Mapping of the anti-adenoviral cytotoxic T cell response mediated by HLA-A*01, -A*02, and -A*24

Kartierung der von HLA-A*01, -A*02 und -A*24 vermittelten anti-adenoviralen zytotoxischen T-Zellantwort

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1. INTRODUCTION

1.1. Adenoviruses

1.1.1. Classification

Adenoviruses are a family of nonenveloped, icosahedral viruses with a linear double-stranded DNA genome, thus belonging to Baltimore class I[1]. They were discovered in 1953 as causative agents of acute respiratory infections[2;3] and christened "adenoviruses" after the original adenoid tissue they were isolated from[4]. Since then, adenoviruses have been identified in a broad range of vertebrate hosts[5]. Recent genomic analysis and reclassification[6] has led to the definition of four genera: *Mastadenovirus* in mammals, *Aviadenovirus* in birds, *Atadenovirus* in reptiles, birds, and mammals, and *Siadenovirus* in reptiles and birds. A new strain isolated from a sturgeon seems to belong to neither of these genera[7]. All adenoviruses infecting humans belong to the genus *Mastadenovirus* and are divided into seven species (species A to G) and further into 53 serotypes (Ad1 to Ad53)[8-10]. Species B is sometimes subdivided into subspecies B1 and B2[11]. Species B and E also contain some simian adenovirus strains. Generally, individual adenovirus serotypes have been found to be pathogenic only within one species[12], although simian species have occasionally been shown to contain antibodies against human strains[13]. Table 1.1 lists the human serotypes and their corresponding species.

Table 1.1: The seven human adenovirus species and their subdivision into serotypes.

Species	Serotypes
A	12, 18, 31
B	3, 7, 11, 14, 16, 21, 34, 35, 50
C	1, 2, 5, 6
D	8 - 10, 13, 15, 17, 19, 20, 22 - 30, 32, 33, 36 - 39, 42 - 49, 51, 53
E	4
F	40, 41
G	52

1.1.2. Virion and genomic structure

All adenoviruses share the same basic structural features (Fig. 1.1). Their virions are icosahedral particles with a diameter of about 900 Å[14] and a mass of 150 MDa. Fibers that vary in length between the different serotypes protrude from the 12 vertices of the icosahedron[15]. Inside the protein shell or capsid lies the DNA-containing core.

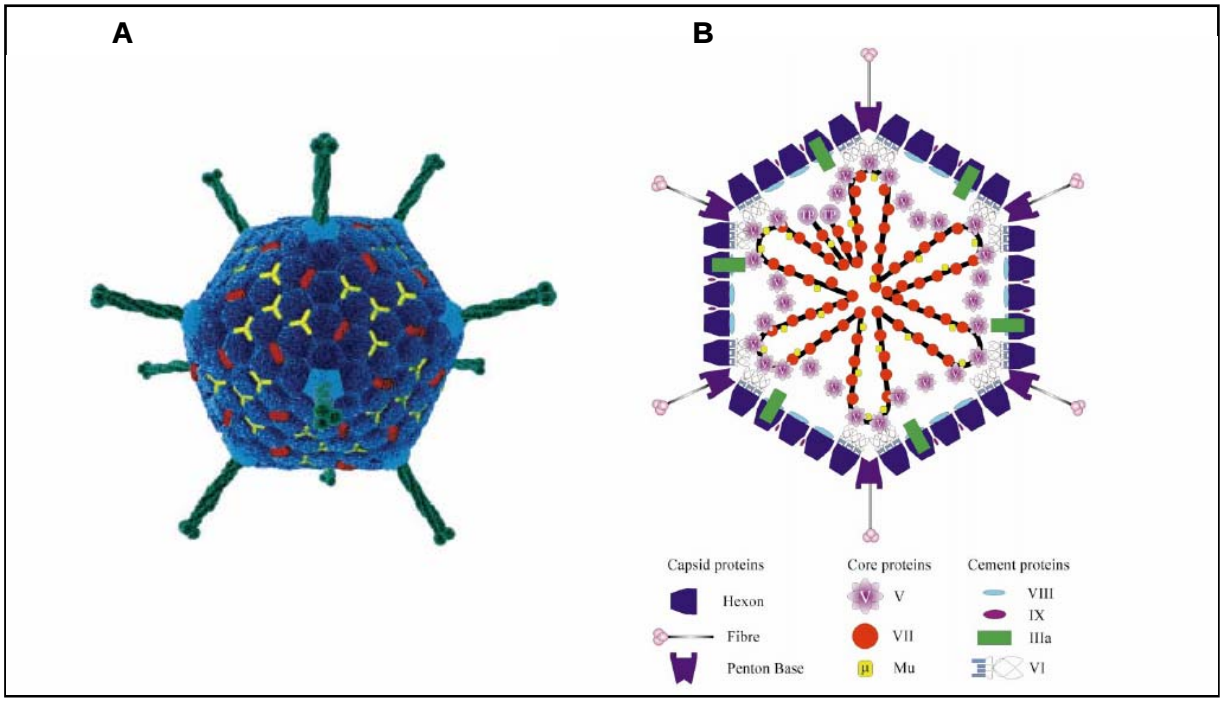


Figure 1.1: A: Three-dimensional drawing of the adenovirus virion. Hexon protein is shown in dark blue, penton base protein in light blue, fiber in green, protein IX in yellow and protein IIIa in red. Adapted from reference [16]. **B:** Cross-section of the virion, showing the DNA-containing core. Adapted from reference [17].

The main structural proteins of the outer shell are 240 hexon capsomers and 12 penton capsomers. Each hexon capsomer is a trimer of protein II (hexon protein) and is surrounded by six neighbors, twelve capsomers making up each of the twenty faces of the icosahedral shell. Penton capsomers form the vertices of the icosahedron and consist of five units of protein III (penton base protein) in the shell and the protruding fiber that is formed by a trimer of protein IV (fiber protein). Pentons are surrounded by five neighboring hexons. The fiber itself has an N-terminal penton base binding domain and a flexible shaft with a C-terminal knob that is the binding site of the primary host cell receptors[18]. Ad40 and Ad41 encode two different fiber proteins[19;20]. The small proteins VIII, IX and IIIa are important for stabilizing the nonequivalent interactions between the individual capsomers. Protein IX ensures the planar configuration of the central hexon trimers of each face, while protein IIIa is located near the vertices and interacts with penton base and neighboring hexons. Little is known about the function of protein VIII[16], but it has been shown to play a role in the structural stability of the capsid[21]. Protein VI seems to be important for the disruption of endosomal membranes during virus uptake and also for the import of protein II into the nucleus for virus assembly[16]. Among the proteins of the viral core, protein VII is the most abundant and serves to condense and package the viral DNA, together with the small peptide μ[22]. Protein V has only been found in mastadenoviruses, where it probably links the core to the shell by binding penton base and protein VI. Terminal protein is attached to the ends of the viral DNA by a phosphodiester bond formed between the β-hydroxyl group of S562 and the 5′ hydroxyl group of the terminal deoxycytidine[23]. Table 1.2 summarizes all structural proteins with their copy numbers and molecular masses.

Table 1.2: Adenovirus structural proteins with their molecular mass, the biochemically determined copy number per virion, and the copy number in the current model (if known). Modified from reference [15].

Protein	Molecular mass [Da]	Biochemical copy number	Copy number in current model
II (hexon)	109,077	720 ± 7	720
III (penton base)	63,296	56 ± 1	60
IIIa	63,535	68 ± 2	60
IV (fiber)	61,960	35 ± 1	36
V	41,631	157 ± 1	-
terminal protein	~55,000	2	2
VI	22,118	342 ± 4	360
VII	19,412	833 ± 19	-
VIII	15,390	127 ± 3	-
IX	14,339	247 ± 2	240
μ	~4,000	~104	-

Adenoviruses have genomes ranging in size between 26 and 45 kbp[6], which is in the medium range for DNA viruses, with inverted terminal repeat sequences of lenghts between 36 and more than 200 bp important for DNA replication. As already mentioned, terminal protein is covalently linked to the 5' ends of both strands in the mature virion. Moreover, a packaging sequence is repeated several times near one end of the genome and is necessary for encapsidation of the DNA into mature virions[24]. Most adenoviral transcription units are transcribed by RNA polymerase II[25], i.e. six early transcription units (E1A, E1B, E2A, E2B, E3, E4), three delayed early units (IX, IVa2, E2 late), and one late unit (major late). From most of these units multiple mRNAs are transcribed using alternative splicing or alternative poly(A) sites. Indeed, the very phenomenon of RNA splicing was discovered in adenoviruses[26]. Additionally, one or two VA RNA genes are transcribed by RNA polymerase III[27]. Fig. 1.2 illustrates the gene layout of simian adenovirus 25, which belongs to species E of human adenoviruses and is closely related to human adenovirus 4.

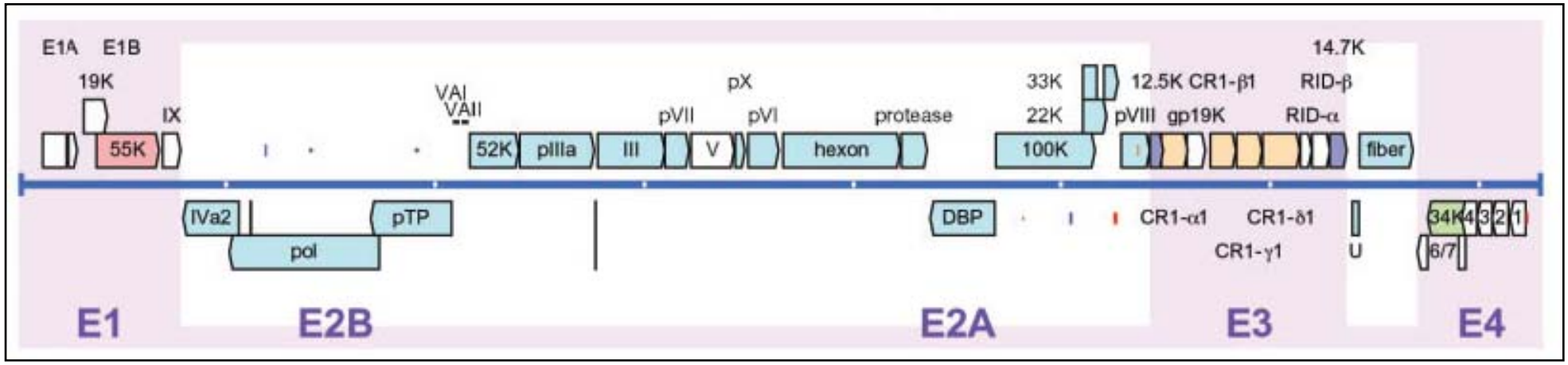


Figure 1.2: Gene map of simian adenovirus 25. Adapted from reference [6]

1.1.3. Entry into host cells

Human adenoviruses use different cellular receptors for entry into target cells. CAR (coxsackievirus-adenovirus receptor) has been found to be used by adenovirus serotypes from all human species except B[28]. It is a component of tight junctions[29;30] and is expressed abundantly in different epithelia, but not in lymphocytes or adult muscle[31]. Other described receptors include CD46 for all species B serotypes except 3 and 7[32], CD80 and CD86 for Ad3[33], sialic acid for Ad8, 19, and 37[34], and heparin sulfate glycosaminoglycans for Ad2 and 5 in addition to CAR[35]. After the initial binding, most adenoviruses bind integrins on the target cell with the penton base protein, which leads to fiber detachment and the endocytosis of fiberless virions[36] in clathrin-coated vesicles that mature into endosomes. After partial disassembly[37], subvirion particles escape from the endosomes into the cytosol approximately 15 minutes after binding to the cell surface[38], probably mediated by protein VI[39], and are transported to the nucleus, where they associate with nuclear pore complexes. The viral DNA with protein VII enters the nucleus about two hours after infection[40].

1.1.4. Early transcription units

E1A

E1A is the first gene transcribed after infection and is expressed in two splice variants, the large and the small E1A protein, that differ in a 46-residue domain present only in the large variant. The large protein can recruit a variety of cellular transcription factors and is thus responsible for the expression of the other viral early genes[41-43]. The small protein specifically activates E2 transcription[44]. The large E1A protein is also responsible for inducing the host cell to enter S phase[45], which is required for optimal virus replication. It activates the expression of several cellular genes (e.g. dihydrofolate reductase, DNA polymerase α, CDK2, several cyclins), blocks the action of CDK inhibitors[46], and influences chromatin structure[47].

Another function is immune evasion, since it can disrupt the signalling pathways of IFN-α and IFN-β[48]. However, p53 levels are also increased by the abnormal cell cycle stimulation E1A provides[49].

E1B

The main task of the E1B unit is the inhibition of apoptosis induced by the effects of E1A. Two different mRNAs are synthesized from the E1B site, encoding the proteins E1B-55K and E1B-19K. E1B-55K associates with E4orf6[50], a protein product of E4, and several cellular proteins to form an ubiquitin ligase complex that marks p53 for degradation in the proteasome[51], and it can inactivate p53 also by binding to it strongly[52]. Moreover, the MRN complex is probably also a target for the E1B-55K/E4orf6 ubiquitin ligase[53]. This is a complex of several cellular proteins engaged in the repair of DNA double-strand breaks and can hence ligate adenovirus genomes randomly into long chains, thus rendering them dysfunctional[54]. The E1B-19K protein has also antiapoptotic effects by binding to the proapoptotic proteins Bak and Bax and preventing their oligomerization[55].

E2A and E2B

The major protein products of these units participate in viral DNA replication, i. e. the viral DNA polymerase (AdPol), pre-terminal protein (pTP), and ssDNA-binding protein (DBP)[56;57].

E3

Six mRNAs are transcribed from the E3 site. The most important of the encoded proteins, E3-19K, is a transmembrane protein in the ER membrane and interferes with peptide presentation on MHC class I molecules by retaining them in the ER[58]. Furthermore, the RID complex in the plasma membrane, formed by two molecules of E3-10.4K and one molecule of E3-14.5K, causes the lysosomal degradation of Fas receptor[59;60] and TRAIL receptors 1 and 2[61], thus protecting the cell from apoptosis *via* these pathways, while E3-14.7K and E3-6.7K also interfere with them

downstream. E3-11.6K, also called adenovirus death protein, accumulates in cells over time and eventually leads to cell death[62].

E4

Of the proteins encoded in E4, E4orf6 has already been mentioned. Apart from its function in the ubiquitin ligase, it can also inhibit p53[63] and p73, another member of the p53 family, on its own. As a dimer with E4orf7 it greatly enhances E2 transcription levels[64]. E4orf1 and E4orf4 raise general protein translation levels in the absence of nutrients[65]. E4orf3 assists in the inactivation of MRN complexes.

VA RNA

Adenoviruses encode one or two (e. g. Ad2 and 5) small RNAs synthesized by RNA polymerase III. VA RNA_I assists in neutralizing the effects of interferons by binding strongly to PKR. PKR, whose expression is induced by INF-α and INF-β, is normally activated by binding to dsRNA in virus-infected cells and drastically reduces protein synthesis by phosphorylation of eIF-2. VA RNA_I binding renders PKR inactive[66]. Independently, both VA RNA_I and VA RNA_{II} inhibit the processing of cellular miRNA and its incorporation into RNA silencing complexes (RISCs)[67].

1.1.5. DNA replication

The replication of adenoviral DNA starts at the inverted terminal repeats of the genome that contain the origins of replication. DNA synthesis is initiated at either end, and only one of the daughter strands is synthesized at first, displacing one parental strand as ssDNA. This strands forms a large loop by the pairing of its two terminal repeats, thus creating a short duplex panhandle region containing a replication origin. Another round of replication is initiated here and the complementary daughter strand is synthesized[68]. Polymerization is carried out by AdPol, with the 80 kDa pTP serving as a protein primer. DBP and two cellular proteins (NF1 and Oct-1) are also required for initiation[69-71]. The priming reaction

involves the forming of an ester bond between the β-hydroxyl group of S580 in pTP and the α-phosphoryl group of dCMP[72]. Cellular topoisomerase I is required for chain elongation[73] and viral DBP for strand separation[74]. pTP remains attached to the 5′-end of each strand and is later proteolytically processed into the 55 kDa TP (terminal protein) by the viral 23K protease[75].

1.1.6. Delayed early and late transcription units

IX

Transcription unit IX is a delayed early unit and encodes a short unspliced mRNA that is translated into protein IX. Apart from being a part of the virion, protein IX also enhances general transcription levels[76].

IVa2

The IVa2 gene encodes a viral transcription factor that contributes to the activation of the major late unit[77]. It is also required for the packaging of DNA into virions[78] and shows ATPase activity.

E2 late

The E2A transcription unit has an alternative promoter that is activated after the onset of DNA replication and helps to supply the large amounts of DBP required during replication[79].

Major late

Five families (L1 to L5) of mRNAs are produced from the major late transcription unit, each containing several mRNA species of different lengths[80]. L1 encodes the 52/55K protein and protein IIIa; L2 proteins III, pVII (precursor of protein VII), and V; L3 proteins pVI and II and the 23K protease; L4 the 100K protein, the 33K protein, the 22K protein, and protein pVIII; and L5 protein IV. Most of these proteins form parts of the virion or are needed for virion assembly. The 100K protein functions to

suppress translation of cellular mRNAs late during infection by dephosphorylating and thus inactivating eIF4E[81]. Viral translation does not depend on eIF4E, and late during infection viral mRNA is translated almost exclusively[82]. Furthermore, 100K protein is also required for the assembly of hexon trimers[83]. E1A is necessary for transcription of the major late unit, and low levels of transcription do already occur in the early phase, but they produce only one mRNA from the L1 family[84]. The promoter becomes several hundred times more active during the late period of infection, and all five mRNA families are produced now[85]. Protein IVa2 is necessary for this activation, as is a modification of the adenoviral chromosome not yet exactly determined.

1.1.7. Virion assembly and release

After its synthesis, protein II is assembled into hexon trimers in the cytosol with the help of 100K protein[86]. Complete penton capsomers consisting of penton base and the fiber also assemble in the cytosol. Capsomers are then transported into the nucleus[87], where virion assembly occurs, protein VI being necessary for this process[16]. The capsid is assembled with 33K protein serving as a scaffold[88]. The viral DNA associates with protein pVII, which binds to protein IVa2 (an ATPase), and 52/55K protein[89]. This complex, together with 22K protein, inserts the DNA into the capsid invariably starting at the end near E1A[24]. The process for the incorporation of the other structural proteins is not yet known in detail. Viral 23K protease, also included in the virions, then cleaves the precursors of protein VI, VII, VIII, and TP, and removes the 52/55K protein, thus rendering the particles infectious[90]. Protein pVI is required for the cleavage to take place, ensuring it to occur only in assembled virions[91]. Finally E3-11.6K or adenovirus death protein, an integral membrane protein localizing to nuclear, ER, and Golgi membranes, promotes cell death and lysis[62], but the mechanism is not known in detail. It is assisted by 23K protease that cleaves cytokeratin K18 and thus restructures the cytoskeleton[92]. Surplus virus fibers released from lysed cells also help the virus

spread, as they bind to CAR in tight junctions, inhibiting its oligomerization and thus helping to break up epithelia[30]. About 10^4 progeny viruses are released from a HeLa cell 24 to 36 hours after infection[93].

1.1.8. Epidemiology and clinical features

Adenoviral infections are widespread and occur frequently worldwide. One study reported 3% of infections in a civilian population to be due to adenovirus[94]. According to another study, 61.3% of adenovirus patients were under 5 years of age, with the most prevalent serotypes being 2 (18.6%), 3 (14.9%), 1 (12.1%), and 41 (10.9%)[95]. Antibodies to Ad1, Ad2, and Ad5 have been reported to be present in 40% to 60% of children[96-98]. Adenovirus infections cause upper and lower respiratory tract illnesses in young children with the usual symptoms being cough, nasal congestion, tonsillitis, and sometimes fever, chills, and headache. In severe cases they can even lead to pneumonia[99], but sporadic cases may be clinically indistinguishable from other respiratory infections (e.g. influenza, parainfluenza, streptococcus). Another group frequently afflicted are military recruits, in whom adenoviruses cause acute respiratory disease (ARD) with similar symptoms to the ones mentioned above[100]. Besides respiratory illnesses, another relevant group of diseases are acute follicular conjunctivitis and the more serious form epidemic keratoconjunctivitis (EKC), also known as “shipyard eye”, the latter being highly contagious. It was first described among shipyard workers in Germany and the U.S.[101] and can lead to prolonged impairment of vision[102]. Gastrointestinal diseases due to adenovirus are also quite common, with one study in Germany reporting adenoviruses detected in 8.3% of stool samples of children suffering from diarrhea[103]. Table 1.3 provides an overview of different diseases caused by adenovirus and the persons most at risk.

Table 1.3: The principle diseases caused by adenovirus, the individuals most at risk, and the most common serotypes. Adapted from reference [104].

Disease	Individuals most at risk	Principal serotypes
Acute febrile pharyngitis	Infants, young children	1, 2, 3, 5, 6, 7
Pharyngoconjunctival fever	School-aged children	3, 7, 14
Acute respiratory disease	Military recruits	3, 4, 7, 14, 21
Pneumonia	Infants, young children	1, 2, 3, 7
Pneumonia	Military recruits	4, 7
Epidemic keratoconjunctivitis	Any age group	8, 11, 19, 37
Pertussislike syndrome	Infants, young children	5
Acute hemorrhagic cystitis	Young children	11, 21
Gastroenteritis	Infants, young children	40, 41
Meningoencephalitis	Children and immunocompromised hosts	7, 12, 32
Hepatitis	Infants and children with liver transplants	1, 2, 5
Myocarditis[105]	Children	2, 5

Another group highly at risk are immunocompromised individuals, especially bone marrow or other transplant patients who receive high doses of immunosuppressive drugs, cancer patients undergoing chemotherapy or radiation, and AIDS patients[106]. In transplant patients, the most prevalent serotypes are Ad2, 5, 11, 31, and 35, cancer patients are more often afflicted by Ad1, 5, and various species B serotypes, and species D serotypes predominate in AIDS patients[107]. Mortality rates are considerable. One study of 86 bone marrow transplant (BMT) patients reported adenovirus infection in 11 cases (13%), five being fatal[108]. In another survey of 2,889 BMT patients, only 85 were found to be infected with adenovirus (3%), but only histopathology and no PCR methods were used in this study. 22 of these patients died[109]. In more recent studies in allogeneic stem cell transplant recipients disseminated adenoviral disease could lead to mortality rates of up to 76%[110;111].

Thus, adenovirus is a serious problem in organ transplantation, although it is not as prevalent as herpesviruses[112]. Immunotherapy with stimulated autologous adenovirus-specific T cells could be a promising method to cope with these infections[113], especially since low T cell counts have been shown to correlate with severity of infection and mortality[114;115]. Adoptive transfer of virus-specific T cells has long been proven to be efficient in the treatment of Epstein-Barr virus or cytomegalovirus infections[116;117]. In recent years it has been shown that in adenoviral infections of immunocompromised children after allogeneic stem cell transplantation, mortality is associated with the absence of Adv-specific T cells, while patients who have successfully cleared the infection have Adv-specific T cell levels comparable to healthy blood donors[118]. Direct adoptive transfer of Adv-specific T cells isolated from the stem cell donors without *in vitro* expansion has shown positive results in such patients[119], while recently a clinical grade method for generating hexon-specific T cells with good cross-reactivity among different adenoviral species has been presented[120].

1.1.9. Persistence and latency

It is known that adenovirus can establish a persistent presence in its host for a long time after clinical symptoms have subsided. About 50% of children are shedding species C adenoviruses in their feces without showing any symptoms of infection[94]. True latency, meaning maintenance of virus DNA in host cells in an episomal state similar to herpesviruses, has not been unambiguously proven yet, but recent findings have supported this possibility. An earlier study has found DNA sequences of adenovirus in tonsillar tissue, but no infectious virus[121]. More recently, quantitative real-time PCR has detected DNA from species C adenoviruses in about 80% of tonsils and adenoids analyzed, with viral copy numbers varying between one copy in 10 to 10^6 cells and T cells being infected preferentially[122]. Later the same group could detect infectious virus only in about 15% of the individuals in whose tonsils viral DNA had been found[123]. Clearly, persistent or

latent adenoviruses could readily give an explanation to the source of infection in immunocompromised patients, who might be infected by endogenous virus once immune system function has been impaired.

1.1.10. Oncogenesis

In 1962, the human serotype Ad12 was found to induce malignant tumors in newborn hamsters[124], and was the first human virus discovered to be oncogenic. All human serotypes that have been tested were shown to transform cultured rodent cells to oncogenic phenotypes, although only some species and strains are outright tumorigenic[125]. One study has reported adenoviral RNA in human neurogenic tumors[126], but since most studies have found no viral nucleic acids or proteins in human malignancies, adenoviruses are not regarded as human tumor viruses at the moment[104].

1.1.11. Adenoviruses as vectors

Adenoviruses have become very popular gene vectors in the last decades, so a short introduction to this topic shall be given here. Adenoviruses have several tangible advantages for use as DNA vectors. The viruses are well known and can be easily produced in cell culture[127]. More importantly, in contrast to most other retroviral vectors they can infect quiescent and non-dividing cells[128;129]. A broad range of tissues are targeted by adenoviruses, facilitating their general use but complicating target-specific delivery. They rarely integrate into the host genome, which reduces risks from oncogenetic mutations but on the other hand limits the duration of transgene expression. One major disadvantage for *in vivo* and therapeutic application is the high immunogenicity of adenoviruses and the derived vectors[130;131], especially since these are mostly derived from Ad5, a strain against which

neutralizing antibodies are very prevalent in the population[132]. These immune responses further limit the timeframe for transgene expression[133].

Basically, adenoviral vectors can be classified into the two main categories of replication-defective and replication-competent. In the former group, one or more essential genes are deleted, usually E1A and E1B, which are absolutely required for viral replication. Consequently, such vectors need to be grown in cell lines that stably express these missing genes, examples being the human kidney line HEK 293[134] or the human embryonic retina line PER.C6[135]. To cope with the problem of host immunity against the vector, which is at least partly due to leaky expression of virion genes[136], vectors with a temperature-sensitive mutant of DBP were introduced, blocking transcription at body temperature[137]. The next generation of replication-defective vectors were the so-called gutless vectors[138], containing only the origins of replication and the packaging sequences, but no possibly immunogenic viral genes whatsoever. They can package much longer DNA sequences (indeed they need to, otherwise "stuffer" DNA has to be employed), but require an adenoviral helper virus for production[139]. Nevertheless, the assumption that the more adenoviral genes are eliminated, the less immunogenic the vector will become, has not been completely validated. Adenoviral vectors induce a broad range of cytokines[140] and the response peaks before any substantial gene expression from the vector[141], suggesting that it is due to the injected capsids themselves.

Replication-deficient adenoviral vectors are employed widely to deliver DNA to cells in culture, especially to cell types that are hard to transfect but susceptible to adenoviral infection. Therapeutic strategies based on direct gene therapy have also been devised, e.g. the induction of proapoptotic or immunostimulatory genes into tumors[142]. Furthermore, vectors have also been employed for vaccination by encoding an antigen of another infectious agent in the vector DNA, such as proteins from hepatitis B[143], HIV[144], or rabies[145].

Replication-competent vectors have been mostly explored as agents to treat cancer, and are hence also referred to as oncolytic vectors[146-148]. The basic strategy in this approach is to utilize the natural life cycle of adenovirus for tumor cell lysis. It is encouraged by the fact that cancer cells with their deregulated cell cycles are more permissive for adenoviral replication than normal or quiescent cells[149;150]. Additionally, oncolytic vectors generally include modifications that are designed to limit replication in noncancerous tissue. Examples are several vectors in clinical studies that have a deletion of the E1B region encoding the 55K protein[151-153]. As mentioned earlier, 55K protein is involved in the inactivation and degradation of p53, a process that was supposed to be essential for viral replication. Accordingly these vectors should have only been able to replicate in tumor cells that lack p53 in the first place. The vector indeed grows better in cancerous than normal cells, but it has been shown that this phenomenon does not depend on p53[154]. Further examples of oncolytic vectors include KD3 with an overexpression of adenovirus death protein (11.6K protein)[155] and delta24 RGD with a partial deletion of the E1A gene[156].

1.2. T lymphocytes

T lymphocytes are the mediators of cellular immunity. They carry highly variable T cell receptors that are able to recognize specific non-self peptides presented on MHC class I or II molecules. Most T cells carry α:β receptors (see 1.2.2.), while a minority with greatly reduced receptor variability has γ:δ receptors[157]. α:β T cells express either CD4 or CD8 on their surface. These are coreceptors mediating recognition of MHC class I (CD8) or MHC class II (CD4) molecules[158]. $CD8^+$ T cells are also called cytotoxic T lymhocytes (CTLs), because their task is to kill infected cells presenting non-native peptides on their surface. $CD4^+$ cells are referred to as T helper (T_H) cells and can be subdivided into several subgroups. When activated, they are in turn responsible for the activation of macrophages and are also efficient activators of antibody secretion by B cells. T_H cells can help in the activation of CTLs as well.

1.2.1. MHC molecules

MHC (major histocompatibility complex) molecules were discovered as the major determinants of histocompatibility in transplantations. In humans, they are also called HLAs (human leukocyte antigens). They are cell surface molecules that can bind peptides from cytosolic or endocytosed proteins and present them to T lymphocytes. There are two major classes of MHC molecules: class I proteins present peptides from the cytosol, mainly with a length of 8 to 10 amino acids, while class II proteins present peptides from intracellular vesicles of variable length (between 9 and 25 amino acids)[159-161]. Class I molecules are expressed on nearly all nucleated cells, while class II molecules are mainly found on specialized professional antigen-presenting cells (APCs) such as B lymphocytes, macrophages, and dendritic cells. Thus, peptide presentation by MHC class I molecules is important for the detection of intracellular parasites, e. g. viruses, by cytotoxic T cells that can subsequently kill these cells. APCs on the other hand take up extracellular antigens and present them to T_H cells which can then activate antibody or macrophage responses.

MHC class I complexes consist of a transmembrane α chain with three extracellular Ig domains (α_1-α_3) and a mass of 45 kDa, and a soluble β_2 microglobulin chain of 12 kDa (Fig. 1.3). A closed peptide binding groove is formed by the interface of domains α_1 and α_2[162] where the peptide ligand is bound in an elongated conformation[163]. MHC class I peptides are usually generated in a complex process involving steps in the cytosol, translocation of intermediates into the ER, and final trimming of future ligands there. Generally, ligands are derived from products of the proteasome, the main enzyme of protein degradation in the cytosol[164]. It is well established that the C-terminus of MHC ligands is determined by the proteasome[165], but proteasomal products mostly range in size from 2 to 25 amino acids[166;167], and the majority do not have the correct length for MHC binding. Longer peptides can be digested at the N-terminus by various cytosolic proteases[168-170] before being transported to the ER by the transporter associated with antigen processing (TAP) complex.

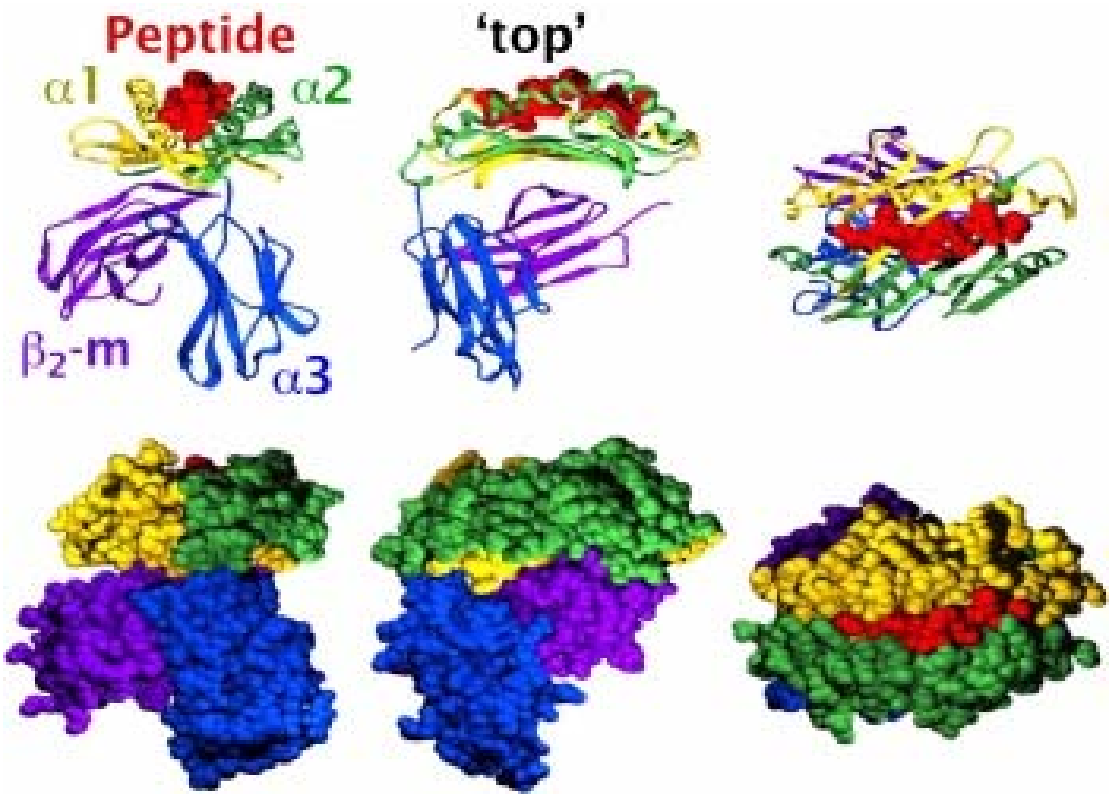


Figure 1.3: Ribbon and space-filling diagrams of an MHC class I molecule in complex with a peptide. Adapted from http://labs.fhcrc.org/strong/projects.html.

This is a heterodimer consisting of TAP1 and TAP2 that actively translocates peptides into the ER and has a length preference of 8 to 13 amino acids, although peptides of up to 40 residues can be transported with low efficiency[171;172]. Final MHC class I assembly and peptide binding occurs in the peptide loading complex (PLC) in the ER. Tapasin and several other ER-resident chaperones are crucial for this process. Tapasin binds TAP and is then linked to a cysteine residue at the active site of the thiol oxidoreductase ERp57[173;174]. This heterodimer then recruits MHC class I molecules and calreticulin to the PLC[175] that receives the peptides translocated by TAP and mediates MHC loading. The final truncation of the peptides is performed by ERAAP[176]. Furthermore, so-called immunoproteasomes with a different subunit composition are formed under the influence of IFN-γ or TNF[177]. The three catalytic subunits of the proteasome are replaced. Numerous studies have shown that immunoproteasomes enable the presentation of ligands poorly produced by proteasomes[178], and their cleavage specificity is altered[179] to enhance the

production of peptide precursors with more suitable C-terminal residues for MHC binding.

