making it highly unlikely that two individuals share the same HLA genes. This variability is even greater since both chromosomes are used for gene transcription.

1.3.5 Peptide processing

The peptides presented by the MHC class I and MHC class II molecules originate from two different sources:

MHC class I ligands: The central role of this pathway is to turn the cell inside out (Figure 1-12). To make the cell interior transparent for the immune system, every cellular protein is sampled and short fragments are presented on the outside of the cell to the $CD8^+$ T cells of the adaptive immune system (Jensen, 2007). Most intracellular proteins end up in the proteasome, where they are digested (Schubert et al., 2000; Yewdell and Bennink, 2001) to peptides 2 to 25 amino acids in size (Kisselev et al., 1999; Nussbaum et al., 1998). The peptides are trimmed by cellular aminopeptidases (Kloetzel, 2004) and are transported into the endoplasmatic reticulum (ER) by the transporter associated with antigen processing (TAP) (Neefjes et al., 1993). TAP is a heterodimer of TAP1 and TAP2. It binds peptides from the cytosol with a length of 8 to 13 amino acids (Momburg et al., 1994). Using ATP it translocates the peptide into the ER lumen. In a final process involving the chaperones calnexin, tapasin and calreticulin the MHC class I molecules are assembled and loaded with the incoming TAP-derived peptide (Ortmann et al., 1997; Wearsch and Cresswell, 2007). Final trimming of the bound peptide occurs by ERAAP (Saric et al., 2002) before the completed MHC/peptide complex is transported to the membrane.

Immunological activation, e.g. by interferons (IFNs) or by reason of tumor necrosis factor (TNF), causes the cell to change the catalytic active subunits and, as a result, the specificity of the proteasome to the immunoproteasome (Aki et al., 1994). This enables the cell to respond to immunological danger signals by enhancing epitope production and MHC loading. This leads to a better representation of pathogens that have invaded the cell (Van den Eynde and Morel, 2001).

While most cells display this processing method, a small subset of immunologically important cells, the APCs, are endowed with the additional ability to process proteins from the extracellular environment and present them on MHC class I molecules (Rock and Shen, 2005). This ability, called “cross-presentation”, is normally restricted to the MHC class II processing pathway. Once deemed to be an exception of minor importance, it is now believed to be essential for the induction of an immune response against tumor antigens

and pathogens not infecting APCs (Matera and Garetto, 2009; Plautz et al., 2000; Winau et al., 2006).

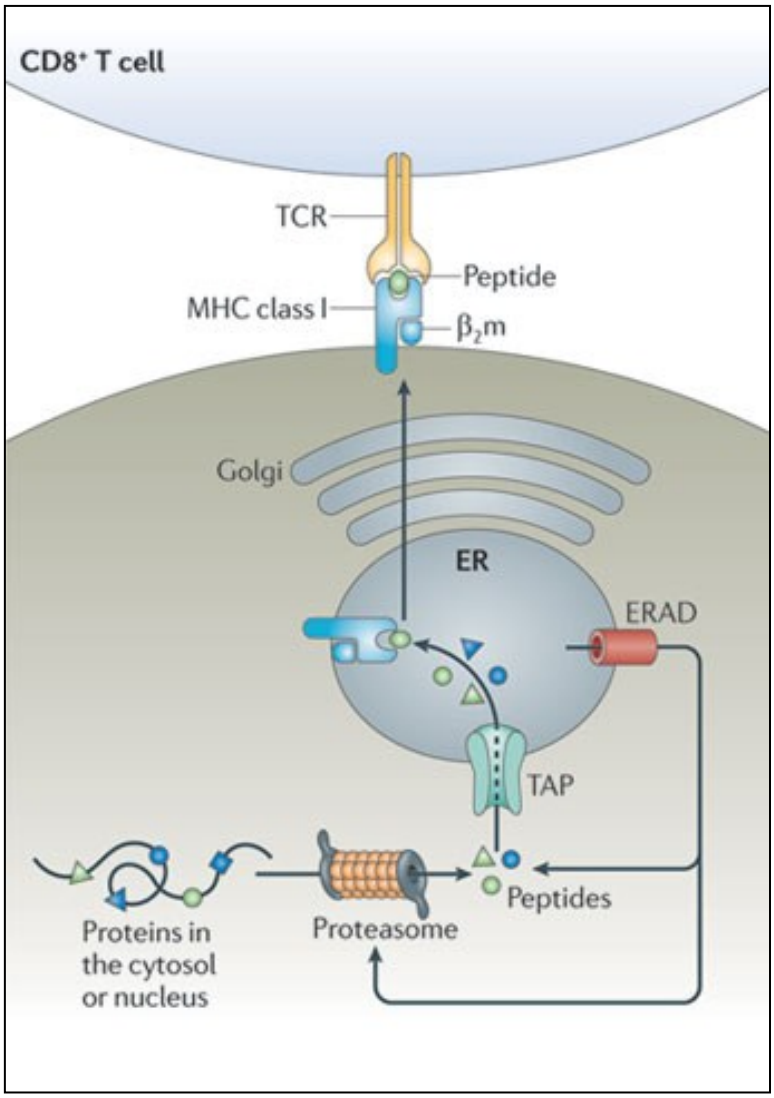

Figure 1-12.The MHC class I antigen presentation pathway (Neefjes et al., 2011). Cellular proteins are degraded by the proteasome. The resulting peptides are transported into the ER and are loaded onto MHC class I molecules. The loaded MHC complex is transported to the cell membrane and scanned by passing $CD8^+$ T cells.

MHC class II ligands: In contrast to the class I presentation pathway, the peptides that finalize the class II processing display the extracellular environment to passing $CD4^+$ T cells (Jensen, 2007). The α and the ß chain of the MHC class II molecule are synthesized into the ER. Together with the invariant chain (Ii or CD74) they form a first MHC class II molecule trimer. Ii fulfills several functions; first, it prevents the binding of ER peptides, second, it stabilizes the forming complex and third, it addresses the complex to endolysosomal compartments, which are also called MHC class II compartments (Bryant and Ploegh, 2004;

Neefjes et al., 2011). Upon acidification of the lysosome, the invariant chain is degraded by lysosomal enzymes. When the lysosome fuses with a phagosome, the remaining fragments of Ii are replaced with the help of the HLA-DM molecule by peptides present in the phagolysosome (Trombetta et al., 2003). In this way the origin of the phagosome affects the composition of presented peptides. The loaded MHC class II molecules are transported in tubular structures that are fused to the cell membrane at the site of T-cell interaction (Boes et al., 2003; Vyas et al., 2007). It is believed that only APCs are able to phagocytose and process proteins that originate from different sources (Figure 1-13. The MHC class II antigen presentation pathway (Neefjes et al., 2011).).The extracellular environment is actively sampled by macropinocytosis and engulfed pathogens are readily processed (Ackerman et al., 2003). Autophagy targets pathogens that live within either the phagosomes or the cytosole. It also plays an important role by delivering self-proteins from cells undergoing stress-induced autophagy to the processing machinery (Dengjel et al., 2005; Levine and Deretic, 2007; Schmid and Münz, 2007).

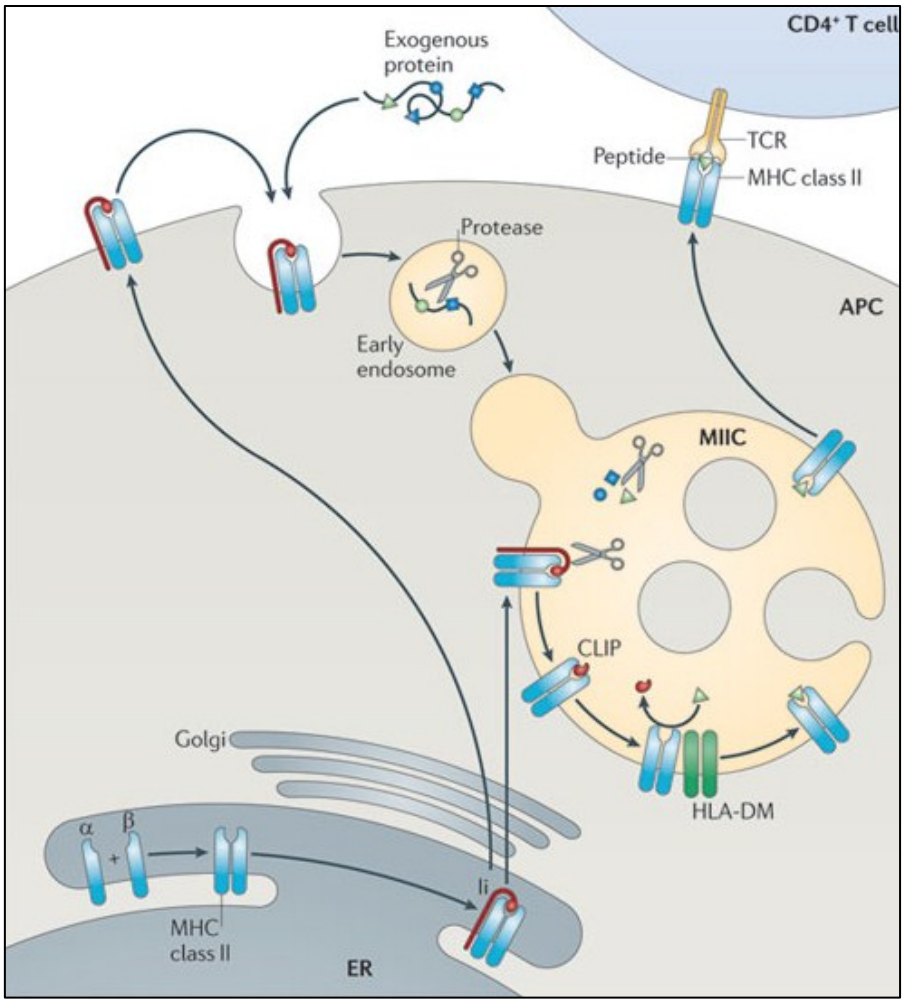

Figure 1-13. The MHC class II antigen presentation pathway (Neefjes et al., 2011). In the ER MHC class II trimers are assembled together with Ii. This complex is transported directly or via the plasma membrane to the MHC class II compartment (MIIC). Ii is degraded and the remaining CLIP fragment is exchanged with the help of HLA-DM. The loaded MHC class II molecule is transported to the cell membrane and is scanned by passing $CD4^+$ T cells.

1.3.6 T-cell development

T cells, as part of the hematopoietic system, are formed in the bone marrow by the division of hematopoietic stem cells. The name T cell derives from the next step in their maturation, which takes place in the thymus (von Boehmer, 1988). When they migrate into the cortex of the thymus, the immature T cells do not carry any coreceptor, neither CD4 nor CD8. These thymocytes are therefore called “double negative” (Scollay and Shortman, 1985; Shortman and Wu, 1996). At the contact region between the medulla and the cortex of the thymus, thymocytes start to express the Rag genes 1 and 2 and gene rearrangement in the TCR genes begins. At this stage the cells can be distinguished by the expression of the surface markers CD25 and CD44 (Zuniga-Pflucker and Lenardo, 1996). The β-TCR locus is rearranged first by combination of a V-, a D- and a J segment (Willerford et al., 1996). The emerging ß chain is

tested together with a pre-TCR-α chain for functional expression. Thymocytes that do not express a useful pre-TCR can be rescued by a successful subsequent rearrangement of the ß locus but will eventually die after repeated futile recombination.

After several cell divisions the successful cells continue with the rearrangement of the α-chain. This stage is characterized by the simultaneous expression of both coreceptors CD4 and CD8 and for that reason the cells are called “double positive” thymocytes (MacDonald et al., 2001).The α-chain rearrangement resembles the ß-locus rearrangement, the difference being the combination of only one V- with one D-segment to heighten the chance of a successful outcome. Recombination is continued until the cell runs out of combinable segments (followed by death) or until a productive TCR can be expressed and positive selection can take place in the cortex of the thymus (Amsen and Kruisbeek, 1996). Only a thymocyte with a TCR that can recognize the surrounding MHC class I or class II molecules will receive a survival signal. This signal also invokes the ceasing of the coreceptor’s expression not engaged in MHC recognition. These single positive thymocytes migrate to the thymic medulla where they interact with APCs and thymic epithelial cells (TECs) to undergo negative selection (Le Borgne et al., 2009; Nitta et al., 2009). Using the transcription factor AIRE (autoimmune regulator) allows TECs the ectopic expression of almost all possible proteins (Liston et al., 2003), thereby ensuring the presentation of all possible self-epitopes in the thymic medulla. A single positive thymocyte receiving a strong signal through its TCR will commit apoptosis, which prevents the emergence of auto-reactive T cells. The whole process of thymic maturation takes about three weeks (Surh and Sprent, 1994).

After thymic maturation the naïve T cells leave the thymus to circulate through the blood and the lymph system. They enter the lymphatic system through the high endothelial venules of the lymph nodes, circulate through the lymphatic vessels and reenter the bloodstream in the subclavical veins. As this circle continues, the naïve T cells patrol between the blood and the lymph (Picker, 1994). In the lymph node they scan the present APCs for their specific MHC/peptide combination (Banchereau and Steinman, 1998) until they eventually find their specific antigen. Naïve T cells can survive for several years in the periphery getting survival signals by low affinity interactions with self-peptide/MHC complexes, similar to those occurring during positive selection (Chen, 2003; Kirberg et al., 1997). Because all T cells compete for these binding sites, the system prevents a single T cell

clone from proliferating without limits and ensures a high variability of circulating T-cell specificities. Dying cells are replaced by cell divisions occasionally or by new cells from the thymus, keeping the total amount of T cells within tight limits (Hayden et al., 1996).

1.3.7 T-cell activation

To become fully activated a naïve T cell requires an additional signal to the TCR triggering by its specific MHC/peptide complex. The sole TCR triggering signals the recognition of self-antigen and pushes the cell into anergy (Mirshahidi et al., 2004), a state in which activation is almost impossible (Andrews et al., 1997). The first activation of a naïve T cell, the so called "priming", occurs only after receipt of the second signal, the costimulus, simultaneously to the TCR triggering (Brinkmann et al., 1996).

When a T cell recognizes its specific MHC/peptide complex, the forming TCR/MHC/peptide trimer becomes stable for a prolonged period and an immunological synapse is formed (Lee et al., 2002). High amounts of TCR, of the major costimulatory molecule CD28 and of the adhesion molecule LFA-1 (lymphocyte function-associated antigen 1) are concentrated in this immunological synapse. The ligands of these molecules are expressed by activated APCs. CD28 is ligated by CD80 and CD86, while LFA-1 binds ICAM-1. There are more such pairs all supporting the activation of the T cell, either by mediation of a costimulus or by stabilization of the immunological synapse. Costimuli are mediated by interaction of 4-1BB (on the T cell) and 4-1BBL (on the APC) and by ICOS (T cell) and ICOSL (APC), while adhesion is increased by LFA-3 (T cell) DC-SIGN (APC) interaction (Batista and Saito, 2010; Bromley et al., 2001; Dustin and Shaw, 1999; Goldstein et al., 2000). Upon formation of the immunological synapse and the associated delivery of stimulus and costimulus, several changes occur in the activating T cell. The T cell produces Interleukin (IL)-2, an important T-cell growth factor, and starts the expression of the α chain of the IL-2 receptor (CD25). This expression enables the association of the high affinity IL-2 receptor, thus increasing the activating effect of the autocrine IL-2 production (Appleman et al., 2000). Furthermore, the activating T cell expresses CD154 that ligates CD40 on the APC and induces an even higher expression of CD80/86v(Tourret et al., 2010). These changes enable the activating T cells to proliferate for several days. In their entirety, the strength of the TCR signal and the costimulus, together with the cytokine environment, deliver a "signal 3" that determines the lineage commitment (see 1.3.9: T-cell populations and T-cell response) of the primed T cell.

After four to seven days the T cells change from naïve to effector status. The cells express CTLA-4 (cytotoxic T lymphocyte activation antigen 4), the counterpart of CD28. CTLA-4 binds the ligands of CD28 with even higher affinity. CTLA-4 transmits not an activation but a deactivation signal (Fife and Bluestone, 2008; Rudd et al., 2009), thereby self-limiting the overall activation of the T cells.

Activated effector T cells can be identified via changes in the expression of several surface molecules. Some of these molecules, for example CD25 (the earlier described α chain of the high affinity IL-2 receptor), prepare the cells for increased activation. Other molecules, for example CD154 (also called CD40L) that can activate macrophages and B cells, enable the cell to express effector functions. Another set of marker molecules is responsible for a changed trafficking behavior of the effector cells.

1.3.8 T-cell trafficking and phenotype

Depending on its degree of maturity, the T cell needs to target different tissues in order to meet an adequate partner cell. A freshly generated progenitor cell needs to target the thymus for receptor rearrangement, while a recently primed T cell must leave the lymph node and travel to the site of infection. Its contact with the APC decides about the further destiny of the T cell (Lanzavecchia and Sallusto, 2000). To perform these different tasks, T cells express different chemokine receptors at different stages of their maturation (Marelli-Berg et al., 2008). The expression of these chemokine receptors, especially of the chemokine receptor CCR7 in combination with CD45 is used to subdivide T cells into distinct phenotypes that differ in their function and trafficking behavior (Sallusto et al., 1999). CD45 is a protein tyrosine phosphatase present on all hematopoietic cells. In T cells it is known to be a regulator of the TCR triggering. In dephosphorylating it activates lck, an integral part of TCR signaling (McNeill et al., 2007). The regulation is not yet fully understood but seems to depend on the expression level of the different splice variants of CD45 (Holmes, 2006). CD45RO is the shortest splice variant as it lacks the RA, RB and RC exons. This variant facilitates the TCR activation better than the longer CD45RA variant (Dornan et al., 2002).

Naïve T cells: These cells have left the thymus as fully matured T cells that have not yet encountered their specific antigen (see 1.3.6). They patrol the lymph and the blood system. The induction of their adhesion molecule L-selectin (CD62L) is the result of CCR7 engagement. The ligands of CCR7 are expressed by the cells of the high endothelial venules.

The CCR7 engagement enables the naïve T cells to enter the lymph nodes through the high endothelial venules and to scan the present APCs for their specific antigen (Miyasaka and Tanaka, 2004; von Andrian and Mempel, 2003). Naïve T cells are characterized by low numbers of activation markers such as CD25 and by the expression of CD45RA.

Antigen experienced T cells: these cells have already encountered their specific antigen presented by an APC; they have been primed. Three distinct subsets can be distinguished by their phenotype:

1. *Effector T cells (T_{EFF})*: These relatively short-lived T cells carry out most effector functions directly after an infection. They produce high amounts of activation markers such as CD25 and effector molecules, like TNF (tumor necrosis factor α) or perforin. They express the RA splice variant of the protein tyrosine phosphatase CD45. But as T_{EFF} cells have already been primed by an activated APC, they lack the expression of CCR7 and do not home to secondary lymphoid organs. On the contrary they must find their antigen in infected tissue. Therefore, they express the chemokine receptors CCR4, CCR6 and CCR9 to enter the skin or the small intestines (Marelli-Berg et al., 2008).

Memory type T cells are long-lived cells that are characterized by the expression of the splice variant CD45RO. The activation requirements of memory cells are not as tightly restricted as the activation of naïve T cells but for most effective activation they also require costimulation (Dutton et al., 1998).

2. *Effector memory T cells (T_{EM})*: T_{EM} represent long-lived versions of T_{EFF}. They express the short RO version of the CD45 phosphatase. As T_{EFF} do, they lack the ability to enter lymph nodes but patrol different tissues in search of their specific antigen. Hence, they do not carry CCR7 but instead CCR4, CCR6 and CCR9 (Marelli-Berg et al., 2008).T_{EM} do proliferate very slowly to compensate for cell loss. This homeostatic proliferation seems to be dependent on continuous TCR-p/MHC contact and IL-15 signaling (Wherry et al., 2003). Upon reactivation they can easily regain full effector functions as displayed by T_{EFF}.

3. *Central memory T cells (T_{CM})*: These long-lived cells also express CD45RO but retain CCR7 and CD62L expression and are therefore located mainly in the secondary lymphoid organs (Sallusto et al., 2004). Their homeostatic proliferation is relying only on IL-7 and 15, not on TCR-p/MHC interaction (Boyman et al., 2007; Swain et al., 1999). Their main function is the stimulation of proliferation of other T and B cells.

1.3.9 T-cell populations and T-cell response

Depending on their coreceptor T cells can be divided into two major groups that are fulfilling quite different functions.

Members of one group express CD8 and, according to their main function, are called T-killer cells or cytotoxic T lymphocytes (CTL). They can identify virus infected cells and cells that harbor intracellular bacteria. CTLs can also differentiate between malignant and non-malignant cells. They can then kill these dangerous cells by any of several mechanisms, including perforin/granzyme or FasLigand mediated apoptosis (Henkart, 1994; Russell and Ley, 2002). Furthermore, activated CTLs produce and release soluble mediators, such as IFN-γ or TNF, upon TCR complex activation (Kuwano et al., 1993).

The other major group, the $CD4^+$ T cells, is divided into several T-Helper (T_H) cell subsets and regulatory T cells. These cells regulate, support or suppress other cells. Depending on their actions the $CD4^+$T cells are divided into T_H1,T_H2,T_H17 and T_{Reg}. The lineage commitment between T_H1, T_H2 and T_H17 takes place during the first priming process. Important determinants of this “signal 3” are the antigen dose, the type and strength of costimulation and the cytokine milieu in the priming environment (Constant et al., 1995; Dong and Flavell, 2000; Fietta and Delsante, 2009; Pfeiffer et al., 1995). T_{Reg} develop in the thymus or in the periphery by recognition of self-peptides, but the exact mechanisms leading to T_{Reg} lineage commitment are controversial (Barnes et al., 2009; Liston et al., 2007; Sakaguchi et al., 2010).

In the past it was thought that this cell lineage commitment was irreversible. Now there is growing evidence that former commitment is reversible and that for example a central memory T cell can change from T_H1 to T_H2 and *vice versa* (Sallusto et al., 2004).

T_H1: These cells communicate with the cellular arm of the immune system. They prepare the immune system to face intracellular pathogens, such as intracellular bacteria or viruses. They

activate macrophages (Stout and Bottomly, 1989) and CTLs (Andreasen et al., 2000). The leading cytokines that mediate the T-cell support are Interferon γ (IFN-γ), IL-2 and IL-3 (Fietta and Delsante, 2009).

T_H2: Under the influence of T_H2 cells, the humoral immune response is enhanced to confront such challenges as extracellular bacteria, viruses and helminths (Fietta and Delsante, 2009). The combined actions of cytokines, such as IL-4, IL-5, IL-6 and IL-13, activate B cells to become plasma cells and produce antibody (Croft and Swain, 1991; Parker, 1993).

T_H17: The presence of TGF-ß and IL-6 induces the formation of T_H17 cells. These cells can produce IL-17, which plays an important role in the recruitment of neutrophils in the immune system's fight against extracellular bacteria, fungi and worms (van de Veerdonk et al., 2009; Wan, 2010).

T_{Reg}: The $CD4^+$ T-cell population also includes a subset of cells that do not activate but downregulate the immune system. These cells are characterized by a high expression of CD25 and the transcription marker FoxP3 (Fontenot and Rudensky, 2005). Natural T_{Reg}s develop in the thymus (Itoh et al., 1999) and reach a population size of around 10% of all $CD4^+$ T cells (Sakaguchi et al., 1995). T_{Reg}s can down modulate CD4 and CD8 T-cell responses. Multiple mechanisms were revealed, but it is uncertain to what extent each alone contributes to the maintenance of self-tolerance or to homeostasis (Sakaguchi et al., 2009). The mechanisms do include soluble suppression factors, such as IL-10 and TGF-ß (Takahashi et al., 1998; Thornton and Shevach, 1998) but are also mediated by direct cell-cell interaction. Direct inhibition is mediated by high level of CTLA-4 (Wing et al., 2008) and a strong competition for available IL-2 (Antony et al., 2006; Setoguchi et al., 2005).

1.4 HPV and the immune system

1.4.1 Immune evasion of HPV

PV have coevolved with primates and their immune system for a long time (Bernard, 2005; Chan et al., 1997). In this long process HPV has developed many strategies to avoid detection and to hinder or prevent successful handling by the immune system. None of HPV's proteins is involved in direct interaction with the immune system. HPV achieves immune evasion by other indirect means:

1. *No "danger signal":* To become activated, APCs need a pro-inflammatory danger signal. In viral infection, the strongest signal is normally provided by the immunogenic death of virus-infected cells caused by lysis (Galluzzi et al., 2010). Since HPV has no lytic phase this strong signal is absent, and no strong APC activation or T-cell migration takes place (Kupper and Fuhlbrigge, 2004).
2. *Hiding*: The viral life cycle has no blood-borne phase. HPV does not produce any secretory proteins but only expresses low amounts of proteins that are mainly located in the nucleus (Greenfield et al., 1991). The most immunogenic L1 and L2 proteins (Rudolf et al., 1999) are only expressed in the terminally differentiated outer layer of the epithelium, which is not accessible to the immune system. There are only very few occasions when the immune system can recognize the virus and direct a proper response against it.
3. *Interference with antigen presentation*: The viral oncogene E7 of HPV types 16 and 18 not only fulfills its mitogenic activity but also represses the promoter for the MHC class I heavy chain (Georgopoulos et al., 2000). E7 of HPV18 additionally represses another bidirectional promoter for the expression of TAP1 (Kanodia et al., 2007). By this means HPV decreases the surface expression of MHC class I and, therefore, the presentation of HPV peptides.
4. *Molecular mimicry*: The HPV16 E7 protein has widespread similarity to several human proteins (Natale et al., 2000). T cells recognizing epitopes, which are shared by the human self-protein and viral E7 are already deleted by negative selection in the thymus. T_{Reg} cells recognizing these shared epitopes can shift the microenvironment into induction of tolerance (Doan et al., 2000). Together with the missing "danger

signal" this might grant E7's peripherally toleriogenic characteristics (Doan et al., 1999; Tindle et al., 2001).

1.4.2 The immune response to HPV

1.4.2.1 Complications in the revelation of the immune response to HPV

Several factors have complicated the revelation of the exact immune response to HPV. The high genetic variability of the different HPV types makes general statements almost impossible. Differences in the viral proteins also influence the immune response. Therefore, insights into the immune response to one specific type of HPV are not readily transferable to other HPV types.

Furthermore, there are only limited *in vitro* data of HPV infection. Most HPV infections do not cause severe symptoms and are cleared by the immune system without ever being diagnosed. Additionally, no mouse or rat animal model does exist because HPV is exclusively adapted to humans and does not infect any other species (Kanodia et al., 2008).

1.4.2.2 Humoral immune response to HPV

The L1 protein is the most immunogenic viral protein of HPV, but antibody levels after natural infections are neither particularly high nor especially long lasting (Carter et al., 2000). Compared to other viral challenges, the approximate 6 months to seroconversion is very long. Between 30-50% of infected individuals do not seroconvert at all (Carter et al., 2000). After virus clearance the antibody levels drop, with about 50% of the seroconverters still having detectable amounts of antibodies after five years (Wang et al., 2004b). In those, who mount an antibody response there is no strong correlation between antibody level and presence of HPV DNA (Andersson et al., 2008).

The antibody response against the L2 protein is less distinctive. The relevant epitopes are only accessible following a change of conformation after the virus has bound to the cell surface (Day et al., 2008).

Antibodies against the viral E proteins can be detected in healthy individuals, but are most consistent in people with cervical cancer. Whether these antibody levels can be used as biomarkers for a developing or established cervix carcinoma is being considered (Rosales et al., 2001).

1.4.2.3 Cellular immune response

Since the continuous expression of the viral oncogenes E6 and E7 is essential for the developing cancer cell, these proteins are expected to be key targets for the immune system. Therefore, the available data are concentrated on these two proteins.

Persistent defects in the cellular immune system due to HIV infection (Nicol et al., 2008; Palefsky, 2007), therapeutic immunosuppression (Paternoster et al., 2008; Ulrich et al., 2008) or inherited partial cellular deficiencies (Bernier-Buzzanga and Su, 1990; Lawrence et al., 2005) cause a higher frequency of HPV persistence and a higher risk of HPV associated diseases (Branca et al., 2003). The application of imiquimod, a Toll like receptor 7 ligand, which functions as an immune danger signal, causes clearance of genital warts in half the reported cases (Brodell et al., 2007). This shows that although the naturally occurring T-cell response is important, it cannot prevent the establishment of a tumor in all cases (Trimble et al., 2010).The presence of E7-specific CD4 T cells correlates with the absence of the virus from the cervix (Seresini et al., 2010). For CD4 T cells specific to E6 and E7 a correlation to prolonged disease free survival in patients with deeply infiltrating tumors has already been proven (Heusinkveld et al., 2011; Kim et al., 2011).These E6 and E7 specific CD4 T cells have a large polyclonal receptor repertoire recognizing multiple epitopes, emphasizing the inability of HPV to avoid detection by the immune system completely (de Vos van Steenwijk et al., 2010).

The occurrence of CD8 T cells specific for the E6 protein seems to be favourable to the patient (Nakagawa et al., 2010). The T cells can recognize and kill cervix carcinoma cells despite partially impaired effector functions (Thomas et al., 2008).

All this suggests an important role of the T-cell compartment in preventing HPV from establishing a persistent infection that may ultimately lead to intraepithelial neoplasia and cancer.

1.4.2.4 Class II restricted T-cell response to L1

Several studies addressed the L1-specific T-cell response to HPV6, 11, 16 and 18 (de Gruijl et al., 1999; Gelder et al., 2003; Hong et al., 1997; Shepherd et al., 1996; Strang et al., 1990; Williams et al., 2002). The identified class II restricted epitopes are summarized in Table 1-1.

Most data rely on proliferative T-cell responses of small groups of a disease population. Williams et al. used an approach of overlapping 15-mer peptides spanning the whole L1 protein of HPV11. These peptides were tested with PBMCs from 25 healthy donors. Besides identifying several epitopes, they also collected first evidence for cross-reactive $CD4^+$ T cells that recognize peptides from different HPV types. They were the first to speculate about whether an existing T-cell response to one HPV type fastens the immune response to a second type. "…, the question of whether T-cell responses selected by one HPV type might modulate the immune response to a second HPV type arises. Even a weak response early in the course of infection might alter the resulting viral load and the subsequent disease process." (Williams et al., 2002)

Table 1-1. Published class II restricted epitopes from L1 HPV6, 11, 16 and 18.

HPV type	position	sequence	reference
6	311-330	KAQGHNNGICWGNQLFVTVV	Hong et al., 1997
6	411-430	DTYRYVQSQAITCQKPTPEK	Hong et al., 1997
11	30-44	TNIFYHASSSRLLAV	Williams et al., 2002
11	68-82	RVFKVVLPDPNKFAL	Williams et al., 2002
11	186-200	ELITSVIQDGDMVDT	Williams et al., 2002
11	204-218	AMNFADLQTNKSDVP	Williams et al., 2002
11	237-257	DPYGDRLFFYLRKEQMFARHF	Williams et al., 2002
11	286-300	SIYVHTPSGSLVSSE	Williams et al., 2002
11	303-317	FNKPYWLQKAQGHNN	Williams et al., 2002
11	356-370	YKEYMRHVEEFDLQF	Williams et al., 2002
11	380-394	SAEVMAYIHTMNPSV	Williams et al., 2002
11	412-426	DTYRYVQSQAITCQK	Williams et al., 2002
16	40-63	PPVPVSKVVSTDEYVARTNIYYHA	Strang et al., 1990
16	91-106	VSGLQYRVFRIHLPDP	Strang et al., 1990
16	219-244	TVIQDGDMVDTGFGAMDFTTLQANKS	Strang et al., 1990
16	279-294	EQMFVRHLFNRAGAVG	Strang et al., 1990
16	311-335	NLASSNYFPTPSGSMVTSDAQIFNK	Shepherd et al., 1996
16	311-335	NLASSNYFPTPSGSMVTSDAQIFNK	de Gruijl et al., 1999

1.4.3 HPV vaccines

1.4.3.1 Preventive vaccines

The L1 protein of HPV readily forms virus like particles (VLPs). If injected intramuscularly, these VLPs are highly immunogenic and induce a very strong immune response. Several trials working with different VLP combinations of different HPV types tested their applicability as

HPV vaccines. The antibody levels reached after vaccination were several fold higher than the antibody levels occurring during natural infection and were sustained for over 4 years (Harper et al., 2004; Harper et al., 2006; Mao et al., 2006; Villa et al., 2006; Villa et al., 2005).These encouraging results led to several phase III trials testing the efficacy of the vaccines.

The results of one phase III trial, that enlisted over 18 000 women, showed a prophylactic efficacy of the adjuvanted, bivalent HPV16/18 vaccine in preventing infection by HPV16 and HPV18 which lead to fewer CIN 2+ lesions (Kreimer et al., 2011; Paavonen et al., 2007).The quadrivalent HPV6/11/16/18 vaccine additionally prevented infection with the two types HPV6 and HPV11 that cause genital warts. (Einstein et al., 2011; Munoz et al., 2009). Both vaccines were licensed: Gardasil (quadrivalent HPV6/11/16/18 vaccine) in 2006 and Cervarix (bivalent HPV16/18 vaccine) in 2007.

The reported periods of protection reach 5 years for Gardasil and over eight years for Cervarix. But these reported times may not be the upper limits since the initial clinical trials are still ongoing (Harper and Williams, 2010; Romanowski, 2011; Schwarz et al., 2011).

Both vaccines are only efficient in preventing the initial primary infection. Therefore, in order to prevent HPV-caused malignancies, they have to be administered before the first contact with the oncogenic HPV types.

1.4.3.2 Therapeutic vaccines

Even with the high coverage rate of the preventive HPV vaccines, it will take several decades until they take full effect. Therefore, therapy for currently HPV-infected patients, who are developing malignancies, is still an important issue.

The development and the maintenance of HPV-infected cancer cells need the constant expression of E6 and E7 (Steenbergen et al., 2005; zur Hausen, 2002). Therefore, many different approaches target cells that express these proteins. These include vaccination with peptides, DNA or protein- or peptide-pulsed DCs (Kenter et al., 2009; Oosterhuis et al., 2011; Pokorná et al., 2009; Seo et al., 2009; Welters et al., 2010; Wu et al., 2010). These vaccinations have elicited immune response to various degrees. Unfortunately, however, so far no clinical success has been observed for patients with established tumors (van der Burg and Melief, 2011).

1.5 Theoretical background: Methods

1.5.1 PBMC isolation

PBMCs (peripheral blood mononuclear cells) are a group of immune cells that are easily prepared from whole blood. PBMCs consist of all mononuclear cell types found in the peripheral blood, namely lymphocytes and monocytes. The other constituents of peripheral blood, erythrocytes, thrombocytes and neutrophil granulocytes are separated by means of a density gradient centrifugation (Ulmer et al., 1984). Therefore, heparinized blood or leukocyte-enriched blood products are layered above a solution of Ficoll-Hypaque. This solution of the polysaccharide Ficoll and the iodine containing Metrizamid has a specific density of 1.077 g/ml. Centrifugation causes the various blood constituents to separate into different phases according to their density. Since the erythrocytes and the granulocytes have a higher density than the Ficoll-Hypaque solution they cross the high density Ficoll layer and pellet on the bottom of the centrifugation tube. Thrombocytes and soluble protein contents in the blood plasma do not move in the centrifugation acceleration and stay in the blood plasma phase above the Ficoll-Hypaque layer. The PBMCs cross the blood plasma layer but cannot enter the Ficoll-Hypaque solution because their specific density is less than 1.077 g/ml. They accumulate instead at the interphase between the plasma and the Ficoll-Hypaque, where they are easily collected.

1.5.2 SYFPEITHI

SYFPEITHI is software used to predict candidate epitopes for MHC class I and class II molecules given the amino acid sequence of a protein of interest (Rammensee et al., 1999). SYFPEITHI divides the amino acid sequence into all possible peptides of the interesting length (HLA class I epitopes: nonamer, decamer; HLA class II: 15mer). SYFPEITHI calculates a total score for each peptide by summing up the individual scores for each single amino acid within the peptide according to a scoring matrix specific for the HLA type of interest. These HLA-specific scoring matrixes are based on known natural ligands isolated from cells bearing the HLA type in question. For each position within the peptide the relative amino acid frequencies are calculated. According to these frequencies scores are assigned to all possible amino acids at all possible positions. To determine the final SYFPEITHI score of a peptide, the scores for the single positions are summed up. The higher the SYFPEITHI score, the greater is the chance that the peptide in question is a natural ligand or T-cell epitope. Because

SYFPEITHI uses natural ligands to calculate the scoring matrices it has the advantage of taking into account all steps of natural epitope processing. Not only binding affinities to the HLA molecule are taken into consideration, but also all other factors (e.g. TAP preferences, proteasome/immunoproteasome, and protein expression) that influence the final epitope presentation.