Apart from the pathway for the presentation of intracellular peptides, it has been shown that some APCs can present exogenous peptides on their MHC class I molecules, too. Since in the canonical model such peptides would have been expected to be presented on MHC class II, the process has been named "cross-presentation"[180;181]. In this pathway, antigens escape from early endosomes to the cytosol, probably through the transporter Sec61b[182], and are subsequently processed in the manner of intracellular antigens.

The MHC class II complex is formed by two transmembrane chains of about 30 kDa (α and β) with two Ig domains each. The peptide binding site lies between domains α_1 and β_1[183]. In contrast to MHC class I molecules, here the peptide binding groove is open and can accommodate peptides of different lengths, usually up to 20 amino acids. Peptides presented on class II are derived from pinocytosed or phagocytosed proteins, and their processing and loading takes place in the same cellular compartment[184]. Proteins are digested in the phagolysosome by acidic proteases and cathepsins, while the MHC class II complex reaches this compartment from the ER together with the so-called invariant chain Ii that blocks the peptide binding groove. Here Ii is degraded and the fragment occupying the binding groove, CLIP, displaced with the assistance of HLA-DM and HLA-DO[185]. *Via* autophagy, peptides from intracellular proteins can also be presented on MHC class II[186].

MHC molecules need the ability to bind and present many different peptides, ideally all possible ones. This required variability of their binding sites is achieved not by somatic recombination, as in T cells, but by polygeny and polymorphism of their genes. There are three *loci* for the MHC class I heavy chain in the human genome, HLA-A, -B, and -C, with several thousand functional alleles being known (1,193 for HLA-A, 1,800 for HLA-B, 829 for HLA-C; August 2010, http://www.ebi.ac.uk/imgt/

hla/stats.html). The β_2m gene is nonpolymorphic. For class II molecules there are also at least three functional *loci* each for the α and the β chains, with a total of 2,123 known functional alleles. The peptide binding groove of each allele is different and has a unique specificity for certain amino acids in defined positions. Especially restricted positions that can only be occupied by a few amino acids are called anchor positions. Considering anchor positions and length preferences, peptide motifs can be described for the different alleles[187;188]. Based on such motifs, the first mouse H2 epitopes could be predicted in 1991[189;190]. This was followed by the development of algorithms that can predict possible epitopes from protein sequences for the different allotypes, e.g. SYFPEITHI[191]. This strategy has been especially successful for class I allotypes because their binding grooves are closed and hence constrain the length of the ligand and the position of anchor amino acids within it.

1.2.2. T cell receptors

The T cell receptor (TCR) is a heterodimer of two transmembrane proteins, each with a mass of about 50 kDa. The receptor is formed either by an α and a β chain (α:β receptor) or a γ and a δ chain (γ:δ receptor). This introduction shall focus on α:β receptors. Both of their chains consist of a short cytoplasmic domain, a transmembrane helix, and two immunoglobuline-like (Ig) domains[192]. The distal Ig domain (the variable or V domain) of each chain contains three (α) or four (β) hypervariable loops (CDR loops) that differ between individual cells and define the peptide and HLA specificity of the receptor[193]. This variability is due to somatic recombination, since each T cell precursor arranges the sequences of its receptor genes individually from many segments. For α chains, one of 70-80 V segments, one of 61 J segments, and the constant C segment are joined to yield the functional gene, while for β chains there are 52 V segments, two D segments, 13 J segments, and even two different "constant" regions[194]. The combinatorial diversity is increased by the possibility of insertion or deletion of nucleotides at the joining sites[195], so that

about 10^{18} different α:β receptors can be formed in humans[196]. This diversity ensures that every possible MHC/peptide complex can be recognized specifically.

Signalling through the T cell receptor is mediated by the CD3 complex. It consists of six transmembrane molecules, i.e. one γ, one δ, two ε, and two ζ chains, and is associated with the T cell receptor[197]. γ, δ, and ε have one extracellular Ig domain and one intracellular ITAM motif for signalling, while the ζ chains have only a very short extracellular portion, but three ITAM motifs. Apart from the CD3 complex, efficient receptor signalling for most ligands also needs the coreceptors CD4 or CD8. Each T cell expresses one of these molecules, depending on the MHC class specificity of its receptor. CD4 binds to MHC class II molecules[198], CD8 is specific for MHC class I molecules[199]. CD4 is a monomer with four extracellular Ig domains. CD8 is usually expressed as an $\alpha\beta$ heterodimer with one Ig domain in each chain[200], but $\alpha\alpha$-homodimers have also been described[201].

1.2.3. T cell development and homeostasis

T lymphocytes are derived from pluripotent hematopoietic stem cells in the bone marrow, but their differentiation and selection processes take place mainly in the thymus. After migrating into the cortex, the outer region of the thymus, T cell progenitors enter a first proliferation phase characterized by the expression of selective surface markers. These cells are also called "double-negative thymocytes", since they express neither CD4 nor CD8 yet. One of the first markers to be expressed is CD44, soon followed by CD25[202]. At this stage, expression of the Rag genes 1 and 2 (recombination activating genes) starts, which enables the somatic recombination in the T cell receptor β *locus*[203]. The γ and δ genes also start recombination at this time point. Since nucleotides can be inserted and deleted randomly in the recombination process, there is only a 33% probability that a functional gene will arise from every given precursor. As a result, cells may arise having functional γ and δ chains, which will eventually develop into γ:δ T cells[204],

or cells having a functional β chain, which develop into α:β T cells. The β chain is sufficient in this case since a surrogate α chain, called pTα, is expressed concomitantly and can form a functional receptor with the β chain, the pre-T-cell receptor. Cells with functional receptors receive survival signals from epithelial cells in the cortex that also end Rag gene expression and recombination[205]. Cells that failed to recombine successfully die. After a proliferation phase, the concomitant expression of CD4 and CD8 starts ("double-positive" phase), as does recombination at the α *locus*. Cells expressing a functional receptor that is able to bind to MHC class I or class II molecules (also expressed in the cortical epithelium) again survive, while those having too low affinity receptors die. This positive selection is accompanied by the shutoff of expression of the coreceptor (CD4 or CD8) not involved in MHC recognition. These "single-positive thymocytes" then migrate to the thymic medulla, where negative selection by APCs and epithelial cells takes place. Thymocytes with too high affinities for MHC complexes of self peptides are committed to apoptosis. Important in this context is also the transcription factor AIRE (autoimmune regulator) which induces ectopic expression of nearly all self-antigens in the thymus[206;207], a key requirement of functional negative selection. In total, less than 2% of precursor cells entering the thymus are released into the blood as mature naive T lymphocytes. Fig. 1.4 outlines the maturation process.

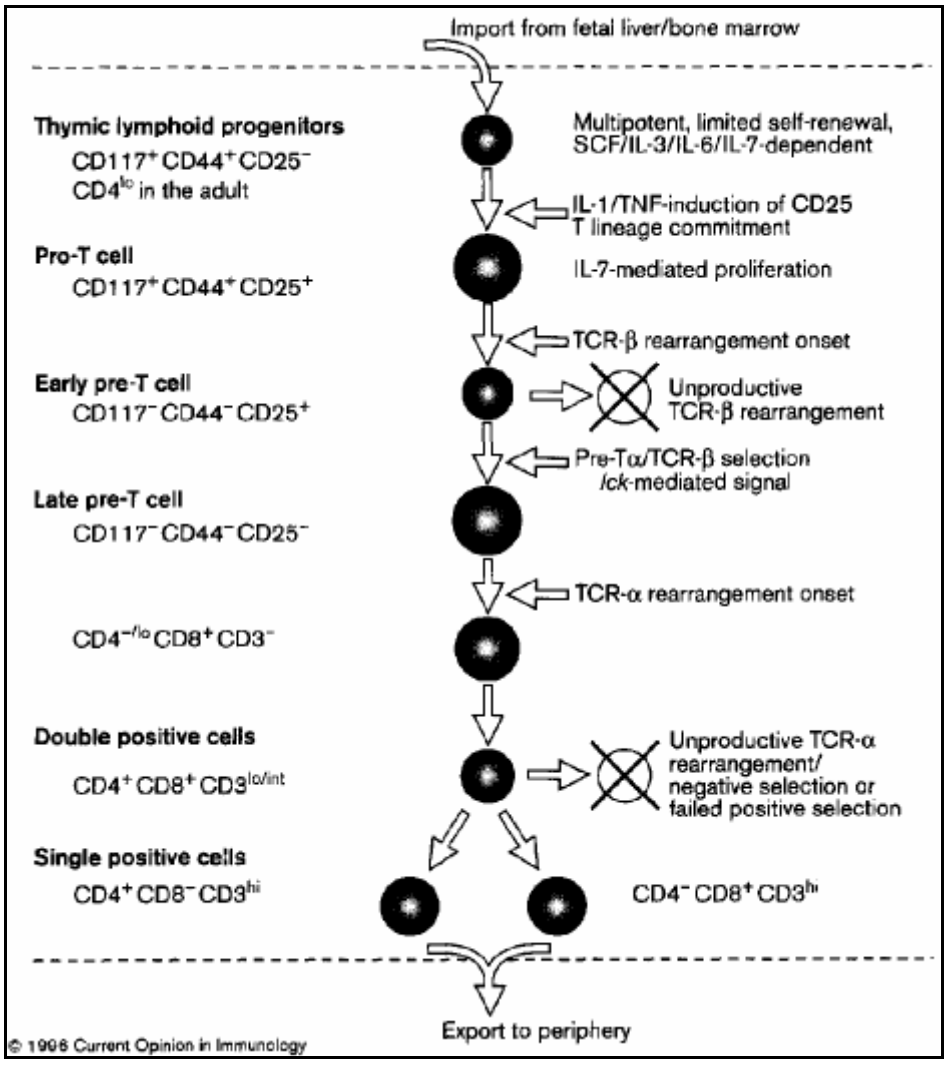


Figure 1.4: Simplified scheme of the stages of T cell differentiation in the thymus. Adapted from reference [202].

After leaving the thymus, naïve T cells circulate through the blood and lymphoid systems, entering the latter *via* the lymph nodes and being returned to the bloodstream in the subclavical veins. Their life-span in the periphery attains several years. Homeostasis of T cell numbers is achieved by the replacement of dead cells by fresh lymphocytes from the thymus or the division of naïve T cells. It has been shown that less than 20% of naïve T cells divide homeostatically over a period of five weeks[208]. T cell numbers are regulated mainly by MHC binding and cytokines. It has been shown that T cell survival in the periphery depends on low-affinity interactions with MHC/self peptide-complexes[209;210], similar to thymic positive selection. Furthermore, naïve T cells can inhibit proliferation of cells with the same

clonal specificity[211]. The general size of the T cell population is regulated by cytokines like IL-7[212].

1.2.4. T cell subsets

α:β T cells are subdivided into two major groups depending on their expression of coreceptor molecules: $CD4^+$ and $CD8^+$ T cells. Generally, the former recognize ligands presented on HLA class II molecules, while the latter are specific for HLA class I-presented ligands. Among $CD8^+$ T cells the main subset are the cytotoxic T lymphocytes (CTLs) that recognize cells infected by viruses or other intracellular pathogens as well as abnormal host cells and are responsible for their killing. Besides, there is a small population of so-called $CD8^+$ T regulatory cells (T_{reg})[213]. In contrast to the "natural" $CD4^+$ $CD25^+$ $Foxp3^+$ T_{reg} discussed below, these cells do not develop their regulatory phenotype in the thymus but are induced by antigen stimulation. They recognize ligands presented by the unusual MHC molecule HLA-E and are important for the suppression of self-reactive $CD4^+$ T cells.

The $CD4^+$ T cells have been named "T helper cells" after their discovery since they generally do not kill infected cells but rather activate other components of the immune system. Two major groups have been defined nearly 20 years ago: T_H1 and T_H2 cells[214]. They differ in their cytokine secretion patterns and the kind of cells they activate. T_H1 cells secrete mainly IL-2, IFN-γ, and lymphotoxin, activating macrophages[215;216] and CTLs[217]. T_H2 responses, on the other hand, are characterized by the secretion of IL-4, IL-5, and IL-13 and lead to the activation of B cells and their antibody production[218-220]. The differentiation of naïve $CD4^+$ T cells into one of these subtypes is guided by the cytokine milieu prevailing during their priming. IL-12 and IFN-γ lead to development of the T_H1 phenotype, while T_H2 cells are induced by IL-4[221;222]. Additionally, T_H1 and T_H2 cells inhibit the activation of each other, thus committing the local immune response to one of the two pathways[223].

Apart from these two classical groups, several other subsets of $CD4^+$ T cells have been identified lately. T_H17 cells are induced early during the immune response under the influence of TGF-β and IL-6[224] and mainly secrete IL-17 (hence their name) and IL-23[225]. They play a role in the activation of neutrophils and are important for immunity against parasitic worms[226]. More recently, another subset dubbed T_H9 has been described[227;228] that originates in the T_H2 lineage, is a strong producer of IL-9, and may be of importance in allergic diseases like asthma[229].

Additionally, several suppressive or regulatory cell populations among $CD4^+$ T cells have been described. The most important of these are the so-called natural T regulatory cells (T_{reg}). They develop in the thymus[230], are capable of suppressing $CD4^+$ as well as $CD8^+$ T cells, and are key players for the maintenance of self-tolerance[231]. They are characterized by the expression of CD25 and especially the transcription factor FoxP3[232]. GITR has been described as a surface marker specific for natural T_{reg}[233].

Adaptive regulatory T cells, in contrast, develop in the periphery from naïve $CD4^+$ T cells and include several subsets with different phenotypes. T_H3 cells are found mainly in the mucosal immune system and are thought to be important for the maintenance of tolerance to the host of non-pathogenic foreign antigens the immune system encounters there[234]. They are $FoxP3^-$ and have a T_H2-like cytokine pattern with the important addition of TGF-β. T_R1 cells resemble T_H3 cells but are characterized by the strong production of IL-10[235]. Finally, an inducible $FoxP3^+$ subtype of regulatory T cells has been described[236] that develops from naïve T cells by antigen priming in the presence of TGF-β. These cells secrete TGF-β themselves and are thus able to inhibit both T_H1 and T_H2 responses.

1.2.5. The T cell response

While circulating in the blood and the peripheral lymphoid organs, naïve T lymphocytes can contact activated APCs that present foreign antigens on their MHC molecules[237]. If the receptor of a T cell is by chance complementary to the MHC-peptide-complex presented on an APC, the T cell is activated. Apart from signalling through the T cell receptor, further costimulatory signals are needed for the T cell to be activated that are provided only by activated APCs, mostly DCs. Signalling through the TCR alone indicates recognition of a self-antigen and induces anergy or apoptosis[238].

The most important costimulatory receptor on T cells is CD28, which can bind CD80 and CD86 expressed on activated APCs[239]. Signalling through CD28 leads to the secretion of IL-2, an autocrine proliferation factor for T cells[240]. At the same time, expression of the α chain of the IL-2 receptor is induced, so that high affinity trimeric $\alpha\beta\gamma$ receptors can be formed, enhancing the effect of IL-2. Other costimulatory signals are transmitted through 4-1BB and its ligand 4-1BBL on the APC[241], or ICOS on T cells and its ligand LICOS on APCs[242]. The physical contact between APC and T cell is reinforced by several cell adhesion molecule pairs like ICAM-1/ LFA-1 or DC-SIGN/ICAM-3, thus forming a so-called "immunological synapse"[243]. It has been shown that only ten cognate MHC/peptide complexes are sufficient for complete activation of a T cell[244]. On the T cell the necessary TCR number is less than 1000, with about 40,000 TCRs present on each cell[245]. Activated $CD4^+$ T cells start expressing CD154 (CD40L), which binds to its receptor CD40 on the APC and in turn induces higher expression of CD80 and CD86. Probably by this mechanism, a $CD4^+$ T cell can facilitate the activation of a $CD8^+$ T cell in contact with the same APC[246].

Activated T cells are transformed from naïve into effector T cells which are characterized by a distinct phenotype. Early activation markers include CD25, CD69,

and CD154, while CD44 expression rises more slowly[247;248]. After four to five days expression of CTLA-4 ensues, a molecule similar to CD28 but with far higher affinity to CD80 and CD86. CTLA-4 binding leads to an inhibitory signal to the T cell[249] which is essential for limiting the initial proliferative response. TCR levels are also slightly down-regulated[250]. Effector CTLs can kill cells expressing the MHC/peptide complex they recognize without further stimulatory signals being needed. They express CD95L (Fas ligand) that can induce apoptosis by binding to its receptor on target cells[251]. Furthermore, CTLs produce effector molecules after activation and store them in cytotoxic granula[252], from which they can be quickly released on antigen contact. These include perforin, which disintegrates the plasma membrane of their targets[253], as well as granzymes, serine proteases that can enter target cells and induce an apoptotic cascade[254]. They also secrete cytokines like INF-γ, which increases the density of MHC molecules on target cells, as well as TNF and lymphotoxin.

1.2.6. Cytotoxic T cell memory

After clearance of an infection, most effector T cells undergo apoptosis, while some remain and form what has been called the immunological memory[255]. After re-infection with the same pathogen, these cells bear the brunt of the immune response since they do not need costimulation for activation and proliferate more rapidly than naïve T cells[256-258]. $CD8^+$ memory T cells have been divided into different subsets[259], the two most important of which are effector memory cells (T_{EM}) and central memory cells (T_{CM}). While naïve T cells express CD45RA and CCR7, the memory subsets show expression of CD45RO, T_{EM} being $CCR7^-$, T_{CM} $CCR7^+$. The differences in CCR7 expression mirror the migration patterns of the subtypes: While central memory cells show similar homing behavior as naïve T cells and circulate in the blood and lymphoid system, effector memory cells are found in the periphery more frequently[260]. The latter group has cytotoxic abilities and can produce IFN-γ, while T_{CM} cells mainly encourage T cell proliferation by the production of IL-2[261].

Another subset are the T_{EMRA} cells, similar to T_{EM} cells but expressing CD45RA instead of CD45RO[262]. Their phenotype is even more "effector-like", and it has been suggested that they are derived from T_{CM} cells upon homeostatic proliferation in the absence of antigen[263].

Which cells will differentiate into memory T cells seems to be determined early during infection[264;265]. Conflicting suggestions have been made as to which factors are responsible for this selection or whether it is merely contingent[266-268], and the question has not been settled yet[269]. For the generation of $CD8^+$ memory cells, $CD4^+$ T cell help has been shown to be essential[270], with the interaction between CD40 and CD154 being involved[271]. The memory cells themselves, however, need no $CD4^+$ help for a recall response.

1.2.7. T cell responses to adenovirus

Although adenoviruses have been known for a long time and have been studied thoroughly, comparatively little is known about T cell responses in humans. In findings consistent with the high frequency of persistent or latent infections, two studies showed that PBMCs of healthy donors exhibited proliferative responses to Ad2 virions in 29 of 30[272] and 20 of 22 cases[273]. E1A of different human serotypes has been long known to be strongly immunogenic in rodents [274-278], and several epitopes have been characterized. In humans, studies have focused on the hexon protein, with the first class I-restricted epitopes being reported in 2004[279]. In one study, the fiber knob has been shown to be the most effective part of the capsid in adenoviral vectors as an incorporation site of foreign antigens to induce T cell responses[280]. There has been considerable progress in the identification of MHC class II-restricted epitopes over the last years (Table 1.4), but regarding $CD8^+$ T cell epitopes the situation is quite different, with only twelve having been described up to now (Table 1.5).

Table 1.4: T cell epitopes from adenovirus inducing $CD4^+$ T cell responses in humans with their proteins of origin. When epitopes have been published in different length variants, the terminal sequences are in brackets. All epitopes are from serotype Ad5.

Sequence	Protein	Position	Reference
(QW)SYMHISGQDA(SEY)	hexon	8 - 22	[281-283]
SGQDASEYLSPGLVQFARATETYFS	hexon	15-39	[282]
(FARATETYFSL)NNKFRNPTVAPTHD	hexon	30-54	[282;284]
THDVTTDRSQRLTLR	hexon	52-66	[281]
(VTTDRSQRL)TLRFIPVDREDTAYS(Y)	hexon	55-79	[281;282]
VDREDTAYSYKARFTLAVGDNRVLD	hexon	70-94	[282]
(PTFKPYS)GTAYNALAPKGAPNP(CEW)	hexon	110-134	[281-283]
(QPEPQIGE)SQWYETEINHAAGRV(LK)	hexon	200-224	[281;282]
GQQSMPNRPNYIAFRDNFIG	hexon	315-334	[282]
LMYYNSTGNMGVLAG	hexon	335-349	[281]
TGNMGVLAGQASQLN	hexon	341-355	[281]
(VDSYD)PDVRIIENHGTEDELPNYCF(PLGGV)	hexon	390-414	[282]
TETLTKVKPKTGQEN	hexon	422-436	[281]
ENGWEKDATEFSDKN	hexon	435-449	[281]
(G)NNFAMEINLNANLW(RNFLYS)	hexon	454-474	[281;282]
ANLWRNFLYSNIALYLPDKLKYSPS	hexon	465-489	[282]
YDYMNKRVVA	hexon	500-509	[282]
VDCYINLGARWSLDY	hexon	513-527	[281;283;284]
GARWSLDYMDNVNPFNHHRNAGLRY	hexon	520-544	[282]
(VPFHIQV)PQKFFAIKNLLLLPG(SYT)	hexon	555-579	[281;282]
(FFAIKNLLLL)PGSYTYEWNFRKDVN	hexon	565-589	[281;282]
EWNFRKDVNMVLQSS	hexon	581-595	[281]
MVLQSSLGNDLRVDGASIKFDSICL	hexon	590-614	[282]
GASIKFDSICLYATF	hexon	604-618	[281]
ATFFPMAHNTASTLE	hexon	616-630	[281]
YLSAANMLYPIPANATNVPISIPSR	hexon	645-669	[282]
(TNVPISIPSR)NWAAFRGWAF	hexon	660-679	[282]
GWAFTRLKTKETPSL	hexon	676-690	[281]
(LGSGY)DPYYTYSGSIPYLDG(TFYLN)	hexon	690-714	[281;282]
(TFYLNHT)FKKVAITFDSSVS(WP)	hexon	710-731	[281;282]
RLLTPNEFEIKRSVDGEGYNVAQCN	hexon	735-759	[282]
VAQCNMTKDWFLVQMLANYN	hexon	755-774	[282]
(SYKDR)MYSFFRNFQPMSRQVVDDTK(YKDQ)	hexon	785-814	[282]
YKDYQQVGILHQHNNSGFVGYLAPT	hexon	810-834	[282]
(VDSIT)QKKFLCDRTLWRIPFSSNFM(SMGAL)	hexon	855-884	[282]
(E)VDPMDEPTLLYVLFE(VFDVVRVHR)	hexon	905-929	[282;284]
(DEP)TLLYVLFEV(FDV)	hexon	910-924	[285;286]
KCGSQILATVSVLAV	fiber	427-441	[284]
IIRFDENGVLLNNSF	fiber	458-472	[284]

Table 1.4 (continued):

Sequence	Protein	Position	Reference
SKEIPTPYMWSYQPQ	pIIIa precursor	2-16	[284]
NRILLEQAAITTTPR	pIIIa precursor	52-66	[284]
RQAILTLQTSSSEPR	pIIIa precursor	165-179	[284]
RPVLPANSTTLTYET	pIIIa	37-51	[284]
DDKLTALLAQLDSLT	pIIIa	97-111	[284]
LLDFLAMHLWRAVVR	E1B	114-128	[284]
QQKVVDGLASGISGV	pVI precursor	77-91	[284]
KPVLPGPTAVVVTRP	pVI precursor	192-206	[284]
IALMFVCLIIMWLIC	E3A-10.5K	39-53	[284]
LYGFVTLTLICSLIT	E3B-10.4K	40-54	[284]
FQRPTISSNSHAIFR	AdPol	475-489	[284]
IDYFDARLLPGVFTV	AdPol	585-599	[284]
VSQIFPDSVMLAVQE	E1A	62-76	[284]
IDLLTFPPAPGSPEP	E1A	78-92	[284]

Table 1.5: T cell epitopes from adenovirus inducing $CD8^+$ T cell responses in humans with their HLA restrictions. All epitopes were found in protein II (hexon) of serotype Ad5.

MHC allele	Sequence	Position	Reference
A*01	TDLGQNLLY	886-894	[279]
A*02	GLRYRSMLL	542-550	[282]
A*02	TFYLNHTFKK	711-721	[279]
A*02	LLYANSAHAL	892-901	[287]
A*02	YVLFEVFDVV	916-925	[287]
A*24	TYFSLNNKF	37-45	[279]
B*07	KPYSGTAYNSL	114-124	[279]
B*07	MPNRPNYIAF	320-329	[279]
B*13/B*49	LFEVFDVVRV	918-927	[287]
B*35	MPNRPNYIA	320-328	[282]
B*53	LPGSYTYEW	575-583	[282]
B*35/B*53	IPYLDGTFY	705-713	[282]

2. MATERIALS AND METHODS

2.1. Theoretical background

2.1.1. SYFPEITHI as a prediction tool for T cell epitopes

SYFPEITHI is a software tool for predicting possible epitopes restricted to certain MHC class I and II alleles from amino acid sequences[191]. Predictions are based on a database of published natural ligands of the respective allotypes, for which peptide motifs have been determined through sequence comparisons. Every amino acid of the candidate epitope is attributed a score depending on its position and the peptide motif of the considered allotype. The aggregate score of all amino acids is then listed as the epitope score. An actually recognized T cell epitope is expected to be found in the best 2% of SYFPEITHI predictions from a given sequence with a reliability of about 80% (http://www.syfpeithi.de).

2.1.2. Refolding of MHC class I/peptide complexes

Although most human proteins need the help of chaperones to attain their native conformation, some denatured proteins can spontaneously refold to their functional form *in vitro* if suitable pH and redox conditions are applied[288]. Protein complexes can be obtained in this manner, too, if all subunits necessary are present[289;290]. Peptide-MHC-monomers are synthetic complexes of peptide, β_2-microglobulin, and a modified MHC class I heavy chain allotype in which the cytoplasmic and transmembrane portion of the protein has been replaced by a biotinylation sequence[291] for the *Escherichia coli*-derived enzyme BirA (also called BHS). For the refolding of MHC class I/peptide complexes, comparably high amounts of denatured heavy chain, β_2 microglobulin, and peptide have to be used, since the complex is stable only if all three components are present, thus requiring improbable ternary

interactions to form. On the other hand, the complex is very stable once it has formed. Another nuisance is the propensity of MHC heavy chains to form aggregates that eventually precipitate from the solution[292]. To keep this process in check, the refolding is carried out in a buffer containing arginine, a weakly chaotropic compound. The component protein chains are stored in a strongly chaotropic buffer containing urea. Before the refolding, an acidic buffer with pH 4.2 is added, and the proteins are injected into stirred refolding buffer through a needle as fast as possible to inhibit aggregation. The redox potential is adjusted by adding oxidized and reduced glutathion, and PMSF is used as a protease inhibitor. The heavy chain has to be added several times over time to compensate for the loss through aggregates. After the refolding, the solution is passed through a filter membrane that holds back proteins and complexes. The flowthrough contains enough peptide for another round of refolding. Nevertheless, in our system only about 1% of original peptide is bound in the complex, which is separated from uncomplexed proteins and aggregates with FPLC.

2.1.3. Fast protein liquid chromatography (FPLC)

FPLC can be used to separate proteins or protein complexes efficiently on chromatographic columns by applying pressure and achieving high flow rates. In this work, the size exclusion variant was used, separating proteins according to their size. The sample is passed over a column containing a gel that consists of small porous beads. Typical bead sizes are 3 – 100 μm with pore sizes of 10 – 100 nm. In this case, a Sephadex 75 column was used, which consists of cross-linked agarose beads coated with dextran. Large molecules are excluded from most pores and are therefore eluted faster, while smaller molecules remain in the beads longer. Thus, the first fractions eluted contain the largest molecules or aggregates. The flowthrough is analyzed with a UV spectrometer at 280 nm to measure the protein concentration.

2.1.4. Biotinylation with BirA and tetramerization

Single MHC class I/peptide complexes can be cross-linked to tetramers with streptavidin after enzymatic biotinylation at the lysine of the inserted biotinylation sequence LHHIFEAQKILWRHR. Streptavidin is a 60 kDa protein from *Streptomyces avidinii* that has four binding sites for biotin with very low dissociation constants. Biotinylation is achieved by BirA ligase, an enzyme from *Escherichia coli* that can catalyze the formation of a peptide bond between the ε-amino group of a lysine residue and the carboxy group of biotin coupled with ATP hydrolysis. Tetramers bind with high affinity to T cells with specific T cell receptors and have been used for over a decade for identifying T cells specific for a peptide presented by a given MHC allotype[293-295]. If the streptavidin used for tetramerization is coupled to a fluorescent dye, the tetramers can be used as probes in flow cytometry (see 2.1.9).

2.1.5. Bradford assay

The Bradford assay is used to determine protein concentrations[296]. It is based on the binding of the dye Coomassie brilliant blue G-250 (Fig. 2.1) to arginine, tryptophan, phenylalanine, histidine, and tyrosine residues in acidic solution. Coomassie dye binds to proteins in its blue anionic form, while free Coomassie dye is red-brown at low pH. The absorption maximum shifts from 470 to 595 nm. Protein concentrations are determined by comparing the OD_{590}/OD_{450} ratios of the samples to those of standard protein solutions (usually BSA)[297].

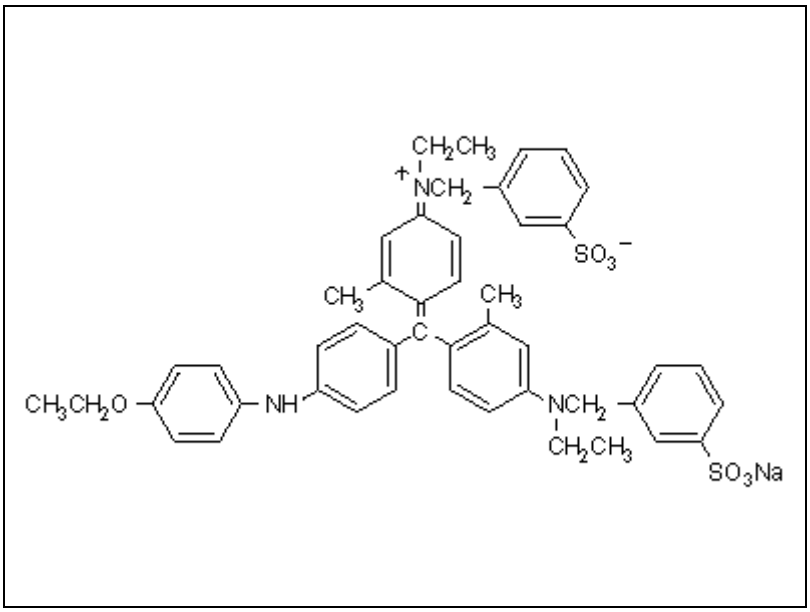


Figure 2.1: Chemical structure of Coomassie brilliant blue G-250. Formula composed by using ISIS Draw.

2.1.6. ELISPOT

The ELISPOT (enzyme-linked immunospot) assay is a method to monitor cytokine secretion of cells after stimulation on a single cell level[298;299] with a detection threshold of about one cell in 10^5[300]. The assay is usually carried out in microtiter plates that have a nitrocellulose membrane at the bottom of the wells. After coating the membrane with a primary antibody against the cytokine to be detected (Fig. 2.2), the target cells are transferred into the wells and stimulated (e.g. by adding peptide to PBMCs). The plates are incubated without any jolts or vibrations so that each cell can settle in a defined position on the membrane. Secreted cytokines are bound by the antibodies in a halo around the secreting cell. After incubation the cells are removed and a biotinylated second antibody against the same cytokine is added, binding only to the spots where activated cells had been. To visualize the spots, an avidin-coupled enzyme is bound to the secondary antibody, which can catalyze the formation of an insoluble colored product. After incubating with the substrate of the enzyme, the spots can be seen and counted, each marking the position of one activated cell.

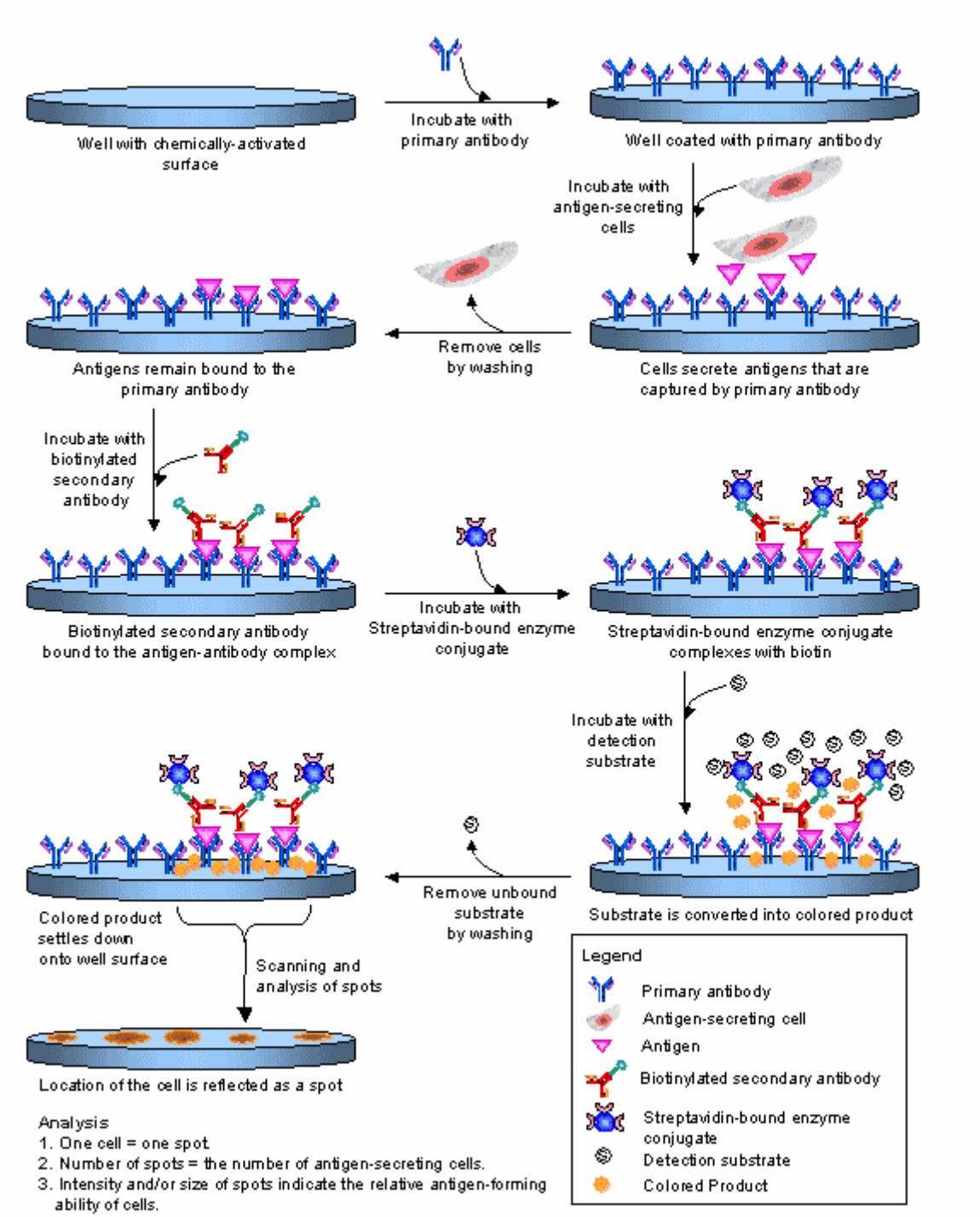


Figure 2.2: Schematic illustration of the different steps in an ELISPOT assay. Adapted from http://www.sigmaaldrich.com/etc/medialib/life-science/custom-dna-oligos/elispot-assay-procedure.Par.0001.Image.573.gif

In this work, the avidin-coupled enzyme was an alkaline phosphatase, and BCIP (5-bromo-4-chloro-3-indolyl phosphate) was used as a substrate together with NBT (nitroblue tetrazolium chloride). After hydrolysis of the phosphate group of BCIP by the enzyme, the resulting indoxyl derivative can be oxidized by NBT, and two insoluble blue products are formed (Fig. 2.3).

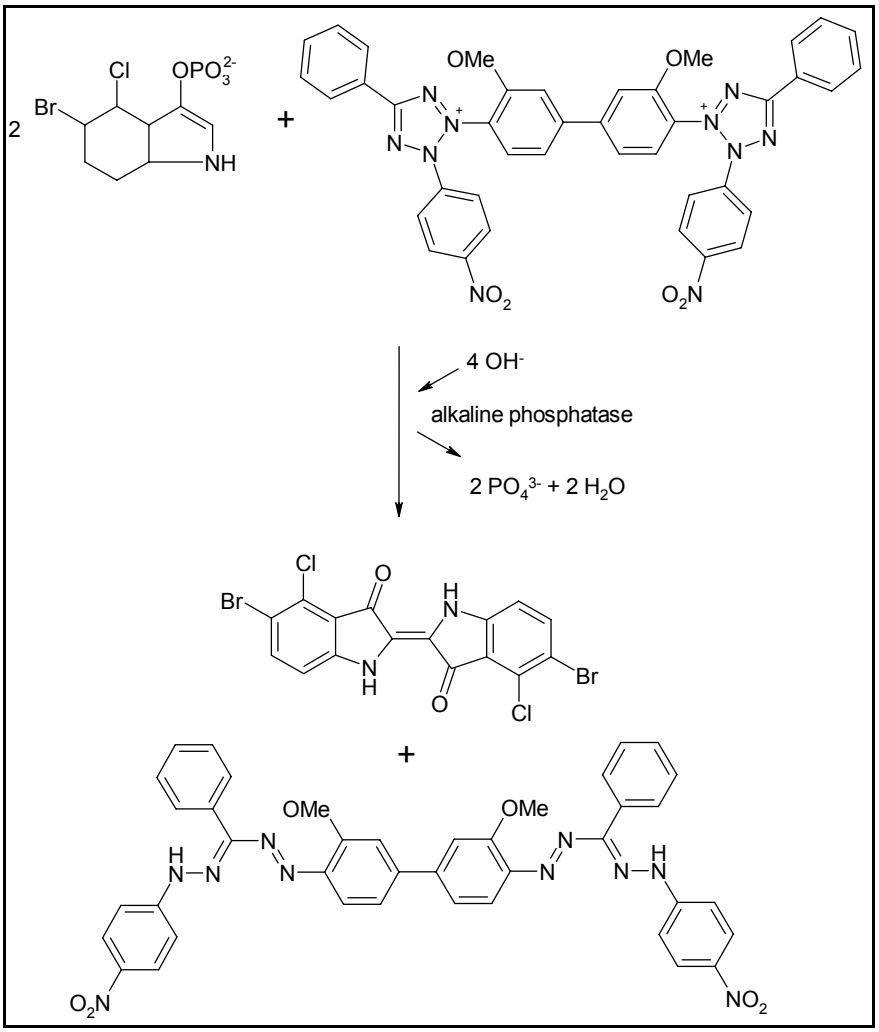


Figure 2.3: Enzymatic reaction yielding the colored products in the ELISPOT assay. Formula composed by using ISIS Draw.

2.1.7. Flow cytometry

Flow cytometry allows for the analysis of cellular properties on the level of single cells. Cell surface molecules are stained with fluorochrome-labelled probes (e.g. antibodies, receptors, receptor ligands) and the cells then analyzed for the fluorescence they emit after excitement with a laser. To achieve analysis on the single cell level, a single cell suspension is passed through a laser beam as a series of drops after hydrodynamic focussing, with each drop containing one cell at most. The intensity of fluorescent light emitted correlates with the amount of fluorochrome bound to the cell. Furthermore, the optical diameter of the cell ("forward scatter",

FSC) and its granularity ("side scatter", SSC) can also be determined. Flow cytometry can be performed with several different fluorochromes at the same time if the excitation or emission wavelengths are not too close to each other. Modern flow cytometry machines sequentially employ three or four lasers with different wavelengths for excitation and up to 18 channels for measurement of emitted light. Of the cytometers used in this work, the FACSCalibur has three lasers and four channels for measurement, while the FACSCanto II has four lasers and eight channels. Fluorescence of different fluorochromes can be distinguished from each other if the fluorochromes are either excited with different lasers or emit light of sufficiently different wavelengths to be measured by different channels (or both, of course). In conjugated fluorochromes (e.g. PE-Cy7 or APC-Cy7) fluorescence resonance energy transfer (FRET) takes place between the two parts of the molecule. Thus they are excited by the excitation wavelength of one part of the conjugate, but emit light at the emission wavelength of the other part, making them especially useful in flow cytometry. Nevertheless, interference between the light emitted by different fluorochromes in an assay routinely takes place, since emission is not confined to single wavelengths but forms spectra that can stretch over the detection wavelengths of more than one channel for some fluorochromes[301]. Consequently, compensation staining for the different channels must be carried out before each measurement.

The fluorochromes used in this work with their excitation and emission wavelengths are summarized in Table 2.1.

Table 2.1: Fluorochromes used in flow cytometry with their optimal excitation and maximum emission wavelengths. Adapted from http://flowcyt.salk.edu/fluo.html

Fluorochrome	**Excitation** (nm)	**Emission** (nm)
Pacific Blue (PB)	403	455
Fluorescein isothiocyanate (FITC)	495	519
LIVE/DEAD® aqua	405	530
R-phycoerythrin (PE)	480	578
Propidium iodide (PI)	536	617
Allophycocyanin (APC)	650	660
AlexaFluor 647	652	668
Peridinium chlorophyll (PerCP)	490	675
R-phycoerythrin-Cy7 (PE-Cy7)	480	767
Allophycocyanin-Cy7 (APC-Cy7)	650	767

2.1.8. Intracellular cytokine staining

Intracellular cytokine staining is a method that enables the analysis of individual cytokine producing cells by flow cytometry. Thus the origin of cytokines in an experimental setup can be assigned to specific cell populations or subtypes without the need for previous separation of cells, and the phenotype and further characteristics of cytokine producing cells can be analyzed. The basic principle is the induction of cytokine production and the inhibition of secretion by the producing cells, leading to an accumulation of the produced cytokines in their cells of origin, and the subsequent staining of these cells with fluorescent antibodies after permeabilization of their cell membranes. In this study, brefeldin A was employed for the inhibition of secretion. This compound is a lactone antibiotic (Fig. 2.4) produced by the fungus *Eupenicillium brefeldianum* that blocks the vesicular transport from the endoplasmic reticulum to the Golgi system[302]. Another widely used reagent for this purpose is monensin, which has the slightly different effect of blocking translocation between the *cis*- and *trans*-Golgi networks[303].

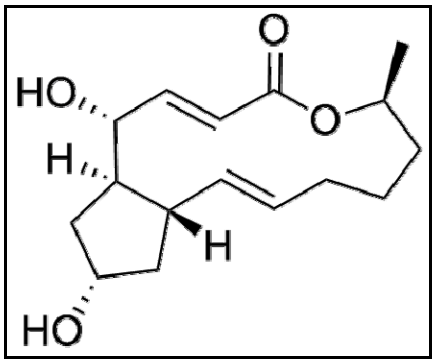


Figure 2.4.: Brefeldin A
Formula composed by using ISIS Draw.

After trapping cytokines inside cells in this manner, a buffer containing detergents and paraformaldehyde is used to permeabilize the cell membrane and fix the cell contents at the same time. Afterwards, fluorescent antibodies for staining can be employed.

Of course, this method can be combined with the staining of surface antigens (e.g. CD4 or CD8) beforehand. In some experiments in this work, staining for the degranulation marker CD107a (LAMP-1) was also performed immediately after cell stimulation. This glycoprotein is present in the membrane of lysosomes[304] and cytotoxic granules of T cells and is detectable on the cell surface immediately after degranulation[305;306].

2.1.9. Tetramer staining

This method utilizes fluorescently labelled MHC class I/peptide tetramers to mark $CD8^+$ T cells that are specific for them. After the first demonstration of the high binding affinity of tetramers and their use for the analysis of cytotoxic T cells[293], it has been shown that tetramer binding is temperature dependent[307] and highly specific[308]. Tetramer staining is very sensitive and easily allows the identification of tetramer-specific T cell populations of as few as 0.1% of $CD8^+$ cells among whole PBMCs, but sensitivities of down to 0.01% of $CD8^+$ cells have been described[309]. As might be expected, the method is also compatible with the staining of surface

antigens. However, care should be taken in the selection of the antibody used for CD8 staining, since some antibodies can block tetramer binding[310], quite contrary to others that increase unspecific binding[311]. On the other hand, binding of tetramers to CD8 alone (and not the TCR and CD8) can lead to a high unspecific staining with tetramers of some allotypes. In this case, heavy chains with the point mutation A245V can be used for tetramer synthesis, decreasing the CD8 binding affinity[312] while maintaining the binding to TCR[313]. Tetramer staining can also be combined with intracellular cytokine staining[314], but in this case T cells that have been stimulated with their specific peptide are likely to have impaired tetramer-binding capability[315].

2.1.10. Cytotoxicity assays

Cytotoxicity assays are used to determine if a certain type of cells, called "effector cells", are capable of killing another type, called "target cells". Several methods can be used to quantify the lysis of target cells and thus the extent of killing. The ^{51}Cr release assay[316] utilizes the fact that chromium in the oxidation state +VI is easily absorbed by most eukaryotic cells and reduced to Cr^{3+}, which in turn cannot cross membranes easily[317]. Target cells are fed with $Na_2{}^{51}CrO_4$ that contains a radioactive chromium atom and is absorbed by the cells. After reduction to Cr^{3+}, the radioactive ions are set free into the surrounding medium only after the lysis of the labelled cells. Thus the radioactive activity of the supernatant after incubation of the targets with effectors is a measure for the amount of previous lysis. The main advantages of this technique are the easy experimental setup and the quick measurement procedure. Drawbacks include the necessity to handle radioactive compounds and to dispose of contaminated materials and the comparatively low sensitivity that requires large numbers of targets and in consequence effectors.

Another method that has been established less than a decade ago is the so-called fluorolysis assay[318]. Here target cells are labelled fluorescently rather than

radioactively, and lysis is calculated by measuring the number of surviving fluorescent target cells after incubation with effectors by flow cytometry. Originally, GFP-transfected target cells were used, but soon the much easier method of labelling the targets with fluorescent dyes has been established[319]. This method is far more sensitive than the chromium release assay and requires lower cell numbers, but has the important disadvantage that for every data point of each triplicate a sample has to be measured by flow cytometry, greatly increasing the time necessary for measurement.

2.2. Materials

2.2.1. Devices

Cell culture hood (Technoflow, Integra Biosciences)
Centrifuge (5415R, Eppendorf)
ELISA reader Spectramax 340 with software Softmax Pro 2.1 (Molecular Devices)
ELISPOT reader Immunospot with software Immunospot 3.2 and Image Acquisition 4.5 (Cellular Technologies Ltd.)
Flow cytometer FACS-Calibur with software Cell Quest Pro (Becton Dickinson)
Flow cytometer FACS-Canto II with software FACSDiva (Becton Dickinson)
FPLC system (ÄKTA prime, Amersham Biosciences)
Incubator for agar plates (Heraeus B6)
Incubator for cell cultures (Labotect 3250) with 5% CO_2 gas supply (Messer)
Light microscope (Leica DMIL)
Magnetic stirrer (RCT basic, IKA Labortechnik)
Membrane pump (KNF Neuberger)
Microcuvettes, 1 cm, plastic (Brand)
Microcuvette, 1 cm, quartz glass (Hellma)
Neubauer counting chamber, depth 0.1 mm (LO-Laboroptik)
Nitrogen pressure bottle (AGA Gas GmbH)
pH-meter (Knick 765)
Shaking incubator (Infors Multitron)
Spinning wheel (Bachofer)
Steam sterilizer (KSG 113)
Steam sterilizer (Sauter 11-6-9 HS1-FD)
Tabletop centrifuges (Heraeus Biofuge fresco; Heraeus Megafuge)
Ultracentrifuge (RC 5C Plus, Sorvall) with rotors (SLA 3000; SS34)

Ultrasonic cell disruptor (Sonifier 250, Branson Ultrasonics)
UV-Vis-spectrometer (Ultraspec 3000, Pharmacia)
Vortex (MS 1, IKA-Works Inc.)
Water bath (Thermomix BM-S, Braun Biotech)
Water distiller (Destamat Bi 18 E, Heraeus)

2.2.2. General materials

96 well plates (3799, Corning)
Cell culture flasks, 50 ml, 250 ml, 500 ml (Bio-one, Greiner)
Concentration tubes, NMWL 10,000 (Amicon Centricon-10, Millipore)
Culture tubes, 14 ml (352059, Becton Dickinson)
Cryotubes, 2 ml (Greiner)
Cryotubes, dark (Biozym)
ELISPOT plates, 96-well (MAHABN4510 opaque, Millipore)
FACS tubes, 0.5 ml (Greiner)
FACS tubes, 5 ml (352058, Becton Dickinson)
Filter membrane, NMWL 30,000 (Biomax 30 PBTK, Millipore)
Glass bottles (Schott)
Glass douncer, 60 ml, with piston "S" (Braun)
Injection needles (Sterican 4657683, Braun)
Reaction vials (1.5 ml, Eppendorf)
Sterilization filters, 0.22 μm (Corning)
Stirred cell, 400 ml (Amicon, Millipore)
Syringes, 1 ml and 10 ml (Becton Dickinson)
Tubes, 15 ml and 50 ml (Falcon)
Ultrafiltration tube, NMWL 10,000 (Amicon Ultra-15, Millipore)
Vacuum filters, 250 ml and 500 ml (Steritop, Millipore)

2.2.3. Buffers and solutions

Ampicillin 1000X:
100 mg/ml ampicillin sulfate (Roth) in ddH_2O

Bradford reagent 5X (Roti-Nanoquant, Roth)

Chloramphenicol 1000X:
34 mg/ml chloramphenicol (Sigma) in 99% ethanol (Merck)

Culture medium:
IMDM with
1% PenStrep
50 μM β-mercaptoethanol (Roth)
2% human serum
sterile

FACS-Clean (Becton Dickinson)

FACS-Fix:
PFEA with
1% 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)
sterile

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

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

Injection buffer:
ddH_2O with
3 M guanidinium chloride (Fluka)
10 mM sodium acetate (Sigma)
10 mM EDTA (Roth)
pH adjusted to 4.2

LB-Amp/Cam:
LB medium with
0.1% Ampicillin 1000X
0.1% Chloramphenicol 1000X
sterile

LB medium:
ddH_2O with
1% Bacto Trypton (Difco)
0.5% Bacto Yeast Extract (Difco)
0.5% NaCl (Merck)
sterilized at 121°C

Leupeptin 1000X:
ddH_2O with
0.1% leupeptin (Roche)

Lymphocyte separation medium (PAA Laboratories)

PBEA:
PBS with
0.5% BSA (Sigma)
2 mM EDTA (Roth)
0.01% NaN_3 (Merck)

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

PBS-BSA:
PBS with
0.5% BSA (Sigma)
sterile filtered

PBS-Tween:
PBS with
0.05% Tween 20 (Serva)
sterile filtered

PenStrep (DE17-602E, BioWhittaker)

Permwash:
PBS with
0.1% saponin (Sigma)
0.5% BSA (Sigma)
0.01% NaN_3 (Merck)

Pepstatin 1000X:
methanol (Merck) with
0.07% pepstatin

PFEA:
PBS with
2% FCS
2 mM EDTA (Roth)
0.01% NaN_3 (Merck)

PMSF 1000X:
methanol (Merck) with
200 mM PMSF

Refolding buffer:
ddH_2O with
6.97% L-arginine (Sigma)
2.38% HEPES (Roth)
2 mM EDTA (Roth)
pH adjusted to 7.76 at 10°C
sterilized at 121°C

Resuspension buffer:
ddH_2O with
50 mM Tris pH 8.0 (Sigma)
100 mM NaCl (Merck)
1 mM DTT (Sigma)
1 mM EDTA (Roth)
0.1% NaN_3 (Merck)

RPMI medium (BioWhittaker)

TBS:
20 mM Tris pH 8.0 (Sigma)
150 mM NaCl (Merck)
sterilized at 121°C

TBSA:
TBS with
0.01% NaN_3 (Merck)

T cell medium:
IMDM with
1% PenStrep
10% human serum
20 μg/ml gentamycin sulfate (BioWhittaker)
sterile

Thawing medium:
IMDM with
1% PenStrep
50 μM β-mercaptoethanol (Roth)
2% human serum
3 μg/ml DNase I
sterile

Triton buffer:
ddH_2O with
50 mM Tris pH 8.0 (Sigma)
100 mM NaCl (Merck)
0.5% Triton X 100 (Sigma)
1 mM DTT (Sigma)
1 mM EDTA (Roth)
0.1% NaN_3 (Merck)

TSB:
50% PBS
50% FCS
2 mM EDTA (Roth)
0.01% NaN_3 (Merck)

Urea buffer:
ddH_2O with
8 M urea (UBC)
10 mM Tris pH 8.0 (Sigma)
10 mM NaH_2PO_4 (Merck)
0.1 mM EDTA (Roth)

X-Vivo 15 medium (Lonza)

2.2.4. Peptides

All peptides had been synthesized in the laboratory using standard Fmoc chemistry, quality control being carried out by HPLC and mass spectrometric analysis. For ELISPOT and peptide stimulations, peptides were stored in aliquots of 1 mg/ml in 10% DMSO at -80°C.

2.2.5. Fluorescent antibodies

Table 2.2: Antibodies used for surface and intracellular stainings for flow cytometry. All antibodies are mouse-derived, except the α-IL-2 antibody, which is rat-derived.

specificity	clone	coupled dye	dilution factor	source
CCR7	3D12	AlexaFluor647	1 : 20	Becton Dickinson
CD3	UCHT1	Pacific Blue	1 : 100	Biolegend
CD4	OKT4	FITC	1 : 400	prepared in house
CD4	SK3	APC	1 : 50	Becton Dickinson
CD4	RPA/T4	APC-Cy7	1 : 100	Becton Dickinson
CD8	OKT8	FITC	1 : 400	prepared in house
CD8	SK1	PerCP	1 : 25	Becton Dickinson
CD8	SFC121Thy2D3	PE-Cy7	1 : 120	Beckman Coulter
CD45RO	UCHL1	FITC	1 : 5	Becton Dickinson
IFN-γ	B27	FITC	1 : 200	Becton Dickinson
IFN-γ	B27	PE	1 : 200	Becton Dickinson
IFN-γ	4S.B3	PE-Cy7	1 : 400	Becton Dickinson
IL-2	MQ1-17H12	PE	1 : 100	Becton Dickinson
TNF	Mab11	Pacific Blue	1 : 120	Biolegend

2.2.6. Other compounds

Agar (Difco)
Alkaline phosphatase, avidin-conjugated (E2636, Sigma)
ATP (Sigma)
BCIP/NBT tablet (B5655, Sigma)
CFSE dye (Invitrogen)
Cytoperm/cytofix solution (Becton Dickinson)
D-biotin (Sigma)
Brefeldin A (Becton Dickinson)
BSA (Sigma)
DMSO (Merck)
DNase I, grade II (Boehringer)
EDTA (Roth)
Ethanol (SAV LP)
Far Red dye (Invitrogen)
Formaldehyde ≥37% (Fluka)
Glutathion, oxidized (Sigma)

Glutathion, reduced (Sigma)
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 (R&D Systems)
Ionomycin (Sigma)
IPTG (Peqlab)
LIVE/DEAD aqua dye (Invitrogen)
$MgCl_2$ (Merck)
$Na_2{}^{51}CrO_4$ (Hartmann Analytik)
NaN_3 (Merck)
L-PHA (Roche)
PMA (Sigma)
Propidium iodide (Fluka)
Streptavidin-APC, 1 mg/ml (S-868, Molecular Probes)
Streptavidin-PE, 1 mg/ml (S-866, Molecular Probes)
Tris (Sigma)
Trypan blue (Merck)

2.2.7. Blood donors

Erythrocyte-depleted, heparinized peripheral blood of healthy blood donors was obtained from the department for transfusion medicine of the university hospital (Abteilung für Transfusionsmedizin, Universitätsklinikum Tübingen). Low resolution HLA typing had also been performed by the same department. All donors and their HLA typing are listed in the Appendix.

2.3. Methods

2.3.1. Synthesis of recombinant MHC heavy chains

Freshly transformed bacteria were used for the expression of MHC heavy chains. The sequences for truncated forms of the HLA alleles to be expressed (without the transmembrane domain and with a BirA biotinylation sequence at the C terminus) had been cloned into the expression vector pET-3d. 0.2 µl of this solution were added to 50 µl of BL21(DE3) *E. coli* carrying the plasmid pLysS and the mixture incubated on ice for 20 min before being transferred to a 42°C water bath for 90 s. After brief chilling on ice, 1 ml of LB medium was added and the samples incubated in a water bath at 37°C for 30 min. Finally, 100 µl of the sample were transferred onto an LB-

Amp/Cam-agar plate (LB medium with 1.5% agar, 100 µg/ml ampicillin sulfate and 50 µg/ml chloramphenicol) and incubated overnight at 37°C.

Colonies from the plates were picked, transferred to 5 ml LB-Amp/Cam and incubated in the shaker at 37°C and 250 rpm for 8 h. The culture was then added to 100 or 200 ml LB-Amp/Cam and shaken overnight at 37°C and 180 rpm, while five or ten 2 l Erlenmeyer flasks containing 1 l LB-medium each were also incubated at 37°C. The next morning, each of the flasks was inoculated with 15 ml of the overnight culture, 1 ml ampicillin 1000X and chloramphenicol 1000X were added, and the cultures were incubated in the shaker at 37°C and 180 rpm. The OD_{600} was measured repeatedly. When it had reached a value between 0.4 and 0.6, 0.5 µl of an 1 M IPTG solution were added to each flask to induce recombinant protein expression and the cultures were incubated for four more hours. Thereafter, they were centrifuged with an SLA 3000 rotor at 4°C and 5000 rpm for 20 min and the supernatant was discarded. The pellets were resuspended in a total of 180 ml PBS and stored in six 30 ml aliquots at -80°C.

After thawing in a water bath at room temperature, the aliquots were treated with ultrasound three times for 2 min each (output 5, duty cycle 50%). Then 150 µl of 10 mg/ml DNAse I and 300 µl 1 M $MgCl_2$ were added and the DNA digested at 37°C for 20 min. Now the lysate was centrifuged in an SS34 rotor at 4°C and 15000 rpm for 20 min and the supernatant was discarded. The pellets consisted of a denser, whiter lower layer (inclusion bodies) and a lighter, grey-brown upper layer (remnants of cell walls and membranes). After scraping off the upper layer carefully, triton buffer was added to the inclusion bodies and the suspension was homogenized in a douncer before being centrifuged as before. Washing with triton buffer was repeated until the inclusion bodies became as white as possible. Afterwards, they were dounced in resuspension buffer, the suspension centrifuged as before, and the pellets taken up together in 20 to 40 ml urea buffer (depending on pellet size). The suspension was rotated on a spinning wheel at 4°C overnight. Insoluble parts were separated by centrifugation the next day (15000 rpm for 20 min, SS34 rotor).

The purity of the protein solution was checked by comparing the OD_{260} to the OD_{280}. The quality was regarded as sufficient if the ratio was below 0.6. The concentration was determined with a Bradford assay (see 2.3.4.). Aliquots containing 8 mg protein were stored in cryotubes at -80°C.

2.3.2. Refolding of MHC/peptide complexes (“monomers”)

2.5 to 7.5 mg of the synthetic peptide to be placed on the MHC complex were weighed out and solved at a concentration of 10 mg/ml in DMSO. An 8 mg aliquot of recombinant MHC α chain protein were thawed at room temperature and urea buffer was added to a total volume of 400 µl. After adding 700 µl injection buffer, the

solution was taken up into a 1 ml syringe with a 26G needle. Likewise, a 7 mg aliquot of recombinant β_2 microglobulin in urea buffer was thawed, mixed with 780 µl injection buffer, and taken up into a syringe, too. Then 250 ml of cold refolding buffer were stirred rapidly at 4°C on a magnetic stirrer, so that a vortex formed. 385 mg reduced glutathion, 77.5 mg oxidized glutathion, and 250 µl PMSF 1000X were added. The peptide solution was added drop by drop, and the heavy and light chain proteins were injected rapidly with the needle to inhibit aggregate forming. Approximately 12 h later, another aliquot of heavy chain was added in the same manner, and a third time again another 12 h later.

After 12 more hours aggregates were filtered out with a 0.22 µm vacuum filter and a membrane pump. A stirring cell was assembled and the solution concentrated with an NMWL 30,000 filter membrane at 4 bar pressure from a nitrogen bottle until about 25 ml of retentate were obtained. The retentate was stored at 4°C, while the permeate was used in a second refolding process. Peptide and glutathion did not need to be added this time. The filter membrane was stored in regeneration buffer and could be reused in the second stirring cell concentration step. After the second refolding, the retentate of the first step was added to the solution and the stirring cell process was repeated. The retentate was then further concentrated in an NMWL 10,000 ultrafiltration tube at 4°C and 4000 rpm (Megafuge) to a final volume of 5 ml.

This retentate was then applied to a Superdex 75 column and separated at a flow rate of 3 ml/min and TBSA. Between 100 and 200 ml flowthrough volume, 5 ml fractions were collected while the absorption at 280 nm was monitored. The fractions containing the monomer were pooled and 0.1% of PMSF 1000X, pepstatin 1000X and leupeptin 1000X each were added.

2.3.3. Biotinylation with BirA

Freshly refolded monomer solution was concentrated in an NMWL 10,000 ultrafiltration tube at 4°C and 4000 rpm (Megafuge) to a final volume of 5 ml. This retentate was mixed with 400 µl 1 M Tris (pH 8.0 at 25°C), 25 µl 1 M $MgCl_2$, 250 µl 100 mM ATP, 28.5 µl 100 mM biotin, and 9.3 µl BirA enzyme solution with a concentration of 2.15 µg/µl. BirA had been expressed in our laboratory using standard recombinant expression procedures. The mixture was incubated in a water bath at 27°C for 12 to 16 h.

After the biotinylation reaction, the mixture was applied to an FPLC system with a Superdex 75 column as after refolding and separated the same way. The fractions containing the biotinylated monomer were pooled. 0.1% of PMSF 1000X, pepstatin 1000X and leupeptin 1000X each were added, as well as 10% NaN_3 and 0.5 M EDTA to final concentrations of 0.1% and 2 mM, respectively. The solution was then again concentrated as before to a volume of about 200 µl. The exact volume was

determined by weighing (density of 1 g/cm^3 assumed), while the protein concentration was measured with a Bradford assay (see 2.3.4.) and adjusted to 2 mg/ml with TBSA. Centricon tubes with NMWL 10,000 were used for concentration in the Megafuge if the initial concentration was too low. The identity of the peptides in the monomers was checked using MALDI mass spectrometry. Aliquots of 50 µg were stored in dark cryotubes at -80°C.

2.3.4. Bradford assay

Protein concentrations of biotinylated monomers and also of recombinant MHC heavy chains were determined using a Bradford assay. Standard solutions of BSA (concentrations: 0 µg/ml; 20 µg/ml; 40 µg/ml; 60 µg/ml; 80 µg/ml; 100 mg/ml) in TBS for monomers or in 8 M urea solution for MHC heavy chains were used as references. The samples were prepared in two different dilutions whose concentrations were expected to be in the range of the references. The assay was carried out in a flat bottom 96 well plate. 50 µl sample volume per well were pipetted in triplicates for every dilution, while one well was used for each reference. Then 1 ml of Bradford reagent 5X was diluted with 4 ml ddH_2O, and 200 µl of this solution added to each well. After 5 min incubation at room temperature the OD_{590}/OD_{450} ratios of the samples were determined using an ELISA reader. The protein concentrations were calculated by comparison with the reference values.

2.3.5. Tetramerization of monomers with streptavidin

To obtain MHC-peptide-complex tetramers from biotinylated monomers for tetramer staining of T cells, conjugation with fluorochrome-labeled streptavidin was applied (concentration 1 mg/ml). The fluorochromes PE (R-phycoerithrin) and APC (allophycocyanin) were used. The labeled streptavidin had to be added to the monomers in a stoichiometric ratio of 1:4. For a monomer aliquot of 50 µg, 78.5 µl streptavidin-PE or 43.5 µl of streptavidin-APC were needed. For this work, always only half of an aliquot was used for tetramerization, the other half was frozen again and stored at -80°C. To achieve maximal saturation of the streptavidin, it was added in ten steps, always 10% of the final amount at a time. Between two steps, the sample was rotated on the spinning wheel at 4°C in the dark for at least 30 min. After the final addition, the tubes were quickly spun down and stored in the dark at 4°C. The tetramers could be used for staining for about 5 weeks.

2.3.6. Isolation of PBMCs

PBMCs were isolated from erythrocyte-depleted, heparinized peripheral blood samples (see 2.2.7). One bag contained about 30 ml. This volume was diluted to 120

ml with PBS. After four 50 ml tubes had been prepared by adding 15 ml of lymphocyte separation medium, 30 ml of the diluted blood were carefully pipetted onto the separation medium layer in order to obtain two phases. The tubes were then centrifuged for 20 min at 20°C and 2000 rpm (Megafuge) with the brake switched off. After centrifugation, PBMCs had formed a layer between the upper plasma phase and the separation medium, while erythrocytes and neutrophils had settled in a pellet at the bottom of the tube. The PBMCs were carefully sucked off with a pipette, pooled, and washed twice with PBS. Finally, they were suspended in freezing medium and stored at -80°C in cryotubes.

2.3.7. Cell counting

Cells were counted using a Neubauer counting chamber with 0.1 mm depth. The counting chamber was cleaned with 70% ethanol and moistened a little. Then the cover-glass was pressed onto the chamber until Newton rings could be seen. After drying the chamber, about 15 µl of cells suspended in 0.05% trypan blue and 0.02% NaN_3 were carefully pipetted under the cover-glass. The cells were counted in two large squares (each 1 mm^2) and the mean value was calculated. The density of the cells in the solution amounted to this multiplied with 10,000 per ml.

2.3.8. Expansion protocol for PBMCs

PBMCs were stimulated with peptide pools for eleven days to reinforce memory T cell responses. PBMCs were thawed by taking up each aliquot into 10 ml thawing medium and washing once at 20°C and 1300 rpm for 6 min (Megafuge). Then they were counted (see 2.3.7.) and sown out into wells of a 24 well plate in T cell medium (about $5 \cdot 10^6$ cells in 500 µl per well). On the second day, 500 µl of T cell medium were added to each well containing the desired peptides at 2 µg/ml each. Pools of up to six adenoviral peptides and a control epitope from HIV were employed. On the fourth day, 500 µl T cell medium were added again containing 6 ng/ml IL-2 (final concentration of 2 ng/ml in the well). Two days later, 500 µl of medium were carefully pipetted off the top of the well in order not to remove any cells, and 500 µl T cell medium were added with 2 ng/ml IL-2. From now on, the wells were carefully monitored, and 500 µl of medium were exchanged once it started to turn yellow, which could be as often as twice a day if cells were proliferating very boisterously. On the thirteenth day, further experiments were performed with the cells.

2.3.9. IFN-γ ELISPOT

IFN-γ ELISPOT assays were carried out in 96-well ELISPOT plates with a nitrocellulose membrane at the bottom of the wells. To coat the plates, they were

incubated with a 1:500 dilution of the primary α-IFN-γ antibody at 4°C at least for 12 h. Incubation could be extended for up to six weeks. For *ex vivo* assays, frozen PBMCs were thawed by taking up each aliquot into 10 ml thawing medium. After washing once more with 10 ml thawing medium (20°C, 6 min, 1300 rpm, Megafuge), the cells were resuspended in 10 ml culture medium and incubated at 37°C overnight. If an ELISPOT assay with a peptide pool was to be carried out, aliquots of the individual peptides were thawed, and the pools mixed and stored at 4°C.

The next day, the plates were flicked out, washed twice with 200 µl culture medium per well and incubated with 50 µl culture medium for at least 1 h at 37°C. Meanwhile, the cells to be tested were counted (see 2.3.7.), spinned down (20°C, 6 min, 1300 rpm, Megafuge), and resuspended in culture medium at a concentration of 10^7 cells per ml. The peptides were thawed if necessary and diluted in culture medium to a concentration of 3 µg/ml (for pools 3 µg/ml each). L-phytohem-agglutinin (L-PHA) was used as a positive control and diluted to 30 µg/ml. A published HLA-matched HIV epitope was used as negative control and diluted in the same manner as the peptides to be tested. The plates were then flicked out again and 50 µl culture medium per well were added. Then, 50 µl cell suspension and 50 µl peptide solution were pipetted into each well. For controls without peptide, 50 µl culture medium were used, and for positive controls 50 µl L-PHA solution. Thus, the final cell number per well was $5 \cdot 10^5$ and the final peptide concentration amounted to 1 µg/ml each (10 µg/ml for L-PHA). Assays were set up in duplicates or triplicates. The plates were incubated for 26 h at 37°C without jolts.