1.5.3 FACS

FACS (Fluorescence Activated Cell Sorting) is a term describing flow cytometry. This technique allows the quantitative analysis of single cells for the expression of surface and intracellular markers. The first "Impulsfluorimeter" (Dittrich and Gohde, 1969) was commercialized by the German company Partec as early as 1969.

The cells of interest are labeled with a fluorescent dye. The cell suspension is separated into single cells using a technique called "hydrodynamic focusing". The single cells then pass through a laser beam and in the process scatter the light according to their physiological properties. The scattered light is detected by photomultipliers and analyzed to determine the Forward Scatter (FSC), which reveals the cell's size and the Sideward Scatter (SSC) that displays the cell's granularity.

Depending on the fluorescent dyes bound to the cell, photons of a dye-specific energy/wavelength are adsorbed and the fluorescent molecule is excited. This excited state decays with a half-life of 0.5-20 ns and emits a photon of slightly less energy (increased wavelength) than the adsorbed one. The amount of energy lost and, therefore, the wavelength of the emitted light quant depends on the fluorochrome. This fluorescence is measured and is proportional to the number of fluorescent molecules bound to the measured cell. When fluorescent dyes with similar excitation but different emission patterns are used, excitation with one laser is possible but signals from the different dyes can be identified. Employing excitation lasers with different wavelengths increases the number of signals that can be measured simultaneously.

The ever increasing variability of accessible fluorescent dyes (Perfetto et al., 2004), including the development of tandem fluorescent dyes using fluorescence resonance energy transfer (FRET), and the usage of semiconductor Qdots (Barroso, 2011; Xu et al., 2007) have increased the amount of parameters that can be measured simultaneously.

Because the fluorescent light emitted also depends on the decay time of the excited dye molecule, which is distributed randomly, the emitted light is not a discrete wavelength but a broad emission peak. This causes the emission spectra of the different dyes to partly overlap. In a process called compensation the spillover is quantified so as to remove it from the collected results. Figure 1-14 exemplarily shows the spectral overlap of the two commonly used fluorescent dyes FITC and PE.

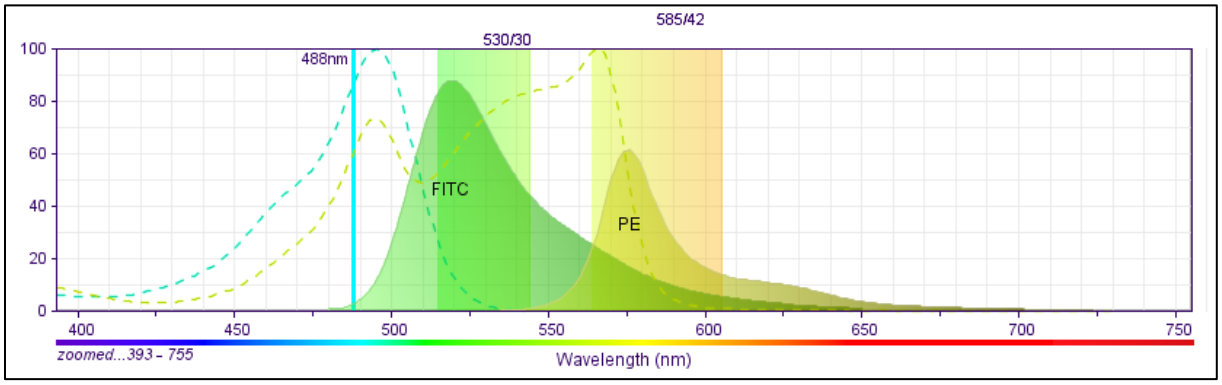

Figure 1-14. Excitation (dotted line) and emission spectra (shaded curve) of the two common fluorochromes FITC and PE. The wavelength ranges used for detection are displayed as shaded blocks. The wavelength of the laser used for excitation (488 nm) is indicated in blue. Taken from BD Fluorescence spectrum viewer www.bdbioscience.com.

1.5.4 Intracellular cytokine staining

Stimulated T cells that have encountered their specific antigen are activated and produce a wide array of different cytokines (Knutson and Disis, 2005). These cytokines can be measured and allocated to the producing cell by a technique called “Intracellular Cytokine Staining” (ICS). This method further allows characterization of the producing cell with population markers, such as CD3, CD4 and CD8. To allocate the cytokine to a cell, the release of the cytokine must be inhibited. This is done by the usage of the Golgi transport system inhibitors brefeldin A (BrefA) or Monensin. BrefA inhibits the transport from the ER to the Golgi apparatus (Klausner et al., 1992), while monensin stops the inter-Golgi apparatus transport (Rosa et al., 1992). Upon antigenic challenge the inhibitors are applied either alone or in combination, and the cells are allowed to continue cytokine production and accumulation. After permeabilization with a detergent and fixation with formaldehyde, the cells can be stained with antibodies against the cytokines. Because the transport inhibitors reduce cell viability the incubation time should not exceed16 h.

1.5.5 ELISPOT

The “Enzyme Linked Immune Spot” (ELISPOT) assay is a technique to identify and quantify cells that react to a challenge with the production and release of a molecule (e.g. a cytokine or an antibody). In this study an IFN-γ ELISPOT was used. IFN-γ is released by activated CD8 and CD4 T cells as well as by NK cells.

In a first step 96-well ELISPOT plates are incubated with a solution of a primary antibody directed against IFN-γ. The wells contain a filter membrane that consists of mixed nitrocellulose esters or polyvinylidene fluoride that allows the binding of the antibody to the membrane. In the next step the cells are given into the wells and the challenge is applied. The cells settle on the filter membrane and activated cells start to produce cytokine. The released cytokine is bound by the immobilized primary antibody in close proximity to the secreting cell. After a production time of 24 h the cells are washed away and the plates are incubated with a secondary biotin-labeled antibody directed against a second epitope of the same cytokine. The biotin label allows the attachment of an enzyme (alkaline phosphatase or horseradish peroxidase) that metabolizes a soluble substrate into an insoluble dye that precipitates in close proximity to the enzyme and forms a visible spot. Each spot identifies the position of a cytokine producing cell beforehand. The number and size of the spots are analyzed using an automated ELISPOT counter.

Because no toxic substance is used in the incubation period of the assay, the incubation time can be extended to over 24 h, thus allowing also weak responses to be detected. If PBMCs are used for an IFN-γ ELISPOT, the produced IFN-γ could originate either from different T-cell subsets or from NK cells, the latter forming smaller spots that can be excluded afterwards. The sensitivity mainly depends on the number of cells producing cytokine without being challenged. A high background complicates the differentiation between responding and non-responding cells. A very weak or marginal background allows a theoretical sensitivity capable of detecting a single cytokine producing cell within 500 000 analyzed cells. Figure 1-15 shows the schematic description of an ELISPOT assay.

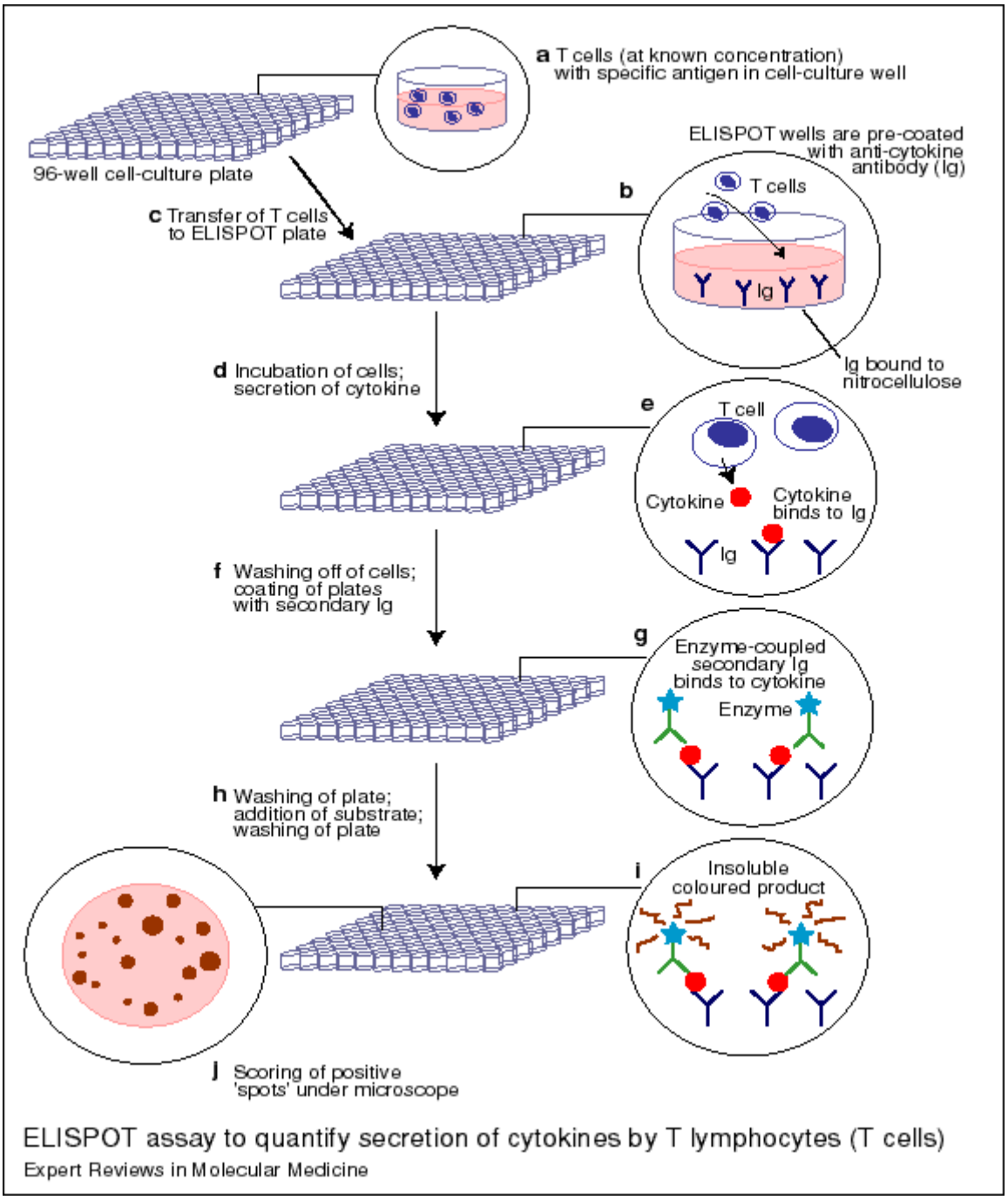

Figure 1-15. The ELISPOT assay. Taken from www.news-in-biotechnology.com.

1.5.6 *In vitro* stimulation and amplification of epitope specific T cells

The almost infinite variability of the TCR enables countless T cells of diverse specificities. The blood of healthy people contains only extremely low frequencies of T cells of a single specificity. To permit the detection of these low frequency cells, the epitope specific cells are activated to proliferate for several days. The peptide epitope in question is added together with low doses of IL-2. The artificial peptides replace the naturally occurring ligands on the MHC molecules on the PBMC, and the artificial peptides will be presented to the T cells in

the PBMCs. The IL-2 dose is kept low to avoid unspecific proliferation of T cells. This dose activates only the T cells that recognized the added peptide. The non-specific cells do not proliferate or even die. Thus, the frequency of present antigen specific T cells is increased above the detection levels of ELISPOT or ICS.

1.5.7 Multiplex bead array serology

Multiplex serology is a further development of the well-established enzyme linked immunosorbent assay (ELISA), developed in 1971 by Engvall and Perlman (Engvall and Perlmann, 1971; Lequin, 2005). The sandwich ELISA quantifies the presence of a molecule of interest using a pair of proteins that bind two different epitopes of the molecule in question. The protein is bound to the test plate and is used to immobilize the target. The target is detected by an antibody coupled to an enzyme. This technique only detects one or two different molecules in a single sample.

Multiplex serology on the other hand does allow the parallel detection of several hundred parameters. The primary binding protein is not fixed to the plastic surface of a microtiter plate but is coupled to tiny polystyrene beads with a diameter of 5.6 µm. The microspheres are impregnated with a mixture of two different fluorescent dyes. By using different ratios of these dyes, an array of several hundred different colored beads can be obtained. Each bead is defined by its internal color code ratio and is loaded separately with one single primary binding molecule each. After loading the bead sets are mixed again and are used together to analyze a sample. The binding of the molecule of interest is confirmed by the binding of a secondary fluorescence labeled antibody to a second epitope of the molecule. The luminex analyser, which resembles a two laser FACS analyser, reassigns the bead-bound florescence (that represents the bead-bound antigen) to the colour code of the bead, thus enabling the simultaneous measurement of over 100 different antigens.

1.6 Outline of the study

The aim of this study was the identification of CD4 T-cell epitopes from the L1 protein of HPV. Four clinically relevant HPV types were chosen for the approach. HPV16 and HPV18 are the most important types that cause HPV associated malignancies. HPV6 and HPV11 are the major cause of genital warts. All four types are included in the therapeutic HPV vaccine Gardasil.

The strategy of reverse immunology (Celis et al., 1994) was applied and candidate epitopes were predicted by SYFPEITHI. The best candidates were chosen for their SYFPEITHI score and their promiscuity. These peptides were tested in ELISPOT screenings with PBMCs from 30 donors. The HPV infection rate for the types of interest in Germany was assumed to be 10 to 25% (Barthell et al., 2009; Michael et al., 2008). We used healthy blood donors' PBMCs with random HLA class II typing. Because of the size and the promiscuity of the approach, we still expected a detectable IFN-γ response. HPV does not actively lyse cells and has no blood born phase so that the immune system rarely gets in contact with the L1 protein (Kupper and Fuhlbrigge, 2004). Therefore the incidence of L1 specific T cells was assumed to be very low. To increase the detection sensitivity of the ELISPOT assay, peptide specific pre-amplifications were performed before using the cells in ELISPOT assays.

Epitopes identified in the ELISPOT screenings were validated by flow cytometry.

2 Materials and Methods

2.1 Materials

2.1.1 Devices

Cell culture hood (Technoflow, Integra Biosciences)

Centrifuge (5415R, Eppendorf)

Cryo freezing container (Nalgene)

ELISPOT reader Immunospot with software Immunospot 3.2 and Image Acquisition 4.5 (Cellular Technologies Ltd.)

Flow cytometer FACS-Canto II with software FACSDiva (Becton Dickinson)

Fluorescence activated cell sorter FACS-Aria with software FACSDiva (Becton Dickinson)

Gammacell, cesium (Gammacell 1000 Elite Nordion International Inc.)

Incubator for cell cultures (Labotect 3250) with 5% CO_2 gas supply (Messer)

Luminex 100 Total system; comprising Luminex 100 analyzer, Luminex XYP plate handler, Luminex SD sheath fluid delivery system with software Luminex IS 2.2 SPI

Light microscope (Leica DMIL)

Membrane pump (KNF Neuberger)

Neubauer counting chamber, depth 0.1 mm (LO-Laboroptik)

Spinning wheel (Bachofer)

Steam sterilizer (KSG 113)

Steam sterilizer (Sauter 11-6-9 HS1-FD)

Tabletop centrifuges (Heraeus Biofuge fresco; Heraeus Megafuge)

Vortex (MS 1, IKA-Works Inc.)

Water bath (Thermomix BM-S, Braun Biotech)

Water distiller (Destamat Bi 18 E, Heraeus)

2.1.2 Materials

2.1.2.1 General materials

Cell culture plates, 6 well, 12 well, 24 well, 96 well (Costar)

Cell culture flasks (red cap), 50 ml, 250 ml, 500 ml (Bio-one, Greiner)

Cell culture flasks (blue cap), 250 ml (Nuk)

Culture tubes, 14 ml (352059, Becton Dickinson)

Cryotubes, 2 ml (Greiner)

Cryotubes, dark (Biozym)

ELISPOT plates, 96-well (MAHABN4510 opaque, Millipore)

FACS tubes, 5 ml (352058, Becton Dickinson)

Glass bottles (Schott)

Reaction vials (1.5 ml, Eppendorf)

Sterilization filters, 0.22 µm (Corning)

Syringes, 1 ml, 5 ml, 10 ml, 50ml (Becton Dickinson)

Tubes, 15 ml and 50 ml (Falcon)

2.1.2.2 Antibodies

Table 2-1. Antibody conjugates used in this work.

antigen	fluorescence	producer	clone	titration
CD3	FITC	inhouse	OKT3	n.a.
	Pacific Blue	Biolegend	UCHT1	100
	FITC	BD	UCHT1	30
CD4	FITC	inhouse	HP2/6	n.a.
	APC Cy7	BD	RPA/T4	100
	APC	BD	RPA/T4	50
	APC	Miltenyi	n.i.	20
CD8	FITC	inhouse	OKT8	n.a.
	PerCP	Biolegend	RPA-T8	50
	PerCP	BD	SK1	25
	PE Cy7	BeckmanCoulter	SFC121Thy2D3	120
CD45RO	FITC	BD	UCHL1	5
CCR7	Alexa647	BD	3D12	20
IFN	PE Cy7	BD	4S.B3	400
	PE	BD	B27	200
	FITC	BD	B27	200
TNF	Pacific Blue	Biolegend	MAb11	120
CD154	FITC	BD	TRAP1	5
IL-2*	PE	BD	MQ1-17H12	100

*all antibodies are mouse derived except for α-IL-2 PE that is rat derived
n.a. one single titration cannot be given because the titration was determined separately for each batch used
n.i. the clone was not indicated by the provider

2.1.2.3 Buffers and solutions

DC medium

RPMI (BioWhittaker) 10% human serum or human plasma

1% PenStrep (BioWhittaker)

FACS-Clean (Becton Dickinson)

FACS-Fix:

PFEA

1.5% formaldehyde

FACS-Flow (Becton Dickinson)

FACS-Rinse (Becton Dickinson)

FCS (PAA Laboratories), heat inactivated at 56°C for 30 min

Freezing medium:

90% FCS

10% DMSO (Merck)

Human serum (PAA Laboratories), heat inactivated at 56°C for 30 min

Human plasma, pooled human plasma of at least 4 different donors, heat inactivated at 56°C for 30 min, tested

IMDM medium BE12-722F + L-glutamin (BioWhittaker)

Lymphocyte separation medium (PAA Laboratories)

MACS buffer:

PBS

0.5% BSA (Sigma)

2 mM EDTA (Roth)

PBS w/o Ca^{2+} and Mg^{2+} (BioWhittaker)

PBS-BSA:

PBS

0.5% BSA (Sigma)

PBS-Tween:

PBS

0.05% Tween 20 (Serva)

PenStrep (DE17-602E, BioWhittaker)

Permwash:

PBS

0.1% saponin (Sigma)

0.5% BSA (Sigma)

0.01% NaN_3 (Merck)

PFEA:

PBS

2% FCS

2 mM EDTA (Roth)

0.01% NaN_3 (Merck)

RPMI medium (BioWhittaker)

TCM (***2%)*** (T-cell medium):

IMDM

1% PenStrep (BioWhittaker)

20 µg/ml gentamycin sulfate (BioWhittaker)

50 µM β-mercaptoethanol (Roth)

2% human serum or human plasma

TCM5%:

IMDM

1% PenStrep (BioWhittaker)

20 µg/ml gentamycin sulfate (BioWhittaker)

50 µM β-mercaptoethanol (Roth)

5% human serum or human plasma

Thawing medium:

IMDM

1% PenStrep (BioWhittaker)

50 µM β-mercaptoethanol (Roth)

3 µg/ml DNase I (Sigma)

X-Vivo 15/20 medium (Lonza)

2.1.2.4 Other materials

Alkaline phosphatase, avidin-conjugated (E2636, Sigma)

BCIP/NBT tablet (B5655, Sigma)

brefeldin A (Sigma)

BSA (Sigma)

Cervarix HPV vaccine (GlaxoSmithKline)

Cytoperm/Cytofix solution (Becton Dickinson)

DMSO (Merck)

DNase I, grade II (Boehringer)

EDTA (Roth)

Engerix-B hepatitis B vaccine (GlaxoSmithKline)

Ethanol (SAV LP)

Formaldehyde ≥37% (Fluka)

Gardasil HPV vaccine (SanofiPasteur MSD)

GM-CSF (Sargramostim/Leukine, Berlex)

Golgi-stop (Monensin, Becton Dickinson)

α-IFN-γ primary antibody, 1 mg/ml (1-D-1k, MabTech)

α-IFN-γ secondary antibody, biotinylated, 1 mg/ml (7-B6-1, MabTech)

IL-2 (Proleukin, Chiron)

IL-4 (PromoKine)

Ionomycin (Sigma)

LIFE/DEAD aqua dye (Invitrogen)

LPS (Sigma)

NaN_3 (Merck)

PHA-L(Sigma)

PHA-P (Sigma)

PMA (Sigma)

AbC anti-mouse bead kit (Invitrogen)

AbC anti-rat/hamster bead kit (Invitrogen)

ArC bead kit (Invitrogen)

2.1.3 Peptides

All used peptides had been synthesized in the Department of Immunology by Stefan Stevanović, Patricia Hrstić or Nicole Zuschke.

2.1.4 Blood products

The PBMCs, abbreviated "BD" were derived from buffy coats (heparinized, erythrocyte and plasma depleted peripheral blood) of healthy donors that were kindly provided by the Department of Transfusion Medicine, University Hospital Tübingen. The buffy coats were collected between October 2006 and June 2008. They were processed one day after collection and stored at -80°C until use. The German Robert-Koch Institute recommends HPV-vaccination of 13 to 17 year old girls since March 2007. The blood donation service requires donors to be over 18 years of age, the average HLA typed donor being by far older. Therefore, we expect the great majority of the recruited donors to be non-HPV vaccinated.

The PBMCs, abbreviated "Sp" were kindly provided by lab members or by Andreas M. Kaufmann, Department of Gynecology, Charité Berlin. They originate from women having received the full regimen of Gardasil vaccination. The PBMCs were prepared on the day of collection and were stored at –80°C until use.

2.1.5 FACS sorting

FACS sorting was done at the FACS core facility of the University Hospital Tübingen. Supervision was provided by Sabrina Treml (Department of Internal Medicine II) and by Cornelia Grimmel (Department of Dermatology).

2.1.6 L1 serology

L1 serology was kindly provided by Michael Pawlita at the Department of Infection and Cancer (DKFZ, Heidelberg). The experiments were supervised by Kristina Michael and Tim Waterboer.

2.2 Methods

2.2.1 Choosing the most promising candidate epitopes

SYFPEITHI epitope prediction was performed from the amino acid sequences of L1HPV6a, L1HPV11, L1HPV16 and L1HPV18 for the following HLA class II DR alleles:

DRB1*01:01, DRB1*03:01, DRB1*04:01, DRB1*07:01, DRB1*11:01 and DRB1*15:01

The 15mer candidate epitopes contain a nine amino acids long “core region” that is essential for the HLA class II binding of the peptide (Figure 2-1).

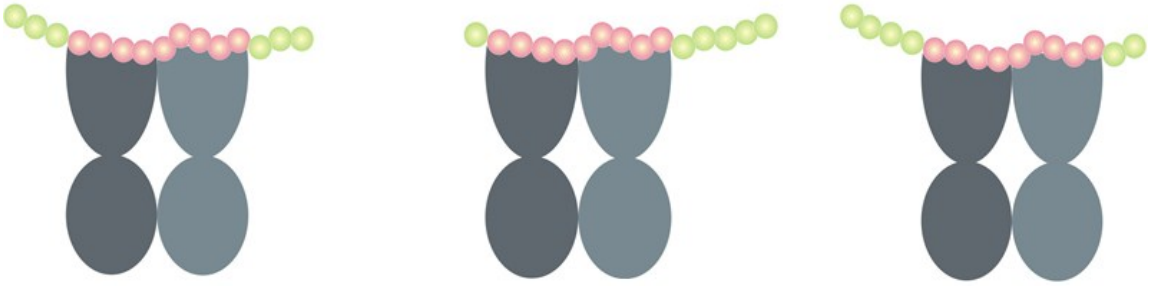

Figure 2-1. Epitope “core region”. The nine amino acids long core region of the HLA class II epitope that is essential for the HLA binding can be located anywhere within the 15mer peptide.

The most important natural epitopes are expected to be contained within the highest scoring 2% of the epitope predictions. In order not to over-represent low-scoring candidate epitopes, a cut-off was introduced at a SYPEITHI score that included candidate epitopes closest to the highest scoring 2% of the predictions (Figure 2-2).

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
477	V D T Y R F V Q S V A I T C Q	33
42	C G H Y I I L F L R N V N V F	30
181	H P F Y N K L D D T E S S H A	30
389	Q L F V T V V D T T P S T N L	30
434	L Q F I F Q L C T I T L T A D	30
9	I L H Y H L L P L Y G P L Y H	29
227	G E H W A K G T A C K S R P L	29
214	Q T Q L C I L G C A P A I G E	28
334	Q S L Y I K G T G M P A S P G	28
447	A D V M S Y I H S M N S S I L	28
450	M S Y I H S M N S S I L E D W	28
500	K D P Y D K L K F W N V D L K	28
4	Y T R V L I L H Y H L L P L Y	27
163	C A G V E I G R G Q P L G V G	27
400	S T N L T I C A S T Q S P V P	27

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
358	G S I V T S D S Q L F N K P Y	30
457	N S S I L E D W N F G V P P P	29
197	T S N V S E D V R D N V S V D	28
100	S S R L L T V G N P Y F R V P	27
205	R D N V S V D Y K Q T Q L C I	27
506	L K F W N V D L K E K F S L D	26
508	F W N V D L K E K F S L D L D	26
306	S M F F C L R R E Q L F A R H	25
427	R H V E E Y D L Q F I F Q L C	25
53	V N V F P I F L Q M A L W R P	24
135	R V Q L P D P N K F G L P D T	24
252	K N T V L E D G D M V D T G Y	24
11	H Y H L L P L Y G P L Y H P R	22
278	K C E V P L D I C Q S I C K Y	22
305	D S M F F C L R R E Q L F A R	22

Figure 2-2. Candidate epitopes for L1HPV18 predicted to be presented by HLA-DRB1*0101 (left) or HLA-DRB1*0301 (right). The red highlight shows the theoretical 2% cutoff. The red line indicates the chosen cutoff according to the number of peptides included and the SYFPEITHI score.

Next, within the lists of the high scoring peptides, we looked for repetitions of an at least nine amino acids long core region. A repetition of such a region in the lists of at least two different HLA-alleles indicates promiscuity (Figure 2-3). Peptides showing high promiscuity were preferentially chosen for synthesis.

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
477	VDTYRFVQSVAITCQ	33
42	CGHYIILFLRNVNVF	30
181	HPFYNKLDDTESSHA	30
389	QLFVTVVDTTPSTNL	30
434	LQFIFQLCTITLTAD	30
9	ILHYHLLPLYGPLYH	29
227	GEHWAKGTACKSRPL	29
214	QTQLCILGCAPAIGE	28
334	QSLYIKGTGMPASPG	28
447	ADVMSYIHSMNSSIL	28
450	MSYIHSMNSSILEDW	28
500	KDPYDKLKFWNVDLK	28

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
53	VNVFPIFLQMALWRP	28
92	TSIFYHAGSSRLLTV	28
149	TSIYNPETQRLVWAC	28
305	DSMFFCLRREQLFAR	28
477	VDTYRFVQSVAITCQ	28
43	GHYIILFLRNVNVFP	26
44	HYIILFLRNVNVFPI	26
197	TSNVSEDVRDNVSVD	26
358	GSIVTSDSQLFNKPY	26
389	QLFVTVVDTTPSTNL	26
431	EYDLQFIFQLCTITL	26

Figure 2-3. Promiscuity: Candidate epitopes for L1HPV18 predicted to be presented by HLA-DRB1*0101 (left) or HLA-DRB1*0401 (right). Promiscuous candidate epitopes are highlighted in color and size.

2.2.2 Cell counting

Cells were counted in a Neubauer counting chamber with a depth of 0.1 mm. After cleaning and drying, the counting chamber was assembled by moisturizing the chamber and sliding the cover glass onto the counting chamber while applying constant pressure. The correct assembly is indicated by the appearance of Newton's rings, caused by thin layer interference. The cell suspension was first pre-diluted with PBS to a concentration of approximately 10^6 cells/ml. 10 µl of the pre-dilution was mixed one to one with 0.05% trypan blue. Trypan blue accumulates in dead cells, staining them blue. This mixture was carefully pipetted into the assembled counting chamber. Viable cells not stained blue were counted in two diagonally positioned large squares, each encasing a volume of 0.1 µl. The mean value was used to calculate the overall cell density.

2.2.3 ELISPOT

All washing steps were performed as follows: 200 µl of the washing solution were pipetted into each well. After at least 30 s incubation time, the plates were sharply inverted into a basin and knocked several times onto a paper towel covered desk to remove remaining washing solution.

IFN-γ ELISPOT was performed in 96-well ELISPOT plates. The filter membranes of the plates were coated with a primary antibody directed against IFN-γ (1-D-1k, dilution 1:500 in PBS). 100 µl of the antibody solution were pipetted into each well. After the antibody was allowed to bind the membrane for 10 h at 4°C, the plates were washed twice with IMDM. Then 50 µl/well TCM were added and the ELISPOT plates were incubated for at least 1 h at 37°C to block unspecific binding sites of the membrane.

Freshly ficollized, thawed or pre-amplified cells were counted and the concentration of the cell suspension was adjusted to 10^7 cells/ml with TCM. Of this cell suspension 50 µl were added to each well of the ELISPOT plate.

Frozen aliquots of the peptides of interest with a concentration of 1 mg/ml were stored at -80°C. The needed aliquots were thawed and diluted with TCM to a concentration of 30 µg/ml. As a positive control a 30 µg/ml solution of PHA-P in TCM was used. As a negative control we used a 30 µg/ml in TCM solution of the peptide filamin $A_{1669\text{-}1683}$. This human self-peptide was found by our working group to be presented on HLA-DR molecules but it should not induce an IFN-γ response. 50 µl of the appropriate challenge solution were added to each well.

After resuspension, the final experiment settings in each well set as duplicates were as follows: 500 000 PBMCs in 150 µl of TCM with a concentration of challenge peptide or PHA of 10 µg/ml. The ELISPOT plates were incubated for 24-26 h at 37°C and 7,5% CO_2 with a special care not to expose them to any shaking.

The cells were removed and the plate was washed once with PBS-Tween, once with ddH_2O and five times with PBS-Tween. A 1:3000 dilution in PBS-BSA of the biotinylated secondary anti-IFN-γ antibody (7-B6-1) was prepared and 100 µl of this solution was added to each plate. The ELISPOT plates were incubated for 2 h at RT. Afterwards, the plates were washed six times with PBS-Tween. A 1:1000 dilution of ExtrAvidin-alkaline phosphatase in PBS-BSA was prepared and 100 µl added to each well. The plates were allowed to incubate for 1 h at RT. They were washed three times with PBS-Tween and three times with PBS to remove remaining detergent that would interfere with the following staining. A BCIP/NBT solution was prepared by dissolving BCIP/NBT tablets in ddH_2O (1 tablet per 10 ml). 50 µl of this solution were added to the empty wells. Afterwards, the spots were developed for 7 minutes in the dark at RT. The reaction was stopped by removing the substrate solution and

washing the plate with running water under the tab. After the plastic cover on the back of the plate was removed and the plates were cleaned, they were dried overnight in the dark and stored at RT in the dark until counted. For counting an automated ELISPOT reader was used. The parameters of the ELISPOT reader were adjusted to detect a maximal spot relation between positive and negative control.

2.2.4 Intracellular cytokine staining

All washing steps were performed as follows: 200 µl of the washing solution were pipetted into each well. The plate was centrifuged for 5 min at 4°C and 1500 rpm (Megafuge). The supernatant was removed from the cells by inverting the 96 well plate into a basin and pressing the inverted plate carefully onto a paper towel to remove remaining supernatant drops. All solutions used were kept at 4°C.

Freshly ficollized, thawed or pre-amplified cells were counted and the concentration of the cell suspension was adjusted to $2*10^7$ cells/ml with TCM. 50 µl of this cell suspension were used for each separate experiment. To this 50 µl brefeldin A solution (30 µg/ml brefeldin A in TCM) was added. In the final step, 50 µl of the challenge solution was added. The challenge solution contained 30 µg/ml peptide in TCM (peptide challenge) or 150 ng/ml PMA and 3 µM ionomycin in TCM (positive control). After resuspension the well contained 10^6 cells in 150 µl TCM with 10 µg/ml brefeldin A and a peptide concentration of 10 µg/ml. The plate was incubated for 12 h at 37°C.

After the 12 h response phase, the cells were stained for cell viability and several intracellular and surface markers according to the requirements of the experiment according to following order:

1. Cell viability: The cells were washed twice with PBS. A 1:400 dilution of the LIFE/DEAD aqua dye in PBS was prepared from a frozen dye aliquot (solution in DMSO) stored at -80°C for no longer than two weeks. Aliquots being stored between two and four weeks were used in a 1:200 dilution. 50 µl of the dilution were added to each well. After the cells were resuspended the plates were incubated for 20 minutes at 4°C in the dark.
2. Surface staining: The cells were washed twice with PFEA. The surface staining master mix was prepared by diluting antibodies directed against surface molecules in PFEA

(dilutions given in Table 2-1). 50 µl of the master mix were given to the cell pellet and the cells were resuspended before being incubated for 20 minutes at 4°C in the dark.

3. Permeabilization: The cells were washed twice with PFEA and then resuspended in 75 µl Cytoperm/Cytofix solution. To allow cell membrane permeabilization and fixation, the cells were incubated for 20 minutes at 4°C in the dark.
4. Intracellular staining: The permeabilized cells were washed twice with Permwash. The intracellular antibody master mix was prepared. The chosen antibodies directed against intracellular molecules were diluted in Permwash in the dilution given in Table 2-1. 50 µl of this master mix were added to each well. The cells were resuspended and incubated for 30minutes at 4°C in the dark.
5. Fixation: The cells were washed twice with PFEA. Finally, the cell pellet was resuspended in 100 µl Facs-Fix and stored at 4°C in the dark until evaluation.
6. Evaluation: Flow cytometric evaluation was done on a FACS Canto II after bead compensation within three days after staining. Data were evaluated using FACSDiva or FlowJo.

2.2.5 Phenotypical T-cell analysis

Freshly ficollized, thawed or pre-amplified cells were counted. Up to 10^6 cells were pipetted into a well of a 96 well round bottom plate. In order to allocate the appropriate gates in the FACS evaluation, unstimulated PBMCs were used as a reference. The cells were stained and measured according to 2.2.4 (2. Surface staining; 5.Fixation; 6.Evaluation). Phenotypes were assigned to the populations according to Sallusto et al. (Sallusto et al., 1999)

2.2.6 DC generation and protein loading

Freshly ficollized PBMCs were counted and resuspended in X-Vivo 20 at a concentration of $7*10^6$ cells/ml. 15 ml of this cell suspension were given into a medium blue cap cell culture flask. The monocytes were allowed to adhere to the plastic surface of the flask for 2 h at 37°C. Supernatant was removed and the remaining non-adherent cells were washed away twice with 10 ml warm PBS. 15 ml DC medium supplied with 800 U/ml GM-CSF and 30 ng/ml IL-4 were added to each flask. On day three and day five GM-CSF and IL-4 was fully replenished (800 U/ml GM-CSF, 30 ng/ml IL-4) and if the medium turned yellow, 5 ml were replaced with fresh DC medium. On day seven, maturation was induced by adding 100 ng/ml

LPS for 24h. Matured DCs were harvested and incubated for 10 h with 10 µg/ml protein. The loaded DCs were washed twice with warm TCM and were used in coculture experiments.

2.2.7 IFN-γ cytokine capture

100 ml cold MACS buffer (4°C), 100 µl cold TCM (4°C) and 10 ml warm TCM (37°C) were prepared. Cells were kept at 4°C and steps were done uninterrupted. 10^7 pre-amplified PBMCs were washed, counted and resuspended in 1 ml TCM with a challenge peptide concentration of 10 µg/ml. The cells were incubated in a 15 ml tube for 6 h at 37°C. After the incubation period, the cells were washed with 10 ml cold MACS buffer and centrifuged (1200 rpm, 10 min, 4°C, Megafuge). The supernatant was removed completely by pipetting and the cells were resuspended in 80 µl cold TCM. 20 µl of IFN-γ catch reagent was added and the cells were resuspended. After incubating for 5 min on ice, 9.9 ml of warm TCM was pipetted to the cell suspension. During the IFN-γ secretion period (45 min, 37°C) the tube was inverted every 5 minutes or rotated on a spinning wheel. Thereafter the cells were put on ice, washed with 10 ml cold MACS buffer and centrifuged (1200 rpm, 10 min, 4°C, Megafuge). The supernatant was carefully removed by pipetting and the cells were resuspended in 100 µl cold MACS buffer with antibodies. These 100 µl contained 20 µl IFN-γ detection antibody and anti-surface marker fluorescence conjugates (e.g. CD3 FITC, CD4-PE) in the appropriate dilutions (see Table 2-1. Antibody conjugates used in this work) according to experimental settings. After the cells were resuspended and incubated for 20 min on ice, they were washed with 10 ml cold MACS-buffer and centrifuged (1200 rpm, 10 min, 4°C, Megafuge). The supernatant was discarded and the cells were deposited in 500 µl cold MACS buffer until cell sorting.

2.2.8 T-cell sorting

Cells that had undergone IFN-γ capture staining were single cell sorted using a FACS-Aria cell sorter. It was important to avoid cell doublets because the preceding IFN-γ capture staining favors the formation of cell agglomerates and the subsequent, successful cloning procedure was based on a successful single cell sorting. Therefore a FSC-A/FSC-W and a FSC-A/SSC-W gating strategy were used to exclude all possible multi-cell aggregates.

The single T cells were sorted into prepared 96 round bottom well plates. These wells already contained 100 µl TCM 5% supplied with 150 U/ml IL-2. Feeder cells had already been prepared and added to the wells.

Feeder cells per well:

- 150 000 PBMCs; these PBMCs must be a mixture from at least 3 different donors. All PBMCs had to be prepared on the day of sorting. Therefore, fresh blood or buffy coats were ficollized and mixed. To ensure they could not outgrow the wanted T-cell clone, they were γ-irradiated (60 Gray, gamma-cell)
- 15 000 LG2-EBV cells. To prevent growth of these cells, they were γ-irradiated (200 Gray, gamma-cell)
- Both cell types were mixed, washed twice with TCM and added to the wells before the sorting procedure.

2.2.9 T-cell long term proliferation

The T cells were cultured in round bottom 96 well plates. Fresh TCM was added as soon as the medium turned yellow, at least 50 µl per week. The IL-2 was totally resupplied every two days to a concentration of 150 U/ml. Every two weeks fresh feeder cells were prepared (as described in 2.2.8) and added to the cell culture wells. The cells were kept in highest possible density. Therefore the wells were split only if the medium turned yellow after one day. T-cell clones used in experiments were given their last feeder cells at least 7 days beforehand. IL-2 is added the last time three days before the next experiment.

2.2.10 PBMC preparation

The PBMCs originated from three different sources:

1. Buffy coat: residuals of 500 ml heparinized peripheral blood, after plasma and erythrocytes were extracted. The donation of blood usually takes place the day before ficollization.
2. Freshly drawn heparinized peripheral blood in various amounts.
3. Leukapheresis: leukocyte-enriched blood product containing up to $3*10^9$ PBMCs.

The buffy coat was opened and extracted into a medium size cell culture flask. The cell suspension (around 40 ml) was diluted with PBS to a volume of 125 ml. Four 50 ml tubes

were filled with 15 ml of lymphocyte separation medium and 30 ml of the cell suspension was carefully overlaid. It was important to keep a sharp phase separation. The tubes were centrifuged for 30 min at RT with breaks turned off. The PBMCs that were located in the interphase between the ficoll and the plasma layer were carefully extracted without carrying over more ficoll or plasma than necessary. The PBMCs of two tubes were joined and the two tubes were filled up to 50 ml with PBS. The tubes were centrifuged for 10 min (RT, 1500 rpm, Megafuge). The supernatant was removed and the two tubes were again refilled with 50 ml PBS. After another centrifugation (RT, 1300 rpm, Megafuge), the cells were joined in one tube, the cell number was counted and the cells were frozen or used for a following experiment.

2.2.11 Freezing and thawing cells

PBMCs were frozen in two different aliquot sizes. Either 20 million cells were stored in 1 ml freezing medium or 100 million cells in 2 ml. Cold freezing medium was used to resuspend the cells. The aliquots were put into a freezing container and were stored at –80°C.

Frozen cell aliquots were thawed and diluted very slowly in 10 ml thawing medium over a 5 min period. Afterwards they were centrifuged (5 min, 4°C, 1500 rpm, Megafuge), washed in 10 ml thawing medium and again centrifuged (5 min, 4°C, 1500 rpm, Megafuge). The cells were taken up in TCM, counted and used in adjacent experiments.

2.2.12 Pre-amplification

To increase the frequency of antigen specific T cells, a peptide specific pre-amplification was performed. This type of *in vitro* amplification is based on the proliferation of memory T cells specific for the stimulated peptide. In opposite to T cell priming, no second signal is given and naïve T cells cannot be activated.

Freshly ficollized or thawed cells were counted and taken up in TCM to a density of $10*10^6$ cells/ml. 2 ml (20 million cells) were seeded into a well of a 6-well plate. The cells were allowed to rest in this high density at 37°C for at least 20 h in order for the TCR to become threshold activated. On day 1, 500 µl of TCM containing up to 10 different peptides in a concentration of 10 µg/ml each and IL-2 (50 U/ml) were added to the well. At this point the final concentration of each peptide was 2 µg/ml, the final IL-2 concentration was 20 U/ml. On day 3, 5, 7 and 9, 500 µl TCM were added and the IL-2 was totally resupplied to

a concentration of 20 U/ml. On day 12, the cells were harvested, washed, counted and used in adjacent experiments.

2.2.13 L1 serology

Antibody titer analysis was kindly done by the group of Prof. Dr. Michael Pawlita at the DKFZ in Heidelberg, Germany. It was performed as described (Waterboer et al., 2005). Briefly, 50 µl of diluted, pre-treated serum were incubated with Luminex beads, each loaded with GST-L1 fusion protein from different HPV strains. After washing, bound antibody was detected by biotinylated secondary antibody and a streptavidin-PE conjugate. The bound fluorescence was measured on a Luminex analyzer and expressed as "Medium Fluorescence Intensity" (MFI) of at least 100 beads. To determine the antigen-specific fluorescence, the GST-tag background and the bead set autofluorescence was subtracted from the measured values. The samples were included into a 3500 sera/plasma containing study to assess reliable cut-off values. Cut-off values for multiplex HPV serology were originally defined 2007 (Clifford et al., 2007). Therefore, a subpanel of 188 Korean sera with predetermined antibody prevalence were included in the study and cut-off values were calculated according to Michael et al (Michael et al., 2008).

3 Results and Discussion

3.1 Epitope identification of L1 HPV6a, 11, 16, 18

3.1.1 Epitope prediction and synthesis

HLA class II DR-restricted candidate epitopes were predicted by SYFPEITHI. In particular, prediction matrices for the HLA DR alleles DRB1*01:01, DRB1*03:01, DRB1*04:01, DRB1*07:01, DRB1*11:01 and DRB1*15:01 were used to screen the amino acid sequences of L1 from HPV type HPV6a, HPV11, HPV16 and HPV18. The exact protein sequences used are given in the appendix (6.2). According to our experience, the most important natural epitopes should be contained in the highest scoring 2% of the predicted epitope candidates. The highest scoring 10 candidate epitopes of all predictions are given in the appendix (6.3). Table 3-1 exemplarily shows the 12 highest scoring peptide candidates (highest scoring 2.3%) from L1 of HPV type 18 predicted for MHC class II DRB1*01:01. Candidate epitopes with the same predicted SYFPEITHI score have the same probability of being natural T-cell epitopes. Any ranking of equally scored peptides in the table is due to the position in the protein.

Table 3-1. Twelve (2.3%) highest scoring 15mers from the L1 protein of HPV type 18 predicted for MHC DRB1*01:01 presentation.

#	position	sequence	SYFPEITHI score
1	477	V D T Y R F V Q S V A I T C Q	33
2	42	C G H Y I I L F L R N V N V F	30
3	181	H P F Y N K L D D T E S S H A	30
4	389	Q L F V T V V D T T P S T N L	30
5	434	L Q F I F Q L C T I T L T A D	30
6	9	I L H Y H L L P L Y G P L Y H	29
7	227	G E H W A K G T A C K S R P L	29
8	214	Q T Q L C I L G C A P A I G E	28
9	334	Q S L Y I K G T G M P A S P G	28
10	447	A D V M S Y I H S M N S S I L	28
11	450	M S Y I H S M N S S I L E D W	28
12	500	K D P Y D K L K F W N V D L K	28

According to 2.2.1 the high-scoring peptides with the highest promiscuity were chosen and synthesized. Out of the 29 peptide candidates chosen, 28 peptides could be synthesized

(Table 3-2). One peptide could not be synthesized in a satisfying purity, most likely due to an accumulation of hydrophobic amino acids in the peptide sequence (Table 3-3).

Table 3-2. Synthesized candidate epitopes from the L1 protein of HPV type 6a, 11, 16 and 18 predicted to bind to HLA-DR with high promiscuity

#	sequence	HPV type	position	mass [Da]
1	VPPPNPVSKVVATDA	**6**, 11	11-25*	1489.81
2	TNIFYHASSSRLLAV	**6**, 11	30-44*	1677.88
3	GHPYFSIKRANKTVV	6	45-59	1715.95
4	FKVVLPDPNKFALPD	**6**, 11	70-84*	1698.93
5	PFLNKYDDVENSGSG	6	118-132	1640.73
6	DRLFFFLRKEQMFAR	6	240-254	2003.06
7	SSIYVNTPSGSLVSS	6	284-298	1496.74
8	QLFVTVVDTTRSTNM	**6**, 16	324-338*	1710.86
9	AEVMAYIHTMNPSVL	**6**, 11	379-393*	1674.81
10	EDTYRYVQSQAITCQ	**6**, 11	410-424*	1803.81
11	GHPYYSIKKVNKTVV	11	45-59	1731.67
12	AMNFADLQTNKSDV	11	204-217	1552.72
13	DRLFFYLRKEQMFAR	11	241-255	2019.05
14	HLFVTVVDTTRSTNM	11	325-339	1719.86
15	PSVLEDWNFGLSPPPN	11	392-406	1767.85
16	VYHIFFQMSLWLPSE	16	20-34	1895.93
17	AMDFTTLQANKSEV	16	233-246	1553.74
18	DSLFFYLRREQMFVR	16	270-284	2006.02
19	VMTYIHSMNSTILED	16	413-427	1752.81
20	NSTILEDWNFGLQPP	16	421-435	1728.83
21	EDTYRFVTSQAIACQ	16	441-455	1730.79
22	YHLLPLYGPLYHPRPL	18	12-27	1948.07
23	CGHYIILFLRNVNVF	18	42-56	1806.96
24	VNVFPIFLQMALWRPS	18	53-68	1917.03
25	GSIVTSDSQLFNKPY	18	358-372	1654.82
26	QLFVTVVDTTPSTNL	18	389-403	1633.86
27	ADVMSYIHSMNSSIL	18	447-461	1666.77
28	VDTYRFVQSVAITCQ	18	477-491	1728.85

* the position in the amino acid sequence of the L1 protein refers to the HPV type written in bold letters

Table 3-3.Candidate epitope chosen for their promiscuity and high SYFPEITHI score that could not be synthesized in satisfying purity

#	sequence	HPV type	position	mass [Da]
29	LQFIFQLCSITLSAE	11	368-382	1711.89

3.1.2 ELISPOT screening

The 28 synthesized candidate epitopes (Table 3-2) were used in IFN-γ ELISPOT screenings with PBMCs from 34 randomly chosen healthy blood donors. The screened HLA-DR haplotypes (DRB1*01:01, DRB1*03:01, DRB1*04:01, DRB1*07:01, DRB1*11:01 and DRB1*15:01) comprise some of the most frequent types in Germany (Table 3-4). The cumulative population proportion that shares at least one of the screened HLA-DRB1 haplotypes is 92.7% according to the algorithm of Schipper et al. (Schipper et al., 1996).

Table 3-4. Population coverage of HLA-DRB1 alleles in Germany. Frequencies were taken from www.allelefrequencies.net (Gonzalez-Galarza et al., 2011), the selected data were from a study comprising 11407 individuals in Germany. The alleles highlighted in bold were used for SYFPEITHI prediction.

allele	phenotype frequency [%]	allele frequency [%]
DRB1*01	**21.0**	**11.1**
DRB1*03	**20.0**	**10.6**
DRB1*04	**24.6**	**13.2**
DRB1*07	**23.6**	**12.6**
DRB1*08	6.4	3.3
DRB1*09	1.9	1.0
DRB1*10	1.7	0.9
DRB1*11	**23.5**	**12.5**
DRB1*12	3.7	1.9
DRB1*13	24.1	12.9
DRB1*14	6.1	3.1
DRB1*15	**26.4**	**14.2**
DRB1*16	4.8	2.4

Because frequencies of antigen specific T cells can be very low, a peptide specific amplification protocol was applied to all PBMCs prior to the ELISPOT experiment. The PBMCs were stimulated once with a cocktail of up to 10 different candidate epitopes. The added peptides displace the natural MHC class II ligands present on APCs contained within the bulk PBMCs. Once the peptides associate with the MHC molecules, they are presented to T cells. A T cell that recognizes its specific antigen gets a low activation stimulus. Because there is no

second activation signal, no naïve T cell is primed. Only already primed epitope-specific memory or effector T cells become activated by this method. The repeated addition of low dose IL-2 together with the TCR signaling drives the activated T cells into a proliferation phase, thereby increasing their frequency. This amplification method needs at least one epitope specific progenitor cell within the stimulated bulk PBMCs. Therefore the number of stimulated PBMCs has to be as high as possible. In order to detect very rare cell specificities without wasting enormous amounts of PBMCs, $20x10^6$ PBMCs for each separate pre-amplification experiment were used. Pools of no more than 10 different peptides were used in the pre-amplifications in order to avoid effects favoring the peptide with the strongest MHC binding.

The pre-stimulated PBMCs were counted on day 12 and 500 000 cells were used per well in an ELISPOT experiment. A medium control showed the unspecific background activation of the used cells, while a 10 µg/ml PHA-P positive control was used to check for general technical reliability of the test.

The background is varying widely and the peptide pre-amplification protocol is effective to a different extent in each blood donor and each separate experiment. To determine if there was a peptide specific cytokine response, the spot count was compared to the negative peptide control. The negative peptide originates from the human protein filamin A. Since the human filamin A protein is ubiquitous, no immune response should be mounted against any fragment of this self-protein. But the peptide filamin$A_{1669\text{-}16683}$ (ETVITVDTKAAGKGK) is an abundant self-ligand presented by multiple HLA-DR allotypes. To define an ELISPOT response as positive, we applied the following empirical criteria:

Positive response (ELISPOT data):

$$Spot\ count\ (epitope\ candidate) \geq Spot\ count\ (negative\ peptide) * 3$$

AND $Spot\ count\ (epitope\ candidate) \geq 10$

An exemplary ELISPOT plate employed in the screening of L1 HPV6a candidate epitopes is shown in Figure 3-1. The ELISPOT plate is analyzed and spot count numbers are collected automatically using an ELISPOT reader. The collected raw data of the exemplary ELISPOT plate is shown in Table 3-5. Mean spot counts are calculated from duplicates and evaluated using the positivity criteria, as shown in Table 3-6.

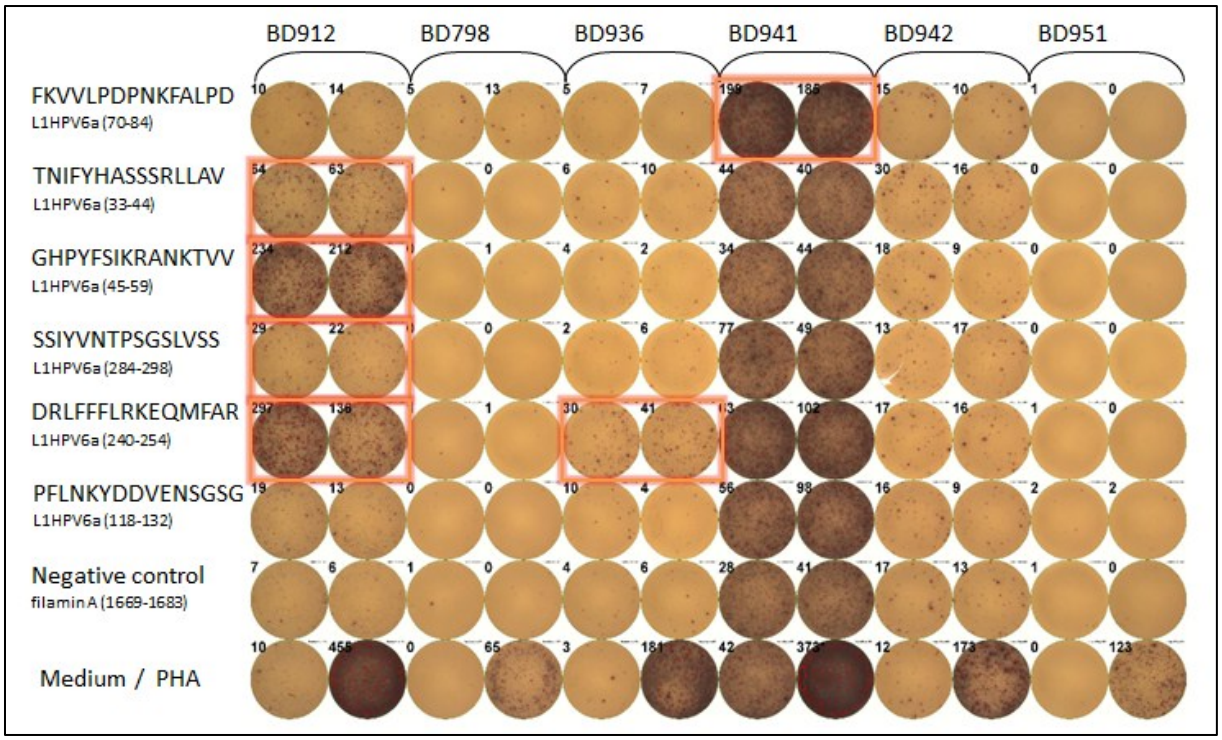

Figure 3-1. Exemplary ELISPOT plate employed in the screening of L1HPV6a candidate epitopes. Pre-amplified PBMCs of 6 different blood donors were used in two columns per donor. The PBMCs were challenged with 10 µg/ml of a single peptide from L1HPV6a or the negative control peptide in duplicates. Single wells were used for medium control and PHA positive control. Positive wells that fulfill the positivity criteria as described earlier (2.2.2) are framed in red.

Table 3-5. Data of exemplary ELISPOT (Figure 3-1) as counted automatically by an ELISPOT reader.

	spots per 500 000 PBMCs											
peptide	**BD912**		**BD798**		**BD936**		**BD941**		**BD942**		**BD951**	
FKVVLPDPNKFALPD	10	14	5	13	5	7	199	185	15	10	1	0
TNIFYHASSSRLLAV	64	63	1	0	6	10	44	40	30	16	0	0
GHPYFSIKRANKTVV	234	212	0	1	4	2	34	44	18	9	0	0
SSIYVNTPSGSLVSS	29	22	0	0	2	6	77	49	13	17	0	0
DRLFFFLRKEQMFAR	297	136	1	1	30	41	63	102	17	16	1	0
PFLNKYDDVENSGSG	19	13	0	0	10	4	56	98	16	9	2	2
Negative control	7	6	1	0	4	6	28	41	17	13	1	0
Medium/PHA	10	455	0	65	3	181	42	373	12	173	0	123

Table 3-6. Data evaluation of exemplary ELISPOT plate (Figure 3-1). Given spot counts are means of two separate ELISPOT experiments in two wells. Mean spot counts that are defined positive are highlighted in orange.

mean spots per 500 000 PBMCs						
peptide	**BD912**	**BD798**	**BD936**	**BD941**	**BD942**	**BD951**
FKVVLPDPNKFALPD	12	9	6	192	12,5	0,5
TNIFYHASSSRLLAV	63,5	0,5	8	42	23	0
GHPYFSIKRANKTVV	223	0,5	3	39	13,5	0
SSIYVNTPSGSLVSS	25,5	0	4	63	15	0
DRLFFFLRKEQMFAR	216,5	1	35,5	82,5	16,5	0,5
PFLNKYDDVENSGSG	16	0	7	77	12,5	2
Negative control	6,5	0,5	5	34,5	15	0,5

The following tables are showing the results of all ELISPOT screening experiments. All screening experiments were performed as described exemplarily. The peptides are organized according to the HPV type they originate from. All peptides were screened with PBMCs from the same 34 randomly chosen healthy blood bank donors.

Table 3-7. Screening results for peptides derived from L1HPV6a. Frequently recognized epitopes (FREPs) are highlighted in bold.

Protein	L1HPV6a										
Position	11-25	30-44	45-59	70-84	118-132	240-254	284-298	324-338	379-393	410-424	
Sequence	VPPPNPVSKVVATDA	TNIFYHASSSRLLAV	GHPYFSIKRANKTVV	FKVVLPDPNKFALPD*	PFLNKYDDVENSGSG	**DRLFFFLRKEQMFAR**	SSIYVNTPSGSLVSS	QLFVTVVDTTRSTNM	AEVMAYIHTMNPSVL*	**EDTYRYVQSQAITCQ***	Summary L1HPV6a
Blood Donor	T-cell reactivity										
782	0	0	0	0	0	0	0	0	0	1	1/10
783	0	0	0	1	0	1	0	0	0	1	3/10
787	0	0	1	0	1	1	1	0	0	0	4/10
789	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	0	0/4
795	0	0	1	0	0	0	0	0	1	1	3/10
797	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	0	0/4
798	0	0	0	0	0	0	0	0	0	0	0/10
805	0	0	0	1	0	1	0	0	0	0	2/10
912	0	1	1	0	0	1	1	0	0	1	5/10
932	0	0	0	1	0	0	0	0	0	0	1/10
936	0	0	0	0	0	1	0	0	0	1	2/10
941	0	0	0	1	0	0	0	0	0	1	2/10
942	0	0	0	0	0	0	0	0	0	1	1/10
951	0	0	0	0	0	0	0	0	0	0	0/10
1192	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	1	1	2/4
1204	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1	0	0	1/4
1242	0	0	0	0	0	0	0	0	0	1	1/10
1244	0	1	0	0	0	0	0	0	1	1	3/10
1245	0	0	0	0	0	1	0	0	0	0	1/10
1246	0	0	0	1	0	1	0	0	0	0	2/10
1247	0	0	0	1	0	0	0	0	0	0	1/10
1248	0	0	0	0	0	0	0	0	0	0	0/10
1249	0	0	0	0	0	1	0	0	0	0	1/10
1250	0	0	0	1	0	0	0	0	0	0	1/10
1251	0	1	0	1	1	1	0	0	0	0	4/10
1252	0	0	0	0	0	1	0	0	0	0	1/10
1253	0	0	0	0	0	0	0	0	0	0	0/10
1256	0	0	0	0	0	0	0	0	1	0	1/10
1262	0	0	0	0	0	1	0	0	0	0	1/10
1263	0	0	0	0	0	0	0	0	0	0	0/10
1265	0	0	0	0	0	1	1	0	1	0	3/10
1266	0	0	0	0	0	0	0	0	0	0	0/10
1276	0	0	0	0	0	0	0	0	0	0	0/10
1277	0	0	1	0	0	1	0	0	0	0	2/10
Summary	0/34	3/30	4/30	8/30	2/30	13/30	3/30	1/34	5/34	10/34	

" ***" this candidate epitope is also part of the L1 protein from HPV11 "n.d." not determined

" 0 " no positive IFN-γ response could be detected" 1 " IFN-γ response could be detected

Table 3-8. Screening results for peptides derived from L1HPV11. Frequently recognized epitopes (FREPs) are highlighted in bold.

Protein	L1HPV11					
Position	45-59	204-217	241-255	325-339	392-406	
Sequence	GHPYYSIKKVNKTVV	AMNFADLQTNKSDV	**DRLFFYLRKEQMFAR**	HLFVTVVDTTRSTNM	PSVLEDWNFGLSPPPN	Summary L1HPV11
Blood donor	T-cell reactivity					
782	0	0	0	0	0	0/5
783	0	0	1	0	1	2/5
787	0	0	1	0	0	1/5
789	0	0	0	0	0	0/5
795	0	0	0	0	1	1/5
797	0	0	0	0	0	0/5
798	0	0	1	0	0	1/5
805	0	0	1	0	0	1/5
912	0	0	1	0	0	1/5
932	0	0	1	0	0	1/5
936	0	0	1	0	0	1/5
941	0	0	0	0	0	0/5
942	0	0	0	0	0	0/5
951	0	0	0	0	0	0/5
1192	0	0	0	0	1	1/5
1204	1	0	0	0	0	1/5
1242	0	1	0	0	0	1/5
1244	0	0	0	0	0	0/5
1245	0	0	0	0	0	0/5
1246	1	0	1	0	1	3/5
1247	0	0	0	0	0	0/5
1248	0	0	0	0	0	0/5
1249	0	0	0	0	0	0/5
1250	0	0	1	0	0	1/5
1251	0	0	1	0	0	1/5
1252	0	0	1	0	0	1/5
1253	0	0	0	1	0	1/5
1256	0	0	0	0	0	0/5
1262	1	0	1	0	0	2/5
1263	1	0	0	0	0	1/5
1265	0	0	1	0	0	1/5
1266	0	0	0	0	0	0/5
1276	0	0	1	0	0	1/5
1277	0	0	1	0	0	1/5
Summary	4/34	1/34	15/34	1/34	4/34	

" 0 " no positive IFN-γ response could be detected " 1 " IFN-γ response could be detected

Table 3-9. Screening results for peptides derived from L1HPV16. Frequently recognized epitopes (FREPs) are highlighted in bold.

Protein	L1HPV16						
Position	20-34	233-246	270-284	413-427	421-435	441-455	
Sequence	VYHIFFQMSLWLPSE	AMDFTTLQANKSEV	**DSLFFYLRREQMFVR**	VMTYIHSMNSTILED	NSTILEDWNFGLQPP	**EDTYRFVTSQAIACQ**	Summary L1HPV16
Blood donor	T-cell reactivity						
782	0	1	0	0	0	1	2/6
783	0	0	1	0	1	0	2/6
787	0	0	1	0		0	1/6
789	0	0	0	0	0	0	0/6
795	0	0	0	0	1	0	1/6
797	0	0	0	0	0	0	0/6
798	0	0	1	1	0	0	2/6
805	0	0	1	1	0	0	2/6
912	0	0	1	0	0	1	2/6
932	0	0	0	1	1	0	2/6
936	0	0	1	0	0	0	1/6
941	0	0	0	0	0	1	1/6
942	0	0	0	0	0	0	0/6
951	0	0	0	0	0	0	0/6
1192	0	0	1	0	0	1	2/6
1204	0	0	0	0	0	1	1/6
1242	0	1	1		0	1	3/6
1244	0	0	0	0	0	0	0/6
1245	0	0	0	0	0	0	0/6
1246	0	0	1	0	1	1	3/6
1247	0	0	0	0	0	0	0/6
1248	0	0	0	0	0	0	0/6
1249	0	0	0	0	0	0	0/6
1250	0	0	0	0	0	0	0/6
1251	0	0	0	0	0	1	1/6
1252	0	0	1	0	0	1	2/6
1253	0	0	0	0	0	0	0/6
1256	0	0	0	0	0	0	0/6
1262	0	0	1	0	0	0	1/6
1263	0	0	1	0	0	0	1/6
1265	0	0	0	0	0	0	0/6
1266	0	0	0	0	0	0	0/6
1276	0	0	0	0	0	1	1/6
1277	0	0	1	1	0	1	3/6
Summary	0/34	2/34	13/34	4/34	4/34	11/34	

" 0 " no positive IFN-γ response could be detected " 1 " IFN-γ response could be detected

Table 3-10. Screening results for peptides derived from L1HPV18. Frequently recognized epitopes (FREPs) are highlighted in bold.

Protein	L1HPV18							
Position	12-27	42-56	53-68	358-372	389-403	447-461	477-491	
Sequence	YHLLPLYGPLYHPRPL	CGHYIILFLRNVNVF	VNVFPIFLQMALWRPS	GSIVTSDSQLFNKPY	QLFVTVVDTTPSTNL	ADVMSYIHSMNSSIL	**VDTYRFVQSVAITCQ**	Summary L1HPV18
Blood donor	T-cell reactivity							
782	0	0	0	0	0	0	1	1/7
783	0	0	0	0	0	0	1	1/7
787	0	0	0	0	0	0	1	1/7
789	0	0	0	0	0	0	0	0/7
795	0	0	0	1	0	0	0	1/7
797	0	0	0	0	0	0	1	1/7
798	0	0	0	0	0	1	1	2/7
805	0	0	0	0	0	0	0	0/7
912	0	0	0	0	0	0	1	1/7
932	0	0	0	0	0	0	0	0/7
936	0	0	0	0	0	0	1	1/7
941	0	0	0	0	0	0	0	0/7
942	0	0	0	0	0	0	0	0/7
951	0	0	0	0	0	0	0	0/7
1192	0	0	0	0	0	0	0	0/7
1204	0	0	0	1	0	0	0	1/7
1242	0	0	0	0	0	0	1	1/7
1244	0	0	0	0	0	0	0	0/7
1245	0	0	0	0	0	0	0	0/7
1246	0	0	0	0	0	0	1	1/7
1247	0	0	0	0	0	0	0	0/7
1248	0	0	0	0	0	0	1	1/7
1249	1	0	0	0	0	0	0	1/7
1250	0	0	0	0	0	0	0	0/7
1251	0	0	0	0	0	0	1	1/7
1252	0	0	0	0	0	0	1	1/7
1253	0	0	0	0	0	0	0	0/7
1256	1	0	0	0	0	1	1	3/7
1262	0	0	0	0	0	0	0	0/7
1263	0	0	0	0	0	1	0	1/7
1265	0	0	0	0	0	0	0	0/7
1266	0	0	0	0	0	0	0	0/7
1276	0	0	0	1	0	0	0	1/7
1277	0	0	0	0	0	0	1	1/7
Summary	2/34	0/34	0/34	3/34	0/34	3/34	14/34	

" 0 " no positive IFN-γ response could be detected " 1 " IFN-γ response could be detected

Table 3-11. Combined summary of Table 3-6, 3-7, 3-8 and 3-9.

	all peptides from L1HPV6a	all peptides from L1HPV11	all peptides from L1HPV16	all peptides from L1HPV18		all peptides
Blood donor	**T-cell reactivity**					
782	1/10	0/5	2/6	1/7		4/28
783	3/10	2/5	2/6	1/7		8/28
787	4/10	1/5	1/6	1/7		7/28
789	0/4	0/5	0/6	0/7		0/22
795	3/10	1/5	1/6	1/7		6/28
797	0/4	0/5	0/6	1/7		1/22
798	0/10	1/5	2/6	2/7		5/28
805	2/10	1/5	2/6	0/7		5/28
912	5/10	1/5	2/6	1/7		9/28
932	1/10	1/5	2/6	0/7		4/28
936	2/10	1/5	1/6	1/7		5/28
941	2/10	0/5	1/6	0/7		3/28
942	1/10	0/5	0/6	0/7		1/28
951	0/10	0/5	0/6	0/7		0/28
1192	2/4	1/5	2/6	0/7		5/22
1204	1/4	1/5	1/6	1/7		4/22
1242	1/10	1/5	3/6	1/7		6/28
1244	3/10	0/5	0/6	0/7		3/28
1245	1/10	0/5	0/6	0/7		1/28
1246	2/10	3/5	3/6	1/7		9/28
1247	1/10	0/5	0/6	0/7		1/28
1248	0/10	0/5	0/6	1/7		1/28
1249	1/10	0/5	0/6	1/7		2/28
1250	1/10	1/5	0/6	0/7		2/28
1251	4/10	1/5	1/6	1/7		7/28
1252	1/10	1/5	2/6	1/7		5/28
1253	0/10	1/5	0/6	0/7		1/28
1256	1/10	0/5	0/6	3/7		4/28
1262	1/10	2/5	1/6	0/7		4/28
1263	0/10	1/5	1/6	1/7		3/28
1265	3/10	1/5	0/6	0/7		4/28
1266	0/10	0/5	0/6	0/7		0/28
1276	0/10	1/5	1/6	1/7		3/28
1277	2/10	1/5	3/6	1/7		7/28
Summary: 34 blood donors	25/34	21/34	20/34	19/34		31/34

A summary of the IFN-γ ELISPOT screening results is given in Table 3-11 and Table 3-12. From the 28 candidate epitopes synthesized, 23 peptides caused a cytokine response within the pre-stimulated PBMC population of at least one donor. Only five of the chosen peptide

candidates never caused any positive reaction. The recognition rates ranged in between 44 and 0% of the 34 randomly chosen healthy donors. The highest ranking six peptides show recognition rates between 29 and 44%. These “Frequently Recognized Epitopes” (FREPs) belong to two different regions in the L1 protein. The three FREPs DRLFFYLRKEQMFAR (L1HPV11$_{241-255}$, recognition rate: 44%), DRLFFFLRKEQMFAR (L1HPV6a$_{240-254}$, recognition rate: 43%) and DSLFFYLRREQMFVR (L1HPV16$_{270-284}$, recognition rate: 38%) are located in “Region 1”. In “Region 2” there are VDTYRFVQSVAITCQ (L1HPV18$_{477-491}$, recognition rate 41%), EDTYRFVTSQAIACQ (L1HPV16$_{441-477}$, recognition rate: 32%) and EDTYRYVQSQAITCQ (L1HPV6a/11$_{410-424}$, recognition rate: 29%). Out of the 31 donors that recognize any of the L1 peptides 29 donors recognize at least one FREP.

There is one additional strong epitope causing an IFN-γ release in approximately one third of the donors: FKVVLPDPNKFALPD (L1HPV6a/11$_{70-84}$, recognition rate: 27%). The remaining 16 reactive peptides show recognition rates below 20%. Three of the peptides caused a cytokine response in only one out of 34 randomly chosen donors. In two of these three cases the single blood donor recognizing the peptide does also respond to FREPs. Peptide AMNFADLQTNKSDV (L1 HPV11$_{204-217}$) was only recognized by BD 1242 (recognizing three FREPs). BD 1204 that recognizes QLFVTVVDTTRSTNM (L1 HPV6a/11$_{324-338}$) does also respond to four other candidate epitopes, including one FREP. Only peptide HLFVTVVDTTRSTNM (L1 HPV11$_{325-339}$) is recognized only by a single donor (BD1253) that does not respond to any other epitope candidates. Despite our efforts to avoid false positive results, this recognition could be caused accidentally.

The detected responses partly overlap with the class II restricted T-cell epitopes reported elsewhere (see 1.4.2.4). Epitopes that overlap with one of the FREPs identified in this study were reported in several publications (Hong et al., 1997; Strang et al., 1990; Williams et al., 2002), emphasizing the importance of the identified FREPs in L1-specific T-cell immunity.

Williams et al. used an approach testing 96 overlapping 15mers spanning the whole L1 protein of HPV11. Seven HLA-DR restricted epitopes were identified. We tested 28 peptides from four different HPV types and identified 23 epitopes. Among these were all seven epitopes identified by Williams et al. or peptides overlapping by at least 9 amino acids. Our approach detected one additional epitope from L1 of HPV11, HLFVTVVDTTRSTNM

(L1HPV11$_{325\text{-}339}$), however, this peptide was suspected to be false positive due to a very low recognition rate (see above).