After the incubation, the plates were flicked off and washed once with 200 µl PBS-Tween, once with 200 µl ddH_2O, and five times with 200 µl PBS-Tween. Afterwards, 100 µl of a 1:3000 dilution of the biotinylated secondary antibody in PBS-BSA was added and the plates were incubated for 2 h at 4°C. After washing six times with 200 µl PBS-Tween, the plates were then incubated with 50 µl of a 1:1000 dilution of the avidin-conjugated alkaline phsophatase in PBS-BSA for 1 h at 4°C. Then they were washed three times with 200 µl PBS-Tween and three times with 200 µl PBS. BCIP/NBT tablets were dissolved in ddH_2O (1 tablet for 10 ml), and 50 µl of this solution were added to each well. After development in the dark for about 5 min, the reaction was stopped with tap water, the plastic coating of the plates was removed, and the were dried and stored in the dark at RT. The evaluation was performed with an ELISPOT reader. Wells the spots of which were too numerous to count were assigned a spot count of 1000.

2.3.10. Intracellular cytokine and tetramer staining and analysis by flow cytometry

For intracellular cytokine stainings, about 10^6 cells per well were pulsed with 10 µg/ml adenoviral or control peptide in 96 well round bottom plates and incubated at

37°C. 50 ng/ml PMA with 1 µM ionomycin were used as positive control. When staining for degranulation was performed, αCD107a-FITC antibody was also added. After 1 h, 10 µg/ml brefeldin A was added to stop translocation from the ER to the Golgi. After 6 h incubation, a multi-step staining process was performed consisting of one or all of the following steps, according to experimental requirements:

1. Staining for viable cells
The plate was centrifuged for 2 min at 4°C and 1800 rpm (Megafuge). The supernatant was discarded, and the cells were washed once more with 200 µl PBS per well in the same manner. Cells were resuspended in 75 µl PBS per well containing LIVE/DEAD aqua dye and incubated at 4°C in the dark for 20 min. Cells were washed with 200 µl PBS per well.

2. Tetramer staining
Tetramers were diluted 1:160 (for PE) or 1:240 (for APC) in TSB and centrifuged for 5 min at 13000 rpm in a tabletop centrifuge to spin down aggregates. 50 µl per well of the supernatant were added to the cells and the plate was incubated for 30 min at 4°C in the dark. Cells were washed with 200 µl PFEA per well.

3. Staining of surface molecules
Surface molecules (e.g. CD4 or CD8) were stained with fluorescent antibodies as experimentally required. Antibodies were diluted in PBEA and cells were incubated for 20 min with 50 µl antibody mix per well at 4°C in the dark. Cells were washed with 200 µl PFEA per well.

4. Staining of intracellular molecules
To permeabilize and fix the cells for intracellular staining, they were resuspended in 100 µl cytoperm/cytofix solution per well and incubated for 20 min at 4°C in the dark. Cells were then washed with 200 µl Permwash per well. The desired antibodies were diluted in Permwash and the cells were incubated for 30 min in 50 µl antibody mix per well at 4°C in the dark. Cells were then washed with 200 µl PFEA per well.

After staining, cells were resuspended in 150 µl FACS-Fix and stored in the dark at 4°C wrapped into aluminium foil. Flow cytometry was performed either on the FACSCalibur or the FACSCanto II cytometer. Data were evaluated using Cell Quest Pro for FACSCalibur data or FACSDiva for FACSCanto data.

2.3.11. Fluorolysis assay

CFSE and Far Red dyes were dissolved to 5 mg/ml in DMSO and stored at -20°C. Target cells were washed twice with RPMI by centrifuging at 1300 rpm for 2 min. 1-$2 \cdot 10^6$ pelleted target cells each were resuspended in 1 ml PBS containing 0.5 µl of CFSE solution and 0.1 µl Far Red solution or 0.5 µl Far Red solution and 0.05 µl CFSE

solution. After incubation for 8 min at 20°C cells were washed with 5 ml RPMI and incubated in 1 ml RPMI with 10 µg/ml adenoviral target peptide (CFSE-labelled cells) or 10 µg/ml HIV control peptide (Far Red-labelled cells). After peptide loading, cells were washed twice in 5 ml TCM and resuspended at 10^6 cells per ml. The desired amount (usually 200 µl each) of the target and control cell suspensions were added to 10 ml TCM and 100 µl per well of this suspension plated in 96-well-plates, usually resulting in 2000 target and control cells per well. Effector cells were washed, counted and suspended in TCM and added to the wells at the desired effector : target ratios, with control wells receiving no effector cells. Triplicates were set up for each value. The plate was incubated for 6 h or 24 h at 37°C, spun down at 1800 rpm for 2 min (Megafuge), and the cells resuspended in 150 µl PBEA per well. Each well was analyzed by flow cytometry and the numbers of the differently stained target and control cell populations were counted. Immediately before flow cytometry, propidium iodide was added to the cell suspensions at a concentration of 1 µg/ml to stain and exclude dead cells. Specific lysis for each well was calculated according to the following formula:

$$\% \text{ specific lysis} = (1 - (t[x] / T) \cdot (C / c[x])) \cdot 100$$

T: mean count of target cells in control wells
C: mean count of control cells in control wells
t[x]: number of target cells in well x
c[x]: number of control cells in well x

2.3.12. Cytotoxicity assay by chromium release

Target cells were counted and washed twice with 5 ml X-Vivo 15 medium by centrifugation at 1300 rpm for 2 min (Megafuge). Cells were then resuspended in 200 µl X-Vivo 15 and the adenoviral target peptide or the HIV control peptide added at 10 µg/ml. Additionally, radioactive $Na_2{}^{51}CrO_4$-solution with an activity of 3.7 MBq was added to each sample. After 1 h incubation at 37°C, cells were washed once with 5 ml X-Vivo 15 and incubated at 37°C for another 30 min. Then cells were washed with 5 ml TCM and 8000 cells per well were plated in a 96-well round bottom plate. Effector cells were counted, washed twice with 5 ml TCM, and added to the target cells at the desired effector-target ratios. For each value, one triplicate with target peptide-loaded and one with control peptide-loaded target cells was set up. Triplicates without effector cells were used for the determination of spontaneous chromium release, and triplicates with 10% Triton X detergent for total release. Plates were then incubated at 37°C for the desired lysis time.

After incubation, 50 µl supernatant were pipetted from the top of each well and transferred to a LUMA plate that was subsequently dried overnight. The plate was

then measured with a 1450 Micorbeta Plus liquid scintillation counter (Wallace). Specific lysis for each well was determined according to the following formula:

$$\% \text{ specific lysis} = (A[x] - A[S]) / (A[T] - A[S]) \cdot 100$$

A[x]: activity in well x
A[S]: mean activity of wells with spontaneous release
A[T]: mean activity of wells with total release

3. RESULTS

3.1. Outline of the study

This study intended to identify CTL epitopes from three adenoviral proteins (proteins II, VIII, and E1A) of two clinically important strains (Ad2 and Ad5) restricted by three frequent MHC class I allotypes (HLA-A*01, -A*02, and A*24) and assess their immunodominance, i.e. the frequency with which they elicit immune responses during infection. An epitope is considered immunodominant if it elicits responses in the majority of infected hosts. The strategy of reverse immunology first described in 1994[320] was employed. Epitope candidates were predicted from the amino acid sequences of the proteins by the SYFPEITHI[191] algorithm, and the top scoring 2% of the resulting peptides for each allotype were synthesized chemically, including already published epitopes as well. PBMCs of HLA-matched healthy blood donors were then screened by ELISPOT for IFN-γ memory responses to possible $CD8^+$ T cell epitopes. Different reports have been published about the prevalence of adenoviral infections in the general population[122;272;273;287;321], with figures for the percentage of adults that have endured adenoviral infection in their life varying between 65% and 100%. Since it could thus be reasonably expected that at least two thirds of healthy blood donors would have cleared a previous infection in their lifetime and possess memory T cells, the ELISPOT screening was carried out without further testing of infection status. Epitope candidates that elicited IFN-γ responses frequently were then also tested on the PBMCs of HLA-mismatched donors, in order to exclude responses mediated by $CD4^+$ T cells or MHC class I allotypes different from the expected one. Finally, fluorescently labelled MHC-peptide tetramers were synthesized for unambiguous staining of T cells specific for the peptide of interest presented on its putative allotype. PBMCs could thus be analyzed by flow cytometry, enabling to verify whether the cytokine-producing cell population were really peptide-specific $CD8^+$ T cells. Additionally, phenotypic and functional

characterization of T cells specific for dominant adenoviral epitopes was also carried out.

3.2. Epitope prediction and peptide synthesis

Two widespread and clinically relevant adenoviral strains were selected for epitope prediction, namely Ad2 and Ad5. Three proteins from these strains were chosen: Protein II or hexon protein, from which all published class I and II epitopes are derived, protein VIII as a representative of the small capsid proteins, and the protein E1A as an example of an early antigen that is not present in the virion itself. Several immunodominant epitopes from this protein have been described in mice[274] and rats[275], but not yet in humans. The frequent MHC allotypes HLA-A*01, -A*02, and -A*24 were selected. Application of the algorithm presented by Schipper *et al.*[322] on published HLA class I gene frequencies[323] leads to a high population coverage of these alleles in all ethnicities, e.g. 38% for African Americans, 51% for Asians, 74% for Caucasians, and 77% for Native Americans. Prediction was carried out with protein sequences from the SwissProt database (www.uniprot.org) and using the SYFPEITHI[191] algorithm. The 2% highest scoring peptides were selected for synthesis, along with epitopes that have already been described on the respective allele. The complete list of peptides entering the screening process is given in Tables 3.1-3.3.

Table 3.1: Epitope screening candidates for HLA-A*01. References for published epitopes are given.

Sequence	SYFPEITHI Score	Protein	Position Ad2	Position Ad5
AQDYSTRINY	27	pVIII	24 – 33 propeptide*	24 – 33 propeptide*
DIETPDTHISY	23	hexon	-	291 – 301
DPDIMCSLCY	28	E1A	166 – 175	166 – 175
EGEEFVLDY	27	E1A	138 – 146	138 – 146
FIEEFVPSVY	24	pVIII	77 – 86	77 – 86
FRDNFIGLMY	28	hexon	340 – 349	328 – 337
GTEDELPNY	29	hexon	416 – 424	404 – 412
IEEFVPSVY	24	pVIII	78 – 86	78 – 86
ISDNPNTYDY	30	hexon	509 – 516	493 – 502
LLDSIGDRTRY	28	hexon	384 – 394	372 – 382
LQDRNTELSY	28	hexon	372 – 381	360 – 369
LTDLGQNLLY	36	hexon	900 – 909	884 – 893
NAETQAKPVY	25	hexon	196 – 205	-
PMDEPTLLY	29	hexon	924 – 932	908 – 916
RSWPAALVY	24	pVIII	72 – 80 propeptide*	72 – 80 propeptide*
TDLGQNLLY[279]	20	hexon	901 – 909	885 – 893
TNDQSFNDY	28	hexon	653 – 661	637 – 645
VLDRGPTFKPY	26	hexon	105 – 115	105 – 115
VVDDTKYKDY	26	hexon	-	804 - 813
VVDDTKYKEY	27	hexon	820 – 829	-
YTYSGSIPY	23	hexon	714 – 722	698 – 706

Table 3.2: Epitope screening candidates for HLA-A*02. References for published epitopes are given.

Sequence	SYFPEITHI Score	Protein	Position Ad2	Position Ad5
ALGPVSMPNL	25	E1A	106 – 115	106 – 115
AVQEGIDLL	24	E1A	73 – 81	73 – 81
FIEEFVPSV	28	pVIII	77 – 85	77 – 85
FLCDRTLWRI	24	hexon	879 – 888	863 – 872
FTPRQAILTL	22	pVIII	51 – 60	51 – 60
HMISRVNGI	24	pVIII	39 – 47 propeptide*	39 – 47 propeptide*
ILRRPTSPV	25	E1A	213 – 221	213 – 221
ILVKQQNGKL	24	hexon	-	248 – 257
LIGKTAVDSI	24	hexon	865 – 874	849 – 858
LLDQLIEEV	29	E1A	19 – 27	19 – 27
LLNEPGQPL	26	E1A	-	272 – 280
LLNESGQPL	25	E1A	272 – 280	-
LLTPNEFEI	23	hexon	752 – 760	736 – 744
LLYANSAHAL[287]	25	hexon	907 – 916	891 – 900
MLLGNGRYV	24	hexon	563 – 571	547 – 555
NLVPEVIDL	26	E1A	114 – 122	114 – 122
QLAGGFRHRV	25	pVIII	107 propeptide* – 5 protein	107 propeptide* – 5 protein
SAGPHMISRV	22	pVIII	35 – 44 propeptide	35 – 44 propeptide
SLLDQLIEEV	31	E1A	18 – 27	18 – 27
SMPNLVPEV	28	E1A	111 – 119	111 – 119
TFYLNHTFKK[279]	3	hexon	726 – 735	710 – 719
TLAVGDNRV	25	hexon	84 – 92	84 – 92
TLLYVLFEV	27	hexon	929 – 937	913 – 921
VINTETLTKV	26	hexon	-	419 – 428
VLAGQASQL	25	hexon	358 – 366	346 – 354
VLFEVFDVV	24	hexon	933 – 941	917 – 925
VLPRDAQAEV	25	pVIII	90 – 99 propeptide*	90 – 99 propeptide*
YLNHTFKKV	24	hexon	728 – 736	712 – 720
YVLFEVFDVV[287]	17	hexon	932 – 941	916 – 925

Table 3.3: Epitope screening candidates for HLA-A*24. References for published epitopes are given.

Sequence	SYFPEITHI Score	Protein	Position Ad2	Position Ad5
AYPANFPYPL	23	hexon	856 – 865	840 – 849
AYSYKARFTL	21	hexon	76 – 85	76 – 85
CYMRTCGMF	20	E1A	174 – 182	174 – 182
DYMDNVNPF	22	hexon	542 – 550	526 – 534
DYLSAANML	22	hexon	660 – 668	644 – 652
EYLSPGLVQF	22	hexon	21 – 30	21 – 30
HYPDQFIPNF	24	pVIII	96 – 105	96 – 105
KYKDYQQVGI	21	hexon	-	809 – 818
KYKEYQQVGI	22	hexon	825 – 834	-
KYNPTNVEI	22	hexon	501 – 509	-
LYSNIALYL	21	hexon	488 – 496	472 – 480
NYIAFRDNF	22	hexon	336 – 344	324 – 332
NYIAFRDNFI	22	hexon	336 – 345	324 – 333
NYMSAGPHMI	21	pVIII	32 – 41 propeptide*	32 – 41 propeptide*
PYLDGTFYL	24	hexon	721 – 729	705 – 713
SFTPRQAIL	18	pVIII	50 – 58	50 – 58
SYDPDVRII	22	hexon	404 – 412	393 – 401
SYKDRMYSF	21	hexon	801 – 809	785 – 793
TYFDIRGVL	23	hexon	98 – 106	98 – 106
TYFSLNNKF[279]	23	hexon	36 – 44	36 – 44
TYSGSIPYL	22	hexon	715 – 723	699 – 707

* Protein pVIII contains a propeptide of 111 amino acids that is cleaved off by an adenoviral 23K protease during processing. Peptides from the propeptide were included in the screening.

3.3. *In vitro* expansion of memory T cells

Previous work during my diploma thesis had shown that adenovirus-specific memory T cells are usually present in very low numbers in peripheral blood. Their low abundance hampers the detection of responses *ex vivo*. Employing standard ELISPOT protocols that use 500,000 PBMCs per well, the number of specific spots in the readout is frequently so low that responses cannot be distinguished from the

background. Therefore I routinely employed an *in vitro* expansion protocol of specific memory T cells before carrying out the ELISPOT screening. This involved stimulating PBMCs once with the peptide(s) they were to be tested against, and culturing them for 12 days in the presence of IL-2 (for details see 2.3.8). Up to six different peptides were used in a single expansion assay. The aim was to entice the proliferation of memory cells specific for the added peptides while excluding *in vitro* priming that would yield false positive responses. This was to be ensured by the short duration of the expansion and by stimulating with peptide only once, while *in vitro* priming protocols routinely employ culturing periods of up to six weeks and repeated stimulations with several cytokines peptides on professional or artificial APCs[324-327].

Figures 3.1 and 3.3 show an example of ELISPOT plates on which PBMCs of the same eight donors positive for HLA-A*02 and -A*24 have been tested against two A*02-restricted and one A*24-restricted peptide *ex vivo* and after stimulation, respectively. Amplification of the spot counts can clearly be recognized, and several responses can be identified after expansion that would have gone unnoticed in an *ex vivo* assay. Figures 3.2 and 3.4 show the mean spot counts per well for each donor and peptide *ex vivo* and after expansion, respectively, together with an A*02-restricted HIV control peptide.

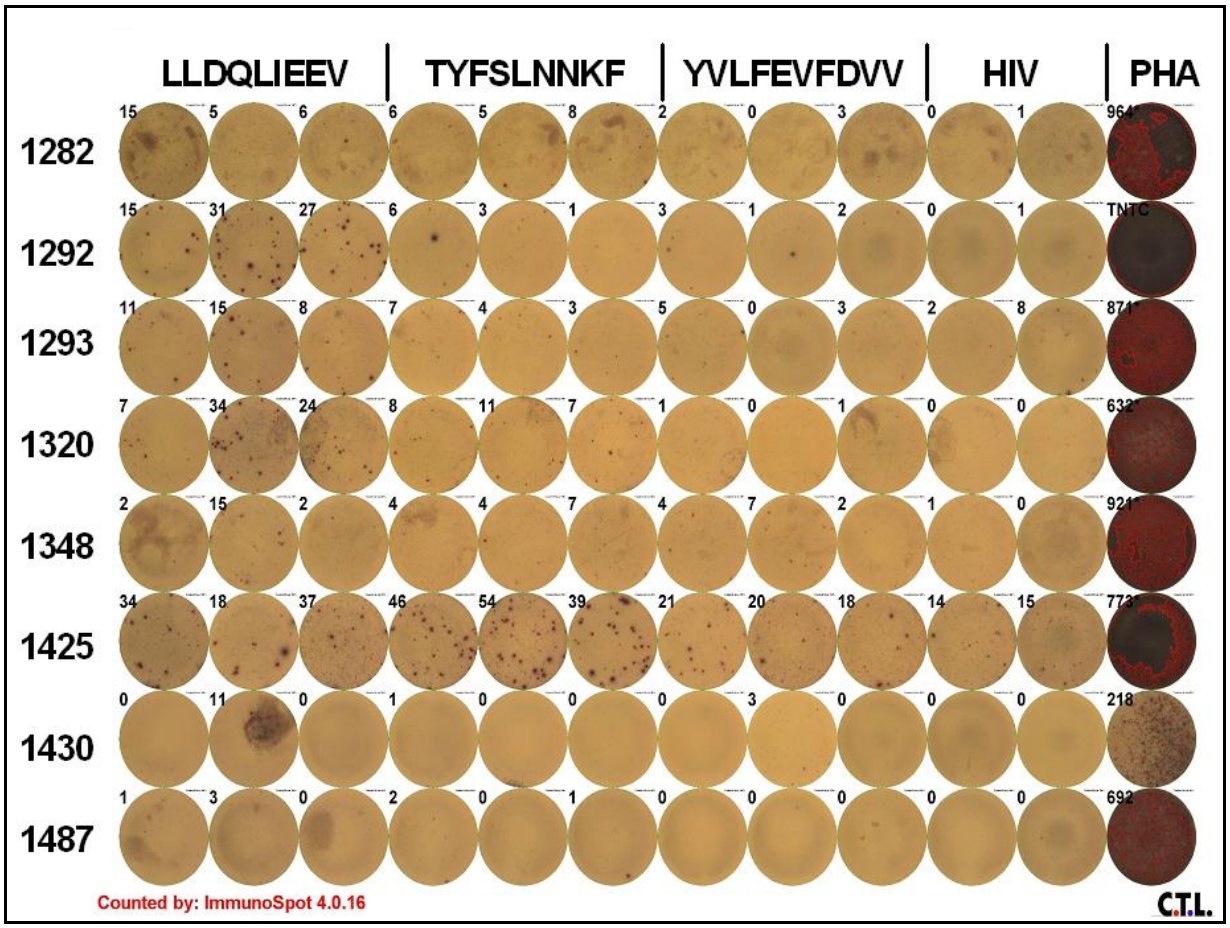


Figure 3.1: ELISPOT plate of 8 donors (one per row, numbers indicated) positive for HLA-A*02 and -A*24 tested ***ex vivo*** against three different peptides (LLDQLIEEV and YVLFEVFDVV restricted by A*02, TYFSLNNKF by A*24) together with the HIV negative control (ILKEPVHGV, A*02-restricted) and the PHA positive control.

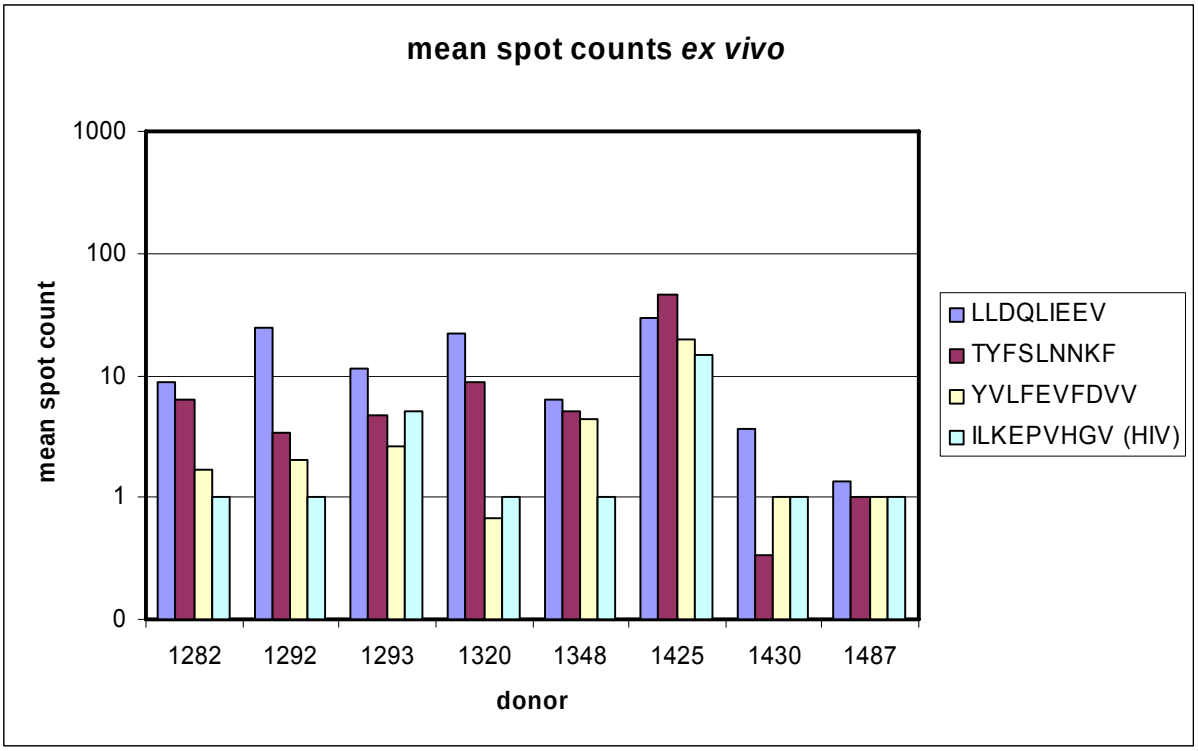


Figure 3.2: Mean spot count of plate in Figure 3.1.

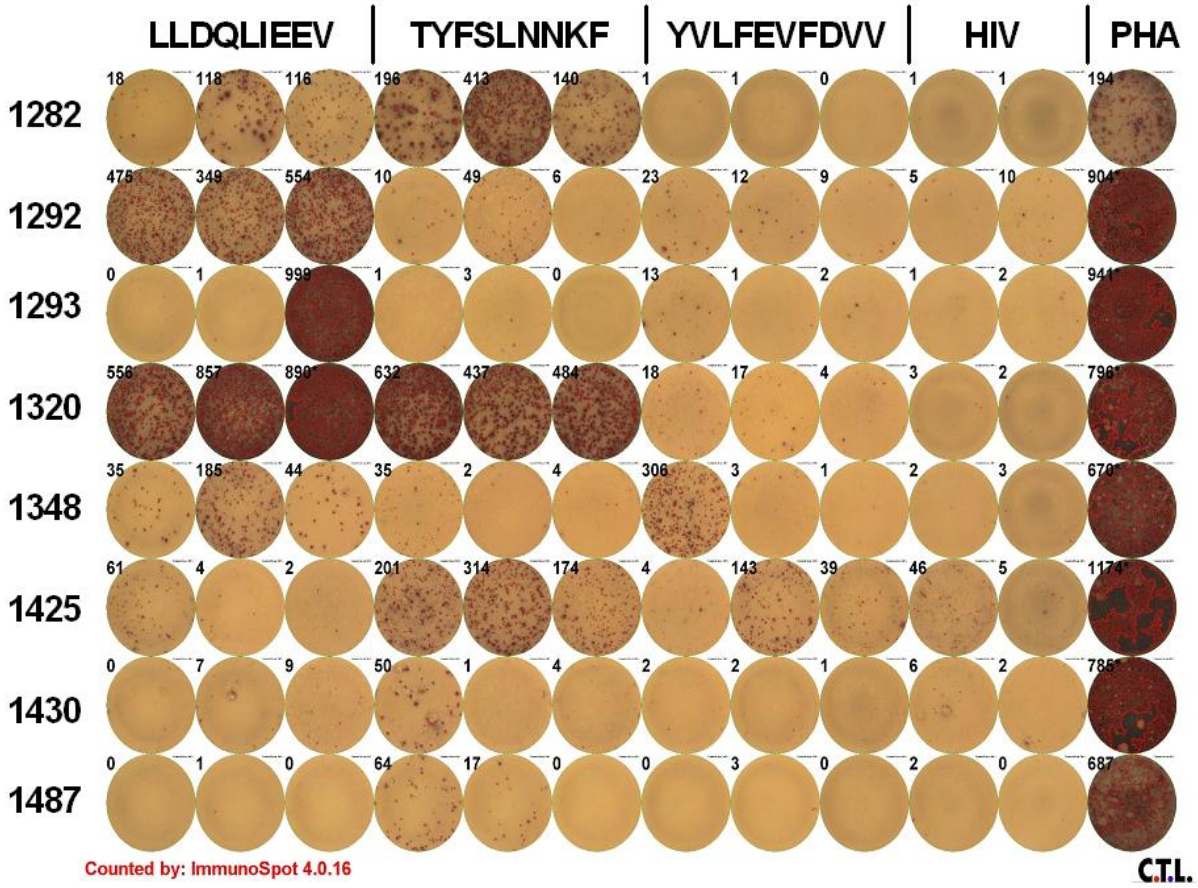


Figure 3.3: ELISPOT plate with the same layout as in Figure 3.1, **after presensitization** with the expansion protocol.

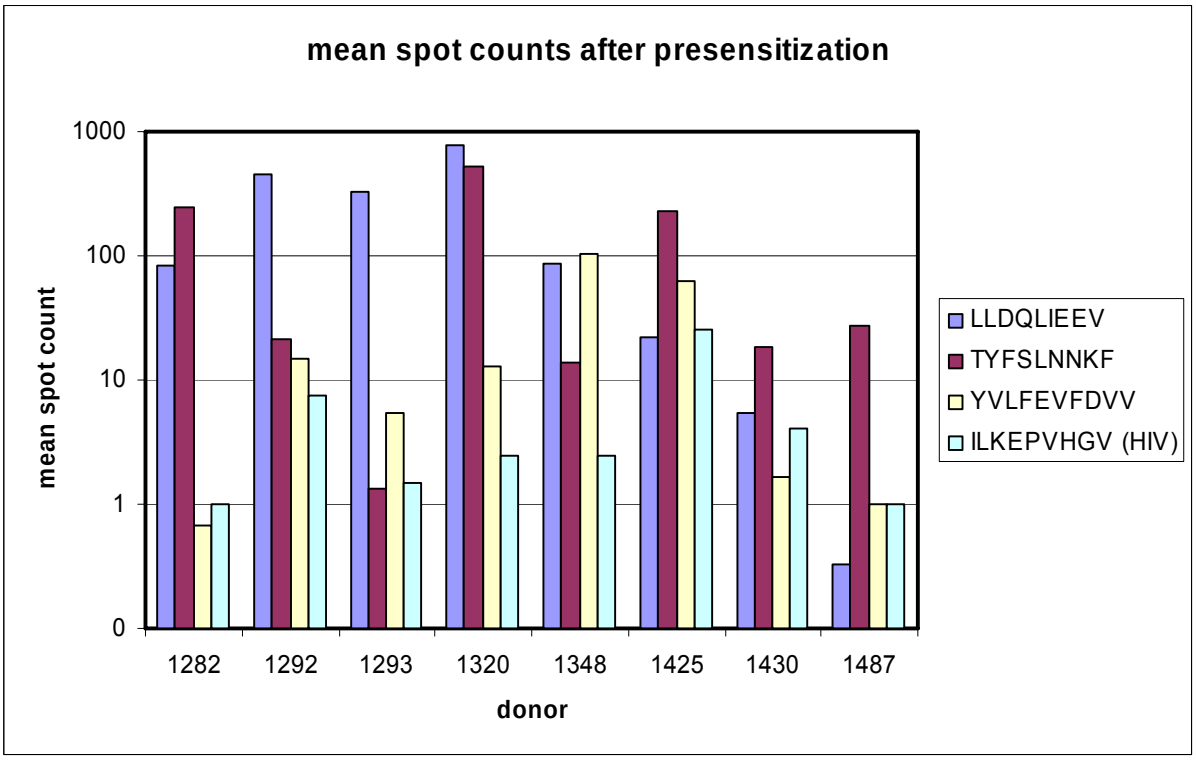


Figure 3.4: Mean spot counts of plate in Figure 3.3.

The effects of memory T cell expansion were tested in this manner for five peptides with several different PBMC donors. Table 3.4 gives a complete summary of the results.

Table 3.4: Amplification of ELISPOT spot counts after application of the 12-day expansion protocol compared to results *ex vivo*. Geometric means of the amplification factors of donors positive when presensitized and the respective maximum amplification factors are given along with the number of responses *ex vivo* or with presensitization.

peptide	HLA restriction	donors			geometric mean of amplification	maximum amplification (-fold)
		tested	positive *ex vivo*	positive presensitized		
LTDLGQNLLY	A*01	14	5	10	9.4 ± 5.9	79.2
LLDQLIEEV	A*02	12	7	9	19.7 ± 2.2	93.8
YVLFEVFDVV	A*02	12	0	6	8.0 ± 3.5	30.8
TYFSLNNKF	A*24	26	4	21	16.6 ± 3.0	68.6
AYPANFPYPL	A*24	14	0	4	9.4 ± 3.5	55.5

The data show that the majority of positive responses would have remained unnoticed without previous T cell expansion. For the peptides YVLFEVFDVV and AYPANFPYPL, none of the responses could be detected *ex vivo*. Expansion ratios of nearly 100fold can be achieved with the protocol.

3.4. ELISPOT screening system

To decrease the number of assays necessary for the screening, expansion and testing were carried out first with peptide pools containing four to six peptides. Pools were created in a manner that each peptide to be tested was present in two pools as shown in Table 3.5. A published and HLA-matched HIV epitope (Table 3.6) was also employed during expansion in each experiment and used as a negative control, since the blood donations were confirmed to be HIV negative by routine testing. Responses to the peptide pools were considered positive if the mean spot count was at least 5 and at least 2.5 times higher than the mean spot count of the HIV control. If

the HIV mean spot count was 0, the value of 1 was assigned for purposes of calculation to avoid division by zero. To ensure consistency of the results, this was also done for HIV mean spot counts of 0.5. This did not lead to false negative results, since a mean spot count of 5 spots per well was required for a positive response anyway. Figure 3.5 shows the results of a pool ELISPOT with the peptides listed in Table 3.5.

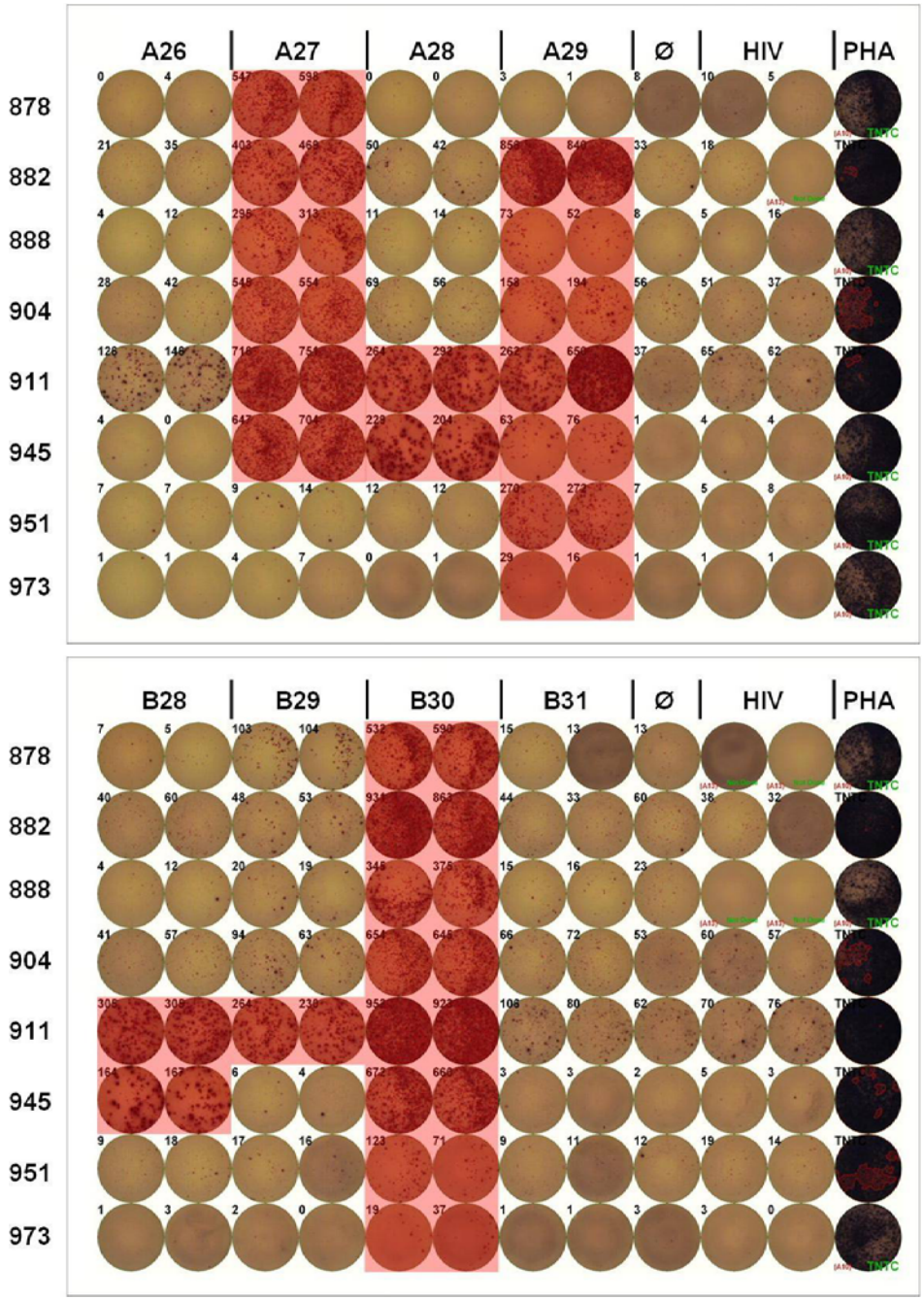
Table 3.5: Example for the composition of twelve pools (A26 to A31 and B26 to B31) containing four to six peptides each, with every peptide being present in two pools. All peptides were screened as candidates for HLA-A*24. Peptides in green are derived from Epstein-Barr virus (EBV).

Pool #	A26	A27	A28	A29	A30	A31
B26	KYKDYQQVGI	AYSYKARFTL	SYDPDVRII	TYFSLNNKF	DYMDNVNPF	
B27	SYKDRMYSF	PYLDGTFYL	NYIAFRDNF	DYLSAANML	LYSNIALYL	HYPDQFIPNF
B28	TYSGSIPYL	EYLSPGLVQF	NYIAFRDNFI	AYPANFPYPL	TYFDIRGVL	NYMSAGPHMI
B29	CYMRTCGMF	KYNPTNVEI	SFTPRQAIL	KYKEYQQVGI	SYKICKAFI	RYSIFFDY
B30	AYAAVAPAYI	TYPVLEEMF	TFLNDECLL	DYNFVKQLF	SYKTLREFF	SYVKQPLCL
B31	NYNPGTLSSL	DFLRLTPEI	SYVKQPLCLL	IFPHPSKPTF		

Table 3.6: Published HIV epitopes used as negative control in the ELISPOT screening. For assays with HLA-A*24, a peptide for each donor was HLA-matched individually to another allele.

sequence	protein	position	HLA-restriction	SYFPEITHI score
GSEELRSLY[328]	HIV-1 gag p17	70-78	A*01	30
ILKEPVHGV[187]	HIV-1 RT	476-484	A*0201	30
KIRLRPGGK[329]	HIV-1 gag p17	18-26	A*03	29
TPGPGVRYPL[330]	HIV-1 nef	128-137	B*07	24

For each donor, peptides that were common to pools with positive responses were tested again in single expansion and ELISPOT assays. This time, more stringent criteria were applied and responses were considered positive if the mean spot count was at least 10 and at least 3 times higher than the mean spot count of the HIV control. Again, HIV mean spot counts below 1 were treated as if they were 1. Peptides eliciting positive responses frequently were then also tested with additional donors in single assays. Each peptide was tested for responses with at least 16 different HLA-matched donors, either in pool or single assays.



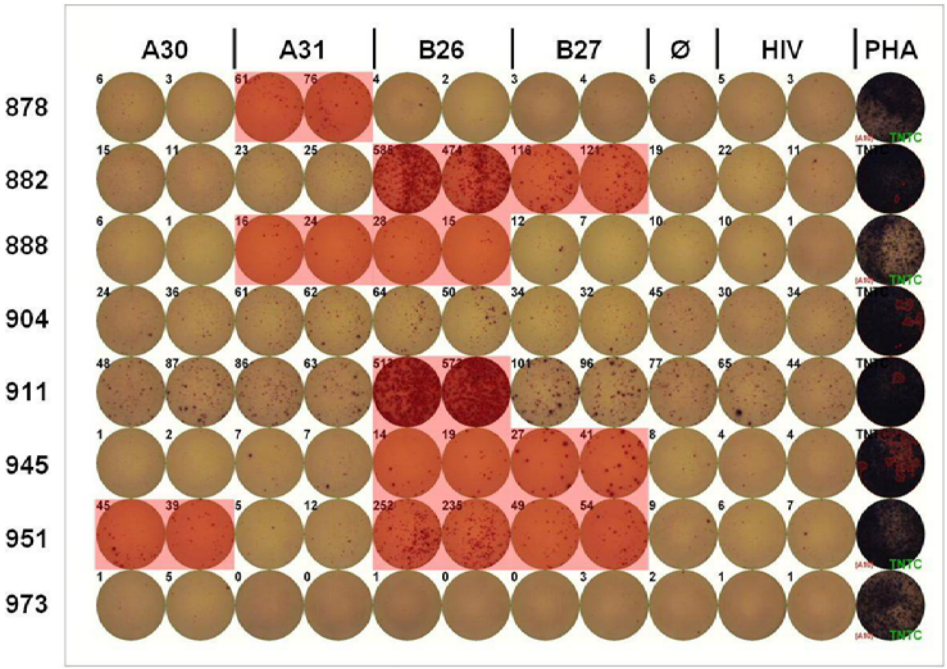


Figure 3.5: Three ELISPOT plates employed in the screening of the twelve peptide pools listed in Table 3.5. PBMCs of eight HLA-A*24-positive donors were tested, with one donor in each row. Each donor was tested for each pool and the HIV control in duplicates. Single wells were used for the medium control and the L-PHA positive control. Positive responses are highlighted in red.

3.5. Results of the ELISPOT screening with HLA-matched donors

The following tables (3.7.1 – 3.9.2) list the results of the ELISPOT screening with PBMCs of donors that carried the supposed restricting allotype. The spot count ratios (mean peptide spot count / mean HIV spot count) are given for positive responses only.

3.5.1. HLA-A*01:

Table 3.7.1: Screening results of two epitope candidates for HLA-A*01. Spot count ratios for positive responses are given. neg.: negative response; n.t.: not tested.

donor #	LTDLGQNLLY	TDLGQNLLY	donor #	LTDLGQNLLY	TDLGQNLLY
790	neg.	n.t.	**1038**	**864**	neg.
806	**21**	n.t.	**1040**	**111**	n.t.
866	**6**	n.t.	**1043**	**115**	n.t.
867	**299**	n.t.	**1050**	**25**	n.t.
878	neg.	n.t.	**1054**	**464**	n.t.
888	neg.	n.t.	**1062**	**11**	n.t.
904	neg.	n.t.	**1067**	**20**	n.t.
945	**58**	n.t.	**1068**	**45**	n.t.
970	**1000**	n.t.	**1069**	**9**	n.t.
984	**47**	**46**	**1072**	**127**	n.t.
988	**32**	**38**	**1075**	**183**	n.t.
991	**87**	**111**	**1078**	**98**	n.t.
992	**9**	**13**	**1083**	**174**	n.t.
995	**379**	**420**	**1085**	**256**	n.t.
998	**178**	**96**	**1086**	**65**	n.t.
1002	**400**	**400**	**1093**	**89**	n.t.
1005	**1000**	**781**	**1098**	**667**	n.t.
1006	**153**	**672**	**1099**	**472**	n.t.
1011	**48**	**109**	**1102**	**76**	n.t.
1012	**115**	**215**	**1106**	**708**	n.t.
1014	neg.	neg.	**1110**	**693**	n.t.
1017	**125**	**125**	**1111**	**132**	n.t.
1025	**667**	**667**	**1115**	**400**	n.t.
1030	**227**	**225**	**1118**	**14**	n.t.

Table 3.7.1 (continued):

donor #	LTDLGQNLLY	TDLGQNLLY
1119	**75**	n.t.
1123	**18**	n.t.
1130	**47**	n.t.
1132	**433**	n.t.
1133	**5**	n.t.
1136	**18**	n.t.
1138	**77**	n.t.
1141	**37**	n.t.
1143	**197**	n.t.
1144	**55**	n.t.
1150	**36**	n.t.
1151	**461**	n.t.
1154	**20**	n.t.
1155	**314**	n.t.
1162	**37**	n.t.
1165	neg.	n.t.
1169	**6**	n.t.
1171	**112**	n.t.
1190	**19**	n.t.
1295	**55**	n.t.
1331	**26**	n.t.
1332	**4.0**	n.t.
1363	**95**	n.t.
1369	**269**	n.t.
1401	**197**	n.t.
total	**93% 68 / 73**	**88% 14 / 16**

Table 3.7.2: Screening results of four epitope candidates for HLA-A*01. Spot count ratios for positive responses are given. neg.: negative response; n.t.: not tested.

donor #	DIETPDTHISY	FRDNFIGLMY	GTEDELPNY	LQDRNTELSY	YTYSGSIPY
970	n.t.	neg.	n.t.	n.t.	neg.
1040	neg.	neg.	neg.	neg.	neg.
1043	neg.	neg.	**4.1**	**295**	neg.
1050	neg.	**3.2**	neg.	neg.	neg.
1054	**80**	neg.	neg.	neg.	**97**
1062	neg.	neg.	neg.	neg.	neg.
1067	neg.	neg.	neg.	neg.	neg.
1068	neg.	neg.	neg.	neg.	neg.
1069	neg.	neg.	neg.	neg.	neg.
1072	neg.	neg.	neg.	neg.	neg.
1075	neg.	neg.	neg.	neg.	neg.
1078	neg.	neg.	neg.	neg.	neg.
1083	neg.	neg.	neg.	neg.	neg.
1085	neg.	neg.	neg.	neg.	neg.
1086	neg.	**3.1**	neg.	neg.	**49**
1093	neg.	neg.	neg.	neg.	neg.
1098	neg.	neg.	neg.	neg.	neg.
1099	neg.	**11.0**	neg.	neg.	**542**
1102	neg.	neg.	neg.	neg.	neg.
1106	neg.	neg.	neg.	neg.	neg.
1110	neg.	neg.	neg.	neg.	neg.
1111	neg.	**4.7**	neg.	neg.	**43**
1115	neg.	neg.	neg.	**400**	neg.
1118	neg.	neg.	neg.	neg.	**11**
1119	neg.	neg.	neg.	neg.	**100**
1123	neg.	neg.	n.t.	neg.	neg.
1130	neg.	neg.	n.t.	neg.	neg.
1132	neg.	neg.	n.t.	neg.	neg.
1133	neg.	neg.	n.t.	neg.	neg.
1136	neg.	neg.	n.t.	neg.	neg.
1138	neg.	**3.6**	n.t.	neg.	neg.
1141	neg.	**6.6**	n.t.	neg.	**42**
1143	neg.	neg.	n.t.	neg.	neg.
1144	neg.	neg.	n.t.	neg.	neg.
1150	neg.	neg.	n.t.	neg.	neg.
1151	neg.	neg.	n.t.	neg.	neg.
1154	neg.	neg.	n.t.	**6**	neg.
1155	neg.	neg.	n.t.	neg.	neg.
1162	neg.	neg.	n.t.	neg.	neg.
1165	neg.	neg.	n.t.	neg.	neg.
1169	neg.	neg.	n.t.	neg.	neg.
1171	n.t.	neg.	n.t.	n.t.	neg.
total	**2.5% 1 / 40**	**14% 6 / 42**	**4.2% 1 / 24**	**7.5% 3 / 40**	**17% 7 / 42**

The 15 remaining peptides from Table 3.1 (AQDYSTRINY, DPDIMCSLCY, EGEEFVLDY, FIEEFVPSVY, IEEFVPSVY, ISDNPNTYDY, LLDSIGDRTRY, NAETQAKPVY, PMDEPTLLY, RSWPAALVY, TNDQSFNDY, VLDRGPTFKPY, VVDDTKYKDY, and VVDDTKYKEY) were all tested with the same 24 donors as GTEDELPNY from Table 3.7.2. No positive responses could be observed.

3.5.2. HLA-A*02:

Table 3.8.1: Screening results of three epitope candidates for HLA-A*02. Spot count ratios for positive responses are given. neg.: negative response; n.t.: not tested.

donor #	LLDQLIEEV	SMPNLVPEV	TLLYVLFEV
752	neg.	neg.	**113**
757	**7**	neg.	neg.
759	**437**	**35**	neg.
765	neg.	neg.	neg.
768	neg.	neg.	**12**
771	neg.	neg.	neg.
773	**9**	neg.	**8**
776	neg.	neg.	neg.
778	**14.6**	neg.	neg.
779	**62**	neg.	**521**
780	**10**	neg.	**37**
781	neg.	neg.	neg.
784	neg.	**7**	**11**
785	neg.	neg.	neg.
788	neg.	neg.	**132**
789	neg.	neg.	**5**
791	**1000**	**45**	**252**
793	**14**	**24**	neg.
794	neg.	neg.	neg.
797	neg.	neg.	**7**
800	**257**	neg.	**512**
801	**636**	**15**	**7**
813	**7**	n.t.	n.t.
829	**114**	n.t.	n.t.
880	**25**	n.t.	n.t.
970	**432**	**1000**	n.t.
984	n.t.	n.t.	**7**
988	n.t.	n.t.	neg.

Table 3.8.1 (continued):

donor #	LLDQLIEEV	SMPNLVPEV	TLLYVLFEV
1006	n.t.	n.t.	neg.
1011	**6**	neg.	**6**
1013	neg.	neg.	**6**
1016	**10**	neg.	**7**
1022	**13**	neg.	**38**
1026	**11**	neg.	**6**
1033	**8**	neg.	**8**
1045	**80**	neg.	**62**
1051	**3**	neg.	**13**
1052	neg.	neg.	**4**
1055	**48**	neg.	**13**
1057	**15**	neg.	neg.
1058	**9**	neg.	**11**
1061	**13**	neg.	**6**
1065	**19**	**29**	**34**
1066	neg.	neg.	**4**
1067	**32**	neg.	**44**
1073	**448**	n.t.	n.t.
1098	**212**	**1000**	n.t.
1099	**283**	**677**	n.t.
1107	**333**	neg.	neg.
1108	**250**	neg.	**24**
1109	neg.	neg.	**24**
1113	**821**	neg.	**1000**
1114	**63**	**4**	**5**
1119	neg.	neg.	**117**
1122	**20**	neg.	neg.
1124	**275**	**27**	**377**
1125	**14**	neg.	neg.
1129	**94**	**7**	neg.
1130	**28**	neg.	neg.
1135	**25**	neg.	**7**
1137	**7**	**85**	**112**
1138	neg.	neg.	**23**
1139	**28**	**5**	**28**
1140	neg.	neg.	neg.
1144	**453**	**195**	n.t.
1154	neg.	**303**	n.t.
1155	**22**	**23**	n.t.

Table 3.8.1 (continued):

donor #	LLDQLIEEV	SMPNLVPEV	TLLYVLFEV
1171	neg.	neg.	n.t.
1238	**72**	n.t.	n.t.
1282	**84**	n.t.	n.t.
1292	**61**	n.t.	n.t.
1293	**222**	n.t.	n.t.
1320	**307**	n.t.	n.t.
1348	**35**	n.t.	n.t.
1425	neg.	n.t.	n.t.
1430	neg.	n.t.	n.t.
1487	neg.	n.t.	n.t.
total	**68% 50 / 74**	**28% 17 / 61**	**67% 38 / 57**

Table 3.8.2 Screening results of three epitope candidates for HLA-A*02. Spot count ratios for positive responses are given. neg.: negative response; n.t.: not tested.

donor #	TFYLNHTFKK	VLFEVFDVV	YVLFEVFDVV
553	**7**	n.t.	n.t.
558	**337**	n.t.	n.t.
560	neg.	n.t.	n.t.
562	neg.	n.t.	n.t.
564	**20**	n.t.	n.t.
570	neg.	n.t.	n.t.
576	**5**	n.t.	n.t.
589	**21**	n.t.	n.t.
602	**83**	n.t.	n.t.
654	**25**	n.t.	n.t.
752	n.t.	neg.	n.t.
757	n.t.	neg.	n.t.
759	n.t.	neg.	n.t.
765	n.t.	neg.	n.t.
768	n.t.	neg.	n.t.
771	n.t.	neg.	n.t.
773	n.t.	neg.	n.t.
776	n.t.	neg.	n.t.
778	n.t.	neg.	n.t.
779	n.t.	neg.	n.t.
780	n.t.	neg.	n.t.
781	n.t.	neg.	n.t.
784	n.t.	neg.	n.t.
785	n.t.	neg.	n.t.
789	n.t.	neg.	n.t.

Table 3.8.2 (continued):

donor #	TFYLNHTFKK	VLFEVFDVV	YVLFEVFDVV
791	n.t.	neg.	n.t.
793	n.t.	neg.	n.t.
794	n.t.	neg.	n.t.
797	n.t.	neg.	n.t.
800	n.t.	**20**	n.t.
813	n.t.	n.t.	neg.
829	n.t.	n.t.	**11**
880	n.t.	n.t.	**25**
951	**5**	neg.	neg.
958	neg.	neg.	neg.
960	neg.	**44**	**38**
968	**5**	neg.	neg.
970	**67**	neg.	neg.
973	**39**	neg.	**5**
983	neg.	**39**	**12**
984	**342**	neg.	**73**
988	**19**	neg.	neg.
989	**8**	**7**	neg.
999	**6**	neg.	**4**
1006	**112**	neg.	**112**
1009	**20**	**4**	**5**
1010	neg.	neg.	neg.
1011	n.t.	neg.	n.t.
1013	n.t.	neg.	n.t.
1016	n.t.	neg.	n.t.
1022	n.t.	neg.	n.t.
1026	n.t.	neg.	n.t.
1033	n.t.	neg.	n.t.
1045	**165**	**6**	**9**
1051	n.t.	neg.	n.t.
1052	n.t.	neg.	n.t.
1055	n.t.	neg.	n.t.
1057	n.t.	neg.	n.t.
1058	neg.	**3**	neg.
1061	n.t.	neg.	n.t.
1065	n.t.	neg.	n.t.
1066	n.t.	neg.	n.t.
1067	n.t.	neg.	n.t.
1073	n.t.	n.t.	**22**
1098	n.t.	neg.	n.t.

Table 3.8.2 (continued):

donor #	TFYLNHTFKK	VLFEVFDVV	YVLFEVFDVV
1099	n.t.	neg.	n.t.
1144	n.t.	neg.	n.t.
1154	n.t.	neg.	n.t.
1155	n.t.	neg.	n.t.
1171	n.t.	neg.	n.t.
1238	n.t.	n.t.	neg.
1282	n.t.	n.t.	neg.
1292	n.t.	n.t.	neg.
1293	n.t.	n.t.	**4**
1320	n.t.	n.t.	**5**
1348	n.t.	n.t.	**41**
1425	n.t.	n.t.	neg.
1430	n.t.	n.t.	neg.
1487	n.t.	n.t.	neg.
total	**69% 18 / 26**	**13% 7 / 56**	**48% 14 / 29**

Table 3.8.3: Screening results of four epitope candidates for HLA-A*02. Spot count ratios for positive responses are given. neg.: negative response; n.t.: not tested.

donor #	FIEEFVPSV	FLCDRTLWRI	FTPRQAILTL	HMISRVNGI
752	neg.	neg.	neg.	neg.
757	neg.	neg.	n.t.	neg.
759	neg.	neg.	**75**	neg.
765	neg.	neg.	n.t.	neg.
768	neg.	neg.	neg.	neg.
771	neg.	neg.	neg.	neg.
773	neg.	neg.	n.t.	neg.
776	neg.	neg.	neg.	neg.
778	neg.	neg.	n.t.	neg.
779	neg.	**120**	neg.	neg.
780	neg.	**14**	neg.	neg.
781	neg.	neg.	n.t.	neg.
784	neg.	neg.	n.t.	neg.
785	neg.	neg.	n.t.	neg.
789	neg.	neg.	n.t.	neg.
791	neg.	neg.	neg.	neg.
793	neg.	neg.	neg.	neg.
794	neg.	neg.	neg.	neg.
797	neg.	neg.	neg.	neg.
800	neg.	neg.	neg.	neg.
1011	neg.	**5**	neg.	neg.
1013	neg.	neg.	neg.	neg.
1016	neg.	neg.	neg.	neg.
1022	neg.	neg.	neg.	neg.
1026	neg.	neg.	neg.	neg.
1033	neg.	**7**	neg.	neg.
1045	neg.	**44**	neg.	neg.
1051	neg.	neg.	neg.	**6**
1052	neg.	neg.	neg.	neg.
1055	**4**	neg.	neg.	neg.
1057	neg.	neg.	neg.	neg.
1058	neg.	neg.	neg.	neg.
1061	neg.	neg.	neg.	neg.
1065	neg.	neg.	neg.	neg.
1066	neg.	neg.	neg.	neg.
1067	neg.	neg.	**69**	neg.
total	**2.8% 1 / 36**	**14% 5 / 36**	**7.1% 2 / 28**	**2.8% 1 / 36**

Table 3.8.4: Screening results of four epitope candidates for HLA-A*02. Spot count ratios for positive responses are given. neg.: negative response.

donor #	ILVKQQNGKL	LLNEPGQPL	LLNESGQPL	LLYANSAHAL
752	neg.	neg.	neg.	neg.
757	neg.	neg.	neg.	neg.
759	neg.	neg.	neg.	neg.
765	neg.	**12**	neg.	neg.
768	neg.	neg.	neg.	neg.
771	neg.	neg.	neg.	neg.
773	neg.	neg.	neg.	neg.
776	neg.	neg.	neg.	neg.
778	neg.	neg.	neg.	**4**
779	neg.	neg.	neg.	neg.
780	neg.	neg.	neg.	neg.
781	neg.	neg.	neg.	neg.
784	neg.	neg.	neg.	neg.
785	neg.	neg.	neg.	**10**
789	neg.	neg.	neg.	neg.
791	neg.	neg.	neg.	neg.
793	neg.	neg.	neg.	neg.
794	neg.	neg.	neg.	neg.
797	neg.	neg.	neg.	neg.
800	neg.	neg.	neg.	neg.
1011	neg.	neg.	neg.	neg.
1013	neg.	neg.	neg.	**6**
1016	neg.	neg.	neg.	neg.
1022	neg.	neg.	neg.	neg.
1026	**5**	neg.	neg.	neg.
1033	neg.	neg.	neg.	neg.
1045	neg.	neg.	neg.	neg.
1051	neg.	neg.	neg.	neg.
1052	neg.	neg.	neg.	neg.
1055	neg.	neg.	**4**	neg.
1057	neg.	neg.	neg.	neg.
1058	neg.	neg.	neg.	neg.
1061	neg.	neg.	neg.	neg.
1065	neg.	neg.	neg.	neg.
1066	neg.	neg.	neg.	neg.
1067	neg.	neg.	neg.	neg.
total	**2.8% 1 / 36**	**2.8% 1 / 36**	**2.8% 1 / 36**	**8.3% 3 / 36**

Table 3.8.5: Screening results of three epitope candidates for HLA-A*02. Spot count ratios for positive responses are given. neg.: negative response.

donor #	SLLDQLIEEV	VLPRDAQAEV	YLNHTFKKV
752	neg.	neg.	neg.
757	**7**	neg.	neg.
759	**480**	neg.	neg.
765	neg.	neg.	neg.
768	neg.	neg.	neg.
771	neg.	neg.	neg.
773	**12**	neg.	neg.
776	neg.	neg.	neg.
778	neg.	neg.	neg.
779	**81**	neg.	neg.
780	**7**	neg.	**11**
781	neg.	neg.	neg.
784	neg.	neg.	neg.
785	neg.	neg.	neg.
789	neg.	neg.	neg.
791	**147**	neg.	neg.
793	**200**	neg.	neg.
794	neg.	**3**	neg.
797	neg.	neg.	neg.
800	**194**	neg.	neg.
1011	**6**	neg.	neg.
1013	neg.	neg.	neg.
1016	**10**	neg.	neg.
1022	neg.	neg.	neg.
1026	**11**	neg.	neg.
1033	**4**	**3**	neg.
1045	**80**	neg.	**7**
1051	**7**	neg.	neg.
1052	neg.	neg.	neg.
1055	**48**	**4**	neg.
1057	**9**	neg.	neg.
1058	**7**	neg.	neg.
1061	**5**	neg.	neg.
1065	**14**	neg.	neg.
1066	**3**	neg.	neg.
1067	neg.	**11**	neg.
total	**56% 20 / 36**	**11% 4 / 36**	**5.6% 2 / 36**

10 of the 12 remaining peptides from Table 3.2 (ALGPVSMPNL, AVQEGIDLL, ILRRPTSPV, LIGKTAVDSI, MLLGNGRYV, NLVPEVIDL, QLAGGFRHRV, TLAVGDNRV, VINTETLNKV, and VLAGQASQL) were tested with the same 36 donors as the peptides in Table 3.8.5. LLTPNEFEI and SAGPHMISRV were tested with 28 or 24 of these donors, respectively. No positive responses could be observed.

3.5.3. HLA-A*24:

Table 3.9.1: Screening results of three epitope candidates for HLA-A*24. Spot count ratios for positive responses are given. neg.: negative response; n.t.: not tested.

donor #	AYPANFPYPL	NYIAFRDNFI	TYFSLNNKF
790	neg.	n.t.	**5**
806	**4**	n.t.	neg.
813	n.t.	n.t.	neg.
829	n.t.	n.t.	**110**
847	**4**	**20**	**6**
866	**9**	neg.	**93**
867	neg.	neg.	**8**
878	neg.	neg.	neg.
880	n.t.	n.t.	**60**
882	neg.	neg.	**37**
888	neg.	neg.	neg.
904	neg.	neg.	**5**
911	neg.	**44**	**28**
940	n.t.	neg.	**8.0**
945	neg.	**172**	**107**
951	neg.	neg.	**25**
961	n.t.	neg.	neg.
973	neg.	neg.	neg.
998	neg.	**106**	neg.
999	neg.	neg.	neg.
1007	**14**	neg.	**90**
1008	neg.	**188**	**69**
1010	neg.	neg.	neg.
1012	**4**	**45**	neg.
1021	neg.	**78**	neg.
1022	**5**	**46**	**19**
1040	**4**	neg.	**14**
1047	n.t.	neg.	**9**

Table 3.9.1 (continued):

donor #	AYPANFPYPL	NYIAFRDNFI	TYFSLNNKF
1049	neg.	neg.	**23**
1052	neg.	neg.	**31**
1071	neg.	neg.	**192**
1073	neg.	neg.	**44**
1082	neg.	neg.	**17**
1101	n.t.	neg.	neg.
1117	neg.	neg.	**105**
1118	neg.	**5**	**4**
1156	**9**	**29**	**227**
1169	**7**	neg.	**125**
1170	n.t.	neg.	**42**
1173	**20**	**68**	**154**
1180	**64**	**7**	**231**
1190	**3**	neg.	**4**
1238	n.t.	n.t.	**114**
1254	n.t.	neg.	**7.0**
1282	n.t.	n.t.	**250**
1292	n.t.	n.t.	neg.
1293	n.t.	n.t.	neg.
1295	neg.	n.t.	**7**
1320	n.t.	n.t.	**207**
1331	neg.	n.t.	**33**
1332	neg.	n.t.	**33**
1348	n.t.	n.t.	**6**
1363	neg.	n.t.	**48**
1369	**4**	n.t.	**38**
1401	**8**	n.t.	**104**
1425	n.t.	n.t.	**9**
1430	n.t.	n.t.	**4.6**
1487	n.t.	n.t.	**27**
total	**35% 14 / 40**	**32% 12 / 38**	**76% 44 / 58**

Table 3.9.2: Screening results of five epitope candidates for HLA-A*24. Spot count ratios for positive responses are given. neg.: negative response.

donor #	AYSYKARFTL	CYMRTCGMF	DYLSAANML	KYKDYQQVGI	KYNPTNVEI
878	neg.	neg.	neg.	neg.	neg.
882	neg.	neg.	neg.	neg.	neg.
888	neg.	neg.	neg.	neg.	neg.
904	neg.	neg.	neg.	neg.	neg.
911	neg.	**19**	neg.	neg.	**13**
945	neg.	neg.	neg.	neg.	neg.
951	neg.	neg.	**6**	neg.	neg.
973	neg.	neg.	neg.	neg.	neg.
998	neg.	neg.	neg.	neg.	neg.
999	neg.	neg.	neg.	neg.	neg.
1007	**3**	neg.	neg.	neg.	neg.
1008	neg.	**136**	neg.	**13**	neg.
1010	neg.	neg.	neg.	neg.	neg.
1012	neg.	neg.	neg.	neg.	neg.
1021	neg.	neg.	neg.	neg.	neg.
1022	neg.	neg.	neg.	neg.	neg.
total	**6.3% 1 / 16**	**13% 2 / 16**	**6.3% 1 / 16**	**6.3% 1 / 16**	**6.3% 1 / 16**

Table 3.9.3: Screening results of four epitope candidates for HLA-A*24. Spot count ratios for positive responses are given. neg.: negative response.

donor #	NYIAFRDNF	NYMSAGPHMI	SFTPRQAIL	TYFDIRGVL
878	neg.	neg.	neg.	neg.
882	neg.	neg.	neg.	neg.
888	neg.	neg.	neg.	neg.
904	neg.	neg.	neg.	neg.
911	neg.	neg.	neg.	neg.
945	**51**	neg.	neg.	neg.
951	neg.	neg.	neg.	neg.
973	neg.	neg.	neg.	neg.
998	neg.	**199**	neg.	neg.
999	neg.	neg.	**13**	neg.
1007	neg.	neg.	neg.	neg.
1008	neg.	neg.	neg.	**155**
1010	neg.	neg.	neg.	neg.
1012	neg.	neg.	neg.	neg.
1021	neg.	**16**	neg.	neg.
1022	neg.	neg.	neg.	neg.
total	**6.3% 1 / 16**	**13% 2 / 16**	**6.3% 1 / 16**	**6.3% 1 / 16**

The 10 remaining peptides from Table 3.3 (DYMDNVNPF, EYLSPGLVQF, HYPDQFIPNF, KYKEYQQVGI, LYSNIALYL, PYLDGTFYL, SYDPDVRII, SYKDRMYSF, and TYSGSIPYL) were tested with the same donors as the peptides in Table 3.9.3. No positive responses could be observed.

3.6. ELISPOTs with HLA-mismatched donors

The ELISPOT screening with donors who are positive for the supposed restricting alleles of the epitope candidates enabled quick detection of IFN-γ immune responses. ELISPOT assays using PBMCs do not, however, allow conclusions as to which cells are responsible for the cytokine secretion. Especially $CD4^+$ T cells, *via* MHC class II molecules, could also be responding to stimuli with short peptides. Even if the IFN-γ secreting cells are in fact $CD8^+$ T cells, this does not necessarily mean that the responses are truly mediated by the MHC allotype in question. Thus, in a first step to weed out responses mediated by other MHC molecules than the desired class I allotype, peptides that had elicited responses frequently were tested with PBMCs of HLA-mismatched donors that did not carry the allotype in question. If a peptide is in fact an epitope restricted by this allotype, responses should be significantly less frequent in the HLA-mismatched cohort compared to the HLA-matched cohort. The ELISPOTs were carried out with single peptides after memory T cell expansion in the same manner as for HLA-matched donors. The following tables list the results of these experiments.