31 of 34 donors (91%) reacted to any peptide. In particular, 25 donors (74%) reacted after challenge with L1HPV6a peptides, 21 donors (62%) responded to L1HPV11, 20 donors (59%) to L1HPV16 peptides and 19 donors (56%) to L1HPV18 peptides. These response rates are high compared to the HPV prevalence in Europe that is estimated between 8 and 15% (Iftner et al., 2010). The difference can be explained by the insensitivity of the former HPV detection methods (Schmitt et al., 2010) and the fact that the presence of specific T cells does not imply an acute HPV infection.

Table 3-12. Summary of IFN-γ ELISPOT screening. The used peptides are listed according to their recognition rate.

#	Sequence	HPV type[a]	position[b]	n[c]	recognition [%][d]
1	DRLFFYLRKEQMFAR	**11**	241-255	34	44
2	DRLFFFLRKEQMFAR	**6**	240-254	30	43
3	VDTYRFVQSVAITCQ	**18**	477-491	34	41
4	DSLFFYLRREQMFVR	**16**	270-284	34	38
5	EDTYRFVTSQAIACQ	**16**	441-455	34	32
6	EDTYRYVQSQAITCQ	**6**, 11	410-424	34	29
7	FKVVLPDPNKFALPD	**6**, 11	70-84	30	27
8	AEVMAYIHTMNPSVL	**6**, 11	380-394	34	15
9	GHPYFSIKRANKTVV	**6**	45-59	30	13
10	VMTYIHSMNSTILED	**16**	413-427	34	12
11	PSVLEDWNFGLSPPPN	**11**	392-406	34	12
12	GHPYYSIKKVNKTVV	**11**	45-59	34	12
13	NSTILEDWNFGLQPP-NH2	**16**	421-435	34	12
14	TNIFYHASSSRLLAV	**6**, 11	30-44	30	10
15	SSIYVNTPSGSLVSS	**6**	284-298	30	10
16	GSIVTSDSQLFNKPY	**18**	358-372	34	9
17	ADVMSYIHSMNSSIL	**18**	447-461	34	9
18	PFLNKYDDVENSGSG	**6**	118-132	30	7
19	AMDFTTLQANKSEV	**16**	233-246	34	6
20	YHLLPLYGPLYHPRPL	**18**	12-27	34	6
21	HLFVTVVDTTRSTNM	**11**	325-339	34	3
22	AMNFADLQTNKSDV	**11**	204-217	34	3
23	QLFVTVVDTTRSTNM	**6**, 16	324-338	34	3
24	VPPPNPVSKVVATDA	**6**, 11	11-25	34	0
25	VYHIFFQMSLWLPSE	**16**	20-34	34	0
26	VNVFPIFLQMALWRPS	**18**	53-68	34	0
27	QLFVTVVDTTPSTNL	**18**	389-403	34	0
28	CGHYIILFLRNVNVF	**18**	42-56	34	0

"a" indicates the HPV types that contain the exact epitope in their L1 amino acid sequence
"b" the numbers given in this column refer to the HPV type given in bold letters
"c" indicates the number of random donors tested for cytokine response after challenge with the peptide
"d" number of donors responding to a challenge with the peptide after stimulation divided by n (x 100)

The number of peptides that was recognized by PBMCs of a single blood donor varied widely (Figure 3-2). Only 7% of the people were not recognizing any of the 28 L1HPV peptides. This indicates widespread coverage of the L1-specific immune response. 7% of the blood donors mounted an immune response against nine of the 28 different peptides and more than half of the donors recognized more than three peptides. The possible reasons for the observed variations are numerous: (I) infection of the donors by several or none of the analyzed HPV types, (II) cross-reaction of T cells, (III) rare HLA-DR haplotype without promiscuous presentation and (IV) time elapsed since HPV infection.

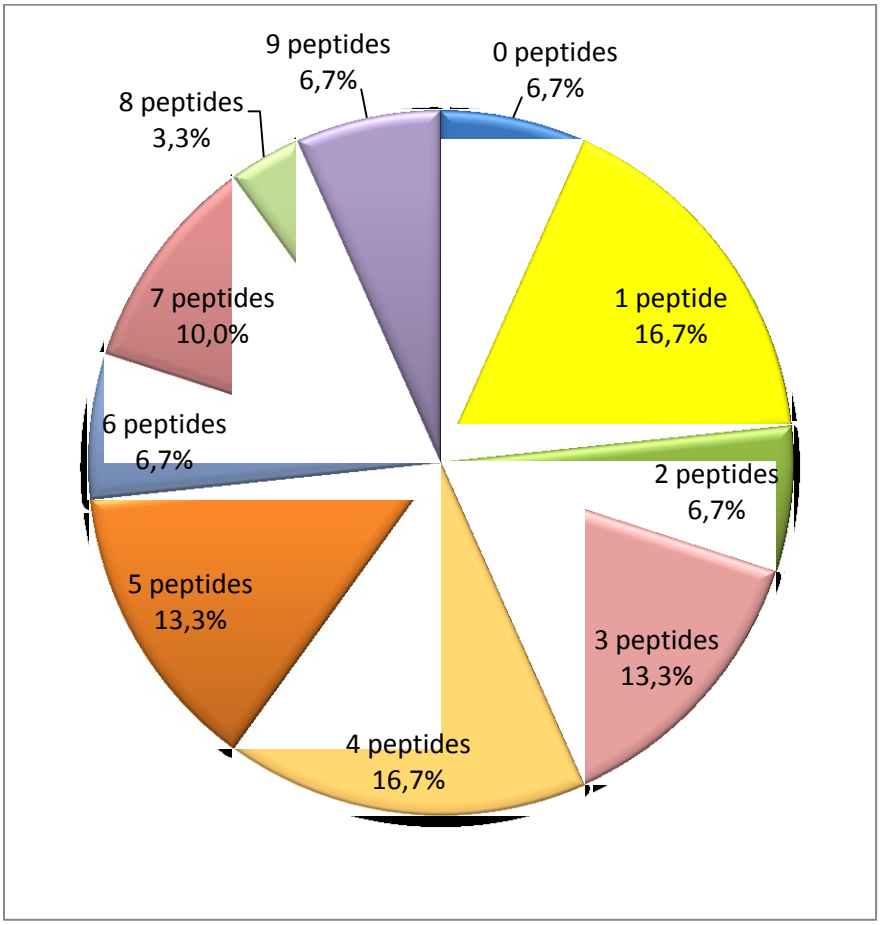

Figure 3-2. Number of peptides recognized by individual donors. Only donors that were tested for all 28 candidate epitopes are included. After peptide specific pre-amplification PBMCs from 30 different blood donors were tested with 28 candidate epitopes from the L1 protein of HPV6/11/16/18 via ELISPOT. The percentage of donors that recognized the indicated number of peptides is given in the slices.

In most cases (83%) an immune response against peptides from several different HPV types could be detected. As it is shown in Table 3-13, only 10% of the donors had a cytokine response restricted to peptides that originate from a single HPV type.

Table 3-13. HPV type specificity of detected T-cell response against L1. Only the donors tested against all candidate epitopes are included (n = 30).

L1 HPV type	type 6	type 11	type 16	type 18	1+types[a]	2+types[b]
donors[n] (T cells detected)	23	25	18	17	28	25
donors [%] (T cells detected)	76.7	83.3	60.0	56.7	93.3	83.3

"a" 1+ types: this column describes the number and percentage of donors, who showed specific T cells against at least one of the four tested HPV types
"b" 2+ types: this column describes the number and percentage of donors, who showed specific T cells against at least two the four tested HPV types

3.2 Intracellular cytokine staining

3.2.1 T-cell populations

To further investigate and identify the cells producing IFN-γ upon peptide stimulation, flow cytometric analysis was performed. PBMCs were pre-amplified with peptide pools and challenged after 12 days with a single peptide in the presence of brefeldin A, according to 2.2.4 (Intracellular cytokine staining). After the cytokine production phase, PBMCs were stained with the dye "LIFE/DEAD aqua" to exclude dead cells. To differentiate between distinct cell populations they were also stained for the surface markers CD4 and CD8. Intracellular cytokine staining (ICS) was performed for IFN-γ to identify cells reacting to the peptide challenge by cytokine response.

Stainings of four exemplary donors are depicted in Figure 3-3, Figure 3-5, Figure 3-6 and Figure 3-7. Figure 3-3 shows the results for BD783 in a more detailed manner. Figure 3-5, Figure 3-6 and Figure 3-7 show the results for the PBMCs of the blood donors Sp1, Sp2 and Sp3.

As gating strategy it was first gated on the lymphocyte cell population according to their size (FSC) and granularity (SSC). This removes most of the cell debris from the data. In a next step single cells were discriminated from cell doublets. This was done by checking the relation between the height (FSC-H) and area (FSC-A) of the FSC signal. Two cells sticking together

have a longer (increase in FSC-A) signal compared to the height of the signal (FSC-H), because they are longer in shape but not thicker. Hence, on the FSC-A/FSC-H plot, cell doublets do not lie on the main line made out of single cells. Next, dead and dying cells were removed from data by a "LIFE/DEAD aqua" plot. LIFE/DEAD aqua is a fluorescent dye that binds to free amino groups. Viable cells are not stained because most free amino groups are contained within the cell and an intact cell membrane prevents the dye from entering the cell. Only dead cells and cell debris are stained with the dye. Data points having a high LIFE/DEAD signal are removed from the analyzed data. Next CD4 was plotted against CD8, thus allowing population analysis of $CD4^+$ T cells and $CD8^+$ T cells separately.

Figure 3-3 shows the analysis of PBMCs from blood donor BD783 in detail. The first CD4 and CD8 population plots show the IFN-γ response after challenge with the negative peptide from human filamin A that was already used in the ELISPOT experiments. The negative peptide stimulates 0.08% of the $CD4^+$ T cells and 0.06% of the $CD8^+$ T cells to produce IFN-γ. The positive control, a mixture of PMA and Ionomycin, causes 42.3% of $CD4^+$ and 37.9% of the $CD8^+$ T cells to produce IFN-γ. These results indicate a good experimental and technical reliability. The next five double plots show the IFN-γ production after challenge with one single peptide. All five CD4 plots show an increased population of IFN-γ producing cells, reaching percentages between 0.23% and 2.24%. None of the five CD8 plots show any increase in the amount of IFN-γ producers (0.03-0.07%). This clearly shows that all five peptides are MHC class II restricted epitopes, recognized by $CD4^+$ T cells and not MHC class I epitopes recognized by $CD8^+$ T cells. For a better overview, the results of BD783 are summarized in Table 3-14. For an explanation how positive and negative responses can be distinguished, see 3.3.2.

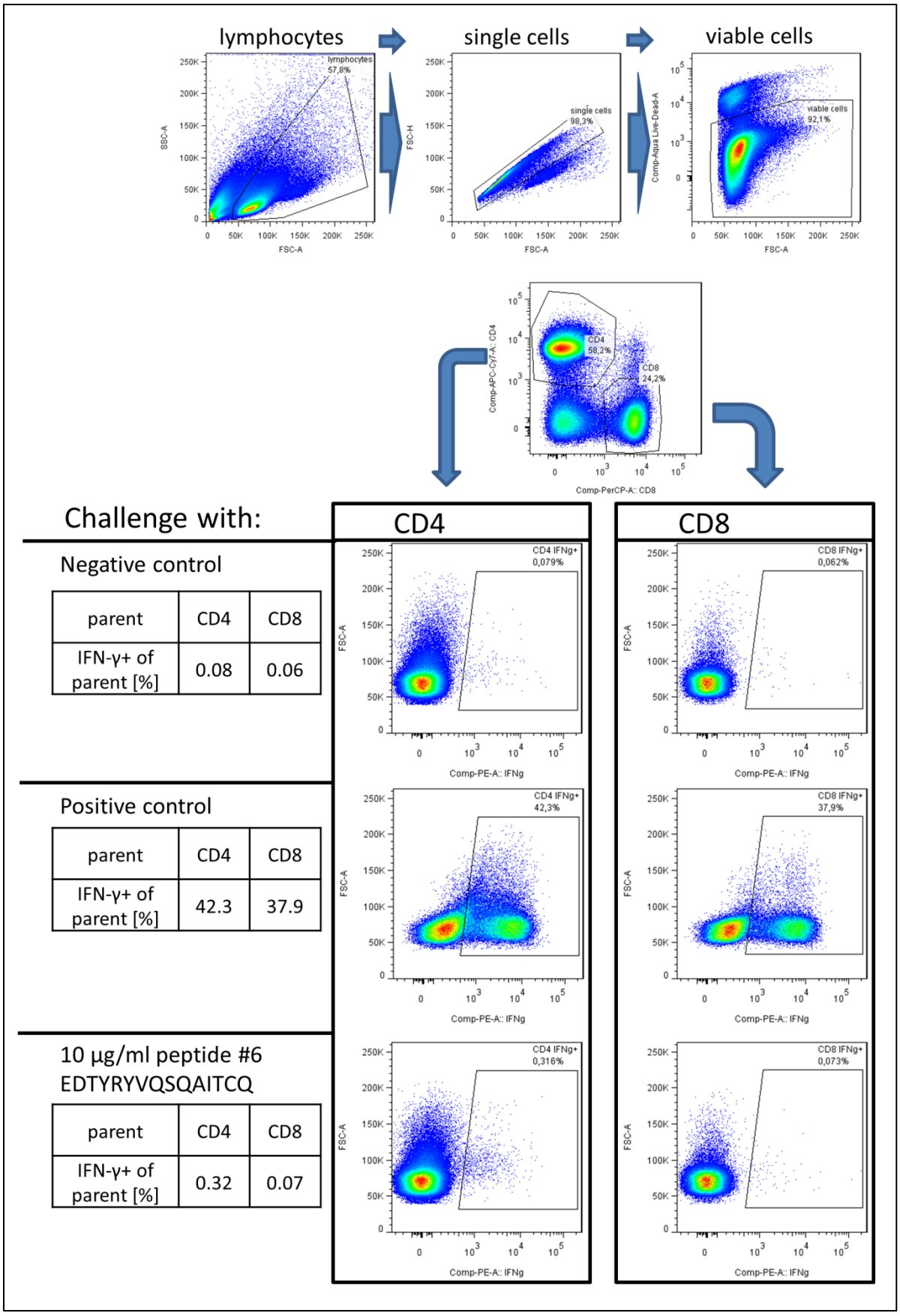

Figure 3-3. Intracellular cytokine staining of BD783. Continues on next page.

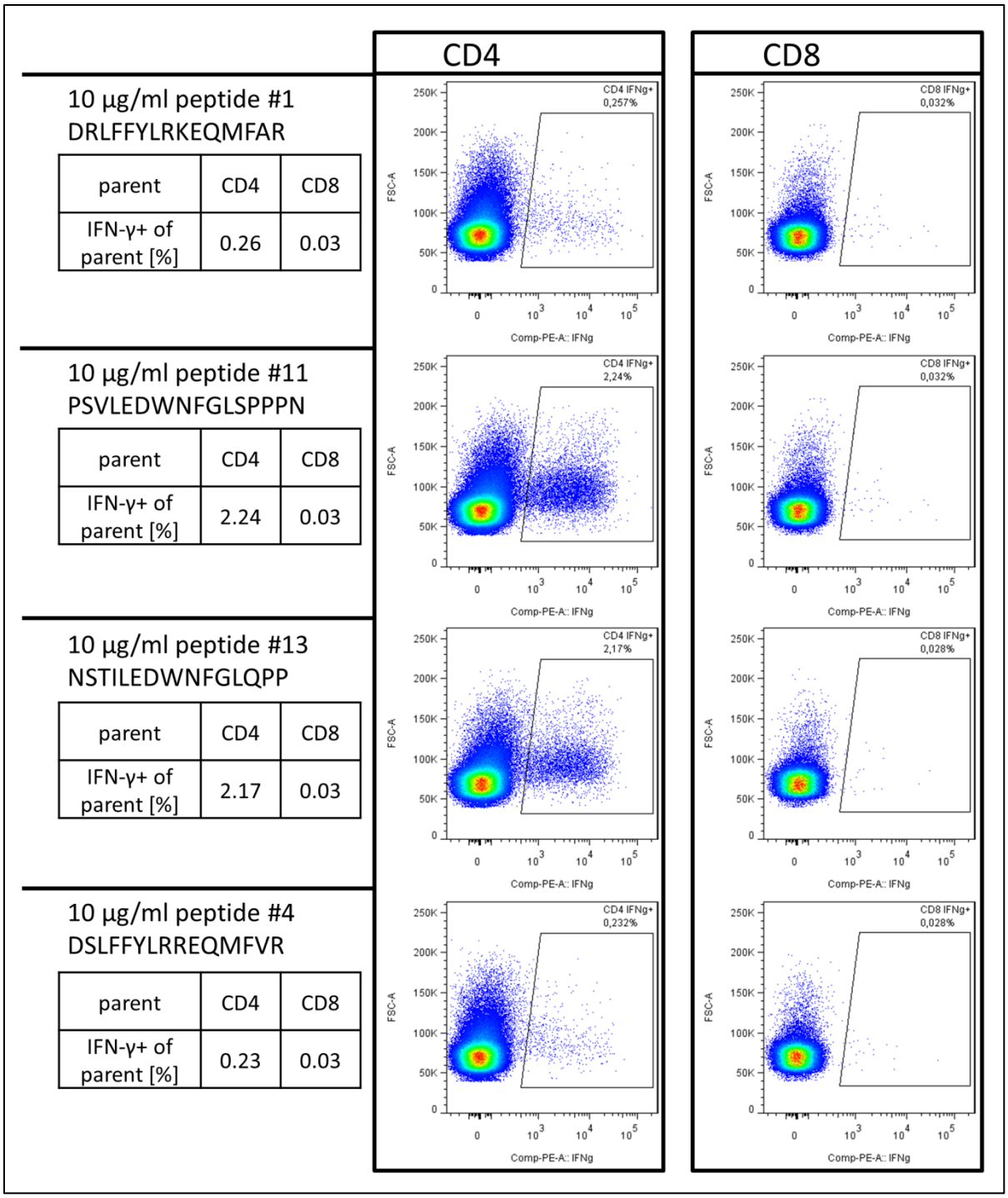

Figure 3-3 continued. Intracellular cytokine staining of BD783. PBMCs were pre-amplified using a pool of up to 10 peptides including the one that was used for challenge. Intracellular IFN-γ levels were measured after 12 h challenge with 10 µg/ml peptide indicated on the left side. Gating was performed as shown (top). First, lymphocytes were gated on by using FSC-A/SSC-A. Second, cell aggregates were excluded by FSC-A/FSC-H. The remaining events were gated for viable cells by usage of LIFE/DEAD aqua-staining. The FACS plots (middle and right) show single, viable lymphocytes divided into $CD4^+$ and $CD8^+$ populations. The plots show IFN-γ levels as a function of cell size (FSC-A). Cells that lie within the gate are cells of the parent cell type that react upon challenge by IFN-γ cytokine production. The percentage of the parent cell type population (single, viable lymphocytes positive for either CD4 or CD8) that produces IFN-γ upon challenge is given in the tables left to the plots.

Table 3-14. Summary of ICS of BD783. Percentage of IFN-γ positive cells of parent population after challenge with a single peptide.

parent population	CD4	CD8
	IFN-γ+ of parent [%]	IFN-γ+ of parent [%]
challenge		
negative control	0.08	0.06
positive control	42.30	37.90
EDTYRYVQSQAITCQ (#6)	0.32	0.07
DRLFFYLRKEQMFAR (#1)	0.26	0.03
PSVLEDWNFGLSPPPN (#11)	2.24	0.03
NSTILEDWNFGLQPP (#13)	2.17	0.03
DSLFFYLRREQMFVR (#4)	0.23	0.03

For the PBMCs of BD783 a backgating analysis was performed (Figure 3-4). The gating included all viable, single cell events that showed increased IFN-γ values. The expression of the surface markers CD3, CD4 and CD8 was analyzed and compared to a negative control of unstained viable, single cells. All five IFN-γ positive populations that were detected after challenge with the single peptide indicated are positive for CD3 and CD4. Most of the IFN-γ producing cells are negative for CD8. In the IFN-γ^+ population after stimulation with the peptides EDTYRYVQSQAITCQ (#6) and DRLFFYLRKEQMFAR (#1) a small population of IFN-γ producers show CD8 expression. Although these populations are not visible in all IFN-γ positive populations and are hence not caused by standard background of the staining they are small and can be ascribed to random unspecific activation. Thus the results clearly indicate that the cells producing IFN-γ after challenge with one of the five used peptides express CD3 and CD4 on their surface but no CD8. The five peptides can therefore be described as MHC class II T-cell epitopes.

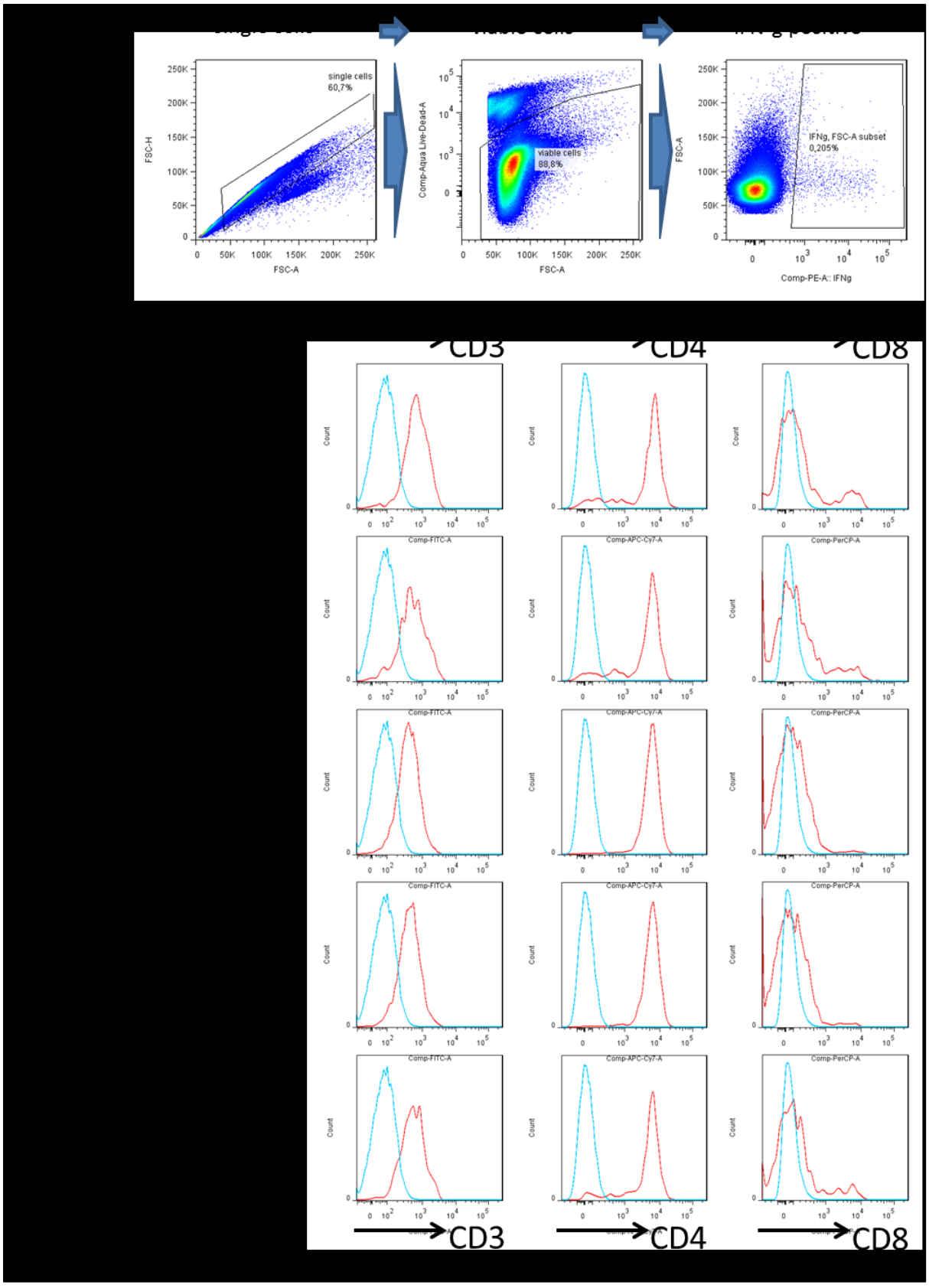

Figure 3-4. Backgating analysis of BD783. Pre- amplified PBMCs of donor BD783 were challenged with a single peptide indicated on the left side and stained for CD3, CD4, CD8 and intracellular IFN-γ. The events gated for the histograms were single, viable cells. The histograms show the surface expression of CD3, CD4 and CD8. IFN-γ producers are depicted in red while blue indicates a negative staining.

Generally, the method of ICS proved to be less applicable than the ELISPOT assay. Despite the use of PBMCs of donors that already reacted to several HPV epitopes in ELISPOT, the amount of cells producing IFN-γ upon challenge was small and the IFN-γ production more difficult to detect than with the ELISPOT. Parallel experiments using the same pre-amplified PBMCs, and comparing ELISPOT and ICS results affirmed nonequivalent results. To enhance the ICS results several parameters in the ICS protocol were varied. Different incubation times were used, a 1 h pre-incubation time (after peptide challenge but before adding brefeldin A) was introduced and different Golgi-apparatus inhibitors (brefeldin A, monensin) and mixtures of both were applied. None of these changes enhanced the ICS results in a significant manner, so that the original ICS protocol as described in 2.2.4 was not changed.

The differences between ELISPOT and ICS most probably arise because of the strong difference in peptide challenge time. The ELISPOT is incubated 24 h while the ICS uses only 12 h incubation time. To prolong the peptide challenge time in the ICS is not practicable because increased incubation periods of BrefA or monensin lead to significant cell loss due to cell death.

As mentioned before, the positive ELISPOT results could not be observed by ICS in most cases. To characterize at least the epitopes with the highest recognition rate, PBMCs of donors that were vaccinated with the HPV vaccine Gardasil (Sp1, Sp2 and Sp3) were used. These persons should have augmented levels of HPV-specific T cells. This should greatly increase the amount of HPV-specific T cells after the pre-amplification and therefore facilitate the detection by ICS.

Figure 3-5, Figure 3-6 and Figure 3-7 show the ICS FACS data of Gardasil vaccinated donors Sp3, Sp1 and Sp2.

As displayed in Figure 3-5, pre-amplified PBMCs from Sp3 recognize six different HPV L1 derived peptides as MHC class II restricted epitopes. The percentage of $CD4^+$ cells, which were reacting with IFN-γ production after peptide challenge range between 0.19% and 0.77%.

These positive results are clearly distinguishable from the very low negative control of 0.03%. The response rate within the $CD8^+$ population varies only between 0.15% and 0.23% and is derived from background (negative control: 0.14%). The positive control is very low.

Only 2.22% of the $CD4^+$ cells and only 2.19% of the $CD8^+$ cells react to a PMA/Ionomycin stimulus. This could be an indicator for unresponsive T cells or could just be caused by a poor quality PMA or Ionomycin batch.

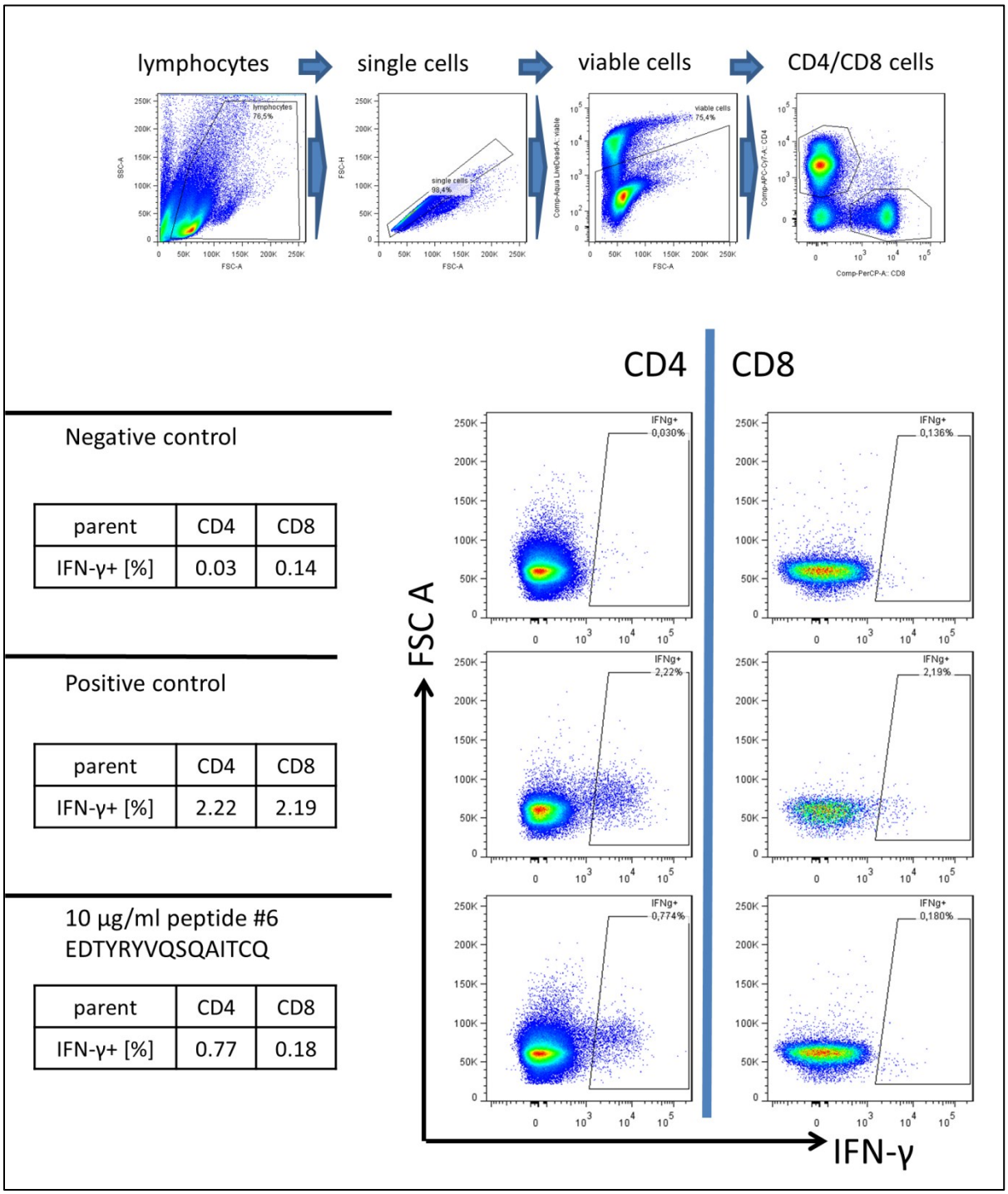

Figure 3-5. ICS FACS data of donor Sp3. Continues on next page.

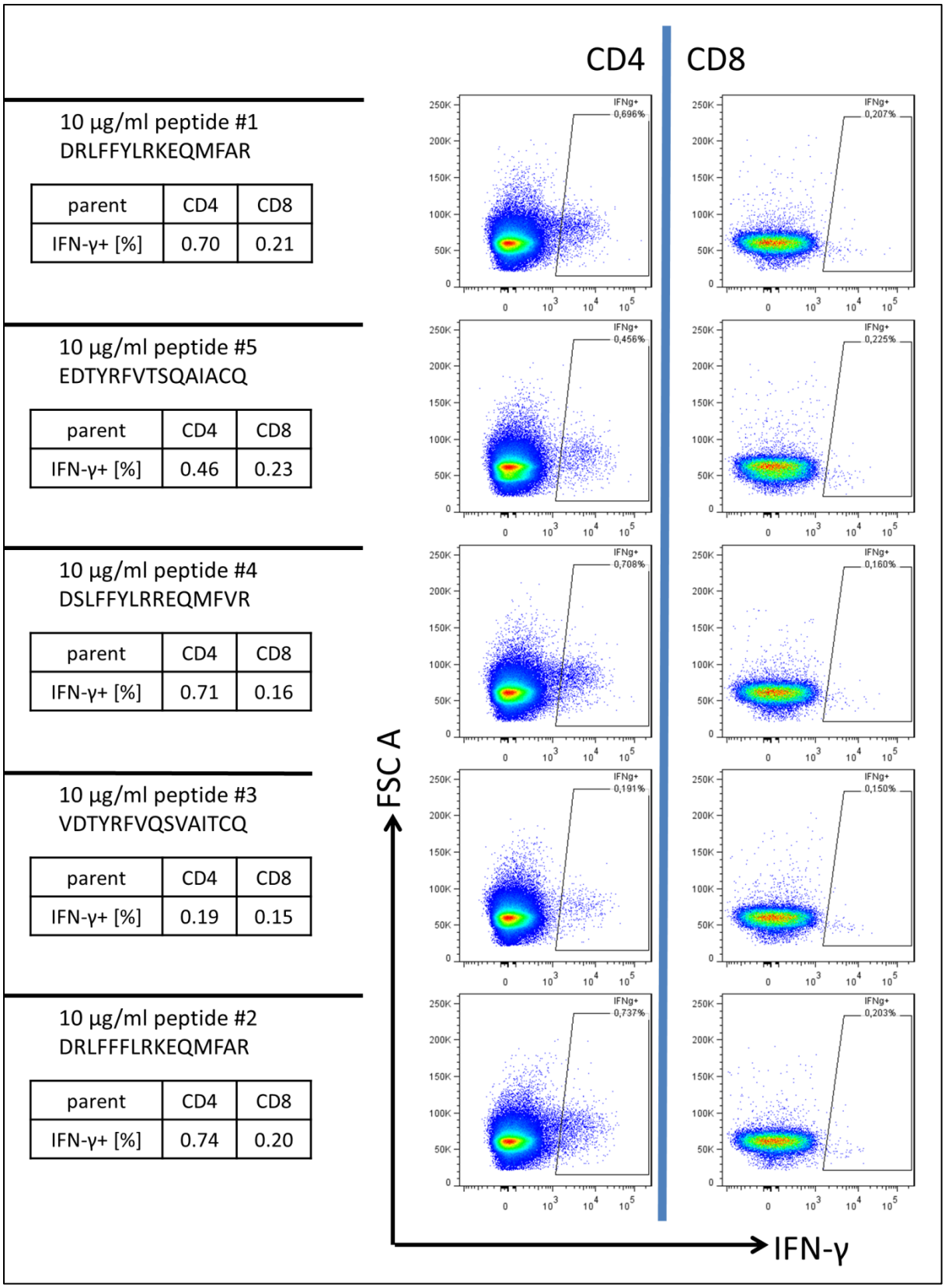

Figure 3-5 continued. ICS FACS data of donor Sp3. PBMCs were pre-amplified using a pool of up to 10 peptides including the one that was used for challenge. Intracellular IFN-γ levels were measured after 12 h challenge with 10 µg/ml peptide indicated on the left side. Gating was performed as shown (top). The FACS plots (middle and right) show single, viable lymphocytes $CD4^+$ (middle) or $CD8^+$ (right).

Figure 3-6 and Figure 3-7 show the FACS data of pre-amplified PBMCs from Gardasil vaccinated donors Sp1 and Sp2. Sp1 shows responses against four different peptides, while the PBMCs from Sp2 react to only two of the challenged peptides.

The background of the $CD4^+$ population of Sp1 is low (0.17%). After challenge with the L1-specific peptides at least twice as many positive-gated events can be detected in comparison to the negative control. The background of the $CD8^+$ population is higher. The negative control shows 0.98% positive cells, while the positive-gated events of the peptide challenged cells reach between 0.72% and 1.33%. All four peptides clearly elicit a response within the $CD4^+$ population and not the $CD8^+$ population.

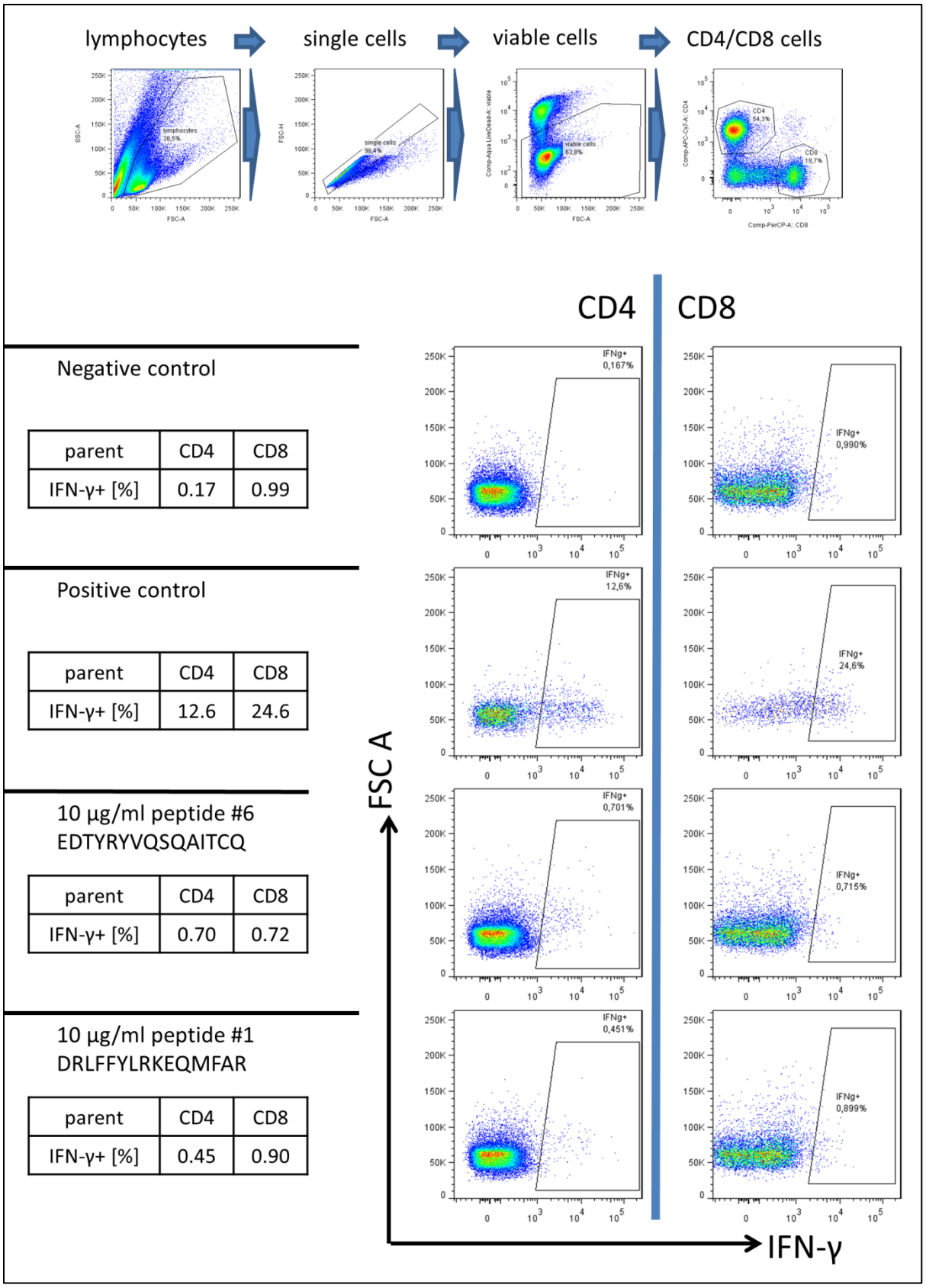

Figure 3-6 ICS FACS data of donor Sp1. Continues on next page.

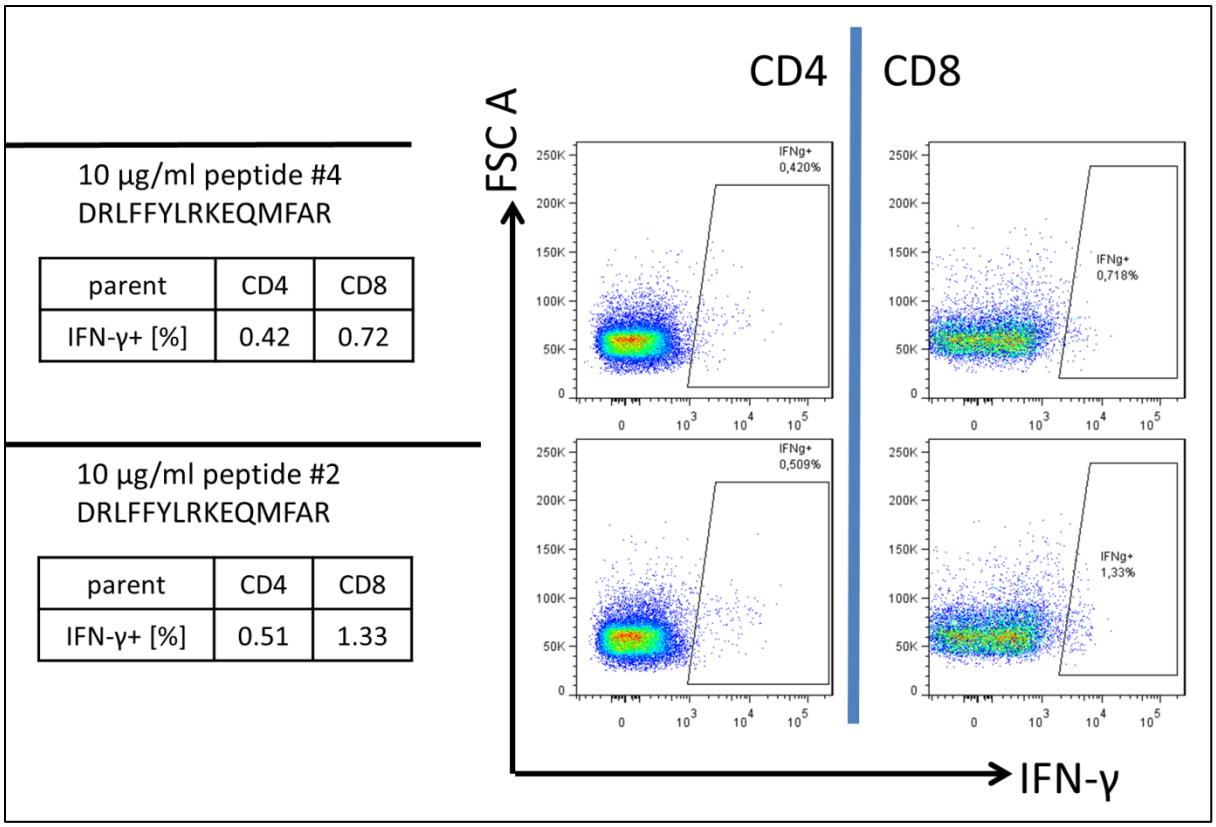

Figure 3-6 continued. ICS FACS data of donor Sp1. PBMCs were pre-amplified using a pool of up to 10 peptides including the one that was used for challenge. Intracellular IFN-γ levels were measured after 12 h challenge with 10 µg/ml peptide indicated on the left side. Gating was performed as shown (top). The FACS plots (middle and right) show single, viable lymphocytes $CD4^+$ (middle) or $CD8^+$ (right).

Despite the donor Sp2 being vaccinated against HPV, the detected IFN-γ responses are only marginal (Figure 3-7). The highest percentage of positive-gated events in the $CD4^+$ population after peptide challenge is 0.39% with a background of 0.14%. Challenge with the peptide EDTYRYVQSQAITCQ causes 0.39% of the $CD4^+$ population to produce IFN-γ. Challenge with EDTYRFVTSQAIACQ causes only 0.18% to respond with IFN-γ production. The response is not distinguishable from the background. Again the $CD8^+$ positive-gated events vary only marginal (0.27% - 0.30%) and stay close to the negative control (0.32%).

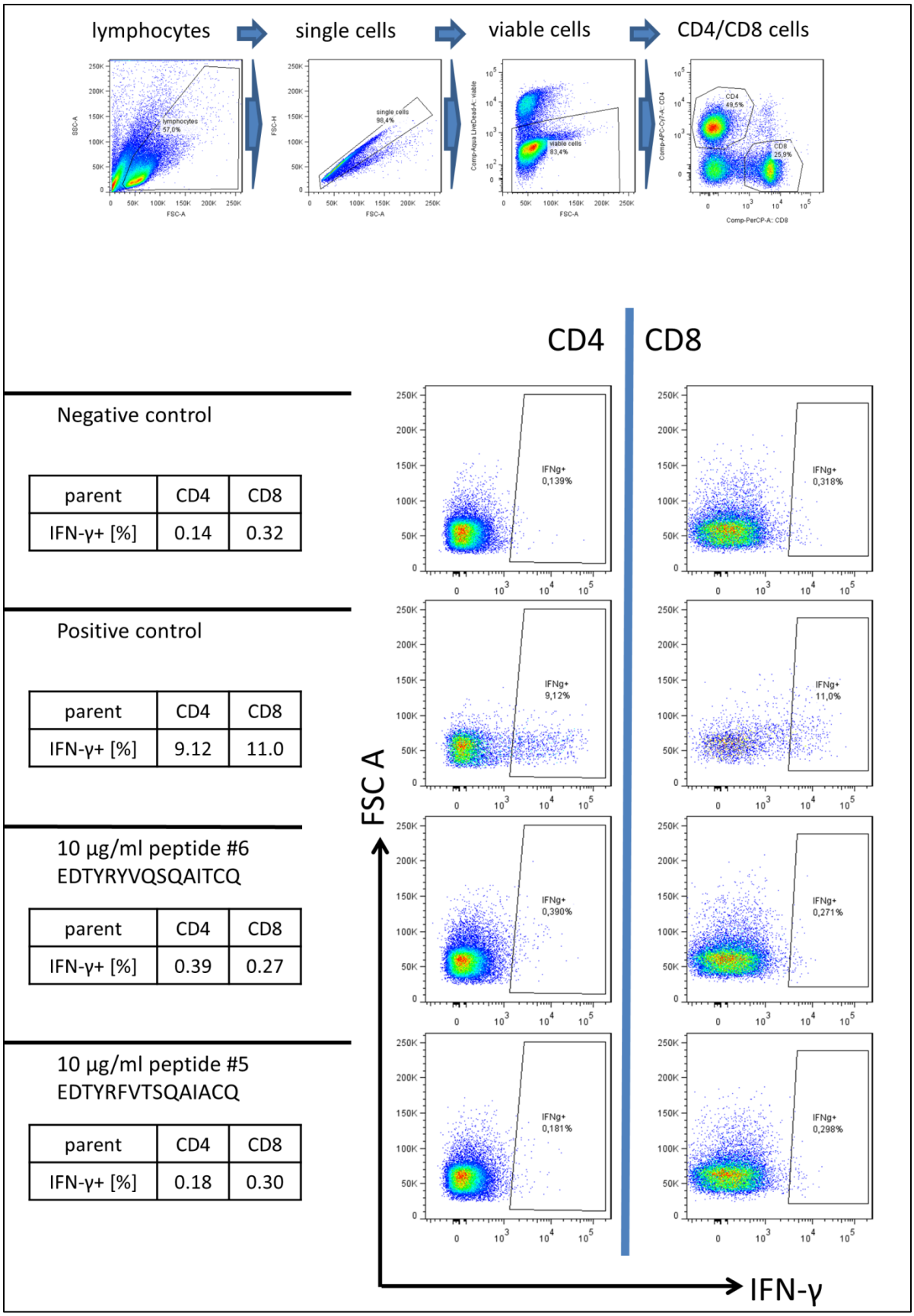

Figure 3-7. ICS FACS data of donor Sp2. PBMCs were pre-amplified using a pool of up to 10 peptides including the one that was used for challenge. Intracellular IFN-γ levels were measured after 12 h challenge with 10 µg/ml peptide indicated (left side). Gating was performed as shown (top). The FACS plots (middle and right) show single, viable lymphocytes $CD4^+$ (middle) or $CD8^+$ (right).

Taken together (see Table 3-15), it was shown that the cells producing IFN-γ after peptide challenge were not $CD8^+$ but $CD4^+$ cells. This was done for all six FREPs and two additional epitopes with a response rate higher than 10%. For five of these 8 peptides it was additionally shown by backgating that the IFN-γ producing cells were $CD3^+$ and $CD4^+$ but $CD8^-$.

All peptides were predicted by SYFPEITHI to be MHC class II restricted. Their size destines them to bind to the open binding cleft of the MHC class II molecules. The binding of a long peptide to the small closed binding cleft of the MHC class I molecule is up to 10 000 fold more difficult than the binding of the short peptide (Rotzschke et al., 1991). All intracellular stainings that showed a strong enough signal to be analyzed validated this presumption. Therefore, it can be assumed that the entire predicted candidate epitopes that cause an IFN-γ response in the ELISPOT experiments are MHC class II restricted T-cell epitopes.

Table 3-15. Summary: T-cell epitope restriction

				CD4 T-cell epitope?	
#	**sequence**	**HPV type[a]**	**recognition [%][b]**	**CD4/CD8 gating[c]**	**backgating[d]**
1	DRLFFYLRKEQMFAR	11	44	**pos**	**pos**
2	DRLFFFLRKEQMFAR	6	43	**pos**	
3	VDTYRFVQSVAITCQ	18	41	**pos**	
4	DSLFFYLRREQMFVR	16	38	**pos**	**pos**
5	EDTYRFVTSQAIACQ	16	32	**pos**	
6	EDTYRYVQSQAITCQ	6, 11	29	**pos**	**pos**
7	FKVVLPDPNKFALPD	6, 11	27	n.d.	
8	AEVMAYIHTMNPSVL	6, 11	15	n.d.	
9	GHPYFSIKRANKTVV	6	13	n.d.	
10	VMTYIHSMNSTILED	16	12	n.d.	
11	PSVLEDWNFGLSPPPN	11	12	**pos**	**pos**
12	GHPYYSIKKVNKTVV	11	12	n.d.	
13	NSTILEDWNFGLQPP	16	12	**pos**	**pos**

"a" indicates the HPV types that contain the exact epitope in their L1 amino acid sequence
"b" percentages of recognition determined by ELISPOT
"c" "pos"(positive):at least one ICS indicates IFN-γ producers to be $CD4^+$ not $CD8^+$
"d" "pos" (positive): IFN-γ producers were backgated to be $CD3^+$, $CD4^+$ and $CD8^-$
"n.d." could not be determined due to too low cell frequency

3.2.2 Intracellular multi-parameter staining

Intracellular multi-parameter staining was performed to assess the function of the responding T cells and to determine if the staining of other activation markers would lead to a higher sensitivity and a higher specificity of the ICS. Therefore PBMCs of donor Sp3 were pre-amplified with peptide pools of up to 10 different peptides. ICS was performed and the cells were stained for CD4, CD8, LIFE/DEAD, the activation marker CD154, and the cytokines IFN-γ, TNF, and IL-2. The FACS data are displayed in Figure 3-8 and the results are summarized in Table 3-16.

Positive response (FACS data):

The **visual inspection** of the FACS plot focuses on the distribution of the positive events of the peptide challenged cells. The distribution and position of all positive events are compared with the distribution of the positive events of the negative peptide control. Of course, this data analysis is very subjective and greatly depends on the investigator. But if done correctly the operator can compensate for random changes (e.g. background activation of the cells, quality of staining).

In a more objective analysis, the number of positive gated events in the experiment is compared to the number of positive gated events of the negative control. The **positive criteria**: "*at least twice the background*" or the like can be used. But this kind of positivity criterion is very susceptible to the setup of the positive gate and the height of background. The lower the background the more sensitive the staining becomes.

Fully automated negative-control based gating tools, that will change the subjectivity of FACS data analysis are being developed (Strain et al., 2009) but were not used in this work.

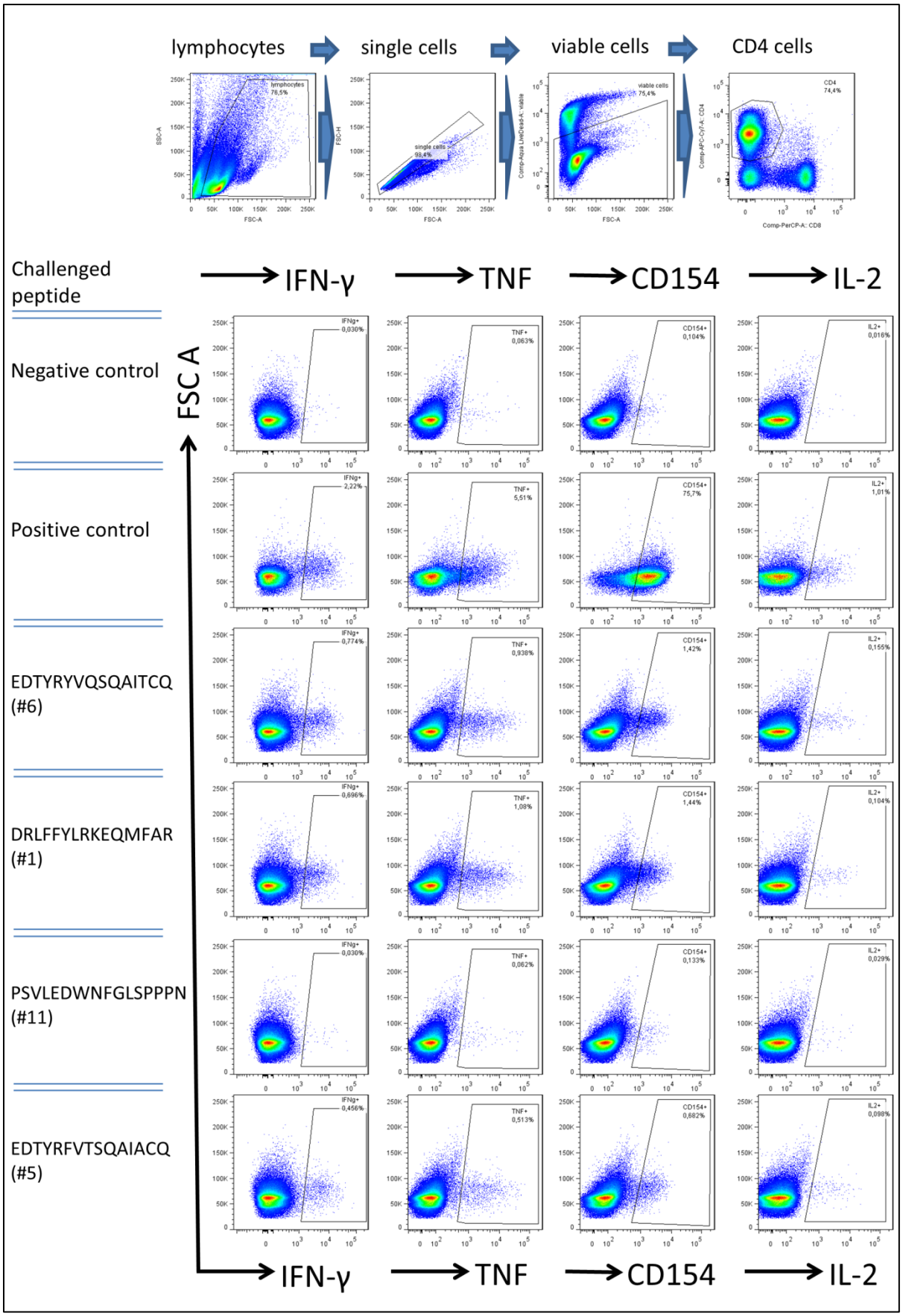

Figure 3-8. Intracellular multi-parameter staining of donor Sp3. Continues on next page.

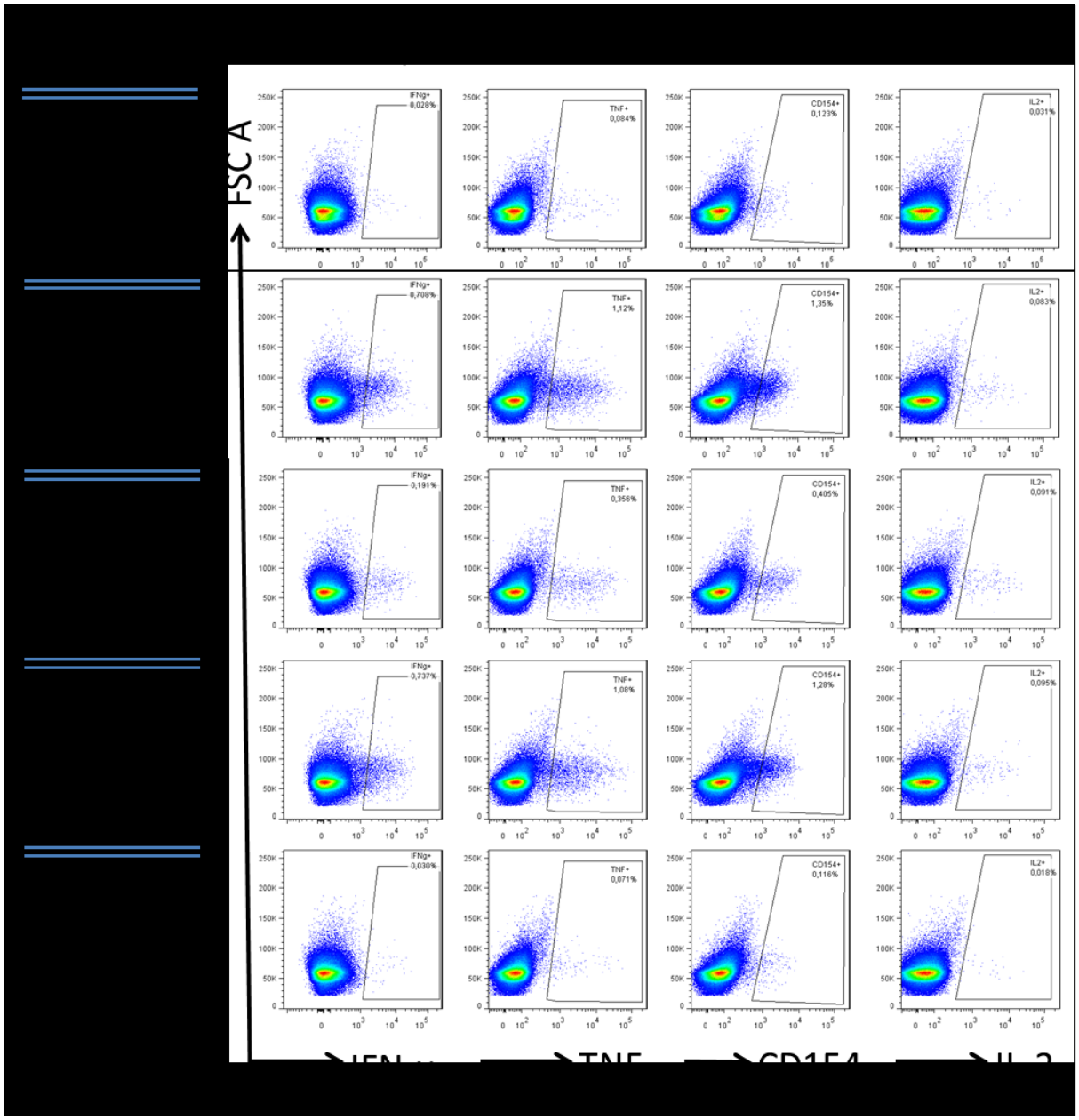

Figure 3-8 continued. Intracellular multi-parameter staining of donor Sp3. PBMCs were pre-amplified using a pool of up to 10 peptides including the one that was used for challenge. Intracellular IFN-γ (left), TNF (middle left), CD154 (middle right) and IL-2 (right) levels were measured after 12 h challenge with 10 µg/ml peptide indicated (left side). Gating was performed as shown (top). The FACS plots show single, viable $CD4^+$ lymphocytes.

As can be seen in the overview (Table 3-16), cells that respond to a peptide challenge produce IFN-γ, TNF, CD154 and IL-2. The positive responses that were detected by visual inspection were at least fourfold above background.

Table 3-16. Summary of intracellular multi-cytokine staining of donor Sp3.

measured marker	IFN-γ		TNF		CD154		IL-2	
	pos** [%]	VI*	pos** [%]	VI*	pos** [%]	VI*	pos** [%]	VI*
challenge								
negative control	0.03		0.06		0.10		0.02	
positive control	**2.22**	**+**	**5.50**	**+**	**75.7**	**+**	**1.01**	**+**
EDTYRYVQSQAITCQ (#6)	**0.77**	**+**	**0.94**	**+**	**1.42**	**+**	**0.16**	**+**
DRLFFYLRKEQMFAR (#1)	**0.70**	**+**	**1.08**	**+**	**1.44**	**+**	**0.10**	**+**
PSVLEDWNFGLSPPPN(#11)	0.03	-	0.06	-	0.13	-	0.03	-
EDTYRFVTSQAIACQ (#5)	**0.46**	**+**	**0.51**	**+**	**0.68**	**+**	**0.10**	**+**
NSTILEDWNFGLQPP (#13)	0.03	-	0.08	-	0.12	-	0.03	-
DSLFFYLRREQMFVR (#4)	**0.71**	**+**	**1.12**	**+**	**1.35**	**+**	**0.08**	**+**
VDTYRFVQSVAITCQ (#3)	**0.19**	**+**	**0.36**	**+**	**0.41**	**+**	**0.09**	**+**
DRLFFFLRKEQMFAR (#2)	**0.74**	**+**	**1.08**	**+**	**1.28**	**+**	**0.10**	**+**
FKVVLPDPNKFALPD (#7)	0.03	-	0.07	-	0.12	-	0.02	-

** pos: column indicates the percentage of $CD4^+$ cells lying in the positive gate of this cytokine. Values that reach more than twice the negative control are termed positive
* VI: visual inspection: column indicates the result by visual inspection: + clearly defined positive population– no positive population visible
bold: Positive results are highlighted in **bold**

In case of the six FREPs EDTYRYVQSQAITCQ (#6), DRLFFYLRKEQMFAR (#1), EDTYRFVTSQAIACQ (#5), DSLFFYLRREQMFVR (#4), VDTYRFVQSVAITCQ (#3) and DRLFFFLRKEQMFAR (#2) a response was detectable with all four activation markers. Therefore, any of these four molecules could be used as a marker for T cells recognizing the challenged epitope. The IFN-γ and IL-2 stainings showed a lower background (negative control IFN-γ: 0.03%; IL-2: 0.02%) than the TNF (negative control TNF: 0.06%) or CD154 (negative control CD154: 0.10) stainings. Generally, the marker CD154 detected the highest percentage of responding cells, followed by TNF, IFN-g and IL-2. The highest measured positive percentage after a peptide challenge was 1.44% for CD154, 1.12% for TNF, 0.77% for IFN-γ and only 0.16% for IL-2. The low background and the relatively high positive populations make the measurement of IFN-γ alone a feasible standard.

3.3 Antibodies

3.3.1 Multiplex serology

From 37 blood donors, plasma samples could be collected. The antibody concentration of anti-L1 antibodies of several HPV types was determined simultaneously by multiplexed L1 serology, kindly provided by Prof. Dr. Michael Pawlita from the DKFZ (Heidelberg, Germany). To ensure reliable data and a proper cut-off value, the samples were measured and analyzed together with 3500 samples from an EPIC study measuring 26 different antigens. The whole measurement lasted three days but continuous data acquirement was ensured by bridging panels adjusting for variability between different days. The cut-off values were determined by inclusion of a subpanel of 188 Korean sera in the measurements. The anti L1 antibody prevalences of these sera were already known and the cut-off values were chosen as to allow maximal consistence. The cut-off values for L1 HPV11 and L1 HPV16 were determined in this manner (Cut-off L1 HPV11: 164 MFI; cut-off L1 HPV16: 145 MFI). The determination of the cut-off for L1 HPV18 resulted in a value below 100. According to Kristina Michael, the person supervising the experiment, such a value would be too low and would result in many false positive results. After visual inspection of the occurring frequency distribution curves, the cut-off was changed to 120 MFI. For the cut-off of L1 HPV6 there exists a problem of very high average values throughout all measurements not limited to this study. This is because of a suspected unspecificity or unsuspected very high seroprevalence of the anti-L1 HPV6 antibodies. According to visual inspection of the occurring frequency distribution curves, the cut-off was changed to 600 MFI. The used cut-off values are summarized in Table 3-17.

Table 3-17.Cut-off values used in this work.

HPV type	6	11	16	18
Cut-off[a]	600	164	145	120

" a " cut-off values are given in MFI

The results of the 37 sera measured in this analysis are shown in Table 3-18 and an overview is given in Table 3-19.

Table 3-18. Results of L1 multiplex serology for 37 healthy blood donors.

	tropism	mucosa	mucosa	mucosa	mucosa
	genus	alpha	alpha	alpha	alpha
	species	10	10	9	7
Blood donor	**HPV type**	**6**	**11**	**16**	**18**
1231		181	31	23	12
1232		358	127	29	137
1233		1789	72	18	14
1234		1857	126	70	9
1235		5343	255	31	35
1239		326	67	43	12
1240		97	72	530	28
1241		523	40	17	7
1242		169	68	33	34
1243		1189	111	92	97
1244		660	96	76	25
1245		84	35	14	9
1246		1408	146	91	72
1247		151	37	49	10
1248		65	54	11	22
1249		199	115	38	122
1250		228	90	48	34
1251		480	97	54	194
1252		235	84	17	21
1253		309	42	27	12
1254		316	66	21	57
1255		320	76	51	13
1256		468	102	20	8
1262		229	54	124	34
1263		511	1001	60	522
1264		749	133	165	50
1265		459	43	7	5
1266		643	46	36	6
1267		261	35	19	15
1272		350	320	71	63
1273		475	1378	117	329
1276		3603	365	23	49
1277		141	42	35	12
1278		3799	2836	7125	1929
1279		3814	3001	9202	2188
1280		198	36	43	8
1281		268	115	183	36

Results are given in net-MFI; bead autofluorescence and the GST tag background are already subtracted; the values that are above the determined cut-off values are highlighted in green.

Out of the 148 data points measured 30 results were positive (20%). The MFI values varied very strong within a single donor. For example, blood donor 1235 had a net MFI-value for anti-L1HPV6 of 4743 above cut-off, while its value for anti-HPV11 is only 91 net-MFI above cut-off. This shows the strong type specificity of the anti-L1 HPV antibodies binding only its specific L1 type and not the one of a closely related HPV type. Also between the different donors the detected anti-L1 antibody level of a specific type differed enormously. For several sera samples the net-MFI for L1 HPV16 is below cut-off, but for blood donor 1297 it reaches 9057 above cut-off. One might speculate that values above 1000 MFI are caused by more recent infections, while values closer to the cut-off could be caused by passed infections already cleared by the immune system.

Antibodies against either one of L1 HPV type 6, 11, 16 or 18 were detected in 51.4% of the tested plasma samples. Most of the detected anti-L1 antibodies (29.7%) were directed against HPV type 6. Antibodies against the remaining three L1 types were detected less frequently (αL1 HPV11: 18.9%; αL1 HPV16: 13.5%;αL1 HPV18: 18.9%). This is despite the artificially increased cut-off of the anti-L1 HPV6 and highlights the unsuspected very high seroprevalence or unspecificity of the anti-L1 HPV6 antibodies already mentioned.

Antibodies against several different L1 proteins from different HPV types were detected in only 18.9% of the plasma. Simultaneous detection of antibodies against the L1 protein of the very closely related HPV types 6 and 11 occurred in only four plasma samples (10.8%). This confirms the known strong type specificity of anti-L1 antibodies associated with the originally antibody-defined type-classification (see 1.1.1.).

Table 3-19. HPV type specificity of detected antibodies directed against L1 (n = 37).

type specificity	type 6	type 11	type 16	type 18	1+ types[a]	2+ types[b]
donors [n] (ab detected)	11	7	5	7	19	7
donors [%] (ab detected)	29.7	18.9	13.5	18.9	51.4	18.9

"a" 1+ types: this column describes the number and percentage of donors, who showed L1 specific antibodies against at least one out of the four tested HPV types

"b" 2+ types: this column describes the number and percentage of donors, who showed L1 specific antibodies against at least two out of the four tested HPV types

3.3.2 Antibody/T-cell correlation

For 18 blood donors whose antibody levels were determined, the antibody results could be correlated with the L1-specific T-cell reactivity found in the PBMCs. This cohort does not include all antibody-tested blood donors, because in 19 cases not enough PBMCs could be collected to perform ELISPOT assays for all candidate epitopes. If the smaller cohort, including 18 blood donors, is examined, the specificity distribution of the found antibody and T-cell reactivity varies from the ones with higher cohort sizes. An overview of this cohort is given in Table 3-20. The exact comparison of the detected L1 specific responses is given in Table 3-21. Most striking, HPV-L1 specific T cells could be detected in more donors than L1-specific antibodies. 94.4% of the PBMCs showed T-cell reactivity against any of the four tested L1 proteins, while antibodies against any of the tested L1 types were only detected in 38.9% of the plasma samples. In BD1242, BD1252 and BD1277 a very diverse T-cell reactivity against peptides originating from different HPV types could be observed despite no detectable antibodies against any of the four analysed HPV types. One reason could be different sensitivities of the two detection methods.

Table 3-20. Type specificity of antibodies and T cells in the 18 donors' cohort.

type specificity	type 6	type 11	type 16	type 18	1+ types[a]	2+ types[b]
Ab detected						
donors [n]	4	2	0	3	7	2
donors [%]	22.2	11.1	0.0	16.7	38.9	11.1
T cells detected						
donors [n]	13	13	8	10	17	13
donors [%]	72.2	72.2	44.4	55.6	94.4	72.2

"a" 1+ types: this column describes the number and percentage of donors, who showed a specific response against at least one out of the four tested HPV types
"b" 2+ types: this column describes the number and percentage of donors, who showed a specific response against at least two out of the four tested HPV types

Furthermore, the T-cell responses are more type diversified than the antibody responses. Only 11.1% of the donors have antibodies against more than one HPV type, but 72.2% have T cells directed against multiple of the analysed HPV types. This suggests another reason for the higher abundance of T-cell responses compared to antibody responses. T cells could cross-react to a challenge by slightly different epitopes from different HPV types. This possibility will be discussed in more detail in the next chapter.

An antibody response without the corresponding T-cell reactivity was only detected in BD1266 and BD1276. In the plasma of both donors antibodies against type 6 L1 could be detected without any T-cell reactivity against any L1 HPV6-specific epitopes. This could be due to the already mentioned unspecificity of the anti-L1 HPV6 antibody detection. Another possibility is that the ELISPOT screening had missed an epitope addressed in these two donors.

Table 3-21. Simultaneous display of detected L1-specific antibodies[a] and L1-specific T cells[b].

	reactivity against HPV type			
BD #	***6***	***11***	***16***	***18***
1242				
1244				
1245				
1246				
1247				
1248				
1249				
1250				
1251				
1252				
1253				
1256				
1262				
1263				
1265				
1266				
1276				
1277				

Fehler! Keine gültige Verknüpfung.

"a" antibody response was determined by multiplex analysis

"b" T-cell reactivity was determined by IFN-γ ELISPOT after pre-amplification of specific T cells; T-cell reactivity was marked positive if at least one type specific peptide was tested positive

The reported T-cell responses are based on IFN-γ, the leading cytokine of T_H1 cells. A T_H1 response supports the cellular arm of the immune system and not the antibody production, as a T_H2 response would do. However, a direct connection between the T-cell response and the antibody levels was drawn because of several reasons: (i) a direct measurement of the T_H2 leading cytokines IL-4 and IL-5 was technically not feasible. Despite major efforts the ICS analysis of IL-4 and IL-5 were not suitable concerning sensitivity and specificity. (ii) The culture conditions used in the peptide specific pre-amplification promote a T_H1 response. Attempts to change the protocol to a more T_H2 friendly environment failed. (iii) As was already mentioned in the introduction (1.3.9 T-cell populations and T-cell response), the lineage commitment of the T-helper compartment is not absolute. Even the commitment of a single cell is reversible (Sallusto et al., 2004). Therefore, the detection of a T_H1 response should be accompanied by a T_H2 response.

3.4 T-cell clones

3.4.1 Possible cross-reactive epitopes

As shown in Table 3-21, the T-cell responses were recognizing more HPV types than the antibody responses. One possible explanation could be inter-type cross-reactive T cells. These cells would simultaneously recognize slightly different epitopes originating from different HPV types. Since the TCR is highly specific, such cross-reactivity is only possible if the presented epitopes display only minor differences.

To identify possible cross-reactive epitopes, the amino acid sequences of the four investigated L1 proteins from HPV type 6a, 11, 16 and 18 were aligned. As can be seen in Figure 3-9, there is considerable conservation between the L1 amino acid sequences of HPV6a and 11. This is expected because both types belong to species 10 of the Alpha-PV. Both types showed less sequence agreement with type 16 belonging to species 9 and HPV type 18 belonging to species 7 (see Figure 1-2).