3.6.1. HLA-A*01:

Table 3.10: ELISPOT results of the three epitope candidates for HLA-A*01 tested with HLA-A*01-negative donors. Spot count ratios for positive responses are given. neg.: negative response.

donor #	LTDLGQNLLY	TDLGQNLLY	YTYSGSIPY
1160	neg.	neg.	**16**
1163	neg.	neg.	neg.
1164	neg.	neg.	neg.
1166	neg.	neg.	neg.
1169	neg.	neg.	neg.
1173	neg.	neg.	neg.
1174	neg.	neg.	neg.
1176	neg.	neg.	neg.
1180	neg.	neg.	neg.
1181	neg.	neg.	**595**
1184	neg.	**3**	neg.
1187	neg.	neg.	**4**
1191	neg.	neg.	neg.
1192	neg.	neg.	neg.
1198	neg.	neg.	neg.
1201	neg.	**9**	neg.
total	**0% 0 / 16**	**13% 2 / 16**	**19% 3 / 16**

Table 3.11: Ratios of positive responses by PBMCs of HLA-matched and –mismatched donors to the peptides of table 3.10 and their p-values by Fisher's exact test. Differences are considered significant for $p < 0.05$.

	responding A*01-positive donors	responding A*01-negative donors	p-value
LTDLGQNLLY	93% 68 / 73	0% 0 / 16	**$1.2 \cdot 10^{-13}$**
TDLGQNLLY	88% 14 / 16	13% 2 / 16	**$2.4 \cdot 10^{-5}$**
YTYSGSIPY	17% 7 / 42	19% 3 / 16	0.73

3.6.2. HLA-A*02:

Table 3.12.1: ELISPOT results of two epitope candidates for HLA-A*02 tested with HLA-A*02-negative donors. Spot count ratios for positive responses are given. neg.: negative response; n.t.: not tested.

donor #	TFYLNHTFKK	TLLYVLFEV	donor #	TFYLNHTFKK	TLLYVLFEV
552	neg.	n.t.	**1170**	**40**	n.t.
555	**13**	n.t.	**1181**	**167**	n.t.
556	**455**	n.t.	**1241**	neg.	n.t.
574	neg.	n.t.	**1246**	**32**	n.t.
623	neg.	n.t.	**1250**	**44**	n.t.
634	**20**	n.t.	**1254**	**89**	n.t.
940	**45**	n.t.	**1265**	**24**	n.t.
961	neg.	n.t.	**1271**	**60**	n.t.
991	n.t.	**11**	**1296**	**4**	n.t.
992	n.t.	neg.	**1321**	**482**	n.t.
995	n.t.	neg.	**1324**	**45**	n.t.
998	n.t.	neg.	**1325**	neg.	n.t.
1002	n.t.	**45**	**1328**	**77**	n.t.
1005	n.t.	**16**	**1331**	**4**	n.t.
1012	n.t.	**11**	**1332**	**16**	n.t.
1014	n.t.	neg.	**1334**	neg.	n.t.
1017	n.t.	**26**	**1338**	**51**	n.t.
1025	n.t.	neg.	**1341**	**63**	n.t.
1030	n.t.	neg.	**1343**	**94**	n.t.
1038	n.t.	**188**	**1347**	**140**	n.t.
1047	**22**	n.t.	**1349**	neg.	n.t.
1101	**63**	n.t.	**1351**	neg.	n.t.
1142	**7**	n.t.	**1352**	**13**	n.t.
1150	neg.	n.t.	**1354**	**13**	n.t.
1153	neg.	n.t.	**1356**	**18**	n.t.
1164	neg.	n.t.	**total**	**69% 27 / 39**	**50% 6 / 12**

Table 3.12.2: ELISPOT results of five epitope candidates for HLA-A*02 tested with HLA-A*02-negative donors. Spot count ratios for positive responses are given. neg.: negative response.

donor #	LLDQLIEEV	LLYANSAHAL	SMPNLVPEV	VLFEVFDVV	YVLFEVFDVV
1321	neg.	neg.	**36**	neg.	neg.
1324	neg.	**4**	**32**	neg.	neg.
1325	neg.	neg.	**56**	neg.	neg.
1328	neg.	neg.	neg.	neg.	neg.
1331	neg.	neg.	neg.	neg.	neg.
1332	neg.	neg.	neg.	neg.	neg.
1334	neg.	neg.	neg.	neg.	neg.
1338	neg.	neg.	neg.	neg.	neg.
1341	neg.	neg.	**431**	neg.	neg.
1343	neg.	neg.	neg.	neg.	neg.
1347	neg.	neg.	neg.	neg.	neg.
1349	neg.	neg.	neg.	neg.	neg.
1351	neg.	neg.	neg.	neg.	neg.
1352	**65**	**9**	**444**	neg.	neg.
1354	neg.	**19**	neg.	neg.	neg.
1356	neg.	neg.	**528**	neg.	neg.
total	**6.3% 1 / 16**	**19% 3 / 16**	**38% 6 / 16**	**0% 0 / 16**	**0% 0 / 16**

Table 3.13: Ratios of positive responses by PBMCs of HLA-matched and –mismatched donors to the peptides of tables 3.12.1 and 3.12.2 and their p-values by Fisher's exact test. Differences are considered significant for $p < 0.05$.

	responding A*02-positive donors	responding A*02-negative donors	p-value
LLDQLIEEV	68% 50 / 74	6.3% 1 / 16	**$6.2 \cdot 10^{-6}$**
LLYANSAHAL	8.3% 3 / 36	19% 3 / 16	0.94
SMPNLVPEV	28% 17 / 61	38% 6 / 16	0.73
TFYLNHTFKK	69% 18 / 26	69% 27 / 39	0.61
TLLYVLFEV	67% 38 / 57	50% 6 / 12	0.22
VLFEVFDVV	13% 7 / 56	0% 0 / 16	0.16
YVLFEVFDVV	48% 14 / 29	0% 0 / 16	**$4.6 \cdot 10^{-4}$**

3.6.3. HLA-A*24:

Table 3.14: ELISPOT results of the three epitope candidates for HLA-A*24 tested with HLA-A*24-negative donors. Spot count ratios for positive responses are given. neg.: negative response; n.t.: not tested.

donor #	AYPANFPYPL	NYIAFRDNFI	TYFSLNNKF
1164	n.t.	neg.	neg.
1181	n.t.	**189**	neg.
1241	n.t.	**191**	neg.
1246	n.t.	neg.	neg.
1250	n.t.	neg.	neg.
1265	n.t.	**34**	neg.
1336	neg.	**83**	neg.
1337	neg.	neg.	neg.
1338	neg.	neg.	neg.
1340	neg.	**3**	neg.
1341	neg.	neg.	neg.
1342	neg.	neg.	neg.
1343	neg.	neg.	neg.
1344	neg.	neg.	neg.
1347	neg.	neg.	neg.
1349	neg.	neg.	neg.
1350	neg.	**93**	neg.
1351	neg.	**146**	**70**
1352	neg.	neg.	neg.
1355	neg.	**424**	neg.
1356	neg.	**350**	neg.
1357	neg.	neg.	neg.
total	**0% 0 / 16**	**41% 9 / 22**	**4.5% 1 / 22**

Table 3.15: Ratios of positive responses by PBMCs of HLA-matched and –mismatched donors to the peptides of table 3.10 and their p-values by Fisher's exact test. Differences are considered significant for $p < 0.05$.

	responding A*24-positive donors	responding A*24-negative donors	p-value
AYPANFPYPL	35% 14 / 40	0% 0 / 16	**$4.0 \cdot 10^{-3}$**
NYIAFRDNFI	32% 12 / 38	41% 9 / 22	0.84
TYFSLNNKF	76% 44 / 58	4.5% 1 / 22	**$3.9 \cdot 10^{-9}$**

In the ELISPOT assays with HLA-mismatched donors, only five epitopes showed significantly less frequent responses than with matched donors. (L)TDLGQNLLY from the hexon protein has already been described as an A*01-restricted epitope in the shorter variant[279]. On HLA-A*02 (Y)VLFEVFDVV, also from the hexon protein, has also been described as an epitope in the longer variant[287], while the two variants of (S)LLDQLIEEV from E1A have not been reported as epitopes yet. On HLA-A*24, the hexon-derived peptide TYFSLNNKF has been published as an epitope as well[279], while the peptide AYPANFPYPL is novel. The other two allegedly A*02-restricted epitopes that have been described in the literature, LLYANSAHAL[287] and TFYLNHTFKK[279], elicited responses in at least as many of the HLA-mismatched donors as of the HLA-matched ones, which made it doubtful whether the responses were really mediated by $CD8^+$ T cells and restricted by HLA-A*02.

3.7. Tetramer and intracellular cytokine stainings

To corroborate the results of the ELISPOT screenings and to identify the cells that are actually responding to the peptide stimuli, flow cytometric analyses were performed with expanded PBMCs. In the first experiments with HLA-A*02-predicted peptides, only peptide-MHC-tetramers were used to identify specific T cells. In later experiments, tetramer staining was combined with intracellular IFN-γ staining - as previously employed in our group[331] - to investigate whether the specific cells were also functional and responsible for cytokine secretion. The HLA-A*01:01-tetramers were carrying the mutation A245V to lower their affinity to CD8[312] and avoid unspecific binding to $CD8^+$ cells. With A*02:01- and A*24:02-tetramers this problem does not arise and hence wild type tetramers were employed. Staining for the coreceptor molecules CD8 and CD4 enabled to determine whether the observed responses were mediated by MHC class I or class II molecules. Finally, a newly available 8-color flow cytometer (FACSCanto II, BD Biosciences) in our institute

allowed the inclusion of an anti-TNF antibody in the staining panel as well, thus enabling to search for responses to a further cytokine.

In the following sections, the results of these flow cytometric experiments will be displayed for each tested peptide. Generally, percentage values of tetramer-specific or cytokine secreting cells will refer to the total number of $CD8^+$ or $CD4^+$ cells (according to context) as 100%. Values were corrected for unspecific staining by subtracting the values of the respective HIV negative control. Values of at least 0.1% were regarded as a positive response.

3.7.1. HLA-A*01

From the A*01-restricted peptides, the epitope (L)TDLGQNLLY in both length variants was selected to confirm its nature as a $CD8^+$ T cell epitope. The shorter variant has already been published as a $CD8^+$ T cell epitope[279].

The longer variant was used in combined tetramer/IFN-γ stainings for 12 A*01-positive donors that had already shown positive responses in ELISPOT. Cells were expanded according to the standard protocol (see 2.3.8) with the adenoviral and the HIV peptide and then stimulated before staining with either the adenoviral or the HIV peptide. Figure 3.6 shows an example of such a staining for one donor.

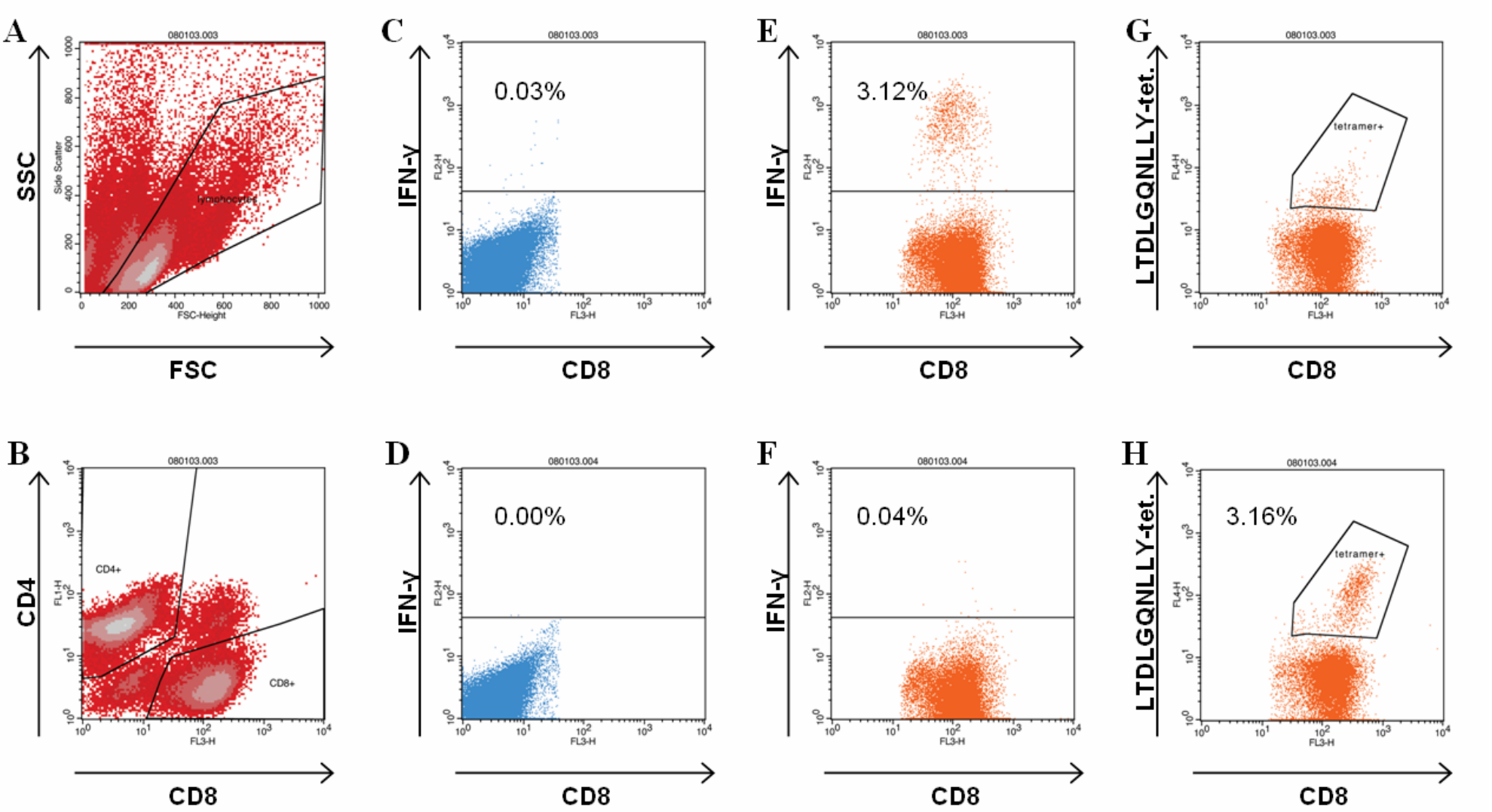


Figure 3.6: Combined tetramer/IFN-γ staining of the HLA-A*01-positive donor #1132 after expansion with the peptide LTDLGQNLLY and an HIV control peptide. **A:** FSC/SSC plot showing the lymphocyte gate. **B:** Plot showing the gating of CD4 and CD8 single positive cells among lymphocytes. **C:** IFN-γ response among $CD4^+$ cells stimulated with Adv peptide. **D:** IFN-γ response among $CD4^+$ cells stimulated with HIV control peptide. **E:** IFN-γ response among $CD8^+$ cells stimulated with Adv peptide. **F:** IFN-γ response among $CD8^+$ cells stimulated with HIV control peptide. **G:** Tetramer staining of $CD8^+$ cells stimulated with Adv peptide. **H:** Tetramer staining of $CD8^+$ cells stimulated with HIV peptide.

In this case, the response is typical for a $CD8^+$ T cell epitope. In the $CD4^+$ T cells, virtually no cytokine secretion can be observed (C, D), while among the $CD8^+$ cells there is a large population (3.12% of the $CD8^+$ cells) that secrete IFN-γ when the cells are stimulated with the adenoviral peptide (E), but not upon stimulation with the HIV control peptide (F). Since the control peptide has also been present during the *in vitro* expansion, the absence of a response can be ascribed to the lack of memory T cells, as should be expected in HIV-negative individuals. An interesting fact is the lower number and fluorescence intensity of tetramer-positive cells after stimulation with the adenoviral peptide (G), as compared to stimulation with the HIV peptide (H). However, it has been described that $CD8^+$ T cells lose their tetramer binding capability for several hours after stimulation with their specific peptide[315]. Thus it is not surprising that the LTDLGQNLLY-tetramer-specific cell population can only be observed after stimulation with the irrelevant HIV peptide. Table 3.16 lists the responses observed in the $CD4^+$ and $CD8^+$ lymphocyte subsets of all 12 tested donors and the IFN-γ ELISPOT screening results of the same donors.

Table 3.16: Results of a combined tetramer/IFN-γ staining of HLA-A*01-positive PBMCs after stimulation with **LTDLGQNLLY** and the ELISPOT screening results of the same donors. Percentages of IFN-γ^+ cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	of $CD4^+$: IFN-γ^+	of $CD8^+$: IFN-γ^+	of $CD8^+$: tetramer$^+$	ELISPOT result
1132	0.03%	**3.08%**	**3.16%**	**433**
1133	0.02%	**0.90%**	**0.85%**	**5**
1136	0.00%	0.02%	0.05%	**18**
1138	0.01%	**0.13%**	**0.58%**	**77**
1141	**0.17%**	**1.52%**	**1.16%**	**37**
1143	0.00%	**1.21%**	**1.38%**	**197**
1144	0.02%	**3.38%**	**3.57%**	**55**
1150	0.00%	**0.15%**	**0.14%**	**36**
1151	0.00%	**0.52%**	**1.62%**	**461**
1154	0.01%	**0.14%**	**0.25%**	**20**
1155	0.00%	0.00%	**1.06%**	**314**
1162	0.00%	0.06%	**0.31%**	**38**

Although all donors showed positive responses in the ELISPOT screening, IFN-γ secretion could be observed only in 9 out of 12 by flow cytometry. Two of the non-responding donors had tetramer-positive cells, however. One of the donors showed, apart from a $CD8^+$ T cell response, also a weak $CD4^+$ T cell response, but the general picture is consistent with what would be expected from a $CD8^+$ T cell epitope.

Additionally, three A*01-positive and one A*01-negative donor were expanded with both length variants and the HIV control and stained for IFN-γ, TNF, and with tetramer. Stimulations and tetramers of both length variants were used in separate assays. Figure 3.7 shows one donor as an example for staining with TDLGQNLLY tetramer. As in Figure 3.6, the response is a typical $CD8^+$ T cell reponse. Tables 3.17 and 3.18 summarize the results of these experiments and the ELISPOT screening results of the same donors.

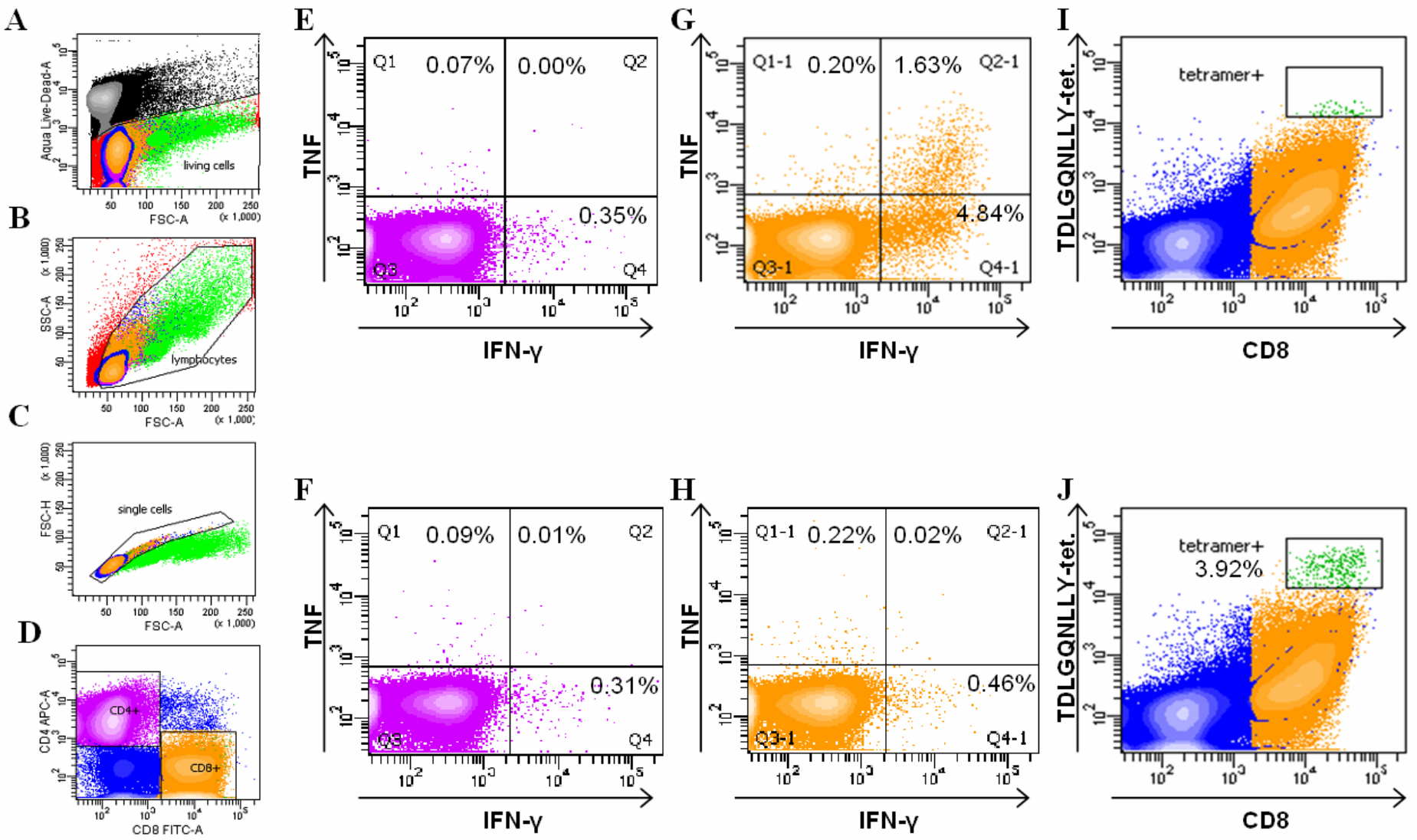


Figure 3.7: Combined tetramer/IFN-γ/TNF staining of the HLA-A*01-positive donor #1017 after expansion with the peptide TDLGQNLLY and an HIV control peptide. **A:** FSC/Live-Dead plot showing the living cell gate. **B:** FSC/SSC plot showing the lymphocyte gate among living cells. **C:** FSC-Area/FSC-Height plot showing the single cell gate among lymphocytes. **D:** CD8/CD4 plot showing the gates for CD4 and CD8 single positive cells among single cells. **E:** IFN-γ and TNF response among $CD4^+$ cells stimulated with Adv peptide. **F:** IFN-γ and TNF response among $CD4^+$ cells stimulated with HIV control peptide. **G:** IFN-γ and TNF response among $CD8^+$ cells stimulated with Adv peptide. **H:** IFN-γ and TNF response among $CD8^+$ cells stimulated with HIV control peptide. **I:** Tetramer staining of single cells stimulated with Adv peptide. **J:** Tetramer staining of single cells stimulated with HIV peptide.

Table 3.17: Results of a combined tetramer/IFN-γ/TNF staining after stimulation with **LTDLGQNLLY** and the ELISPOT screening results of the same donors. Percentages of cytokine-positive cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	HLA-A*01	of CD4+:			of CD8+:			of CD8+: tetramer+	ELISPOT result
		TNF+	IFN-γ+	TNF+ IFN-γ+	TNF+	IFN-γ+	TNF+ IFN-γ+		
1002	+	0.00%	0.06%	0.04%	0.07%	**1.66%**	**5.28%**	**2.83%**	**400**
1005	+	**0.24%**	0.00%	0.00%	**1.20%**	**1.22%**	**3.37%**	**4.28%**	**1000**
1017	+	0.00%	0.03%	0.00%	0.07%	**5.18%**	**2.89%**	**5.07%**	**125**
1201	-	0.00%	0.06%	0.01%	**0.18%**	0.00%	0.00%	0.01%	neg.

Table 3.18: Results of a combined tetramer/IFN-γ/TNF staining after stimulation with **TDLGQNLLY** and the ELISPOT screening results of the same donors. Percentages of cytokine-positive cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	HLA-A*01	of CD4+:			of CD8+:			of CD8+: tetramer+	ELISPOT result
		TNF+	IFN-γ+	TNF+ IFN-γ+	TNF+	IFN-γ+	TNF+ IFN-γ+		
1002	+	0.00%	0.00%	0.00%	**0.11%**	**2.06%**	**3.07%**	**2.74%**	**400**
1005	+	0.00%	0.00%	0.00%	**1.53%**	**0.92%**	**1.30%**	**6.99%**	**781**
1017	+	0.00%	0.04%	0.00%	0.00%	**4.38%**	**1.61%**	**3.92%**	**125**
1201	-	0.07%	0.00%	0.00%	**0.12%**	0.01%	0.02%	0.02%	**8.7**

A few weak cytokine responses can be observed to the longer peptide in CD4+ T cells, but generally the cytokine producers are CD8+ T cells. In the case of the HLA-A*01-negative donor #1201, some weak TNF responses in both CD8+ and CD4+ T cells are present, but no IFN-γ responses and no tetramer staining. This contrasts with the fact that this donor showed a weak response in the IFN-γ-ELISPOT to the shorter peptide variant. Generally, IFN-γ/TNF double producers seem to be the most reliable indicators of a response. Such cells are only observed in the CD8+ population and only in the HLA-A*01-positive donors.

3.7.2. HLA-A*02

Several of the epitope candidates predicted for HLA-A*02 and of the published epitopes were tested in tetramer and cytokine staining assays. The data will be presented here grouped by peptides.

LLDQLIEEV

This peptide was first tested after PBMC expansion in 18 A*02-positive donors by tetramers without concomitant cytokine staining. Conversely, 13 other A*02-positive donors were stained for IFN-γ but not with tetramers. 10 donors were stained for both IFN-γ and with tetramer. Tables 3.19 - 3.21 summarize the results of these experiments.

Table 3.19: Results of a tetramer staining of A*02-positive PBMCs after stimulation with **LLDQLIEEV** and the ELISPOT screening results of the same donors.

donor #	of $CD8^+$: $tetramer^+$	ELISPOT result
757	**0.37%**	**57**
765	**0.56%**	neg.
773	**0.41%**	**9**
778	**3.79%**	**15**
784	**0.82%**	neg.
1011	**0.89%**	**6**
1013	0.01%	neg.
1016	**1.67%**	**10**
1022	0.02%	**13**
1026	**0.74%**	**11**
1033	0.07%	**8**
1045	**0.33%**	**80**
1051	0.02%	**3**
1052	**0.18%**	neg.
1055	**0.49%**	**48**
1057	**0.25%**	**15**
1058	**0.24%**	**9**
1061	0.03%	**13**

Table 3.20: Results of an IFN-γ staining of A*02-positive PBMCs after stimulation with **LLDQLIEEV** and the ELISPOT screening results of the same donors. Percentages of IFN-γ^+ cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	of CD4⁺: IFN-γ⁺	of CD8⁺: IFN-γ⁺	ELISPOT result
1107	**0.23%**	**4.76%**	**333**
1108	**0.18%**	**6.80%**	**250**
1109	0.02%	0.06%	neg.
1113	0.04%	**0.88%**	**821**
1114	0.06%	**1.65%**	**63**
1119	0.06%	0.05%	neg.
1122	**0.12%**	**1.33%**	**20**
1124	0.05%	**1.56%**	**275**
1129	**0.35%**	**0.38%**	**94**
1130	0.08%	**2.65%**	**28**
1135	0.04%	**0.74%**	**25**
1137	**0.11%**	**0.14%**	**7**
1139	0.04%	**0.82%**	**28**

Table 3.21: Results of a combined tetramer/IFN-γ staining of HLA-A*02-positive PBMCs after stimulation with **LLDQLIEEV** and the ELISPOT screening results of the same donors. Percentages of IFN-γ^+ cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	of CD4⁺: IFN-γ⁺	of CD8⁺: IFN-γ⁺	of CD8⁺: tetramer⁺	ELISPOT result
759	0.00%	**1.40%**	**1.56%**	**437**
765	0.01%	**0.54%**	**2.49%**	neg.
778	0.00%	**2.08%**	**4.79%**	**15**
780	0.00%	**0.49%**	**0.68%**	**10**
791	0.00%	**3.63%**	**4.83%**	**1000**
794	0.00%	**1.26%**	0.06%	neg.
1013	0.01%	**0.15%**	0.04%	neg.
1022	0.00%	0.00%	0.00%	**13**
1033	**0.13%**	**5.78%**	**8.29%**	**8**
1051	0.00%	**0.81%**	0.02%	**3**

Additionally, combined tetramer and IFN-γ/TNF stainings were carried out with PBMCs of seven A*02-positive and two A*02-negative donors. Table 3.22 summarizes the results of these experiments, while Figure 3.8 shows the staining of one donor as an example.

Table 3.22: Results of a combined tetramer/IFN-γ/TNF staining after stimulation with **LLDQLIEEV** and the ELISPOT screening results of the same donors. Percentages of cytokine-positive cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	HLA-A*02	of CD4+:			of CD8+:			of CD8+: tetramer+	ELISPOT result
		TNF+	IFN-γ+	TNF+ IFN-γ+	TNF+	IFN-γ+	TNF+ IFN-γ+		
970	+	0.00%	0.00%	0.06%	0.00%	**0.33%**	**1.20%**	**1.13%**	**342**
984	+	0.00%	0.00%	0.00%	**0.24%**	0.00%	0.08%	0.07%	n.t.
1013	+	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	neg.
1026	+	**0.25%**	**0.46%**	**0.42%**	**1.02%**	**1.06%**	**0.14%**	**2.40%**	**11**
1113	+	0.00%	0.00%	0.07%	**0.11%**	**1.20%**	**2.42%**	**3.16%**	**821**
1124	+	0.00%	0.00%	0.00%	0.00%	0.07%	**0.31%**	**0.38%**	**275**
1137	+	0.00%	0.00%	0.00%	0.02%	0.00%	**0.16%**	**0.21%**	**7**
1324	-	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.05%	neg.
1352	-	0.00%	0.00%	0.00%	0.00%	0.04%	0.00%	0.04%	neg.

In the cytokine staining assays, some responses by $CD4^+$ T cells can be seen, but these are weak in comparison to the $CD8^+$ T cell responses. Furthermore, some donors with tetramer-positive cells do not exhibit IFN-γ responses and *vice versa*. The IFN-γ responses observed in the flow cytometry assays do not always correspond with those observed in the ELISPOT screening, but this can be explained by the fact that the assays were performed after different rounds of stimulation. The results thus depend on the presence of a sufficient number of memory precursor cells in the batch selected for stimulation, which may not always be given if the frequency of the precursor cells among the PBMCs is very low. Generally, the responses are typical for $CD8^+$ T cell epitope, as can be also seen in Figure 3.8.

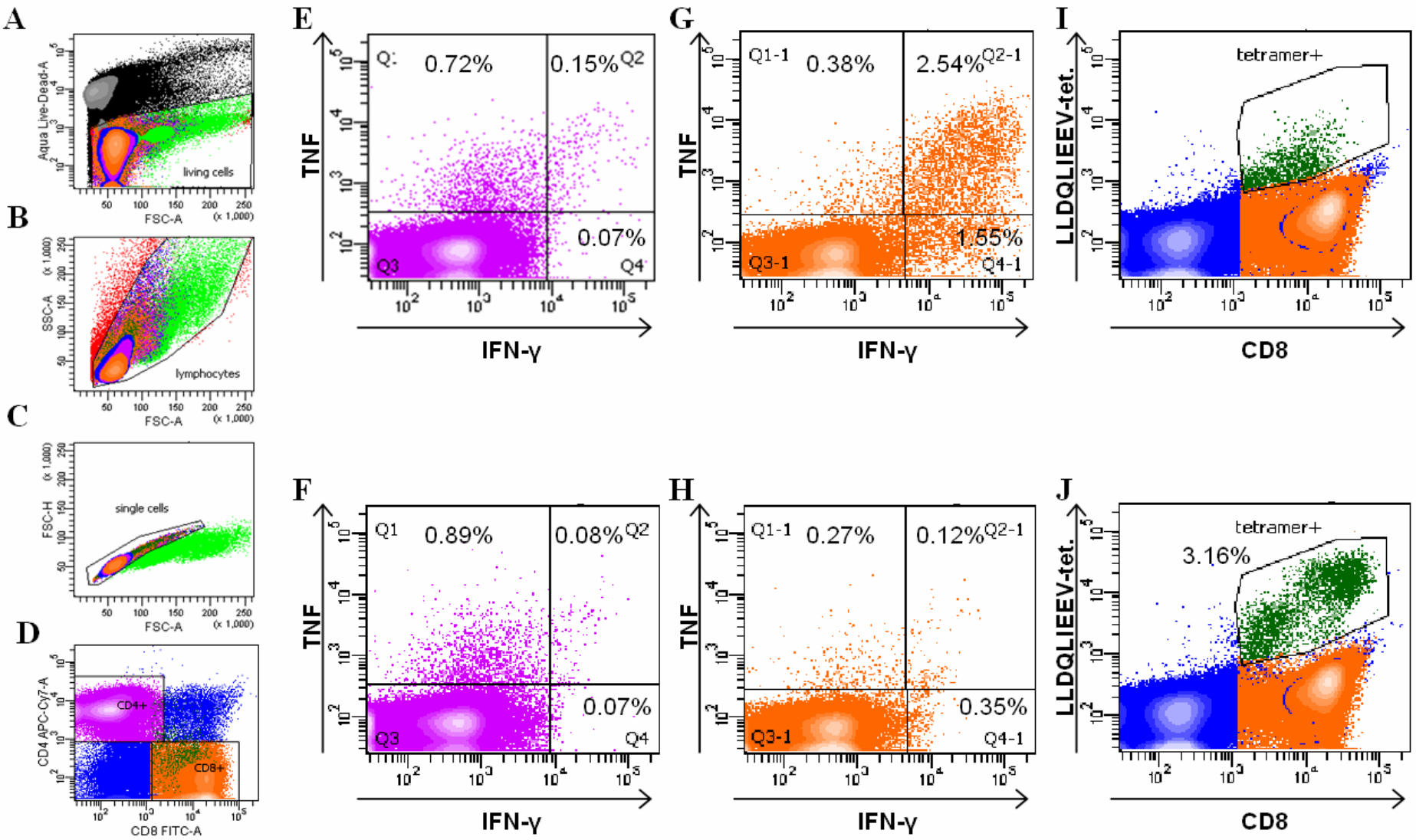


Figure 3.8: Combined tetramer/IFN-γ/TNF staining of the HLA-A*02-positive donor #1113 after expansion with the peptide LLDQLIEEV and an HIV control peptide. **A:** FSC/Live-Dead plot showing the living cell gate. **B:** FSC/SSC plot showing the lymphocyte gate among living cells. **C:** FSC-Area/FSC-Height plot showing the single cell gate among lymphocytes. **D:** CD8/CD4 plot showing the gates for CD4 and CD8 single positive cells among single cells. **E:** IFN-γ and TNF response among $CD4^+$ cells stimulated with Adv peptide. **F:** IFN-γ and TNF response among $CD4^+$ cells stimulated with HIV control peptide. **G:** IFN-γ and TNF response among $CD8^+$ cells stimulated with Adv peptide. **H:** IFN-γ and TNF response among $CD8^+$ cells stimulated with HIV control peptide. **I:** Tetramer staining of single cells stimulated with Adv peptide. **J:** Tetramer staining of single cells stimulated with HIV peptide.

LLYANSAHAL

For this peptide, no functional tetramers could be produced. Tetramers were obtained applying the standard refolding protocol, but no staining in any donor could ever be observed. Nevertheless, cytokine responses could be detected in several donors. With PBMCs of seven A*02-positive donors, only IFN-γ staining was performed (Table 3.23), while an IFN-γ/TNF double staining was performed with cells from seven A*02-positive and two A*02-negative donors (Table 3.24).

Table 3.23: Results of an IFN-γ staining of A*02-positive PBMCs after stimulation with **LLYANSAHAL** and the ELISPOT screening results of the same donors. Percentages of IFN-γ^+ cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	of $CD4^+$: IFN-γ^+	of $CD8^+$: IFN-γ^+	ELISPOT result
778	0.00%	0.00%	neg.
780	0.00%	0.00%	neg.
791	0.00%	**0.33%**	neg.
794	0.00%	**0.69%**	neg.
1013	**0.31%**	0.00%	**6**
1022	0.00%	0.00%	neg.
1033	0.00%	0.00%	neg.