The analysis of the ELISPOT screening revealed two FREP containing regions, “Region 1” (DSLFFYLRREQMFVR: $L1HPV16_{270\text{-}284}$ and corresponding) and “Region 2“ (EDTYRFVTSQAIACQ: $L1HPV16_{441\text{-}455}$ and corresponding). The screening approach identified multiple CD4 T-cell epitopes originating from different HPV types in each of these regions.

The maximal agreement for a 15mer in this setting would be 13 fully conserved amino acids. “Region 1” has nine fully conserved residues while “Region 2” has ten.

Region 1 is part of the β-sheet F while Region 2 forms the α-helix 4 of the L1 protein (Chen et al., 2000). Both regions are not located in any highly variable regions, such as the variable loops, where most variability is concentrated because of permanent pressure by the immune system (see 1.1.3.6). The observed medium level of sequence agreement could allow T-cell mediated cross-reaction.

Figure 3-9. Multiple alignment of the amino acid sequences of L1 from HPV6, 11, 16 and 18 (Clustal Omega www.ebi.ac.uk). Two regions, where at least two FREPs were found are framed in green (Region 1) and red (Region 2).The legend of the alignment is given in Figure 3-10.

An * (asterisk) indicates positions which have a single, fully conserved residue.
A : (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.
A . (Period) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix.

Residue	Colour	Property
AVFPMILW	RED	Small (small+ hydrophobic (incl.aromatic -Y))
DE	BLUE	Acidic
RK	MAGENTA	Basic - H
STYHCNGQ	GREEN	Hydroxyl + sulfhydryl + amine + G

Figure 3-10. Legend for amino acid sequence alignment in Figure 3-9 and 3-11 (taken from www.ebi.ac.uk).

After identifying “Region 1” and “Region 2” as FREP containing regions, positioned at sites of considerable sequence agreement, the search for epitopes allowing cross-reaction was extended to two additional clinically relevant HPV types.

Thus, the HPV types 31 and 45 were included, being clinically important types strongly associated with cancer (Smith et al., 2007). Additionally, HPV type 45 belongs to the same species 7 as HPV18 and HPV31 shares its species 9 with HPV16. This type selection includes three pairs of closely related HPV types that display a high L1-sequence conservation.

In an alignment of “Region 1” and “Region 2” of the types HPV6a, 11, 16, 18, 31 and 45 (Figure 3-11), both regions showed considerable sequence agreement. Out of 15 amino acids, nine positions show fully conserved residues. Additionally, three positions were conserved between groups of strongly similar properties. “Region 1” includes an additional minor conservation between groups of weakly similar properties. Therefore, both regions were equally promising concerning the existence of inter-type cross-reactive T cells. “Region 2” was further analyzed, while the peptides of “Region 1” were further investigated by the group of Andreas Kaufmann at the Charité, Berlin.

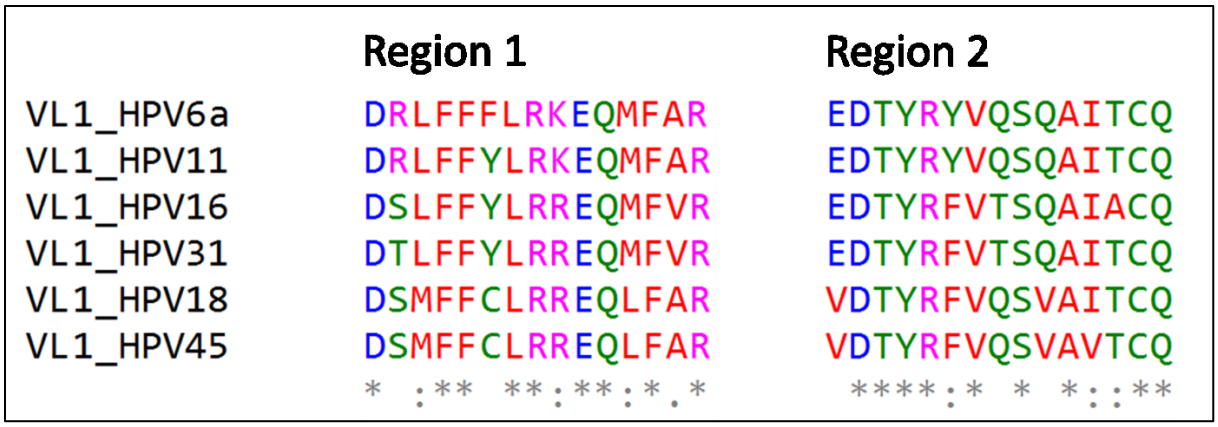

Figure 3-11. Amino acid sequence alignment of "Region 1" and "Region 2" of HPV6a, 11, 16, 18, 31 and 45.The legend is given in Figure 3-10.

3.4.2 Cloning

To determine, if T cells specific for a peptide from HPV "*type A*" are able to recognize and react to a slightly different peptide derived from HPV "*type B*", T-cell clones were generated. Each clone was derived from a single PBMC derived T cell. Therefore, all cells of a clonal population have the same TCR and can be used to analyze cross-reactivity against different epitopes.

PBMCs from donors that responded to one of the epitopes of "Region 2" were pre-amplified, using epitopes from HPV type 6a, 11, 16 and 18 of "Region 2" (*4 HPV type Region 2 mix*, see Table 3-22). After 12 days the T cells specific for one of these four peptides were stained for IFN-γ release (see 2.2.7IFN-γ cytokine capture), single cell sorted (see 2.2.8 T-cell single sorting) and cultured (see 2.2.9 T-cell long-term proliferation) until appearance of a visible cell colony. Half of the cells were used to confirm the peptide specificity by an ICS using *4 HPV type Region 2 mix*.

Table 3-22. Peptides contained in "4 HPV type Region 2 mix".

Sequence	HPV type[a]	position[b]
EDTYRYVQSQAITCQ	**6**, 11	410-424
EDTYRFVTSQAIACQ	**16**	441-455
VDTYRFVQSVAITCQ	**18**	477-491

"a" indicates the HPV types that contain the exact epitope in their L1 amino acid sequence
"b" the numbers given in this column refer to the HPV type given in bold letters

The size of the three cloning approaches, the origin of the PBMCs used and the amount of resulting clones are summarized in Table 3-23. The first approach used three different donors (BD783, BD912 and BD1252) that were found in the ELISPOT screening to be strongly positive for at least one of the „Region 2“ epitopes. The cells were pre-amplified using the *4 HPV type Region 2 mix*. For the single cell sorting they were challenged with the same peptides. 14 96-well plates were loaded with one cell per well. 1344 single cells were sorted. Shortly after the first cell pellets were emerging, they were lost to bacterial contamination.

Not to expend the limited supply of already screened PBMCs, 24 new donors (BD1457, 1458, 1459, 1460, 1461, 1462, 1463, 1464, 1465, 1466,1467, 1468, 1469, 1470, 1471, 1472, 1473, 1474, 1475, 1476, 1478, 1479, 1480, 1491)were tested for their response against the *4 HPV type Region 2 mix* by ELISPOT. The best two responders, BD1463 and BD1478 were used in the second approach.

The PBMCs were pre-amplified and challenged with the *4 HPV type Region 2 mix*. This time 864 single cells were sorted. After the proliferation period 24 growing cell colonies could be detected. The ICS analysis showed no cytokine response above background after challenge with the *4 HPV type Region 2 mix*. Therefore it was concluded that the gate in the preceding IFN-γ capture experiment, distinguishing the responding cells from the background was set too close to the non-responders.

Because the IFN-γ capture is not as sensitive as the standard ICS and sensitivity problems were already encountered with the standard ICS, PBMCs from two freshly Gardasil vaccinated women (Sp1 and Sp2) were used. These cells were kindly provided by Andreas Kaufmann from the Charité in Berlin, Germany. The cells were pre-amplified and challenged with the *4 HPV type Region 2 mix*. The positive gate of the IFN-γ capture experiment was set well apart from the negative population. This resulted in much lower positive gated cell numbers but the probability of the sorted cells to be peptide specific and not randomly activated would be elevated. 960 single cells were sorted. Only six cell colonies developed. Four of the growing clone populations showed a strong cytokine response after challenge with the *4 HPV type Region 2 mix*, as measured by ICS.

Table 3-23. Cloning approaches.

#	PBMC donors[a]	pre-amplification[b]	approach size[c]	cell colonies developing[d]	peptide specific clones[e]
1	responded in ELISPOT screening	yes	1344	Not determined	-
2	responded in additional ELISPOT	yes	864	24	0
3	vaccinated with Gardasil	yes	960	6	4

"a" indicates the choice of PBMC donor used for the cloning approach
"b" 12-day pre-amplification of the PBMCs with *4 HPV type Region 2 mix*
"c" number of single IFN-γ positive T cells sorted into single wells
"d" number of visually detectable cell colonies developing after three weeks
"e" number of different clones that reacted to a challenge with *4 HPV type Region 2 mix*

These four clonal populations, gained in the third round of cloning were named Clone 1.41, Clone 2.21, Clone 2.24 and Clone 2.3 according to the donor and the position in the 96-well plates (donor.position). These clonal populations were proliferating robustly and were kept in long term culture according to 2.2.9 (T-cell long-time proliferation).

3.4.3 Clone analysis

3.4.3.1 Clone phenotype

Clones were analyzed via flow cytometry for the expression of CCR7, CD45RO, CD4 and CD8. The expression levels of these marker proteins were compared to the ones of freshly isolated PBMCs.

As can be seen in Figure 3-12, the sampled cell colonies contained almost completely $CD4^+$ $CD8^-$ lymphocytes. Less than 1% of the gated single, viable lymphocytes were not $CD4^+$ $CD8^-$ cells. Therefore, the only cells in the culture that were able to proliferate were the single sorted $CD4^+$ T cells and their daughter cells. The feeder cells, that were added every two weeks to the T cell culture were gamma irradiated so that they were unable to proliferate. The mentioned background of about 1% might be derived from feeder cells that were not yet degraded.

The quadrant gate in the CD45RO/CCR7 staining was chosen so that the four distinct subpopulations mentioned in 1.3.8 (T-cell trafficking and phenotype) were distinguishable in the fresh PBMC sample. Figure 3-12 shows all clones to have the same phenotype. Almost all

cells have a high expression of CD45RO and a low expression of the lymph node homing receptor CCR7. According to Sallusto et al. (Sallusto et al., 2004) this highly activated T-cell subset is called T_{EM}. Clone 2.21 and clone 2.24 also have a small population of $CD45RO^-$ and $CCR7^-$ cells called T_{EMRA}.

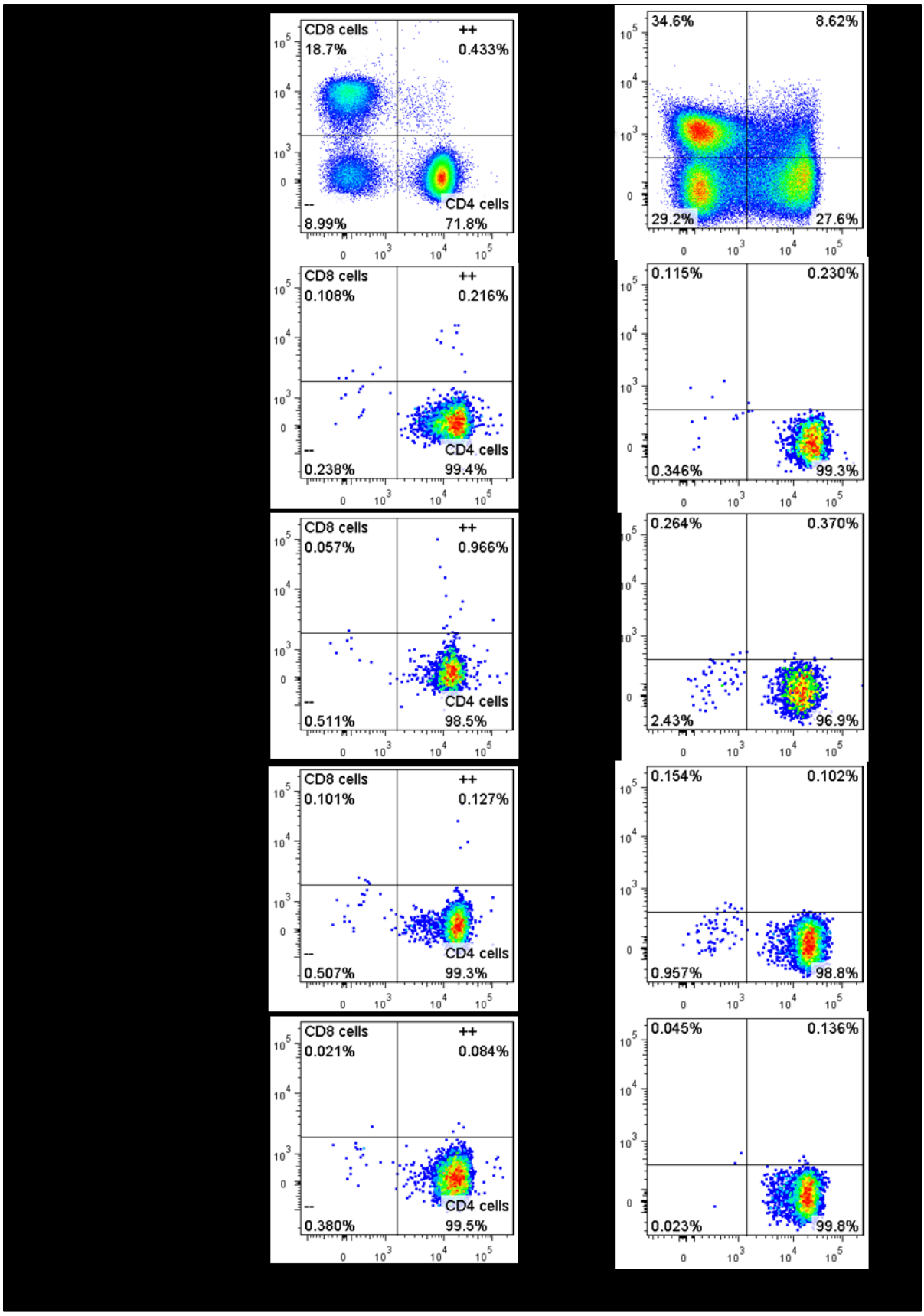

Figure 3-12. Phenotype analysis of T-cell clones. Clone 1.41, clone 2.21, clone 2.24 and clone 2.3 were stained with LIFE/DEAD to exclude dead cells and for CD4, CD8 (left plot column) and the phenotype markers CD45RO and CCR7 (right plot column). All plots show single, viable lymphocytes. The expression was compared to fresh PBMCs (top).

3.4.3.2 Epitope specificity of the T-cell clones

The peptide specificity of the clones was analyzed via ICS. The clones were challenged with a single peptide out of the five different “Region 2”peptides from HPV type 6a, 11, 16, 18, 31 and 45. The acquired FACS data are shown for clone 1.41 in Figure 3-13, for clone 2.21 in Figure 3-14, for clone 2.24 in Figure 3-15 and for clone 2.3 in Figure 3-16.

The amount of T cells reacting to the peptide challenge by cytokine production varied strongly. For example clone 2.3 reacted to the challenge with the “Region 2” peptide from HPV6/11 with a very weak response. Only 1.2% of cells produced any cytokine compared to 0.48% after challenge with the negative peptide but the reactions are still distinguishable from each other by visual inspection. The same clone 2.3 reacts to the “Region 2” peptides derived from HPV16 and HPV45 by a very strong response. Over 40% of the clone reacted by production of cytokines in both cases.

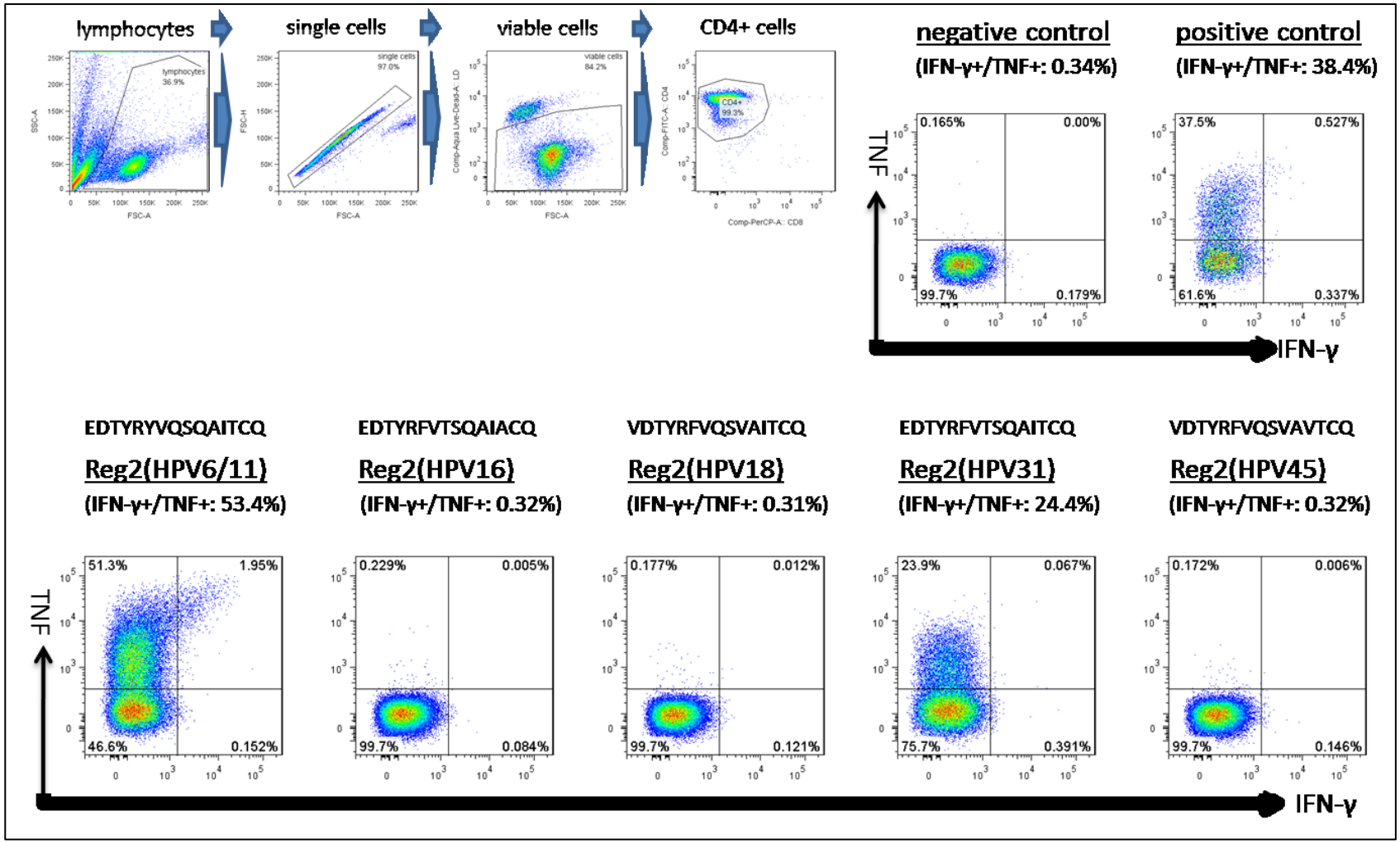

Figure 3-13. Epitope specificity of clone 1.41. ICS was performed with clone 1.41 after challenge with 10 µg/ml of a single peptide out of "Region 2" from the indicated HPV type, a PMA/Ionomycin positive control or a peptide negative control. Cells were stained for CD4, CD8, LIFE/DEAD aqua and the activation markers IFN-γ and TNF. The gating was performed as displayed (upper left). The percentage given in brackets under the challenge indicates the sum of IFN-γ positive, TNF positive and double positive cells.

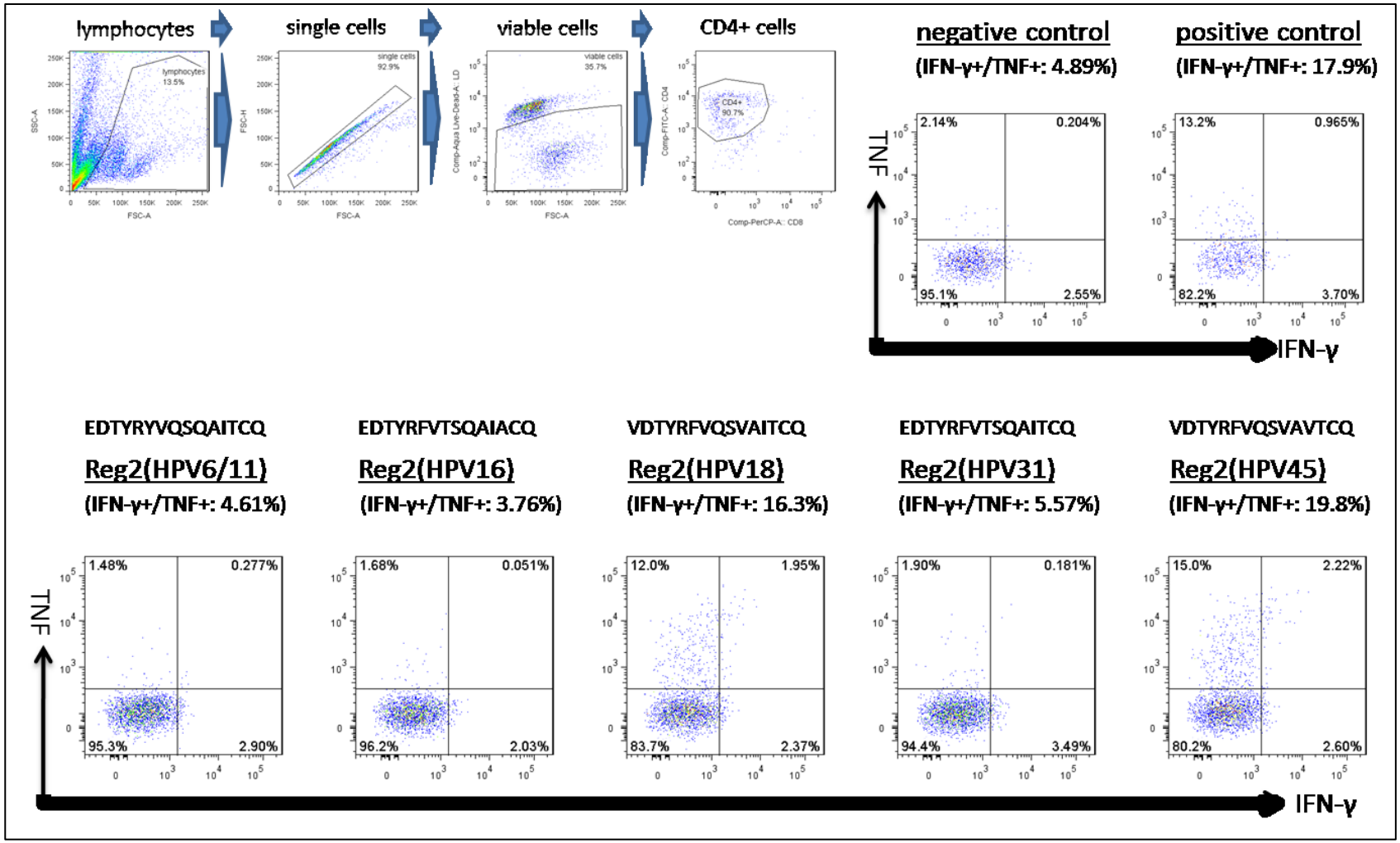

Figure 3-14. Epitope specificity of clone 2.21. ICS was performed with clone 2.21 after challenge with 10 µg/ml of a single peptide out of "Region 2" from the indicated HPV type, a PMA/Ionomycin positive control or a peptide negative control. Cells were stained for CD4, CD8, LIFE/DEAD aqua and the activation markers IFN-γ and TNF. The gating was performed as displayed (upper left). The percentage given in brackets under the challenge indicates the sum of IFN-γ positive, TNF positive and double positive cells.

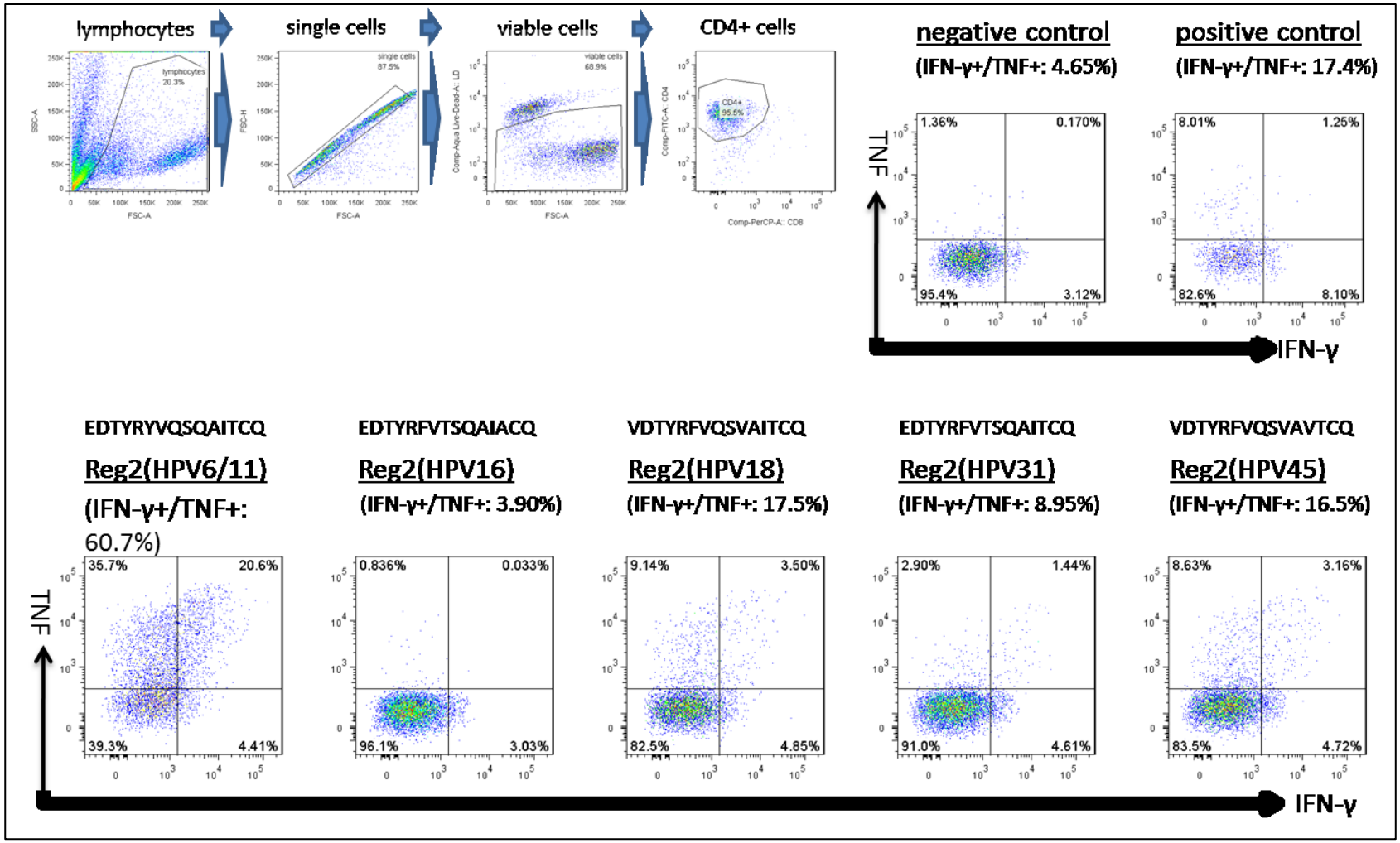

Figure 3-15. Epitope specificity of Clone 2.24. ICS was performed with clone 2.24 after challenge with 10 µg/ml of a single peptide out of "Region 2" from the indicated HPV type, a PMA/Ionomycin positive control or a peptide negative control. Cells were stained for CD4, CD8, LIFE/DEAD aqua and the activation markers IFN-γ and TNF. The gating was performed as displayed (upper left). The percentage given in brackets under the challenge indicates the sum of IFN-γ positive, TNF positive and double positive cells.

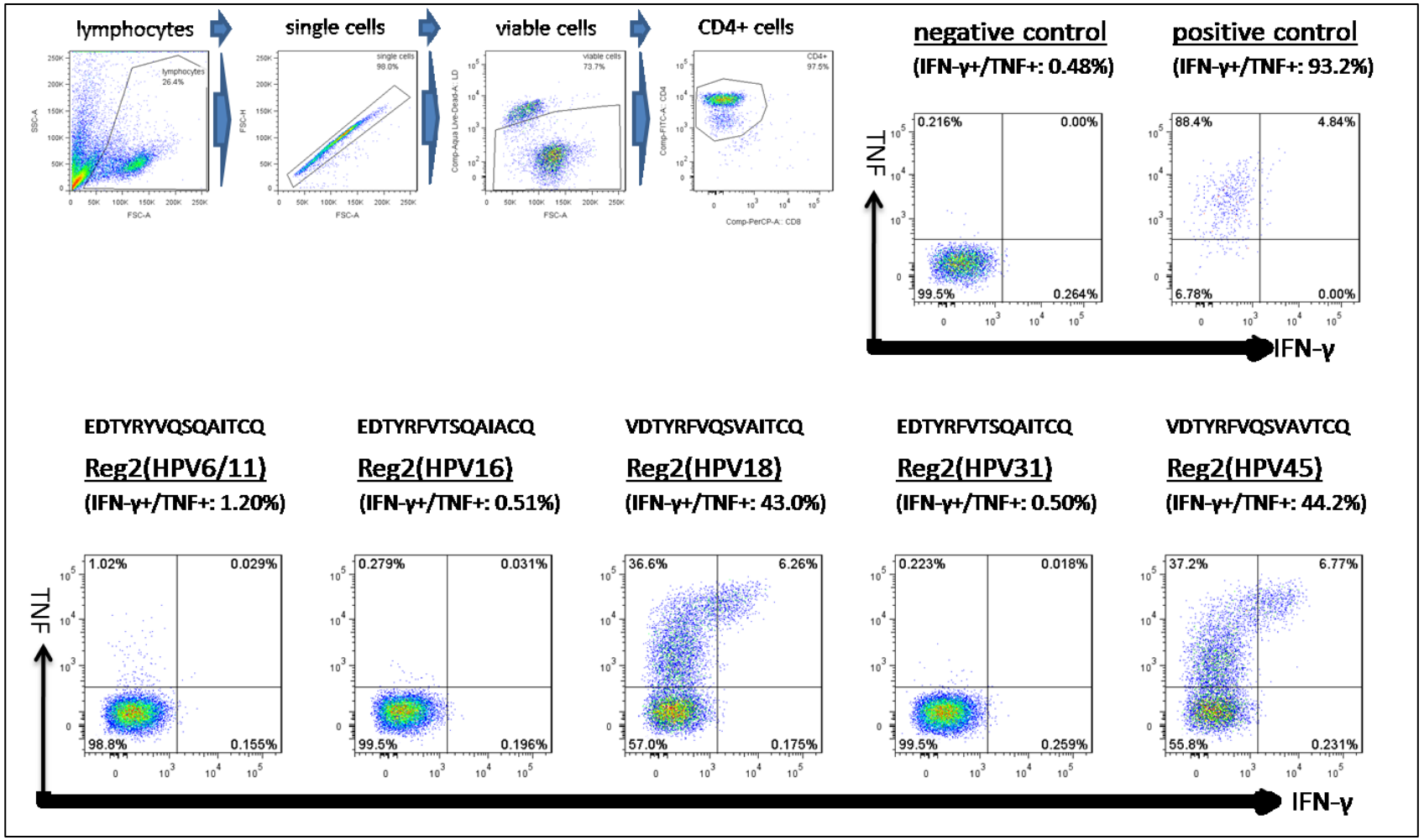

Figure 3-16. Epitope specificity of clone 2.3. ICS was performed with clone 2.3 after challenge with 10 µg/ml of a single peptide out of "Region 2" from the indicated HPV type, a PMA/Ionomycin positive control or a peptide negative control. Cells were stained for CD4, CD8, LIFE/DEAD aqua and the activation markers IFN-γ and TNF. The gating was performed as displayed (upper left). The percentage given in brackets under the challenge indicates the sum of IFN-γ positive, TNF positive and double positive cells.

A summary of the resulting epitope specificities and the strengths of the cytokine production is given in Table 3-24. Most important, all clones recognize more than one epitope and are therefore cross-reactive. The clones show different combinations of epitope specificities and recognition strengths. Clones 1.41 recognize two different epitopes, while clone 2.3 recognizes three and clone 2.24 recognizes even four. As expected, the cross-reactivity within a HPV species is stronger than across the species border. All clones that recognize type 18 also recognize the other HPV type from species 7, HPV45. The amino acid exchange of an isoleucine to a valine at position 12 is well tolerated. It is suspicious that no clone recognized the peptide derived from high-risk strain HPV16. Even the clones 1.41 and 2.24 that recognize the very closely related peptide derived from HPV31 (EDTYRFVTSQAITCQ) do not recognize the peptide derived from HPV16 (EDTYRFVTSQAIACQ). These two peptides differ only at position 13.The change from polar threonine to hydrophobic alanine inhibits recognition by both clones. The clones 2.24 and 2.3 also cross-react between the epitope from HPV6/11 and the epitopes from HPV18 and HPV45. The massive exchange of negatively charged glutamic acid to hydrophobic valine is well tolerated in these two clones. It can be speculated that this position is not part of the recognized zone of the epitope. On the contrary, clone 2.21 does not tolerate this exchange in amino acids and the HPV 6/11 epitope is not recognized.

Table 3-24. Summary of clone epitope specificity.

type sequence	HPV 6a/11 EDTYRYVQSQAITCQ	HPV 16 EDTYRFVTSQAIACQ	HPV 18 VDTYRFVQSVAITCQ	HPV 31 EDTYRFVTSQAITCQ	HPV 45 VDTYRFVQSVAVTCQ
Clone 1.41	++	-	-	++	-
Clone 2.21	-	-	++	-	++
Clone 2.24	++	-	++	+	++
Clone 2.3	+	-	++	-	++

"-" no cytokine response detectable
"+" IFN-γ positive and/or TNF positive: 1.5-3 fold higher than negative control
"++" IFN-γ positive and/or TNF positive : >3 fold higher than negative control

One could argue that a T cell should react to a specific peptide challenge if it recognizes the peptide or should not react, if it does not recognize the peptide. Therefore one might expect

percentages of reacting cells to be more extreme, being close to 0% (negative) or 100% (positive). In the experiment, no professional APCs were added. The only cells bearing MHC class II molecules are the CD4 T-cell clones themselves. Because even activated CD4 T cells bear only little MHC class II on the surface, the number of MHC class II molecules that can present the added peptide is very limited. This could complicate the T-cell activation. Additionally, the cells culture condition used causes the cells to proliferate violently. As a result a proportion of the cells is still undergoing mitosis at the time of the experiment and cannot be activated.

The described cross-reactivity has an important implication. First, no conclusions concerning the infection rate can be drawn from the detected T-cell epitope recognition rates (see 3.1.2 ELISPOT screening). The observed response of 91% of the donors to any epitopes of the HPV types 6, 11, 16 and 18 does not mean that all these donors were infected by one of the HPV types 6, 11, 16 or 18. The T cells that recognize the epitopes could also be caused by the infection with any other HPV type. It is impossible to differentiate between multiple infections and cross-reactivity by analyzing only T-cell reactivities. The detection of specific antibody levels is more convenient to analyze the type of cleared HPV infections because the level of cross-reactivity is low.

3.4.3.3 Clone responsiveness

To check if the clones were responding to the peptides in a dose dependent manner and if the percentage of activated T cells reaches a plateau at the 10 µg/ml standard challenge peptide concentration, peptide titrations were performed. Only clones 2.24 and 2.3 proliferated strong enough to reach the cell numbers needed for this experiment. Clones were challenged over night with escalating amounts of peptide (10 ng/ml – 10 µg/ml) in the presence of BrefA. The IFN-γ and TNF production was analyzed by ICS.

Clone 2.24 was challenged with “Region 2” peptides derived from HPV6/11, HPV18, HPV 31 and HPV45 (see Figure 3-15). Against these four peptides the clone had reacted in the precedent experiment.

The TNF data for clone 2.24 are displayed in Figure 3-17. The IFN-γ data were not included due to a very high background. As can be seen, clone 2.24 reacted to challenges with all four peptides in a dose dependent manner. The TNF response started to rise between a concentration of 100 ng/ml and 1 µg/ml. Even at the high concentration of 10 µg/ml the

response rate did not reach a plateau, remaining below 10%. This is by far less than the 50% TNF^+ cells measured in the previous experiment (Figure 3-15). This could be due to the effects described in 3.4.3.2. This unexpected weak response could also be caused by a general low activation status of the clones. The strong activation by PHA-L that was applied repeatedly in the culture condition might have driven the cells into exhaustion (Wherry, 2011; Yi et al., 2010).

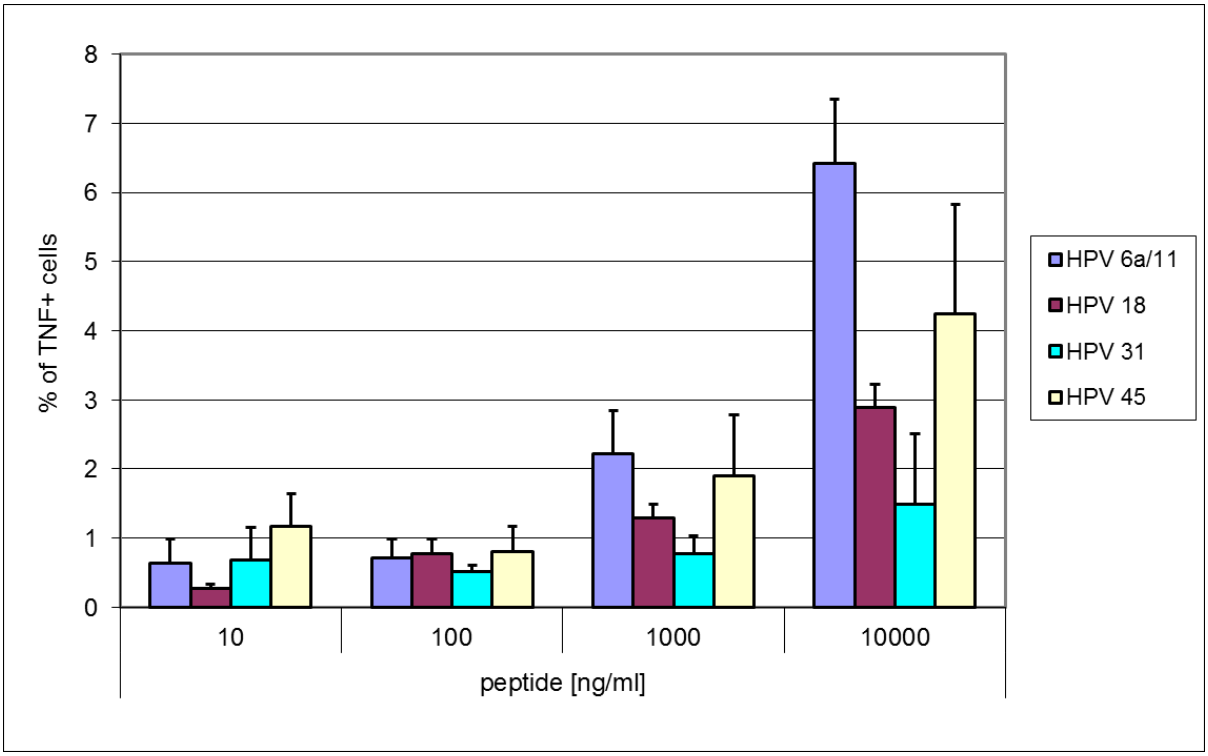

Figure 3-17. Peptide titration of clone 2.24. Cells were challenged with different concentrations of the "Region 2" epitope of the indicated HPV types. The TNF response of viable $CD4^+$ cells was determined by ICS. The bars show the mean values of three separate experiments.

Clone 2.3 was challenged with the peptides from "Region 2" of the HPV types 6/11, 18 and 45, against which the clone had reacted in previous experiments (see Figure 3-16).

The peptide titration of clone 2.3 is displayed in Figure 3-18 for the TNF responses and in Figure 3-19 for the IFN-γ responses. Both figures show a dose dependent increase of the response rate for all challenged peptides. Comparable with clone 2.24, no saturation can be detected at the high challenge concentration of 10 µg peptide per ml. The TNF data correlate with the IFN-γ data in qualitative issues. But the IFN-γ figure shows a strong background and large alterations of the replicates. In Figure 3-18 an emerging TNF response is detectable for the "Region 2" peptides at challenge concentrations as low as 10-100 ng peptide per ml.

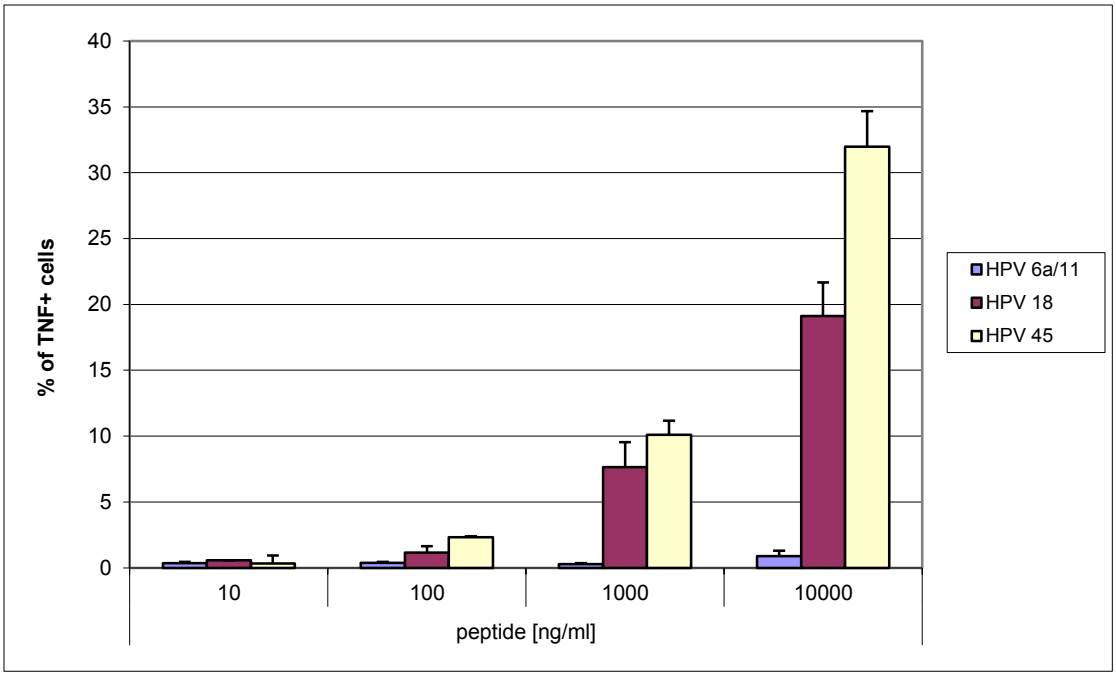

Figure 3-18. Peptide titration of clone 2.3. Cells were challenged with different concentrations of the "Region 2" epitope of the indicated HPV types. The TNF response of viable $CD4^+$ cells was determined by ICS. The bars show the mean values of three separate experiments.

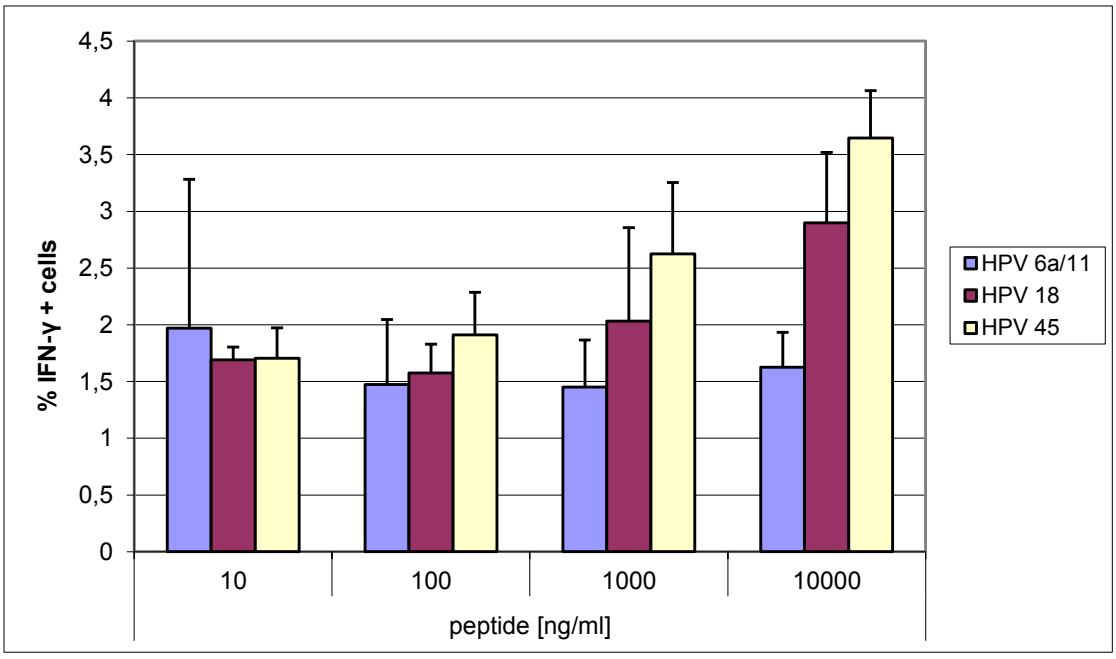

Figure 3-19. Peptide titration of clone 2.3. Cells were challenged with different concentrations of the "Region 2" epitope of the indicated HPV types. The IFN-γ response of viable $CD4^+$ cells was determined by ICS. The bars show the mean values of three separate experiments.

3.4.3.4 Clone protein challenge

To check if the T-cell clones react to the naturally processed protein or if they only recognize artificial peptides, the following coculture experiment was performed. First, autologous DCs were generated from frozen PBMCs of Sp1 according to 2.2.6. The matured DCs were loaded with 10 µg/ml of Gardasil (containing L1 of HPV6a, 11, 16 and 18) or Cervarix (containing L1 of HPV16 and 18), respectively. As a negative control, DCs were loaded with 10 µg/ml of the Hepatitis-B vaccine Engerix-B. Engerix-B also consist of a viral surface antigen (HBsAg) adsorbed on aluminum hydroxide like Gardasil. Therefore, it was used as negative control containing the $Al(OH)_3$ but including a completely different irrelevant protein. The Cervarix vaccine additionally includes the adjuvants AS04. AS04 consists of the Toll-like receptor (TLR) antagonist MPL (3-O-desacyl-4'-monophosphoryl lipid A), a detoxified derivative of the LPS isolated from the Gram-negative bacterium *Salmonella Minnesota* (Didierlaurent et al., 2009). By its nature it cannot directly interact with T cells, but signals through the TLR4 pathway and activates NFκB in cells of the innate immunity to provide a proinflammatory cytokine milieu (Iwasaki and Medzhitov, 2004). This enhances the cytokine response of $CD4^+$ T cells but does not initiate a response on its own (Casella and Mitchell, 2008).

Clone 1.41 was cultured with BrefA overnight in the presence of protein-loaded DCs or in the presence of protein alone. On the next day cells were stained for LIFE/DEAD, CD4 and the cytokines IFN-γ and TNF.

The IFN-γ and TNF production of single, viable, $CD4^+$ lymphocytes are displayed in Figure 3-20. A massive cytokine response can be observed when the clone is challenged with Gardasil in the presence of DCs (upper middle). Over 95% of the cells reacted with the production of TNF and/or IFN-γ. This is not the case, however, when challenged with Engerix-B-loaded DCs (0.68% TNF/IFN-γ^+). When challenged with Cervarix-loaded DCs (upper left), 2.17% of the clones responded with cytokine production. If no DCs were present, the percentage of cytokine producers was lower. Challenge by Cervarix or the Engerix-B caused no cytokine production at all (Cervarix: 0.37%; negative protein: 0.63%). A challenge by Gardasil without additional DCs caused 18.51% of the cells to produce TNF and/or IFN-γ. Theoretically, the clones can only be activated if processed protein is presented to them in the form of an epitope associated with a MHC molecule. If there are no APCs present, there should not be any T-cell activation. But despite $CD4^+$ T cells are no APCs, they express MHC

class II molecules on their surface after activation (Barnaba et al., 1994; Hewitt and Feldmann, 1989) and they exhibit limited processing capabilities (Barnaba et al., 1994) However, these T-APCs were described to provide down-regulatory signals (Holling et al., 2004; Pichler and Wyss-Coray, 1994). The limited cytokine response of the clones after challenge with Gardasil without addition of DCs could be due to this limited, intrinsic capability of activated T-cells to take up and process protein.

As can be seen, mainly TNF was produced in response to the challenge. In the case of Gardasil loaded DCs, 95% of the clones produced TNF and only 4% produced IFN-γ. Additionally, IFN-γ was produced only in addition to TNF. No cells were producing only IFN-γ. The same TNF focus was already observed when the clones were challenged with the peptides (see 3.4.3.2 Epitope specificity of the T-cell clones).

The clone 1.41 reacts strongly to a challenge with Gardasil-loaded but only weakly to Cervarix-loaded DCs. This is consistent with the detected epitope specificity of clone 1.41 (see Figure 3-13). The clone responded only after challenge with peptides derived from L1 of HPV6/11 and HPV31. Because Cervarix contains only VLPs from L1 of HPV16 and 18, but Gardasil contains also VLPs from the types HPV6 and HPV11, only a response to Gardasil challenge was expected. The low percentage (2.17%) of responders after challenge with Cervarix loaded DCs could be caused by a low cross-reactivity supported by the adjuvant, which was not detected in the epitope challenge experiments.

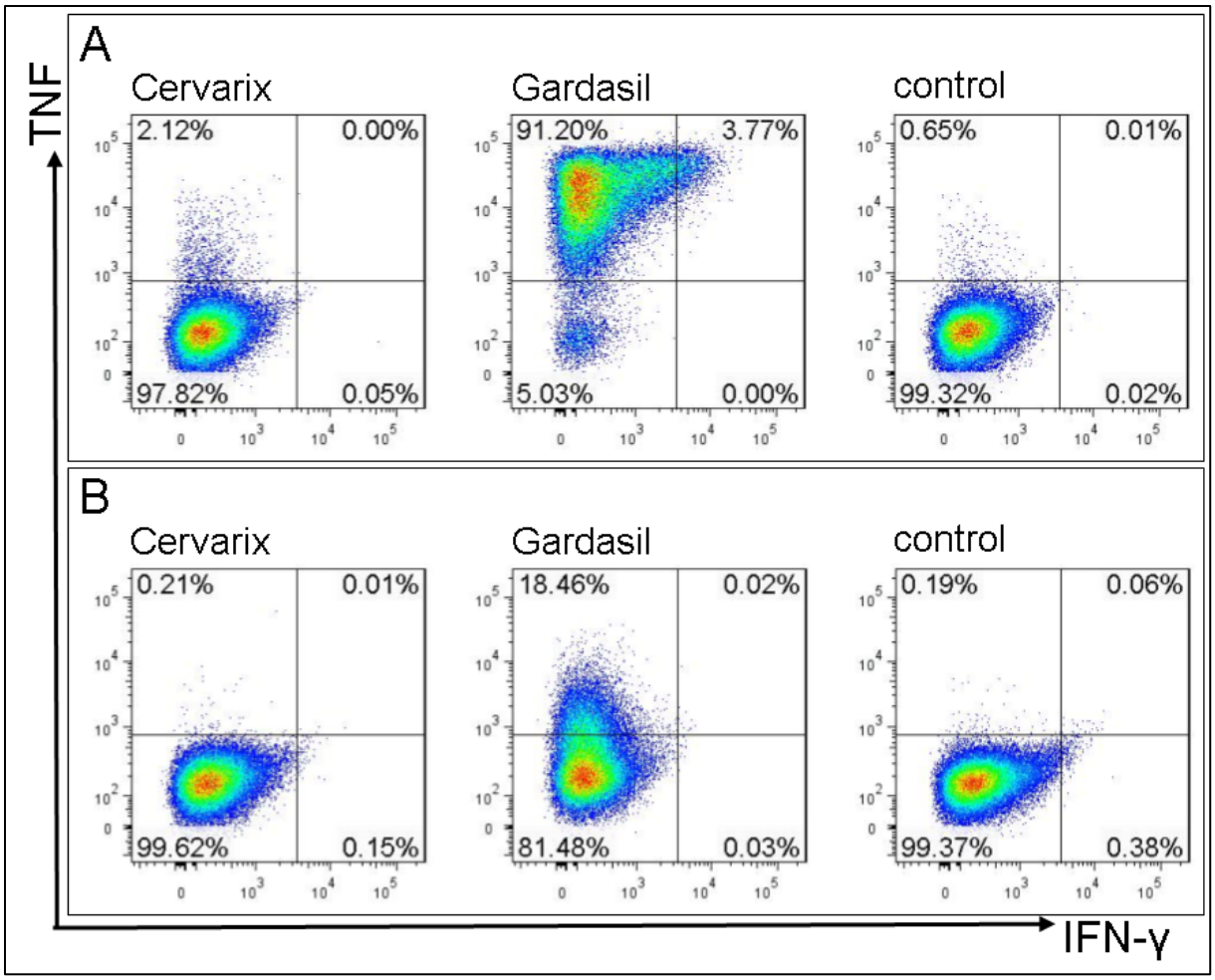

Figure 3-20. Protein challenge of clone 1.41. Clone 1.41 with (A) or without (B) autologous DCs was challenged with 10 µg protein per ml of Cervarix or Gardasil or a control protein. After 12 h incubation time in the presence of BrefA, the cells were stained for LIFE/DEAD, CD4 and the cytokines IFN-γ and TNF. Events were gated for viable, single, $CD4^+$ lymphocytes. The plots show their IFN-γ and TNF production measured by flow cytometry.

3.5 Epitope prominence

To asses the prominence of the defined HPV L1 epitopes, Gardasil was used as stimulus in protein specific pre-amplification to enlarge the L1-specific T-cell population. The L1-specific T-cell responses before and after this amplification were compared in PBMCs from six healthy donors. To analyse the L1-specific response the cells were challenged with either 10 µg/ml Gardasil or with 10 µg/ml of a mixture of all 23 HPV-L1 epitopes defined in Table 3-12.

TNF and IFN-γ producing T cells were detected by flow cytometry. Figure 3-21 shows the HPV specific reactivities in percentage of TNF and/or IFN-γ positive cells within the parent population. For correction the appropriate background control was subtracted.

While there are no detectable cytokine producing cells neither in the $CD4^+$ nor in the $CD8^+$ population *ex vivo*, there was cytokine production detected after the Gardasil-amplification. The cytokine producers were exclusively $CD4^+$ cells and not $CD8^+$. This cytokine response was detected by challenge with Gardasil as well as by challenge with the peptide mix.

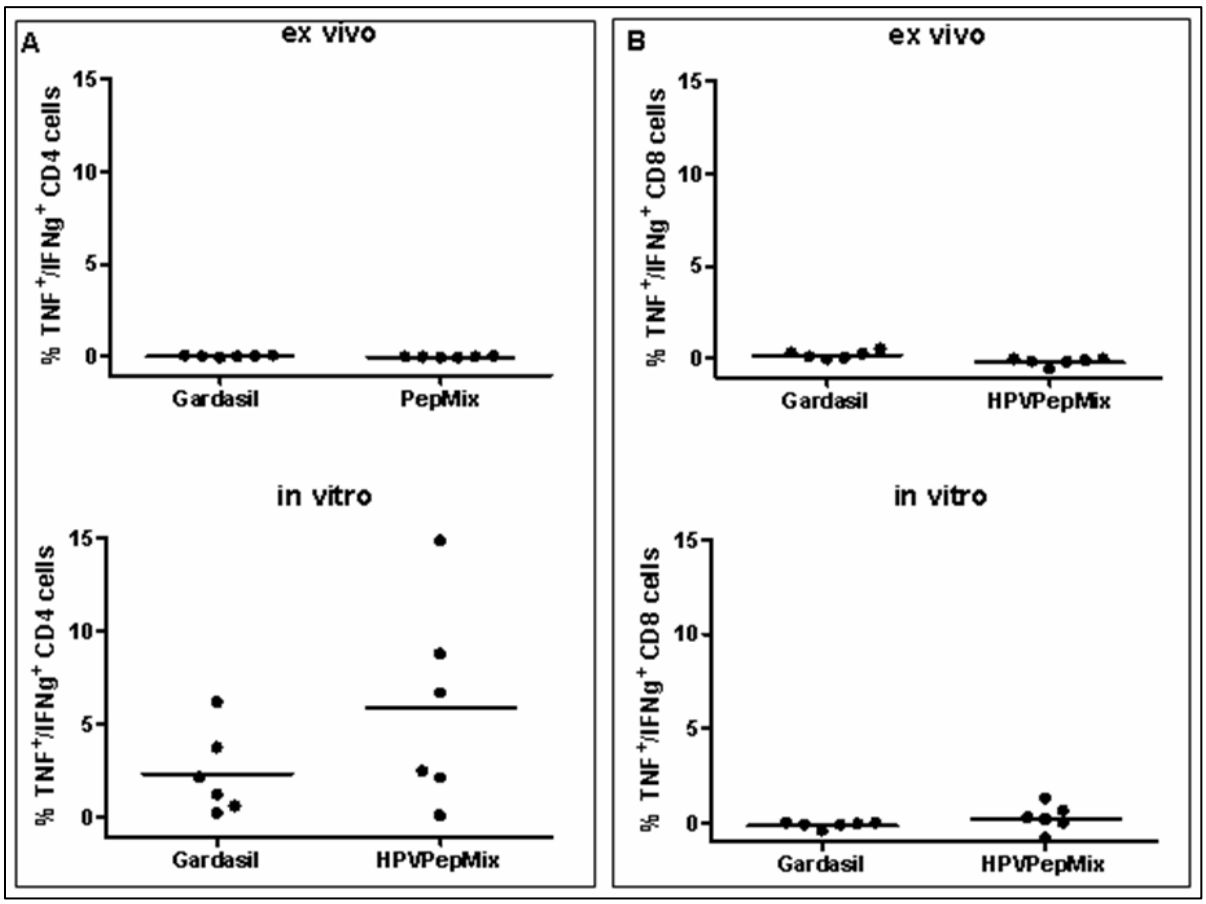

Figure 3-21. Peptide challenge after Gardasil specific proliferation. Thawed PBMCs of six healthy donors were challenged after 1 day culture (*ex vivo*) or after Gardasil specific amplification (*in vitro*) with either 10 µg/ml Gardasil (Gardasil) or 10 µg/ml peptide pool (HPVPepMIX). HPVPepMIX contained the 23 epitopes identified in the ELISPOT screening. The graph shows percentage of TNF/IFN-γ positive, single, viable cells of the parent population. (A) Shows the $CD4^+$ population, (B) shows the $CD8^+$ population.

The challenge results were qualitatively comparable. The mean of the percentage of producers after challenge with the peptide mix was even higher than after Gardasil challenge. Both challenges were done with the same challenge concentration (10 µg/ml). But the protein must first be taken up and processed by APCs before being presented by MHC class II molecules. The added peptides only need to displace the natural ligands on the available MHC class II molecules. Therefore, the results should not be compared quantitatively.

The results of this experiment clearly demonstrate that *in vitro* amplification of Gardasil-specific T cells leads to a proliferation of T cells specific for one or more of the 23 epitopes. Thus, the defined peptides play an important role in the T-cell immune response against the L1 protein of HPV.

4 Summary

The human *papillomaviridae* (HPV) are DNA viruses that infect the human mucosa and the skin. Over 130 genetically different HPV types are described. The amino acid sequence of L1, the major capsid protein, differs up to 60%. The persistent infection with a high-risk HPV (e.g. HPV16 and 18) is the necessary cause for the development of several malignancies, among these cervix carcinoma. The low-risk types (e.g. HPV6 and 11) are causing bothersome warts at the site of infection but are not associated with the development of cancer.

This study was directed at identifying CD4 T-cell epitopes derived from the L1 protein of HPV6, HPV11, HPV16 and HPV18. Therefore, the strategy of reverse immunology was applied. Most promising candidate epitopes were chosen for their SYFPEITHI score and their promiscuity. These candidates were used to screen PBMCs of healthy donors for T-cell responses. The found epitopes were validated by flow cytometry. The anti-L1-serostatus of the blood donors was analyzed and correlated with the T-cell data. The emerging assumption of inter-HPV type cross-reactivity of the T cell response was further investigated by an analysis of L1 specific T-cell clones.

23 T-cell epitopes were identified by ELISPOT. Among these were six FREPs (frequently recognized epitopes) that were recognized by between 29 and 44% of healthy donors. The MHC class II restriction of eight epitopes was verified by flow cytometry. The correlation of the type specificity of T-cell and antibody responses indicated a cross-reactivity of the L1 specific T cells. Four clones, specific for a prominent region of the L1 protein could be obtained by single-cell sorting. The T-cell clones showed cross-reactivity between the L1 proteins of HPV6, HPV11, HPV18, HPV31 and 45. The cross-reactivity was strongest within one HPV species. While the antibody response was type specific, the T cells recognized a set of strongly related epitopes that originate from different HPV types.

It can be speculated that this T-cell cross-reactivity allows the immune system to fasten the identification of a new serotype and to overcome the infection.

Zusammenfassung

Die humanen Papillomaviridae (HPV) sind menschliche Mukosa und Haut infizierende DNA-Viren. Es gibt über 130 genetisch unterschiedliche HPV-Typen, deren Aminosäuresequenz des Hauptkapsidproteins L1 sich um bis zu 60% unterscheidet. Eine persistente Infektion mit einem der Hochrisikotypen (z.B. HPV16 und 18), ist unabdingbare Voraussetzung für die Entwicklung mehrerer bösartiger Tumorarten, darunter Gebärmutterhalskrebs. Die risikoarmen HPV Typen (z.B. HPV6 und 11) verursachen störende Warzen an der Infektionsstelle, sind aber nicht an der Entstehung von Krebs beteiligt.

Während dieser Arbeit sollten CD4 T-Zellepitope der L1-Proteine der Typen HPV6, HPV11, HPV16 und HPV18 identifiziert werden. Hierfür wurde ein Ansatz der reversen Immunologie angewandt. Aufgrund ihres SYFPEITHI-Wertes und ihrer Promiskuität wurden die vielversprechendsten Epitopkandidaten ausgewählt. Mononukleäre Zellen des peripheren Blutes gesunder Spender wurden auf T-Zellantworten gegen diese Kandidaten untersucht. Die gefundenen Epitope wurden mittels Durchflusszytometrie validiert. Der anti-L1 Serostatus der Spender wurde bestimmt und mit den Daten der T-Zellantworten verglichen. Die dabei aufkommenden Hinweise auf Kreuzreaktivität zwischen HPV-Typen wurden mittels der Untersuchung L1-spezifischer T-Zellklone weiter verfolgt.

23 T-Zellepitope wurden per ELISPOT identifiziert, darunter sechs FREPs (häufig erkannte Epitope), die von 29-44% der gesunden Spender erkannt wurden. Die MHC Klasse II Restriktion acht der Epitope wurde mittels Durchflusszytometrie validiert. Ein Vergleich der Typenspezifität der Antikörper- und der T-Zellantwort deutete auf eine Kreuzreaktivität L1-spezifischer T-Zellen hin. Durch Einzelzellsortierung konnten vier T-Zellklone gewonnen werden, die eine wichtige Region des L1 Proteins erkannten. Die Klone zeigten eine Kreuzreaktivität zwischen den L1 Proteinen von HPV6, HPV11, HPV18, HPV 31 und 45. Innerhalb einer HPV Spezies war die Kreuzreaktivität am stärksten. Während die Antikörperantwort typenspezifisch war, erkannten die T-Zellen eine Gruppe eng verwandter Epitope, die von verschieden HPV Typen stammten.

Dies legt die Vermutung nahe, dass diese T-Zellkreuzreaktivität die Erkennung eines neuen HPV Serotyps durch das Immunsystem beschleunigt und dadurch hilft diese Neuinfektion erfolgreich zu bekämpfen.

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6 Appendix

6.1 Abbreviations

aa	aminoacid
APC	antigen presenting cell or allophycocyanin
ATP	adenosine triphosphate
BPV	bovine papilloma virus
BrefA	brefeldin A
CIN	cervical intraepithelial neoplasia
CTL	cytotoxic lymphocyte
DC	dendritic cell
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
EGFR	epidermal growth factor receptor
ELISPOT	enzyme linked immune spot
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FFC	forward scatter
FRET	fluorescence resonance energy transfer
FREP	frequently recognized epitope
GM-CSF	granulocyte macrophage-colony stimulating factor
HLA	human leukocyte antigen
HPV	human papilloma virus
ICS	intracellular cytokine staining
IFN-γ	interferon gamma
Ig	immunoglobulin
ITAM	immune receptor tyrosine-based activation motif
LCR	long control region
LPS	lipopolysaccharide
MFI	medium fluorescence intensity
MHC	major histocompatibility complex
MPL	3-O-desacyl-4'-monophosphoryl lipid A

ORF	open reading frame
Pap	Papanicolaou
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PDGF	platelet derived growth factor receptor
PHA	phytohemagglutinin
PMA	phorbol myristate acetate
PV	papilloma viridae
rb	retinoblastoma
RNA	ribonucleic acid
RT	room temperature
Sp	special Gardasil vaccinated donor
SSC	sideward scatter
TAP	transporter associated with antigen processing
T_{cm}	T-central memory cell
TCR	T-cell receptor
TEC	thymic epithelial cells
T_{eff}	T-effector cell
T_{em}	T-effector memory cell
T_{emra}	T-effector memory cell, $CD45RA^{+}$
T_H	T-helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor alpha
T_{reg}	T-regulatory cell
VLP	virus like particle

6.2 L1 amino acid sequences (taken from www.uniprot.org)

P69898 (VL1_HPV6A) length: 500 amino acids, mass: 55 597 Da

```
        10         20         30         40         50         60
MWRPSDSTVY VPPPNPVSKV VATDAYVTRT NIFYHASSSR LLAVGHPYFS IKRANKTVVP

70         80         90        100        110        120
KVSGYQYRVF KVVLPDPNKF ALPDSSLFDP TTQRLVWACT GLEVGRGQPL GVGVSGHPFL

       130        140        150        160        170        180
NKYDDVENSG SGGNPGQDNR VNVGMDYKQT QLCMVGCAPP LGEHWGKGKQ CTNTPVQAGD

       190        200        210        220        230        240
CPPLELITSV IQDGDMVDTG FGAMNFADLQ TNKSDVPIDI CGTTCKYPDY LQMAADPYGD

       250        260        270        280        290        300
RLFFFLRKEQ MFARHFFNRA GEVGEPVPDT LIIKGSGNRT SVGSSIYVNT PSGSLVSSEA

       310        320        330        340        350        360
QLFNKPYWLQ KAQGHNNGIC WGNQLFVTVV DTTRSTNMTL CASVTTSSTY TNSDYKEYMR

       370        380        390        400        410        420
HVEEYDLQFI FQLCSITLSA EVMAYIHTMN PSVLEDWNFG LSPPPNGTLE DTYRYVQSQA

       430        440        450        460        470        480
ITCQKPTPEK EKPDPYKNLS FWEVNLKEKF SSELDQYPLG RKFLLQSGYR GRSSIRTGVK

       490        500
RPAVSKASAA PKRKRAKTKR
```

P04012 (VL1_HPV11) length: 501 amino acids, mass: 55 835 Da

```
        10         20         30         40         50         60
MWRPSDSTVY VPPPNPVSKV VATDAYVKRT NIFYHASSSR LLAVGHPYYS IKKVNKTVVP

        70         80         90        100        110        120
KVSGYQYRVF KVVLPDPNKF ALPDSSLFDP TTQRLVWACT GLEVGRGQPL GVGVSGHPLL

       130        140        150        160        170        180
NKYDDVENSG GYGGNPGQDN RVNVGMDYKQ TQLCMVGCAP PLGEHWGKGT QCSNTSVQNG

       190        200        210        220        230        240
DCPPLELITS VIQDGDMVDT GFGAMNFADL QTNKSDVPLD ICGTVCKYPD YLQMAADPYG

       250        260        270        280        290        300
DRLFFYLRKE QMFARHFFNR AGTVGEPVPD DLLVKGGNNR SSVASSIYVH TPSGSLVSSE

       310        320        330        340        350        360
AQLFNKPYWL QKAQGHNNGI CWGNHLFVTV VDTTRSTNMT LCASVSKSAT YTNSDYKEYM

       370        380        390        400        410        420
RHVEEFDLQF IFQLCSITLS AEVMAYIHTM NPSVLEDWNF GLSPPPNGTL EDTYRYVQSQ

       430        440        450        460        470        480
AITCQKPTPE KEKQDPYKDM SFWEVNLKEK FSSELDQFPL GRKFLLQSGY RGRTSARTGI

490        500
KRPAVSKPST APKRKRTKTK K
```

P03101 (VL1_HPV16) length: 531 amino acids, mass: 59 474 Da

```
        10         20         30         40         50         60
MQVTFIYILV ITCYENDVNV YHIFFQMSLW LPSEATVYLP PVPVSKVVST DEYVARTNIY

        70         80         90        100        110        120
YHAGTSRLLA VGHPYFPIKK PNNNKILVPK VSGLQYRVFR IHLPDPNKFG FPDTSFYNPD

       130        140        150        160        170        180
TQRLVWACVG VEVGRGQPLG VGISGHPLLN KLDDTENASA YAANAGVDNR ECISMDYKQT

       190        200        210        220        230        240
QLCLIGCKPP IGEHWGKGSP CTNVAVNPGD CPPLELINTV IQDGDMVDTG FGAMDFTTLQ

       250        260        270        280        290        300
ANKSEVPLDI CTSICKYPDY IKMVSEPYGD SLFFYLRREQ MFVRHLFNRA GAVGENVPDD

       310        320        330        340        350        360
LYIKGSGSTA NLASSNYFPT PSGSMVTSDA QIFNKPYWLQ RAQGHNNGIC WGNQLFVTVV

       370        380        390        400        410        420
DTTRSTNMSL CAAISTSETT YKNTNFKEYL RHGEEYDLQF IFQLCKITLT ADVMTYIHSM

       430        440        450        460        470        480
NSTILEDWNF GLQPPPGGTL EDTYRFVTSQ AIACQKHTPP APKEDPLKKY TFWEVNLKEK

       490        500        510        520        530
FSADLDQFPL GRKFLLQAGL KAKPKFTLGK RKATPTTSST STTAKRKKRK L
```

P06794 (VL1_HPV18)length: 568 amino acids, mass: 63 624 Da

```
        10         20         30         40         50         60
MCLYTRVLIL HYHLLPLYGP LYHPRPLPLH SILVYMVHII ICGHYIILFL RNVNVFPIFL

         70         80         90        100        110        120
QMALWRPSDN TVYLPPPSVA RVVNTDDYVT PTSIFYHAGS SRLLTVGNPY FRVPAGGGNK

       130        140        150        160        170        180
QDIPKVSAYQ YRVFRVQLPD PNKFGLPDTS IYNPETQRLV WACAGVEIGR GQPLGVGLSG

       190        200        210        220        230        240
HPFYNKLDDT ESSHAATSNV SEDVRDNVSV DYKQTQLCIL GCAPAIGEHW AKGTACKSRP

       250        260        270        280        290        300
LSQGDCPPLE LKNTVLEDGD MVDTGYGAMD FSTLQDTKCE VPLDICQSIC KYPDYLQMSA

       310        320        330        340        350        360
DPYGDSMFFC LRREQLFARH FWNRAGTMGD TVPQSLYIKG TGMPASPGSC VYSPSPSGSI

       370        380        390        400        410        420
VTSDSQLFNK PYWLHKAQGH NNGVCWHNQL FVTVVDTTPS TNLTICASTQ SPVPGQYDAT

       430        440        450        460        470        480
KFKQYSRHVE EYDLQFIFQL CTITLTADVM SYIHSMNSSI LEDWNFGVPP PPTTSLVDTY

       490        500        510        520        530        540
RFVQSVAITC QKDAAPAENK DPYDKLKFWN VDLKEKFSLD LDQYPLGRKF LVQAGLRRKP

       550        560
TIGPRKRSAP SATTSSKPAK RVRVRARK
```