Table 3.24: Results of a combined IFN-γ/TNF staining after stimulation with **LLYANSAHAL** and the ELISPOT screening results of the same donors. Percentages of cytokine-positive cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	HLA-A*02	of $CD4^+$:			of $CD8^+$:			ELISPOT result
		TNF^+	IFN-γ^+	TNF^+ IFN-γ^+	TNF^+	IFN-γ^+	TNF^+ IFN-γ^+	
970	+	**0.61%**	0.04%	**0.76%**	0.00%	0.00%	0.00%	n.t.
984	+	0.00%	**0.39%**	**1.46%**	0.00%	**0.57%**	**0.11%**	n.t.
1013	+	0.00%	0.01%	**0.20%**	0.00%	0.00%	0.00%	**6**
1026	+	**0.11%**	**0.20%**	0.03%	0.00%	0.04%	0.00%	neg.
1113	+	0.05%	0.00%	0.03%	0.00%	0.03%	**2.72%**	n.t.
1124	+	**0.10%**	0.00%	0.00%	0.00%	0.00%	0.00%	n.t.
1137	+	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	n.t.
1324	-	**0.11%**	0.00%	0.00%	0.00%	0.00%	0.00%	**4**
1352	-	0.05%	0.07%	**0.22%**	0.00%	0.00%	0.00%	**9**

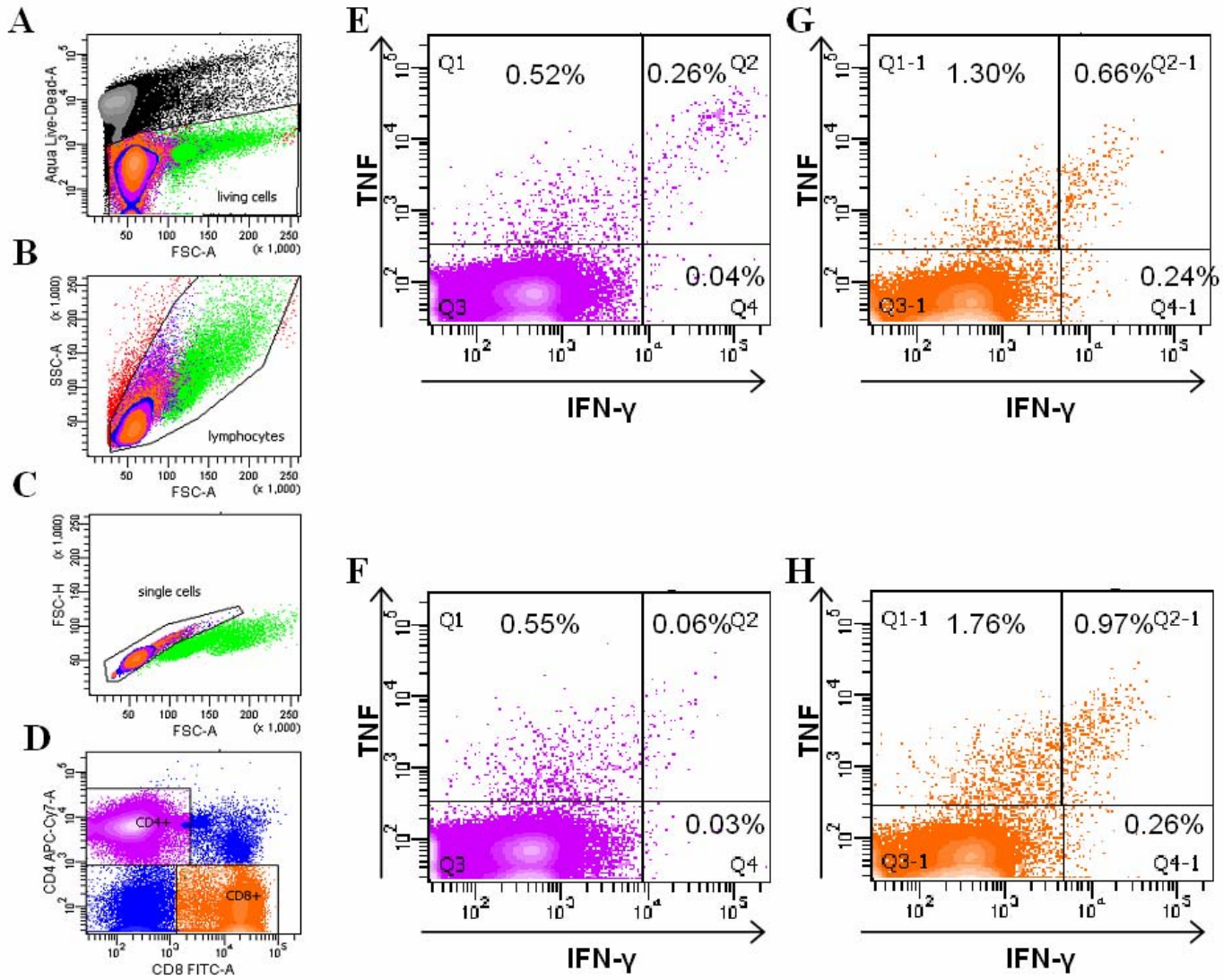


Figure 3.9: Combined IFN-γ/TNF staining of the HLA-A*02-positive donor #1013 after expansion with the peptide LLYANSAHAL and an HIV control peptide. **A:** FSC/Live-Dead plot showing the living cell gate. **B:** FSC/SSC plot showing the lymphocyte gate among living cells. **C:** FSC-Area/FSC-Height plot showing the single cell gate among lymphocytes. **D:** CD8/CD4 plot showing the gates for CD4 and CD8 single positive cells among single cells. **E:** IFN-γ and TNF response among $CD4^+$ cells stimulated with Adv peptide. **F:** IFN-γ and TNF response among $CD4^+$ cells stimulated with HIV control peptide. **G:** IFN-γ and TNF response among $CD8^+$ cells stimulated with Adv peptide. **H:** IFN-γ and TNF response among $CD8^+$ cells stimulated with HIV control peptide.

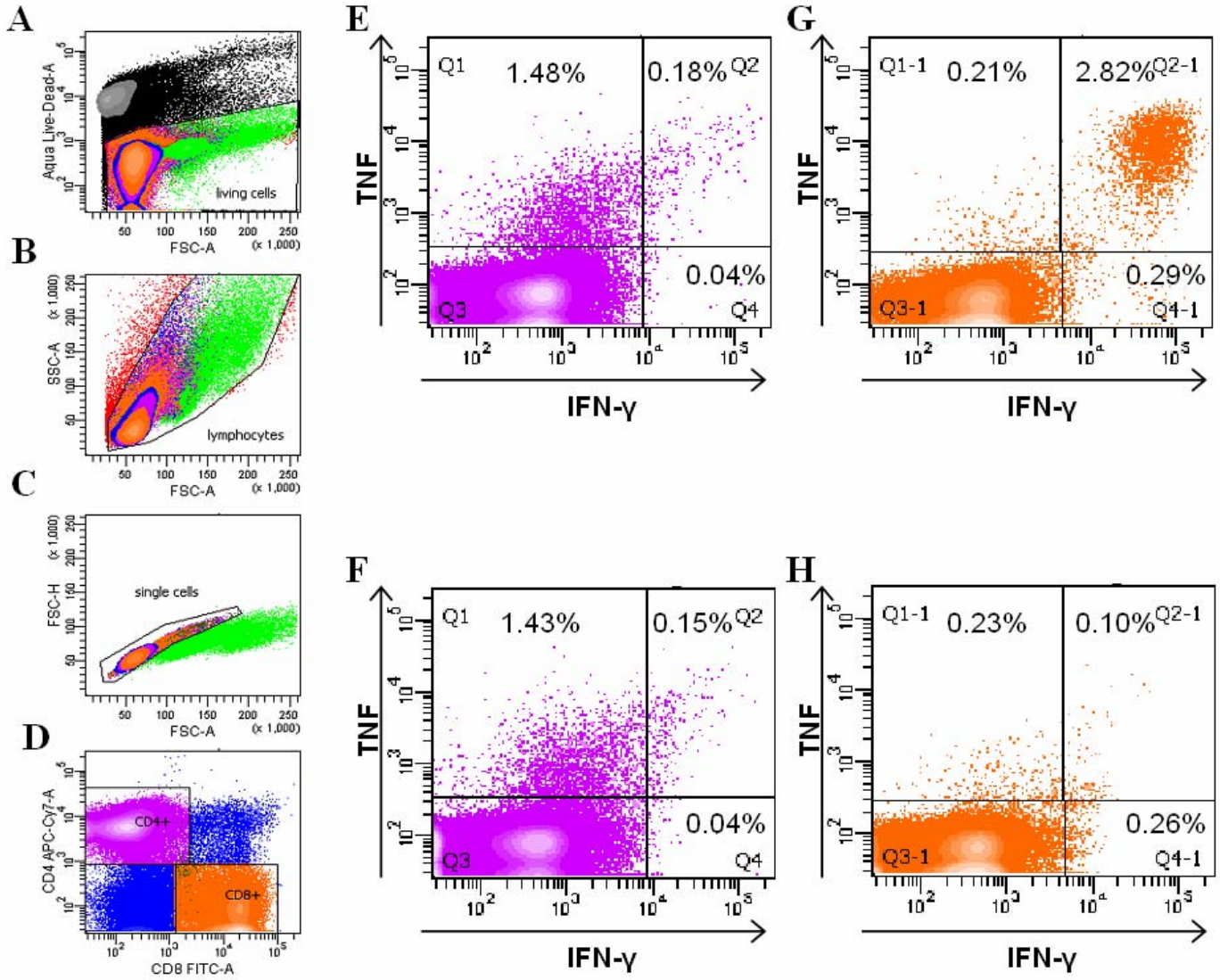


Figure 3.10: Combined IFN-γ/TNF staining of the HLA-A*02-positive donor #1113 after expansion with the peptide LLYANSAHAL and an HIV control peptide. **A:** FSC/Live-Dead plot showing the living cell gate. **B:** FSC/SSC plot showing the lymphocyte gate among living cells. **C:** FSC-Area/FSC-Height plot showing the single cell gate among lymphocytes. **D:** CD8/CD4 plot showing the gates for CD4 and CD8 single positive cells among single cells. **E:** IFN-γ and TNF response among $CD4^+$ cells stimulated with Adv peptide. **F:** IFN-γ and TNF response among $CD4^+$ cells stimulated with HIV control peptide. **G:** IFN-γ and TNF response among $CD8^+$ cells stimulated with Adv peptide. **H:** IFN-γ and TNF response among $CD8^+$ cells stimulated with HIV control peptide.

Several substantial $CD4^+$ T cell responses can be seen to the peptide. Figure 3.9 shows one example. Although the background staining for TNF is quite high, a distinct and strongly double-positive population can be seen in plot E that is absent from the HIV control plot (F). Nevertheless, the peptide also elicited a very strong $CD8^+$ T cell response that is shown in Figure 3.10. Thus, apart from being a $CD8^+$ T cell epitope as previously published[287], this peptide is most likely also part of a yet unknown $CD4^+$ T cell epitope.

SMPNLVPEV

This peptide was first tested after PBMC expansion in five A*02-positive donors by tetramers without concomitant cytokine staining. Conversely, eight other A*02-positive donors were stained for IFN-γ but not with tetramers. Seven donors were stained for both IFN-γ and with tetramer. Tables 3.25 - 3.27 summarize the results of these experiments.

Table 3.25: Results of a tetramer staining of A*02-positive PBMCs after stimulation with **SMPNLVPEV** and the ELISPOT screening results of the same donors.

donor #	of $CD8^+$: $tetramer^+$	ELISPOT result
757	**0.31%**	neg.
765	0.07%	neg.
773	**0.22%**	neg.
778	0.08%	neg.
784	**0.83%**	**7**

Table 3.26: Results of an IFN-γ staining of A*02-positive PBMCs after stimulation with **SMPNLVPEV** and the ELISPOT screening results of the same donors. Percentages of IFN-γ^+ cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	of $CD4^+$: IFN-γ^+	of $CD8^+$: IFN-γ^+	ELISPOT result
1113	**0.34%**	**0.27%**	neg.
1114	**0.27%**	**0.56%**	**4**
1122	0.00%	**0.12%**	neg.
1124	0.03%	**0.39%**	**67**
1129	0.05%	**0.10%**	**7**
1135	**1.30%**	**2.17%**	neg.
1137	0.02%	**0.24%**	**85**
1139	0.02%	0.04%	**5**

Table 3.27: Results of a combined tetramer/IFN-γ staining of HLA-A*02-positive PBMCs after stimulation with **SMPNLVPEV** and the ELISPOT screening results of the same donors. Percentages of IFN-γ^+ cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	of $CD4^+$: IFN-γ^+	of $CD8^+$: IFN-γ^+	of $CD8^+$: $tetramer^+$	ELISPOT result
759	0.00%	0.00%	0.07%	**35**
778	0.00%	0.00%	0.07%	neg.
791	0.00%	0.00%	0.06%	**45**
794	0.00%	0.02%	**2.39%**	neg.
1013	0.00%	0.00%	0.09%	neg.
1022	0.00%	0.00%	**0.42%**	neg.
1065	0.02%	**1.89%**	**2.24%**	**29**

Additionally, combined tetramer and IFN-γ/TNF stainings were carried out with PBMCs of seven A*02-positive and two A*02-negative donors. Table 3.28 summarizes the results of these experiments.

Table 3.28: Results of a combined tetramer/IFN-γ/TNF staining after stimulation with **SMPNLVPEV** and the ELISPOT screening results of the same donors. Percentages of cytokine-positive cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	HLA-A*02	of CD4+:			of CD8+:			of CD8+: tetramer+	ELISPOT result
		TNF+	IFN-γ+	TNF+ IFN-γ+	TNF+	IFN-γ+	TNF+ IFN-γ+		
970	+	0.00%	0.04%	0.00%	0.00%	**0.81%**	**7.14%**	0.06%	**1000**
984	+	0.00%	0.00%	0.00%	0.00%	**0.29%**	**0.14%**	0.09%	n.t.
1013	+	0.00%	0.01%	0.00%	0.00%	0.09%	0.00%	0.03%	neg.
1026	+	0.00%	0.00%	**0.79%**	0.05%	0.00%	**5.65%**	**5.32%**	neg.
1113	+	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.06%	neg.
1124	+	0.00%	0.02%	0.03%	0.00%	**0.26%**	**1.31%**	**1.22%**	**67**
1137	+	0.00%	0.01%	0.00%	**0.14%**	**0.99%**	**1.37%**	**1.51%**	**85**
1324	-	0.00%	0.03%	0.00%	**0.19%**	**0.32%**	**17.70%**	0.05%	**32**
1352	-	0.00%	0.00%	0.00%	**0.16%**	**0.70%**	**9.57%**	0.06%	**444**

The cytokine secretion patterns for this peptide are rather unusual. There are several weak and one substantial (donor #1135) IFN-γ responses by $CD4^+$ T cells. Apart from that, cytokine secretion by $CD8^+$ T cells and tetramer affinity appear to be quite independent of each other. The following figures show examples for cytokine secretion accompanied by tetramer binding (donor #1137, Fig. 3.11), cytokine secretion without tetramer binding by cells of an A*02-negative (donor 1352, Fig. 3.12), and the reverse case, a strong tetramer-positive population without any IFN-γ release (donor #794, Fig. 3.13).

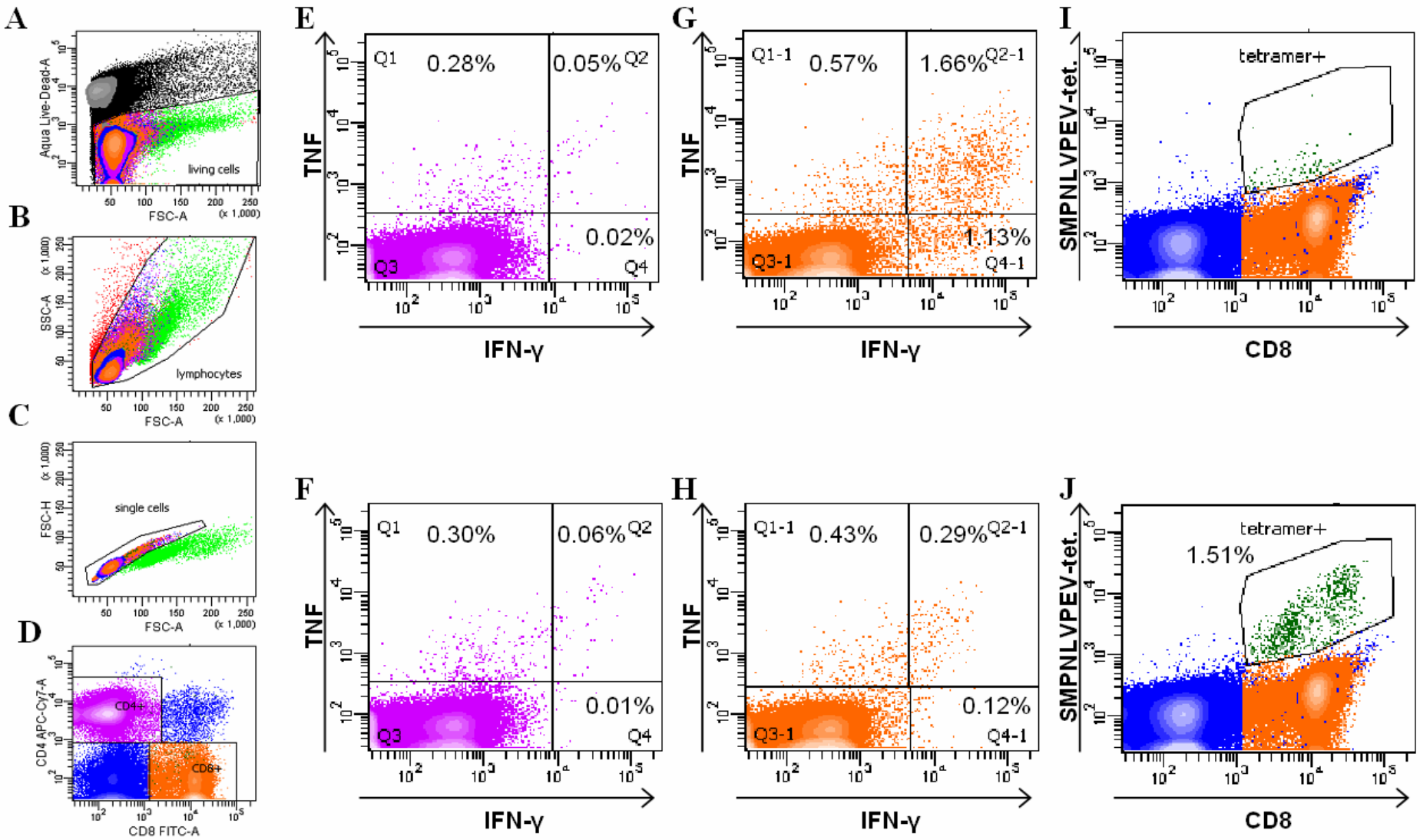


Figure 3.11: Combined tetramer/IFN-γ/TNF staining of the HLA-A*02-positive donor #1137 after expansion with the peptide SMPNLVPEV and an HIV control peptide. **A:** FSC/Live-Dead plot showing the living cell gate. **B:** FSC/SSC plot showing the lymphocyte gate among living cells. **C:** FSC-Area/FSC-Height plot showing the single cell gate among lymphocytes. **D:** CD8/CD4 plot showing the gates for CD4 and CD8 single positive cells among single cells. **E:** IFN-γ and TNF response among $CD4^+$ cells stimulated with Adv peptide. **F:** IFN-γ and TNF response among $CD4^+$ cells stimulated with HIV control peptide. **G:** IFN-γ and TNF response among $CD8^+$ cells stimulated with Adv peptide. **H:** IFN-γ and TNF response among $CD8^+$ cells stimulated with HIV control peptide. **I:** Tetramer staining of single cells stimulated with Adv peptide. **J:** Tetramer staining of single cells stimulated with HIV peptide.

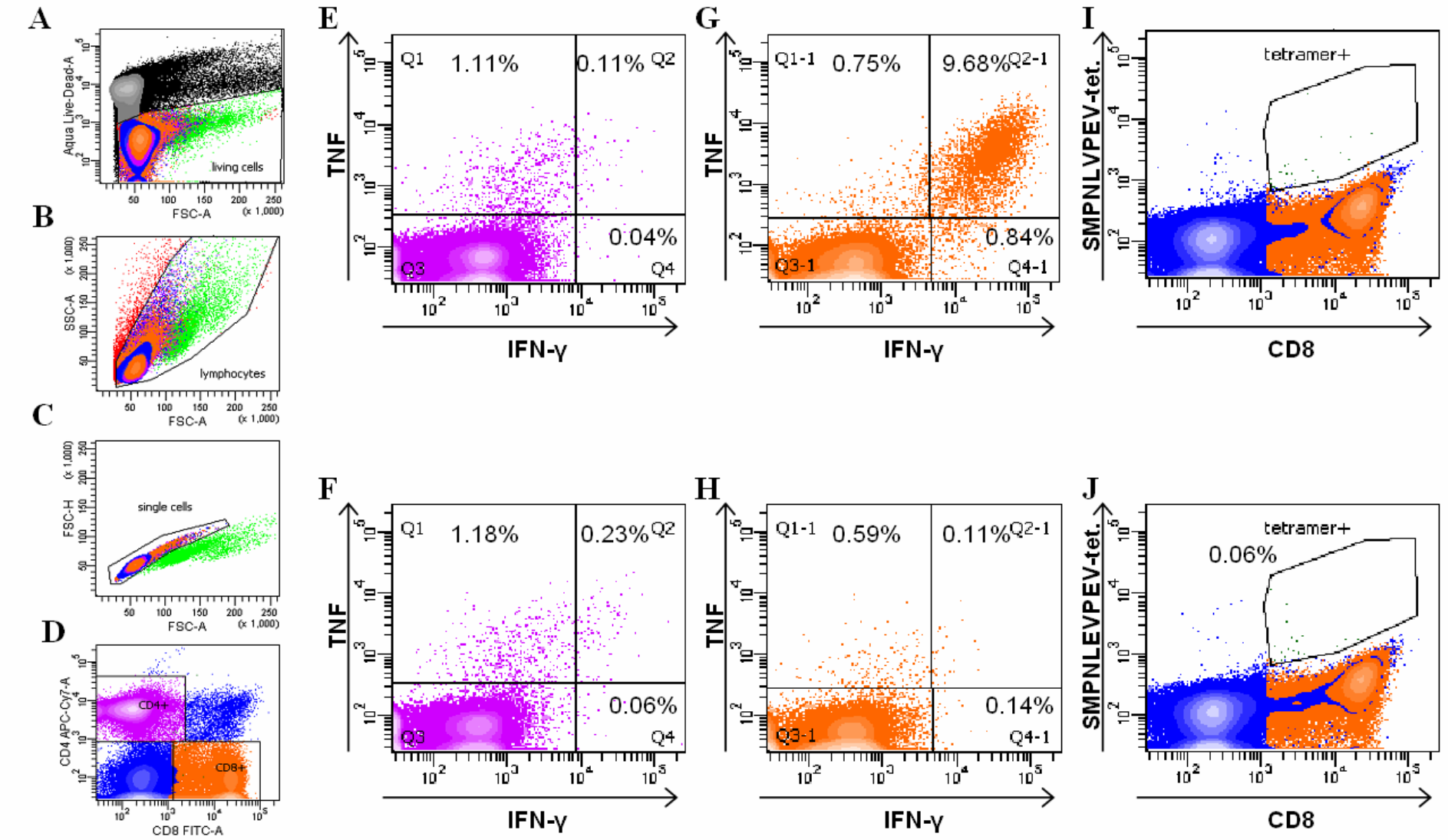


Figure 3.12: Combined tetramer/IFN-γ/TNF staining of the HLA-A*02-negative donor #1352 after expansion with the peptide SMPNLVPEV and an HIV control peptide. **A:** FSC/Live-Dead plot showing the living cell gate. **B:** FSC/SSC plot showing the lymphocyte gate among living cells. **C:** FSC-Area/FSC-Height plot showing the single cell gate among lymphocytes. **D:** CD8/CD4 plot showing the gates for CD4 and CD8 single positive cells among single cells. **E:** IFN-γ and TNF response among $CD4^+$ cells stimulated with Adv peptide. **F:** IFN-γ and TNF response among $CD4^+$ cells stimulated with HIV control peptide. **G:** IFN-γ and TNF response among $CD8^+$ cells stimulated with Adv peptide. **H:** IFN-γ and TNF response among $CD8^+$ cells stimulated with HIV control peptide. **I:** Tetramer staining of single cells stimulated with Adv peptide. **J:** Tetramer staining of single cells stimulated with HIV peptide.

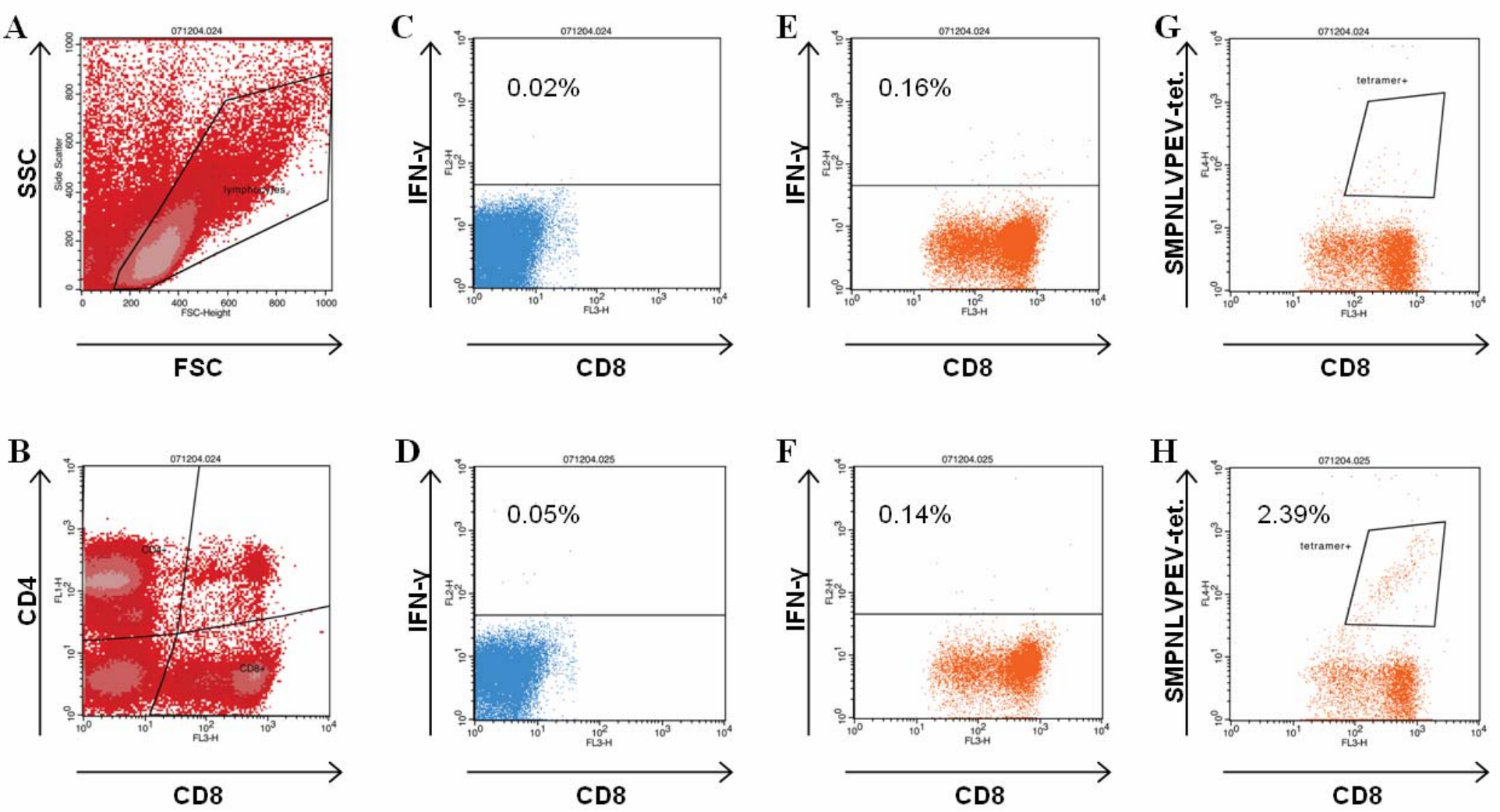


Figure 3.13: Combined tetramer/IFN-γ staining of the HLA-A*02-positive donor #794 after expansion with the peptide SMPNLVPEV and an HIV control peptide. **A:** FSC/SSC plot showing the lymphocyte gate. **B:** Plot showing the gating of CD4 and CD8 single positive cells among lymphocytes. **C:** IFN-γ response among $CD4^+$ cells stimulated with Adv peptide. **D:** IFN-γ response among $CD4^+$ cells stimulated with HIV control peptide. **E:** IFN-γ response among $CD8^+$ cells stimulated with Adv peptide. **F:** IFN-γ response among $CD8^+$ cells stimulated with HIV control peptide. **G:** Tetramer staining of $CD8^+$ cells stimulated with Adv peptide. **H:** Tetramer staining of $CD8^+$ cells stimulated with HIV peptide.

TLLYVLFEV

This peptide was first tested after PBMC expansion in 18 A*02-positive donors by tetramers without concomitant cytokine staining. Conversely, 12 other A*02-positive donors were stained for IFN-γ but not with tetramers. Four donors were stained for both IFN-γ and with tetramer. Tables 3.29 - 3.31 summarize the results of these experiments.

Table 3.29: Results of a tetramer staining of A*02-positive PBMCs after stimulation with **TLLYVLFEV** and the ELISPOT screening results of the same donors.

donor #	of $CD8^+$: $tetramer^+$	ELISPOT result
757	0.04%	neg.
765	0.02%	neg.
773	0.09%	**8**
778	0.06%	neg.
784	0.09%	**11.2**
1011	0.05%	**6**
1013	0.01%	**6**
1016	0.00%	**7**
1022	0.00%	**38**
1026	0.00%	**5.5**
1033	0.02%	**8**
1045	0.00%	**62**
1051	0.00%	**13**
1052	0.00%	**4**
1055	0.01%	**13**
1057	0.00%	neg.
1058	0.01%	**11**
1061	0.00%	**6**

Table 3.30: Results of an IFN-γ staining of A*02-positive PBMCs after stimulation with **TLLYVLFEV** and the ELISPOT screening results of the same donors. Percentages of IFN-γ^+ cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	of CD4$^+$: IFN-γ^+	of CD8$^+$: IFN-γ^+	ELISPOT result
1107	**0.14%**	**0.47%**	neg.
1108	0.03%	0.07%	**24**
1109	0.07%	0.08%	**24**
1113	**0.39%**	0.02%	**1000**
1114	0.01%	0.01%	**5**
1119	0.08%	0.04%	**117**
1122	**0.35%**	0.07%	neg.
1124	0.04%	0.06%	**377**
1135	0.09%	0.06%	**7**
1137	**0.75%**	**0.33%**	**112**
1138	**0.54%**	**0.43%**	**23**
1139	**0.33%**	0.09%	**28**

Table 3.31: Results of a combined tetramer/IFN-γ staining of HLA-A*02-positive PBMCs after stimulation with **TLLYVLFEV** and the ELISPOT screening results of the same donors. Percentages of IFN-γ^+ cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	of CD4$^+$: IFN-γ^+	of CD8$^+$: IFN-γ^+	of CD8$^+$: tetramer$^+$	ELISPOT result
1013	0.00%	0.03%	0.09%	**6**
1033	**1.24%**	**0.20%**	0.07%	**8**
1051	0.03%	0.00%	0.08%	**13**
1067	0.03%	0.00%	0.04%	**44**

Additionally, combined tetramer and IFN-γ/TNF stainings were carried out with PBMCs of seven A*02-positive and two A*02-negative donors. Table 3.32 summarizes the results of these experiments, while Figure 3.14 shows the staining of one A*02-negative donor as an example.

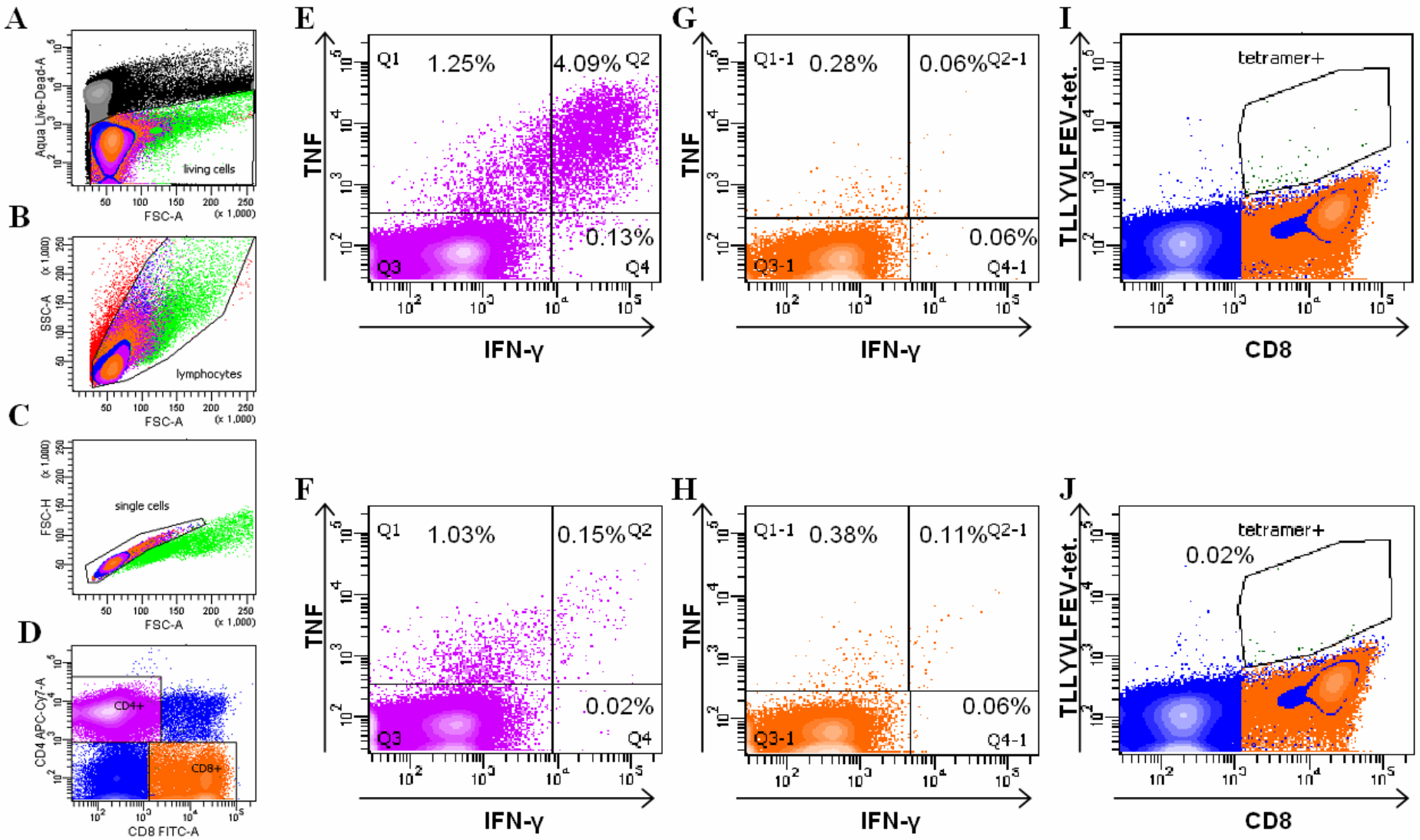


Figure 3.14: Combined tetramer/IFN-γ/TNF staining of the HLA-A*02-negative donor #1324 after expansion with the peptide TLLYVLFEV and an HIV control peptide. **A:** FSC/Live-Dead plot showing the living cell gate. **B:** FSC/SSC plot showing the lymphocyte gate among living cells. **C:** FSC-Area/FSC-Height plot showing the single cell gate among lymphocytes. **D:** CD8/CD4 plot showing the gates for CD4 and CD8 single positive cells among single cells. **E:** IFN-γ and TNF response among $CD4^+$ cells stimulated with Adv peptide. **F:** IFN-γ and TNF response among $CD4^+$ cells stimulated with HIV control peptide. **G:** IFN-γ and TNF response among $CD8^+$ cells stimulated with Adv peptide. **H:** IFN-γ and TNF response among $CD8^+$ cells stimulated with HIV control peptide. **I:** Tetramer staining of single cells stimulated with Adv peptide. **J:** Tetramer staining of single cells stimulated with HIV peptide.