P17388 (VL1_HPV31) length: 504 amino acids, mass: 56 352 Da

```
        10         20         30         40         50         60
MSLWRPSEAT VYLPPVPVSK VVSTDEYVTR TNIYYHAGSA RLLTVGHPYY SIPKSDNPKK

70         80         90        100        110        120
IVVPKVSGLQ YRVFRVRLPD PNKFGFPDTS FYNPETQRLV WACVGLEVGR GQPLGVGISG

       130        140        150        160        170        180
HPLLNKFDDT ENSNRYAGGP GTDNRECISM DYKQTQLCLL GCKPPIGEHW GKGSPCSNNA

       190        200        210        220        230        240
ITPGDCPPLE LKNSVIQDGD MVDTGFGAMD FTALQDTKSN VPLDICNSIC KYPDYLKMVA

       250        260        270        280        290        300
EPYGDTLFFY LRREQMFVRH FFNRSGTVGE SVPTDLYIKG SGSTATLANS TYFPTPSGSM

       310        320        330        340        350        360
VTSDAQIFNK PYWMQRAQGH NNGICWGNQL FVTVVDTTRS TNMSVCAAIA NSDTTFKSSN

       370        380        390        400        410        420
FKEYLRHGEE FDLQFIFQLC KITLSADIMT YIHSMNPAIL EDWNFGLTTP PSGSLEDTYR

       430        440        450        460        470        480
FVTSQAITCQ KTAPQKPKED PFKDYVFWEV NLKEKFSADL DQFPLGRKFL LQAGYRARPK

490        500
FKAGKRSAPS ASTTTPAKRK KTKK
```

P36741 (VL1_HPV45) length: 539 amino acids, mass: 60 311 Da

```
        10         20         30         40         50         60
MAHNIIYGHG IIIFLKNVNV FPIFLQMALW RPSDSTVYLP PPSVARVVST DDYVSRTSIF

        70         80         90        100        110        120
YHAGSSRLLT VGNPYFRVVP NGAGNKQAVP KVSAYQYRVF RVALPDPNKF GLPDSTIYNP

       130        140        150        160        170        180
ETQRLVWACV GMEIGRGQPL GIGLSGHPFY NKLDDTESAH AATAVITQDV RDNVSVDYKQ

       190        200        210        220        230        240
TQLCILGCVP AIGEHWAKGT LCKPAQLQPG DCPPLELKNT IIEDGDMVDT GYGAMDFSTL

       250        260        270        280        290        300
QDTKCEVPLD ICQSICKYPD YLQMSADPYG DSMFFCLRRE QLFARHFWNR AGVMGDTVPT

       310        320        330        340        350        360
DLYIKGTSAN MRETPGSCVY SPSPSGSIIT SDSQLFNKPY WLHKAQGHNN GICWHNQLFV

       370        380        390        400        410        420
TVVDTTRSTN LTLCASTQNP VPSTYDPTKF KQYSRHVEEY DLQFIFQLCT ITLTAEVMSY

       430        440        450        460        470        480
IHSMNSSILE NWNFGVPPPP TTSLVDTYRF VQSVAVTCQK DTTPPEKQDP YDKLKFWTVD

       490        500        510        520        530
LKEKFSSDLD QYPLGRKFLV QAGLRRRPTI GPRKRPAAST STASTASRPA KRVRIRSKK
```

6.3 SYFPEITHI predictions

A

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
14	P N P V S K V V A T D A Y V T	33
203	A M N F A D L Q T N K S D V P	33
410	E D T Y R Y V Q S Q A I T C Q	32
324	Q L F V T V V D T T R S T N M	30
367	L Q F I F Q L C S I T L S A E	30
380	A E V M A Y I H T M N P S V L	28
383	M A Y I H T M N P S V L E D W	28
120	L N K Y D D V E N S G S G G N	27
240	D R L F F F L R K E Q M F A R	27
304	N K P Y W L Q K A Q G H N N G	27
433	P D P Y K N L S F W E V N L K	27
186	L I T S V I Q D G D M V D T G	26
292	S G S L V S S E A Q L F N K P	26
394	L E D W N F G L S P P P N G T	26
31	N I F Y H A S S S R L L A V G	25
69	V F K V V L P D P N K F A L P	25
77	P N K F A L P D S S L F D P T	25
284	S S I Y V N T P S G S L V S S	25
285	S I Y V N T P S G S L V S S E	25
356	K E Y M R H V E E Y D L Q F I	25
362	V E E Y D L Q F I F Q L C S I	25
460	G R K F L L Q S G Y R G R S S	25
30	T N I F Y H A S S S R L L A V	24
56	K T V V P K V S G Y Q Y R V F	24
66	Q Y R V F K V V L P D P N K F	24

B

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
390	N P S V L E D W N F G L S P P	37
70	F K V V L P D P N K F A L P D	30
140	R V N V G M D Y K Q T Q L C M	28
356	K E Y M R H V E E Y D L Q F I	26
398	N F G L S P P P N G T L E D T	26
71	K V V L P D P N K F A L P D S	25
241	R L F F F L R K E Q M F A R H	25
360	R H V E E Y D L Q F I F Q L C	25
292	S G S L V S S E A Q L F N K P	23
69	V F K V V L P D P N K F A L P	22
187	I T S V I Q D G D M V D T G F	22
428	P E K E K P D P Y K N L S F W	22
18	S K V V A T D A Y V T R T N I	21
38	S S R L L A V G H P Y F S I K	21
230	Y L Q M A A D P Y G D R L F F	21
213	K S D V P I D I C G T T C K Y	20
443	E V N L K E K F S S E L D Q Y	20
56	K T V V P K V S G Y Q Y R V F	19
84	D S S L F D P T T Q R L V W A	19
99	C T G L E V G R G Q P L G V G	19
109	P L G V G V S G H P F L N K Y	19
260	A G E V G E P V P D T L I I K	19
373	L C S I T L S A E V M A Y I H	19
435	P Y K N L S F W E V N L K E K	19
59	V P K V S G Y Q Y R V F K V V	18

C

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
30	T N I F Y H A S S S R L L A V	28
45	G H P Y F S I K R A N K T V V	28
120	L N K Y D D V E N S G S G G N	28
203	A M N F A D L Q T N K S D V P	28
410	E D T Y R Y V Q S Q A I T C Q	28
70	F K V V L P D P N K F A L P D	26
181	C P P L E L I T S V I Q D G D	26
239	G D R L F F F L R K E Q M F A	26
293	G S L V S S E A Q L F N K P Y	26
324	Q L F V T V V D T T R S T N M	26
364	E Y D L Q F I F Q L C S I T L	26
367	L Q F I F Q L C S I T L S A E	26
390	N P S V L E D W N F G L S P P	26
7	S T V Y V P P P N P V S K V V	22
31	N I F Y H A S S S R L L A V G	22
67	Y R V F K V V L P D P N K F A	22
85	S S L F D P T T Q R L V W A C	22
162	G E H W G K G K Q C T N T P V	22
224	T C K Y P D Y L Q M A A D P Y	22
240	D R L F F F L R K E Q M F A R	22
284	S S I Y V N T P S G S L V S S	22
382	V M A Y I H T M N P S V L E D	22
433	P D P Y K N L S F W E V N L K	22
438	N L S F W E V N L K E K F S S	22
14	P N P V S K V V A T D A Y V T	20

D

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
284	S S I Y V N T P S G S L V S S	34
30	T N I F Y H A S S S R L L A V	32
31	N I F Y H A S S S R L L A V G	32
327	V T V V D T T R S T N M T L C	32
382	V M A Y I H T M N P S V L E D	32
85	S S L F D P T T Q R L V W A C	26
144	G M D Y K Q T Q L C M V G C A	26
410	E D T Y R Y V Q S Q A I T C Q	26
77	P N K F A L P D S S L F D P T	24
84	D S S L F D P T T Q R L V W A	24
193	D G D M V D T G F G A M N F A	24
367	L Q F I F Q L C S I T L S A E	24
371	F Q L C S I T L S A E V M A Y	24
373	L C S I T L S A E V M A Y I H	24
443	E V N L K E K F S S E L D Q Y	24
22	A T D A Y V T R T N I F Y H A	22
23	T D A Y V T R T N I F Y H A S	22
48	Y F S I K R A N K T V V P K V	22
92	T Q R L V W A C T G L E V G R	22
109	P L G V G V S G H P F L N K Y	22
123	Y D D V E N S G S G G N P G Q	22
181	C P P L E L I T S V I Q D G D	22
241	R L F F F L R K E Q M F A R H	22
254	R H F F N R A G E V G E P V P	22
337	N M T L C A S V T T S S T Y T	22

Figure 6-1. 25 highest scoring candidate epitopes from L1 HPV6a predicted for (A) HLA-DRB1*01:01, (B) HLA-DRB1*03:01, (C) HLA-DRB1*04:01 and (D) HLA-DRB1*07:01.

A

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
45	G H P Y F S I K R A N K T V V	25
240	D R L F F F L R K E Q M F A R	25
53	R A N K T V V P K V S G Y Q Y	23
203	A M N F A D L Q T N K S D V P	23
324	Q L F V T V V D T T R S T N M	23
472	R S S I R T G V K R P A V S K	23
120	L N K Y D D V E N S G S G G N	22
266	P V P D T L I I K G S G N R T	22
462	K F L L Q S G Y R G R S S I R	22
11	V P P P N P V S K V V A T D A	21
66	Q Y R V F K V V L P D P N K F	21
14	P N P V S K V V A T D A Y V T	20
38	S S R L L A V G H P Y F S I K	20
98	A C T G L E V G R G Q P L G V	20
303	F N K P Y W L Q K A Q G H N N	20
439	L S F W E V N L K E K F S S E	20
441	F W E V N L K E K F S S E L D	20
478	G V K R P A V S K A S A A P K	20
352	N S D Y K E Y M R H V E E Y D	19
454	L D Q Y P L G R K F L L Q S G	19
64	G Y Q Y R V F K V V L P D P N	18
149	Q T Q L C M V G C A P P L G E	18
249	E Q M F A R H F F N R A G E V	18
253	A R H F F N R A G E V G E P V	18
323	N Q L F V T V V D T T R S T N	18

B

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
117	H P F L N K Y D D V E N S G S	30
8	T V Y V P P P N P V S K V V A	24
42	L A V G H P Y F S I K R A N K	24
93	Q R L V W A C T G L E V G R G	24
101	G L E V G R G Q P L G V G V S	24
181	C P P L E L I T S V I Q D G D	24
239	G D R L F F F L R K E Q M F A	24
335	S T N M T L C A S V T T S S T	24
359	M R H V E E Y D L Q F I F Q L	24
367	L Q F I F Q L C S I T L S A E	24
379	S A E V M A Y I H T M N P S V	24
380	A E V M A Y I H T M N P S V L	24
390	N P S V L E D W N F G L S P P	24
391	P S V L E D W N F G L S P P P	24
436	Y K N L S F W E V N L K E K F	24
451	S S E L D Q Y P L G R K F L L	24
18	S K V V A T D A Y V T R T N I	20
24	D A Y V T R T N I F Y H A S S	20
59	V P K V S G Y Q Y R V F K V V	20
61	K V S G Y Q Y R V F K V V L P	20
64	G Y Q Y R V F K V V L P D P N	20
71	K V V L P D P N K F A L P D S	20
79	K F A L P D S S L F D P T T Q	20
111	G V G V S G H P F L N K Y D D	20
152	L C M V G C A P P L G E H W G	20

C

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
14	P N P V S K V V A T D A Y V K	33
204	A M N F A D L Q T N K S D V P	33
411	E D T Y R Y V Q S Q A I T C Q	32
325	H L F V T V V D T T R S T N M	30
368	L Q F I F Q L C S I T L S A E	30
381	A E V M A Y I H T M N P S V L	28
384	M A Y I H T M N P S V L E D W	28
305	N K P Y W L Q K A Q G H N N G	27
48	Y Y S I K K V N K T V V P K V	26
187	L I T S V I Q D G D M V D T G	26
241	D R L F F Y L R K E Q M F A R	26
293	S G S L V S S E A Q L F N K P	26
395	L E D W N F G L S P P P N G T	26
31	N I F Y H A S S S R L L A V G	25
69	V F K V V L P D P N K F A L P	25
77	P N K F A L P D S S L F D P T	25
285	S S I Y V H T P S G S L V S S	25
357	K E Y M R H V E E F D L Q F I	25
363	V E E F D L Q F I F Q L C S I	25
434	Q D P Y K D M S F W E V N L K	25
461	G R K F L L Q S G Y R G R T S	25
30	T N I F Y H A S S S R L L A V	24
56	K T V V P K V S G Y Q Y R V F	24
66	Q Y R V F K V V L P D P N K F	24
93	Q R L V W A C T G L E V G R G	24

D

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
391	N P S V L E D W N F G L S P P	37
70	F K V V L P D P N K F A L P D	30
141	R V N V G M D Y K Q T Q L C M	28
357	K E Y M R H V E E F D L Q F I	26
399	N F G L S P P P N G T L E D T	26
71	K V V L P D P N K F A L P D S	25
242	R L F F Y L R K E Q M F A R H	25
361	R H V E E F D L Q F I F Q L C	25
455	L D Q F P L G R K F L L Q S G	24
265	G E P V P D D L L V K G G N N	23
293	S G S L V S S E A Q L F N K P	23
18	S K V V A T D A Y V K R T N I	22
69	V F K V V L P D P N K F A L P	22
188	I T S V I Q D G D M V D T G F	22
38	S S R L L A V G H P Y Y S I K	21
214	K S D V P L D I C G T V C K Y	21
231	Y L Q M A A D P Y G D R L F F	21
109	P L G V G V S G H P L L N K Y	20
444	E V N L K E K F S S E L D Q F	20
56	K T V V P K V S G Y Q Y R V F	19
84	D S S L F D P T T Q R L V W A	19
99	C T G L E V G R G Q P L G V G	19
261	A G T V G E P V P D D L L V K	19
270	D D L L V K G G N N R S S V A	19
374	L C S I T L S A E V M A Y I H	19

Figure 6-2. 25 highest scoring candidate epitopes from L1 HPV6a predicted for (A) HLA-DRB1*11:01 and (B) HLA-DRB1*15:01 and 25 highest scoring candidate epitopes from L1 HPV11 predicted for (C) HLA-DRB1*01:01 and (D) HLA-DRB1*03:01.

A

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
30	T N I F Y H A S S S R L L A V	28
204	A M N F A D L Q T N K S D V P	28
411	E D T Y R Y V Q S Q A I T C Q	28
70	F K V V L P D P N K F A L P D	26
182	C P P L E L I T S V I Q D G D	26
294	G S L V S S E A Q L F N K P Y	26
325	H L F V T V V D T T R S T N M	26
365	E F D L Q F I F Q L C S I T L	26
368	L Q F I F Q L C S I T L S A E	26
391	N P S V L E D W N F G L S P P	26
7	S T V Y V P P P N P V S K V V	22
31	N I F Y H A S S S R L L A V G	22
45	G H P Y Y S I K K V N K T V V	22
67	Y R V F K V V L P D P N K F A	22
85	S S L F D P T T Q R L V W A C	22
120	L N K Y D D V E N S G G Y G G	22
163	G E H W G K G T Q C S N T S V	22
225	V C K Y P D Y L Q M A A D P Y	22
241	D R L F F Y L R K E Q M F A R	22
255	R H F F N R A G T V G E P V P	22
285	S S I Y V H T P S G S L V S S	22
383	V M A Y I H T M N P S V L E D	22
439	D M S F W E V N L K E K F S S	22
14	P N P V S K V V A T D A Y V K	20
38	S S R L L A V G H P Y Y S I K	20

B

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
285	S S I Y V H T P S G S L V S S	34
30	T N I F Y H A S S S R L L A V	32
31	N I F Y H A S S S R L L A V G	32
328	V T V V D T T R S T N M T L C	32
383	V M A Y I H T M N P S V L E D	32
85	S S L F D P T T Q R L V W A C	26
145	G M D Y K Q T Q L C M V G C A	26
338	N M T L C A S V S K S A T Y T	26
411	E D T Y R Y V Q S Q A I T C Q	26
51	I K K V N K T V V P K V S G Y	24
77	P N K F A L P D S S L F D P T	24
84	D S S L F D P T T Q R L V W A	24
194	D G D M V D T G F G A M N F A	24
255	R H F F N R A G T V G E P V P	24
368	L Q F I F Q L C S I T L S A E	24
372	F Q L C S I T L S A E V M A Y	24
374	L C S I T L S A E V M A Y I H	24
444	E V N L K E K F S S E L D Q F	24
23	T D A Y V K R T N I F Y H A S	22
92	T Q R L V W A C T G L E V G R	22
109	P L G V G V S G H P L L N K Y	22
182	C P P L E L I T S V I Q D G D	22
242	R L F F Y L R K E Q M F A R H	22
278	N N R S S V A S S I Y V H T P	22
342	C A S V S K S A T Y T N S D Y	22

C

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
241	D R L F F Y L R K E Q M F A R	25
45	G H P Y Y S I K K V N K T V V	24
53	K V N K T V V P K V S G Y Q Y	23
204	A M N F A D L Q T N K S D V P	23
325	H L F V T V V D T T R S T N M	23
120	L N K Y D D V E N S G G Y G G	22
11	V P P P N P V S K V V A T D A	21
48	Y Y S I K K V N K T V V P K V	21
66	Q Y R V F K V V L P D P N K F	21
14	P N P V S K V V A T D A Y V K	20
38	S S R L L A V G H P Y Y S I K	20
98	A C T G L E V G R G Q P L G V	20
304	F N K P Y W L Q K A Q G H N N	20
440	M S F W E V N L K E K F S S E	20
442	F W E V N L K E K F S S E L D	20
353	N S D Y K E Y M R H V E E F D	19
455	L D Q F P L G R K F L L Q S G	19
64	G Y Q Y R V F K V V L P D P N	18
150	Q T Q L C M V G C A P P L G E	18
250	E Q M F A R H F F N R A G T V	18
254	A R H F F N R A G T V G E P V	18
324	N H L F V T V V D T T R S T N	18
365	E F D L Q F I F Q L C S I T L	18
381	A E V M A Y I H T M N P S V L	18
461	G R K F L L Q S G Y R G R T S	18

D

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
117	H P L L N K Y D D V E N S G G	30
8	T V Y V P P P N P V S K V V A	24
42	L A V G H P Y Y S I K K V N K	24
93	Q R L V W A C T G L E V G R G	24
101	G L E V G R G Q P L G V G V S	24
182	C P P L E L I T S V I Q D G D	24
216	D V P L D I C G T V C K Y P D	24
222	C G T V C K Y P D Y L Q M A A	24
240	G D R L F F Y L R K E Q M F A	24
336	S T N M T L C A S V S K S A T	24
360	M R H V E E F D L Q F I F Q L	24
368	L Q F I F Q L C S I T L S A E	24
380	S A E V M A Y I H T M N P S V	24
381	A E V M A Y I H T M N P S V L	24
391	N P S V L E D W N F G L S P P	24
392	P S V L E D W N F G L S P P P	24
437	Y K D M S F W E V N L K E K F	24
452	S S E L D Q F P L G R K F L L	24
18	S K V V A T D A Y V K R T N I	20
24	D A Y V K R T N I F Y H A S S	20
59	V P K V S G Y Q Y R V F K V V	20
61	K V S G Y Q Y R V F K V V L P	20
64	G Y Q Y R V F K V V L P D P N	20
71	K V V L P D P N K F A L P D S	20
79	K F A L P D S S L F D P T T Q	20

Figure 6-3. 25 highest scoring candidate epitopes from L1 HPV11 predicted for (A) HLA-DRB1*04:01, (B) HLA-DRB1*07:01, (C) HLA-DRB1*11:01 and (D) HLA-DRB1*15:01.

A

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
233	A M D F T T L Q A N K S E V P	36
491	G R K F L L Q A G L K A K P K	34
441	E D T Y R F V T S Q A I A C Q	33
354	Q L F V T V V D T T R S T N M	30
20	V Y H I F F Q M S L W L P S E	29
411	A D V M T Y I H S M N S T I L	29
414	M T Y I H S M N S T I L E D W	29
299	D D L Y I K G S G S T A N L A	28
81	P N N N K I L V P K V S G L Q	27
188	K P P I G E H W G K G S P C T	27
334	N K P Y W L Q R A Q G H N N G	27
216	L I N T V I Q D G D M V D T G	26
270	D S L F F Y L R R E Q M F V R	26
315	S N Y F P T P S G S M V T S D	26
57	T N I Y Y H A G T S R L L A V	25
58	N I Y Y H A G T S R L L A V G	25
88	V P K V S G L Q Y R V F R I H	25
178	K Q T Q L C L I G C K P P I G	25
179	Q T Q L C L I G C K P P I G E	25
228	D T G F G A M D F T T L Q A N	25
322	S G S M V T S D A Q I F N K P	25
393	G E E Y D L Q F I F Q L C K I	25
427	D W N F G L Q P P P G G T L E	25
21	Y H I F F Q M S L W L P S E A	24
85	K I L V P K V S G L Q Y R V F	24

B

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
421	N S T I L E D W N F G L Q P P	37
170	R E C I S M D Y K Q T Q L C L	28
323	G S M V T S D A Q I F N K P Y	28
387	K E Y L R H G E E Y D L Q F I	26
497	Q A G L K A K P K F T L G K R	26
391	R H G E E Y D L Q F I F Q L C	25
100	R I H L P D P N K F G F P D T	24
271	S L F F Y L R R E Q M F V R H	24
398	L Q F I F Q L C K I T L T A D	24
485	L D Q F P L G R K F L L Q A G	24
45	S K V V S T D E Y V A R T N I	23
217	I N T V I Q D G D M V D T G F	22
294	G E N V P D D L Y I K G S G S	22
459	P P A P K E D P L K K Y T F W	22
65	T S R L L A V G H P Y F P I K	21
83	N N K I L V P K V S G L Q Y R	21
98	V F R I H L P D P N K F G F P	21
243	K S E V P L D I C T S I C K Y	21
406	K I T L T A D V M T Y I H S M	21
474	E V N L K E K F S A D L D Q F	21
28	S L W L P S E A T V Y L P P V	20
85	K I L V P K V S G L Q Y R V F	20
99	F R I H L P D P N K F G F P D	20
138	P L G V G I S G H P L L N K L	20
148	L L N K L D D T E N A S A Y A	20

C

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
18	V N V Y H I F F Q M S L W L P	28
57	T N I Y Y H A G T S R L L A V	28
114	T S F Y N P D T Q R L V W A C	28
270	D S L F F Y L R R E Q M F V R	28
441	E D T Y R F V T S Q A I A C Q	28
28	S L W L P S E A T V Y L P P V	26
211	C P P L E L I N T V I Q D G D	26
323	G S M V T S D A Q I F N K P Y	26
354	Q L F V T V V D T T R S T N M	26
395	E Y D L Q F I F Q L C K I T L	26
411	A D V M T Y I H S M N S T I L	26
421	N S T I L E D W N F G L Q P P	26
4	T F I Y I L V I T C Y E N D V	22
11	I T C Y E N D V N V Y H I F F	22
58	N I Y Y H A G T S R L L A V G	22
72	G H P Y F P I K K P N N N K I	22
113	D T S F Y N P D T Q R L V W A	22
233	A M D F T T L Q A N K S E V P	22
314	S S N Y F P T P S G S M V T S	22
413	V M T Y I H S M N S T I L E D	22
469	K Y T F W E V N L K E K F S A	22
503	K P K F T L G K R K A T P T T	22
5	F I Y I L V I T C Y E N D V N	20
8	I L V I T C Y E N D V N V Y H	20
17	D V N V Y H I F F Q M S L W L	20

D

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
314	S S N Y F P T P S G S M V T S	34
57	T N I Y Y H A G T S R L L A V	32
357	V T V V D T T R S T N M S L C	32
413	V M T Y I H S M N S T I L E D	30
44	V S K V V S T D E Y V A R T N	26
58	N I Y Y H A G T S R L L A V G	26
113	D T S F Y N P D T Q R L V W A	26
149	L N K L D D T E N A S A Y A A	26
174	S M D Y K Q T Q L C L I G C K	26
441	E D T Y R F V T S Q A I A C Q	26
20	V Y H I F F Q M S L W L P S E	24
28	S L W L P S E A T V Y L P P V	24
106	P N K F G F P D T S F Y N P D	24
223	D G D M V D T G F G A M D F T	24
257	Y P D Y I K M V S E P Y G D S	24
265	S E P Y G D S L F F Y L R R E	24
378	E T T Y K N T N F K E Y L R H	24
402	F Q L C K I T L T A D V M T Y	24
414	M T Y I H S M N S T I L E D W	24
474	E V N L K E K F S A D L D Q F	24
7	Y I L V I T C Y E N D V N V Y	22
26	Q M S L W L P S E A T V Y L P	22
27	M S L W L P S E A T V Y L P P	22
50	T D E Y V A R T N I Y Y H A G	22
75	Y F P I K K P N N N K I L V P	22

Figure 6-4. 25 highest scoring candidate epitopes from L1 HPV16 predicted for (A) HLA-DRB1*01:01, (B) HLA-DRB1*03:01, (C) HLA-DRB1*04:01 and (D) HLA-DRB1*07:01.

A

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
72	G H P Y F P I K K P N N N N K I	25
233	A M D F T T L Q A N K S E V P	25
254	I C K Y P D Y I K M V S E P Y	25
270	D S L F F Y L R R E Q M F V R	25
21	Y H I F F Q M S L W L P S E A	24
123	R L V W A C V G V E V G R G Q	23
354	Q L F V T V V D T T R S T N M	23
502	A K P K F T L G K R K A T P T	23
38	Y L P P V P V S K V V S T D E	21
398	L Q F I F Q L C K I T L T A D	21
493	K F L L Q A G L K A K P K F T	21
65	T S R L L A V G H P Y F P I K	20
82	N N N K I L V P K V S G L Q Y	20
127	A C V G V E V G R G Q P L G V	20
333	F N K P Y W L Q R A Q G H N N	20
411	A D V M T Y I H S M N S T I L	20
449	S Q A I A C Q K H T P P A P K	20
470	Y T F W E V N L K E K F S A D	20
472	F W E V N L K E K F S A D L D	20
503	K P K F T L G K R K A T P T T	20
95	Q Y R V F R I H L P D P N K F	19
280	Q M F V R H L F N R A G A V G	19
485	L D Q F P L G R K F L L Q A G	19
17	D V N V Y H I F F Q M S L W L	18
93	G L Q Y R V F R I H L P D P N	18

B

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
15	E N D V N V Y H I F F Q M S L	30
8	I L V I T C Y E N D V N V Y H	28
24	F F Q M S L W L P S E A T V Y	28
20	V Y H I F F Q M S L W L P S E	24
28	S L W L P S E A T V Y L P P V	24
69	L A V G H P Y F P I K K P N N	24
122	Q R L V W A C V G V E V G R G	24
130	G V E V G R G Q P L G V G I S	24
211	C P P L E L I N T V I Q D G D	24
230	G F G A M D F T T L Q A N K S	24
245	E V P L D I C T S I C K Y P D	24
251	C T S I C K Y P D Y I K M V S	24
269	G D S L F F Y L R R E Q M F V	24
365	S T N M S L C A A I S T S E T	24
410	T A D V M T Y I H S M N S T I	24
411	A D V M T Y I H S M N S T I L	24
421	N S T I L E D W N F G L Q P P	24
422	S T I L E D W N F G L Q P P P	24
464	E D P L K K Y T F W E V N L K	24
482	S A D L D Q F P L G R K F L L	24
443	T Y R F V T S Q A I A C Q K H	22
491	G R K F L L Q A G L K A K P K	22
1	M Q V T F I Y I L V I T C Y E	20
18	V N V Y H I F F Q M S L W L P	20
39	L P P V P V S K V V S T D E Y	20

C

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
477	V D T Y R F V Q S V A I T C Q	33
42	C G H Y I I L F L R N V N V F	30
181	H P F Y N K L D D T E S S H A	30
389	Q L F V T V V D T T P S T N L	30
434	L Q F I F Q L C T I T L T A D	30
9	I L H Y H L L P L Y G P L Y H	29
227	G E H W A K G T A C K S R P L	29
214	Q T Q L C I L G C A P A I G E	28
334	Q S L Y I K G T G M P A S P G	28
447	A D V M S Y I H S M N S S I L	28
450	M S Y I H S M N S S I L E D W	28
500	K D P Y D K L K F W N V D L K	28
4	Y T R V L I L H Y H L L P L Y	27
163	C A G V E I G R G Q P L G V G	27
400	S T N L T I C A S T Q S P V P	27
15	L P L Y G P L Y H P R P L P L	26
107	G N P Y F R V P A G G G N K Q	26
305	D S M F F C L R R E Q L F A R	26
333	P Q S L Y I K G T G M P A S P	26
388	N Q L F V T V V D T T P S T N	26
461	L E D W N F G V P P P P T T S	26
533	Q A G L R R K P T I G P R K R	26
35	Y M V H I I I C G H Y I I L F	25
44	H Y I I L F L R N V N V F P I	25
92	T S I F Y H A G S S R L L T V	25

D

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
358	G S I V T S D S Q L F N K P Y	30
457	N S S I L E D W N F G V P P P	29
197	T S N V S E D V R D N V S V D	28
100	S S R L L T V G N P Y F R V P	27
205	R D N V S V D Y K Q T Q L C I	27
506	L K F W N V D L K E K F S L D	26
508	F W N V D L K E K F S L D L D	26
306	S M F F C L R R E Q L F A R H	25
427	R H V E E Y D L Q F I F Q L C	25
53	V N V F P I F L Q M A L W R P	24
135	R V Q L P D P N K F G L P D T	24
252	K N T V L E D G D M V D T G Y	24
11	H Y H L L P L Y G P L Y H P R	22
278	K C E V P L D I C Q S I C K Y	22
305	D S M F F C L R R E Q L F A R	22
516	K F S L D L D Q Y P L G R K F	22
4	Y T R V L I L H Y H L L P L Y	21
6	R V L I L H Y H L L P L Y G P	21
24	P R P L P L H S I L V Y M V H	21
80	A R V V N T D D Y V T P T S I	21
133	V F R V Q L P D P N K F G L P	21
295	Y L Q M S A D P Y G D S M F F	21
357	S G S I V T S D S Q L F N K P	21
442	T I T L T A D V M S Y I H S M	21
510	N V D L K E K F S L D L D Q Y	21

Figure 6-5. 25 highest scoring candidate epitopes from L1 HPV16 predicted for (A) HLA-DRB1*11:01 and (B) HLA-DRB1*15:01 and 25 highest scoring candidate epitopes from L1 HPV18 predicted for (C) HLA-DRB1*01:01 and (D) HLA-DRB1*03:01.

A

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
53	V N V F P I F L Q M A L W R P	28
92	T S I F Y H A G S S R L L T V	28
149	T S I Y N P E T Q R L V W A C	28
305	D S M F F C L R R E Q L F A R	28
477	V D T Y R F V Q S V A I T C Q	28
43	G H Y I I L F L R N V N V F P	26
44	H Y I I L F L R N V N V F P I	26
197	T S N V S E D V R D N V S V D	26
358	G S I V T S D S Q L F N K P Y	26
389	Q L F V T V V D T T P S T N L	26
431	E Y D L Q F I F Q L C T I T L	26
434	L Q F I F Q L C T I T L T A D	26
447	A D V M S Y I H S M N S S I L	26
457	N S S I L E D W N F G V P P P	26
15	L P L Y G P L Y H P R P L P L	22
42	C G H Y I I L F L R N V N V F	22
93	S I F Y H A G S S R L L T V G	22
181	H P F Y N K L D D T E S S H A	22
289	I C K Y P D Y L Q M S A D P Y	22
319	R H F W N R A G T M G D T V P	22
349	S C V Y S P S P S G S I V T S	22
449	V M S Y I H S M N S S I L E D	22
500	K D P Y D K L K F W N V D L K	22
505	K L K F W N V D L K E K F S L	22
4	Y T R V L I L H Y H L L P L Y	20

B

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
92	T S I F Y H A G S S R L L T V	32
349	S C V Y S P S P S G S I V T S	32
392	V T V V D T T P S T N L T I C	32
449	V M S Y I H S M N S S I L E D	30
62	M A L W R P S D N T V Y L P P	28
184	Y N K L D D T E S S H A A T S	28
357	S G S I V T S D S Q L F N K P	28
93	S I F Y H A G S S R L L T V G	26
209	S V D Y K Q T Q L C I L G C A	26
477	V D T Y R F V Q S V A I T C Q	26
85	T D D Y V T P T S I F Y H A G	24
141	P N K F G L P D T S I Y N P E	24
148	D T S I Y N P E T Q R L V W A	24
258	D G D M V D T G Y G A M D F S	24
300	A D P Y G D S M F F C L R R E	24
319	R H F W N R A G T M G D T V P	24
325	A G T M G D T V P Q S L Y I K	24
414	P G Q Y D A T K F K Q Y S R H	24
434	L Q F I F Q L C T I T L T A D	24
438	F Q L C T I T L T A D V M S Y	24
450	M S Y I H S M N S S I L E D W	24
465	N F G V P P P P T T S L V D T	24
510	N V D L K E K F S L D L D Q Y	24
26	P L P L H S I L V Y M V H I I	22
44	H Y I I L F L R N V N V F P I	22

C

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
15	L P L Y G P L Y H P R P L P L	32
305	D S M F F C L R R E Q L F A R	25
56	F P I F L Q M A L W R P S D N	24
388	N Q L F V T V V D T T P S T N	24
30	H S I L V Y M V H I I I C G H	23
107	G N P Y F R V P A G G G N K Q	23
9	I L H Y H L L P L Y G P L Y H	22
181	H P F Y N K L D D T E S S H A	22
529	K F L V Q A G L R R K P T I G	22
5	T R V L I L H Y H L L P L Y G	21
389	Q L F V T V V D T T P S T N L	21
117	G G N K Q D I P K V S A Y Q Y	20
162	A C A G V E I G R G Q P L G V	20
368	F N K P Y W L H K A Q G H N N	20
506	L K F W N V D L K E K F S L D	20
539	K P T I G P R K R S A P S A T	20
52	N V N V F P I F L Q M A L W R	19
130	Q Y R V F R V Q L P D P N K F	19
419	A T K F K Q Y S R H V E E Y D	19
521	L D Q Y P L G R K F L V Q A G	19
1	M C L Y T R V L I L H Y H L L	18
46	I I L F L R N V N V F P I F L	18
128	A Y Q Y R V F R V Q L P D P N	18
214	Q T Q L C I L G C A P A I G E	18
314	E Q L F A R H F W N R A G T M	18

D

pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
12	Y H L L P L Y G P L Y H P R P	34
6	R V L I L H Y H L L P L Y G P	30
50	L R N V N V F P I F L Q M A L	30
474	T S L V D T Y R F V Q S V A I	30
53	V N V F P I F L Q M A L W R P	28
59	F L Q M A L W R P S D N T V Y	28
18	Y G P L Y H P R P L P L H S I	24
29	L H S I L V Y M V H I I I C G	24
43	G H Y I I L F L R N V N V F P	24
44	H Y I I L F L R N V N V F P I	24
55	V F P I F L Q M A L W R P S D	24
157	Q R L V W A C A G V E I G R G	24
165	G V E I G R G Q P L G V G L S	24
280	E V P L D I C Q S I C K Y P D	24
286	C Q S I C K Y P D Y L Q M S A	24
426	S R H V E E Y D L Q F I F Q L	24
434	L Q F I F Q L C T I T L T A D	24
446	T A D V M S Y I H S M N S S I	24
447	A D V M S Y I H S M N S S I L	24
457	N S S I L E D W N F G V P P P	24
458	S S I L E D W N F G V P P P P	24
502	P Y D K L K F W N V D L K E K	24
503	Y D K L K F W N V D L K E K F	24
510	N V D L K E K F S L D L D Q Y	24
518	S L D L D Q Y P L G R K F L V	24

Figure 6-6. 25 highest scoring candidate epitopes from L1 HPV18 predicted for (A) HLA-DRB1*04:01, (B) HLA-DRB1*07:01, (C) HLA-DRB1*11:01 and (D) HLA-DRB1*15:01.

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6.5 Curriculum vitae

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