Table 3.32: Results of a combined tetramer/IFN-γ/TNF staining after stimulation with **TLLYVLFEV** and the ELISPOT screening results of the same donors. Percentages of cytokine-positive cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	HLA-A*02	of CD4+:			of CD8+:			of CD8+: tetramer+	ELISPOT result
		TNF+	IFN-γ+	TNF+ IFN-γ+	TNF+	IFN-γ+	TNF+ IFN-γ+		
970	+	0.02%	**0.12%**	**3.63%**	0.00%	0.07%	0.00%	0.08%	n.t.
984	+	0.00%	**0.20%**	**0.28%**	**0.20%**	0.00%	0.09%	0.09%	**7**
1013	+	0.03%	0.06%	**0.57%**	0.00%	**0.12%**	0.00%	0.03%	**6**
1026	+	0.00%	**0.31%**	**0.42%**	0.05%	**0.19%**	**0.30%**	0.09%	**6**
1113	+	**4.81%**	0.04%	**2.36%**	0.00%	0.09%	0.01%	0.07%	**1000**
1124	+	**0.14%**	0.08%	**0.93%**	0.00%	0.00%	0.00%	0.05%	**377**
1137	+	**0.95%**	**0.17%**	**1.22%**	0.00%	0.00%	0.00%	0.03%	**112**
1324	-	**0.22%**	**0.11%**	**3.94%**	0.00%	0.00%	0.00%	0.02%	n.t.
1352	-	0.06%	0.09%	**1.70%**	0.00%	0.07%	0.00%	0.05%	n.t.

For this peptide, tetramer binding could never be observed. Some cytokine staining of CD8+ T cells can be observed, but these always accompany CD4+ T cell responses. The great majority of and the strongest cytokine responses are mediated by CD4+ T cells, and there are strong responses by PBMCs of A*02-negative donors as well (Fig. 3.14). Moreover, this peptide has already been described as a CD4+ T cell epitope in this form[286] as well as in an extended version[285]. This fits well the data presented here.

TFYLNHTFKK

This peptide has also been published as an HLA-A*02-restricted MHC class I epitope[279]. The sequence, however, is completely different from the binding motif of HLA-A*02, which precludes positive charges at the C-terminus. Consequently the SYFPEITHI score of the peptide for this allotype is only 3. No HLA-A*02-tetramer could be refolded with this peptide, presumably because the binding affinity is too low.

PBMCs of 10 A*02-positive and six A*02-negative donors were tested for this peptide after expansion in an intracellular IFN-γ staining. Table 3.33 summarizes the results.

Table 3.33: Results of an IFN-γ staining of A*02-positive and A*02-negative PBMCs after stimulation with **TFYLNHTFKK** and the ELISPOT screening results of the same donors. Percentages of IFN-γ^+ cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	HLA-A*02	of $CD4^+$: IFN-γ^+	of $CD8^+$: IFN-γ^+	ELISPOT result
552	-	0.00%	0.00%	neg.
553	+	0.00%	0.00%	**7.3**
555	-	0.00%	0.02%	**13**
556	-	**0.41%**	0.00%	**455**
558	+	**0.35%**	0.00%	**337**
560	+	0.01%	0.06%	neg.
562	+	0.01%	0.01%	neg.
564	+	0.00%	0.00%	**20**
570	+	0.00%	0.00%	neg.
574	-	0.00%	0.00%	neg.
576	+	0.00%	0.00%	**4.7**
589	+	0.04%	0.00%	**21**
602	+	**0.32%**	0.00%	**83**
623	-	0.00%	0.00%	neg.
634	-	**0.23%**	0.00%	**20**
654	+	**0.12%**	0.09%	**28**

Additionally, combined tetramer and IFN-γ/TNF stainings were carried out with PBMCs of seven A*02-positive and two A*02-negative donors. Table 3.34 summarizes the results of these experiments, while Figure 3.15 shows the staining of one A*02-negative donor as an example.

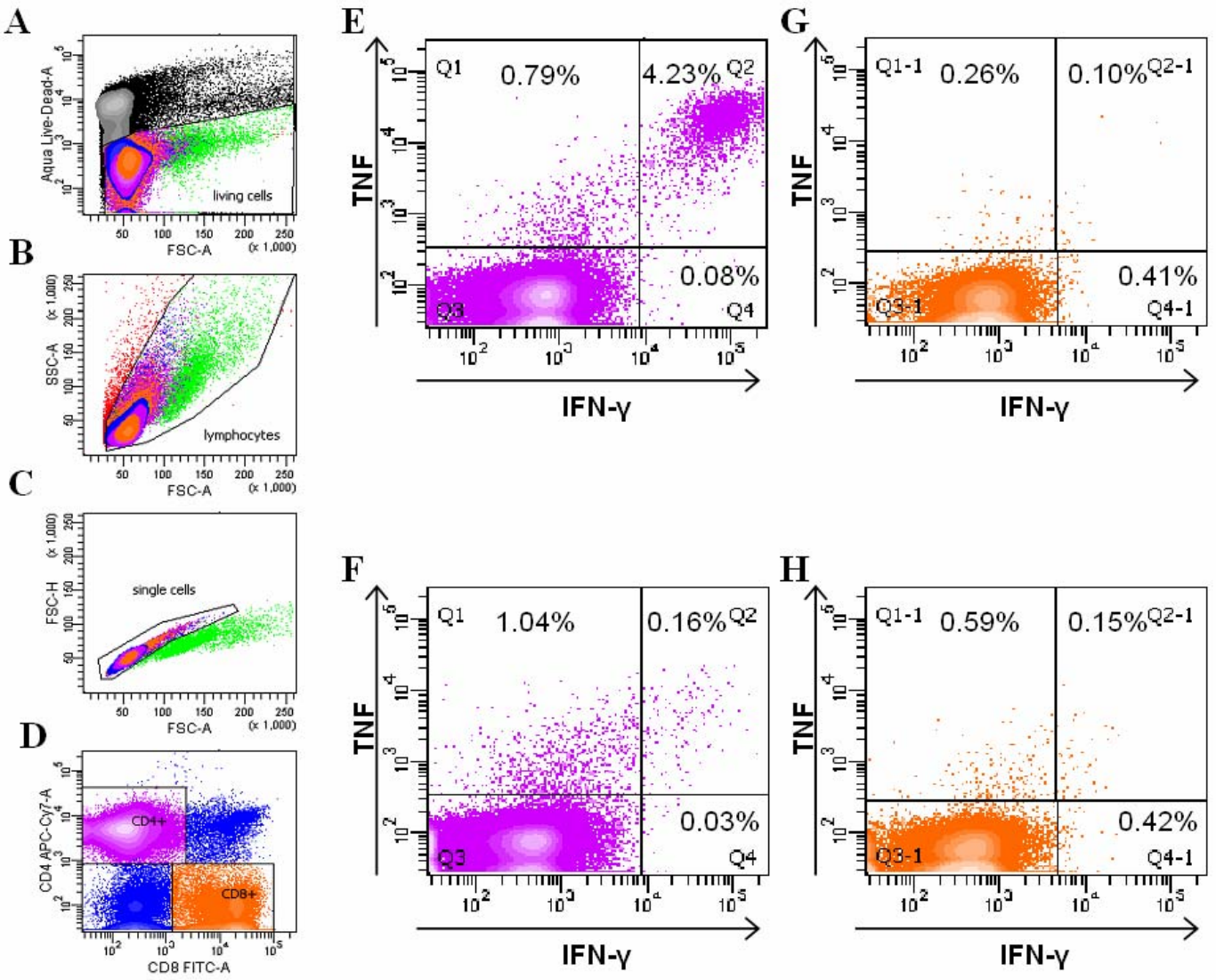


Figure 3.15: Combined IFN-γ/TNF staining of the HLA-A*02-negative donor #1324 after expansion with the peptide TFYLNHTFKK and an HIV control peptide. **A:** FSC/Live-Dead plot showing the living cell gate. **B:** FSC/SSC plot showing the lymphocyte gate among living cells. **C:** FSC-Area/FSC-Height plot showing the single cell gate among lymphocytes. **D:** CD8/CD4 plot showing the gates for CD4 and CD8 single positive cells among single cells. **E:** IFN-γ and TNF response among $CD4^+$ cells stimulated with Adv peptide. **F:** IFN-γ and TNF response among $CD4^+$ cells stimulated with HIV control peptide. **G:** IFN-γ and TNF response among $CD8^+$ cells stimulated with Adv peptide. **H:** IFN-γ and TNF response among $CD8^+$ cells stimulated with HIV control peptide.

Table 3.34: Results of a combined IFN-γ/TNF staining after stimulation with **TFYLNHTFKK** and the ELISPOT screening results of the same donors. Percentages of cytokine-positive cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	HLA-A*02	of CD4+:			of CD8+:			ELISPOT result
		TNF+	IFN-γ+	TNF+ IFN-γ+	TNF+	IFN-γ+	TNF+ IFN-γ+	
970	+	0.09%	0.00%	0.06%	0.00%	0.00%	0.00%	**67**
984	+	**0.45%**	0.00%	0.00%	0.08%	0.00%	0.00%	**342**
1013	+	0.00%	0.01%	**0.20%**	0.00%	0.00%	0.00%	n.t.
1026	+	**0.28%**	**0.37%**	0.09%	**0.41%**	0.00%	0.00%	n.t.
1113	+	0.07%	0.00%	**3.82%**	0.02%	0.00%	0.00%	n.t.
1124	+	0.00%	0.01%	**0.79%**	0.06%	0.00%	0.00%	n.t.
1137	+	0.03%	0.02%	**2.20%**	0.00%	0.00%	0.00%	n.t.
1324	-	0.00%	0.05%	**4.07%**	0.00%	**0.16%**	0.00%	**45**
1352	-	0.00%	0.00%	**0.87%**	0.00%	0.08%	0.00%	**13**

With this peptide as well, only cytokine responses by $CD4^+$ T cells were observed, apart from some weak TNF and IFN-γ stainings in $CD8^+$ T cells. Figure 3.15 shows a very strong $CD4^+$ T cell response by an A*02-negative donor. This is not surprising, since the peptide is the N-terminal part of an already published MHC class II epitope, TFYLNHTFKKVAITVDSSVS[282]. The hardly fitting peptide motif of HLA-A*02 and our results make it seem highly unlikely that this peptide actually is an HLA-A*02 epitope.

ILRRPTSPV

With this peptide, only some tetramer stainings of PBMCs from A*02-positive donors were performed. Curiously, some tetramer-positive cells could be observed, but in the ELISPOT screening no response was ever elicited by this peptide. As in the case of the peptide SMPNLVPEV, this is again an example for the presence of non-functional tetramer-positive T cells after expansion that do not secrete IFN-γ. Table 3.35 summarizes these data.

Table 3.35: Results of a tetramer staining of A*02-positive PBMCs after stimulation with **ILRRPTSPV** and the ELISPOT screening results of the same donors.

donor #	of $CD8^+$: $tetramer^+$	ELISPOT result
1011	**0.20%**	neg.
1013	0.02%	neg.
1016	**1.01%**	neg.
1022	0.07%	neg.
1026	**1.41%**	neg.
1033	**0.62%**	neg.
1045	**0.16%**	neg.
1051	0.07%	neg.
1052	**0.34%**	neg.
1055	**0.28%**	neg.
1057	0.06%	neg.
1058	0.08%	neg.
1061	0.07%	neg.

LLNEPGQPL, LLTPNEFEI, VINTETLTKV

Because for these peptides HLA-A*02-tetramers were already at hand and did not need to be produced, PBMCs of the same donors were stained as for ILRRPTSPV, but no tetramer-positive population could be observed in any experiment.

3.7.3. HLA-A*24

TYFSLNNKF

This peptide is already published as an HLA-A*24-restricted MHC class I epitope[279]. A combined IFN-γ/tetramer staining has been performed with the PBMCs of 16 donors after expansion, but unfortunately in this experiment the αCD4 antibody was omitted. Thus only the cytokine responses for the $CD8^+$ T cells can be given (Table 3.36).

Table 3.36: Results of a combined tetramer/IFN-γ staining of HLA-A*24-positive PBMCs after stimulation with **TYFSLNKKF** and the ELISPOT screening results of the same donors. Percentages of IFN-γ^+ cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	of CD8$^+$: IFN-γ^+	of CD8$^+$: tetramer$^+$	ELISPOT result
847	**0.12%**	**0.26%**	**6.4**
866	**0.85%**	**1.25%**	**93**
945	**0.88%**	**1.24%**	**107**
998	0.00%	0.04%	neg.
1007	**6.89%**	**6.08%**	**90**
1008	**0.28%**	**0.39%**	**69**
1012	**0.68%**	**0.85%**	neg.
1021	0.03%	0.06%	neg.
1022	**1.65%**	**3.36%**	**19**
1071	**1.19%**	**1.14%**	**192**
1117	0.03%	**0.14%**	**105**
1118	0.02%	0.03%	**4.1**
1156	**0.18%**	**0.28%**	**227**
1169	**0.55%**	**0.37%**	**125**
1173	**0.22%**	**0.92%**	**154**
1180	0.00%	**1.12%**	**231**

Additionally, combined tetramer and IFN-γ/TNF stainings were carried out with PBMCs of three A*24-positive and two A*24-negative donors. Table 3.37 summarizes the results of these experiments, while Figure 3.16 shows the staining of one A*24-positive donor as an example.

Table 3.37: Results of a combined tetramer/IFN-γ/TNF staining after stimulation with **TYFSLNNKF** and the ELISPOT screening results of the same donors. Percentages of cytokine-positive cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	HLA-A*24	of CD4$^+$:			of CD8$^+$:			of CD8$^+$: tetramer$^+$	ELISPOT result
		TNF$^+$	IFN-γ^+	TNF$^+$ IFN-γ^+	TNF$^+$	IFN-γ^+	TNF$^+$ IFN-γ^+		
1156	+	0.03%	0.01%	0.00%	0.05%	0.04%	**0.20%**	**0.21%**	**227**
1173	+	0.04%	0.03%	0.01%	**0.10%**	**0.24%**	**0.36%**	**0.48%**	**154**
1180	+	0.01%	0.00%	0.00%	**0.36%**	**0.27%**	**1.76%**	**1.36%**	**231**
1351	-	0.00%	0.00%	0.02%	0.06%	**0.10%**	**0.95%**	**0.45%**	**70**
1355	-	0.00%	0.02%	0.00%	0.00%	0.01%	0.02%	0.01%	neg.

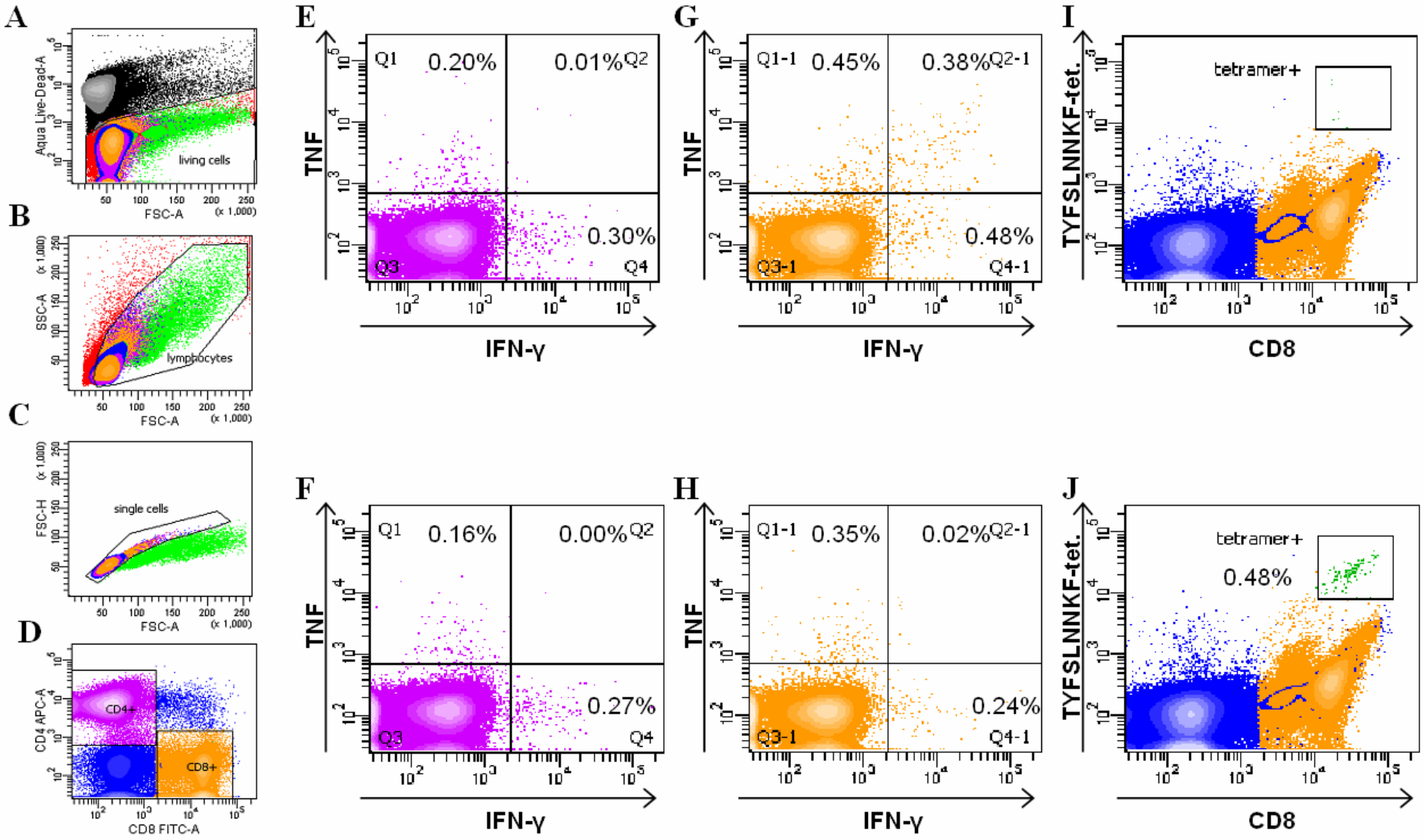


Figure 3.16: Combined tetramer/IFN-γ/TNF staining of the HLA-A*24-positive donor #1173 after expansion with the peptide TYFSLNNKF and an HIV control peptide. **A:** FSC/Live-Dead plot showing the living cell gate. **B:** FSC/SSC plot showing the lymphocyte gate among living cells. **C:** FSC-Area/FSC-Height plot showing the single cell gate among lymphocytes. **D:** CD8/CD4 plot showing the gates for CD4 and CD8 single positive cells among single cells. **E:** IFN-γ and TNF response among $CD4^+$ cells stimulated with Adv peptide. **F:** IFN-γ and TNF response among $CD4^+$ cells stimulated with HIV control peptide. **G:** IFN-γ and TNF response among $CD8^+$ cells stimulated with Adv peptide. **H:** IFN-γ and TNF response among $CD8^+$ cells stimulated with HIV control peptide. **I:** Tetramer staining of single cells stimulated with Adv peptide. **J:** Tetramer staining of single cells stimulated with HIV peptide.

The responses are typical for an MHC class I epitope, and the tetramer-positive cell populations confirm the restriction by HLA-A*24. There is a curious case of one A*24-negative donor (#1351) whose $CD8^+$ T cells nevertheless respond to the peptide and are stained by tetramer. This donor, however, is A*23-positive, and the peptide motif of these two alleles is quite similar. Thus a possible explanation is presentation of the peptide on A*23 as well and cross-reaction of the specific T cells with the A*24-TYFSLNNKF-tetramer.

AYPANFPYPL

For this peptide, only the combined tetramer/IFN-γ/TNF stainings were performed with the same donors as for TYFSLNNKF. Table 3.38 summarizes the results and Figure 3.17 shows one staining as an example.

Table 3.38: Results of a combined tetramer/IFN-γ/TNF staining after stimulation with **AYPANFPYPL** and the ELISPOT screening results of the same donors. Percentages of cytokine-positive cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	HLA-A*24	of $CD4^+$:			of $CD8^+$:			of $CD8^+$: tetramer$^+$	ELISPOT result
		TNF^+	IFN-γ^+	TNF^+ IFN-γ^+	TNF^+	IFN-γ^+	TNF^+ IFN-γ^+		
1156	+	0.00%	0.02%	0.00%	0.04%	0.05%	0.00%	0.00%	**9.0**
1173	+	0.04%	0.00%	0.01%	**0.20%**	0.00%	0.05%	0.03%	**20**
1180	+	0.00%	0.00%	0.01%	0.06%	**0.10%**	**0.55%**	**1.16%**	**64**
1351	-	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	neg.
1355	-	0.06%	0.02%	0.00%	0.09%	0.00%	0.00%	0.01%	neg.

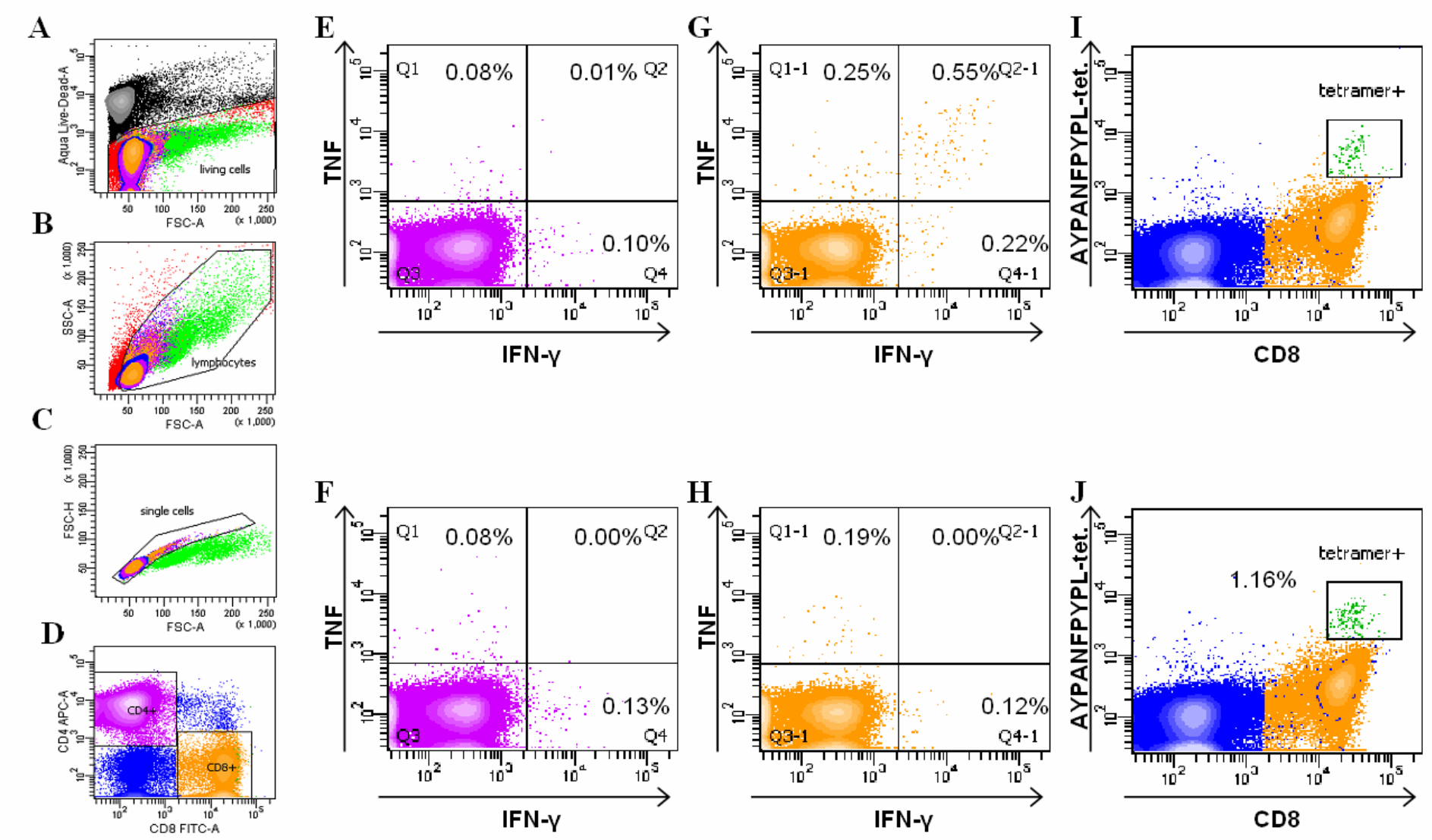


Figure 3.17: Combined tetramer/IFN-γ/TNF staining of the HLA-A*24-positive donor #1180 after expansion with the peptide AYPANFPYPL and an HIV control peptide. **A:** FSC/Live-Dead plot showing the living cell gate. **B:** FSC/SSC plot showing the lymphocyte gate among living cells. **C:** FSC-Area/FSC-Height plot showing the single cell gate among lymphocytes. **D:** CD8/CD4 plot showing the gates for CD4 and CD8 single positive cells among single cells. **E:** IFN-γ and TNF response among $CD4^+$ cells stimulated with Adv peptide. **F:** IFN-γ and TNF response among $CD4^+$ cells stimulated with HIV control peptide. **G:** IFN-γ and TNF response among $CD8^+$ cells stimulated with Adv peptide. **H:** IFN-γ and TNF response among $CD8^+$ cells stimulated with HIV control peptide. **I:** Tetramer staining of single cells stimulated with Adv peptide. **J:** Tetramer staining of single cells stimulated with HIV peptide.

This peptide has elicited IFN-γ responses in the cells of A*24-positive donors in the ELISPOT screening, albeit with low frequency. A tetramer-positive cell population can be observed in one donor, and the cytokine response of this donor is typical for an MHC class I epitope. There are no responses in $CD4^+$ T cells. These findings confirm that AYPANFPYPL is a novel non-immunodominant MHC class I epitope restricted by HLA-A*24.

NYIAFRDNFI

Also for this peptide, only the combined tetramer/IFN-γ/TNF stainings were performed with the same donors as for TYFSLNNKF. Table 3.39 summarizes the results and Figure 3.18 shows one staining as an example.

Table 3.39: Results of a combined tetramer/IFN-γ/TNF staining after stimulation with **NYIAFRDNFI** and the ELISPOT screening results of the same donors. Percentages of cytokine-positive cells are calculated by subtracting the value of the HIV stimulation from the respective value of Adv stimulation.

donor #	HLA-A*24	of $CD4^+$:			of $CD8^+$:			of $CD8^+$: tetramer$^+$	ELISPOT result
		TNF^+	IFN-γ^+	TNF^+ IFN-γ^+	TNF^+	IFN-γ^+	TNF^+ IFN-γ^+		
1156	+	0.09%	0.03%	**0.36%**	0.01%	0.00%	0.00%	0.01%	**29**
1173	+	**0.28%**	0.05%	**1.58%**	**0.17%**	0.00%	0.03%	0.00%	**68**
1180	+	**0.27%**	0.02%	**0.67%**	0.00%	0.00%	0.00%	0.00%	**7.3**
1351	-	**0.19%**	0.05%	**2.13%**	0.00%	0.00%	0.00%	0.02%	**146**
1355	-	**0.67%**	0.04%	**1.64%**	**0.10%**	0.07%	0.01%	0.01%	**424**

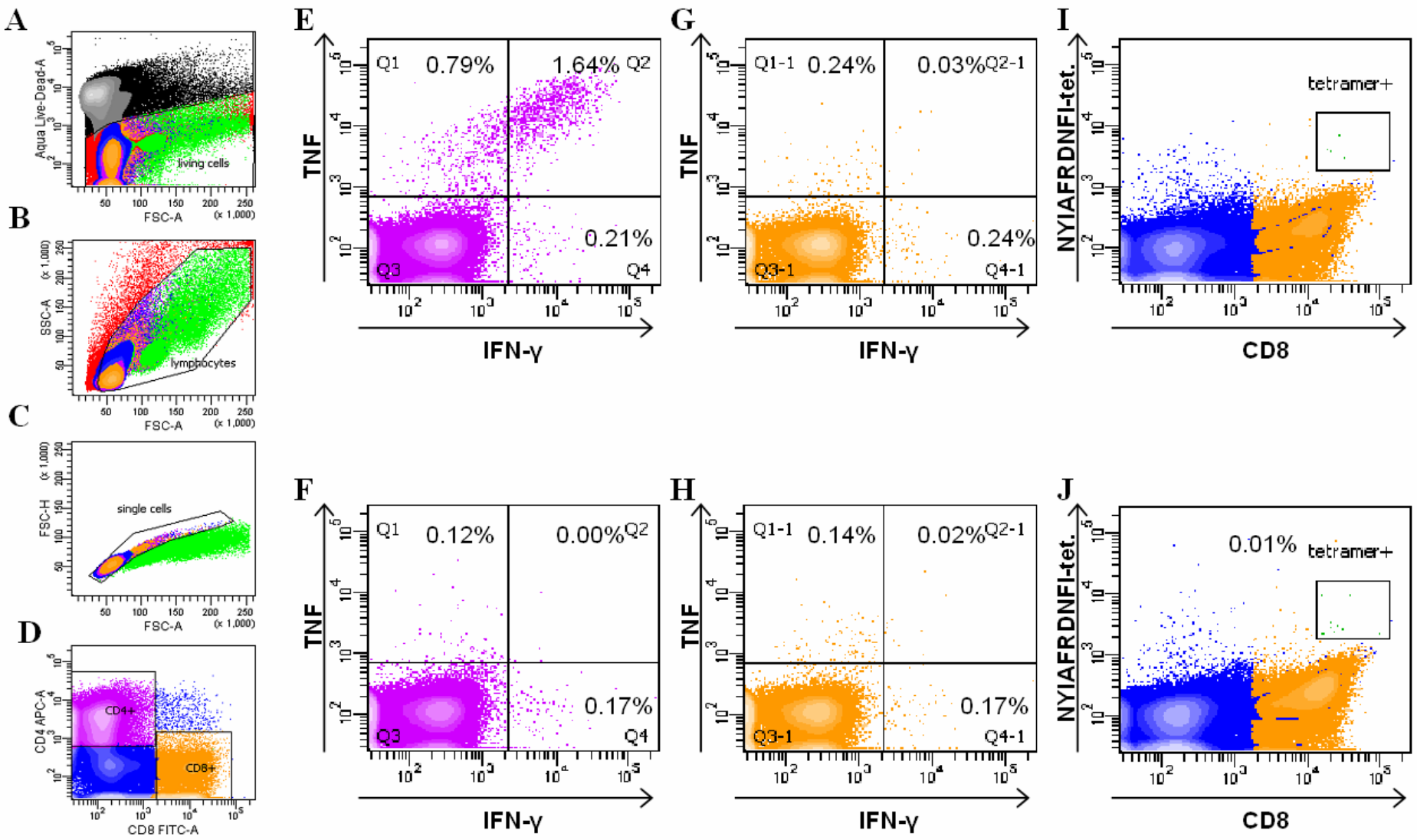


Figure 3.18: Combined tetramer/IFN-γ/TNF staining of the HLA-A*24-negative donor #1355 after expansion with the peptide NYIAFRDNFI and an HIV control peptide. **A:** FSC/Live-Dead plot showing the living cell gate. **B:** FSC/SSC plot showing the lymphocyte gate among living cells. **C:** FSC-Area/FSC-Height plot showing the single cell gate among lymphocytes. **D:** CD8/CD4 plot showing the gates for CD4 and CD8 single positive cells among single cells. **E:** IFN-γ and TNF response among $CD4^+$ cells stimulated with Adv peptide. **F:** IFN-γ and TNF response among $CD4^+$ cells stimulated with HIV control peptide. **G:** IFN-γ and TNF response among $CD8^+$ cells stimulated with Adv peptide. **H:** IFN-γ and TNF response among $CD8^+$ cells stimulated with HIV control peptide. **I:** Tetramer staining of single cells stimulated with Adv peptide. **J:** Tetramer staining of single cells stimulated with HIV peptide.

The data are typical for a $CD4^+$ T cell response. The donor in Fig. 3.18 exhibits a strong cytokine response but is A*24-negative. No staining with the tetramer can be observed either. Fittingly, the peptide is part of a larger MHC class II epitope that has already been published (GQQSMPNRPNYIAFRDNFIG)[282].

3.8. Phenotypical characterization of precursor T cells

Keeping in mind the eventual goal of *in vitro* expansion of adenovirus-specific T cells for adoptive transfer to immunocompromised patients, an attempt was made to characterize the precursor T cells present in the PBMCs of healthy donors before expansion. Several publications have claimed that T cells expanded from central memory cells have higher functional capacity than effector memory-derived cells[332-335]. Analyzing adenovirus-specific memory T cell *ex vivo* is hampered by the very low frequency of these cells. After testing several A*02-positive donors, four were found that had detectable amounts of LLDQLIEEV-specific T cells without expansion. Staining for the differentiation markers CD45RO and CCR7 was performed to determine the subtype of the precursors according to the system of Sallusto *et al*[259]. Figure 3.19 shows the staining of one donor, while table 3.40 summarizes the results.

Table 3.40: Characterization of Adv specific T cells *ex vivo*. Percentage of LLDQLIEEV-HLA-A*02:01 tetramer-specific cells in the $CD8^+$ T cell population of different donors and their phenotypical distribution as characterized by CD45RO/CCR7 staining.

donor #	tetramer-specific cells in $CD8^+$ population	of these:			
		naive	CM	EM	EMRA
793	0.064%	7.9%	**60.5%**	31.6%	0.0%
1016	0.043%	20.0%	**60.0%**	16.7%	3.3%
1026	0.063%	4.4%	11.5%	**73.5%**	10.6%
1045	0.053%	6.1%	**48.5%**	45.5%	0.0%

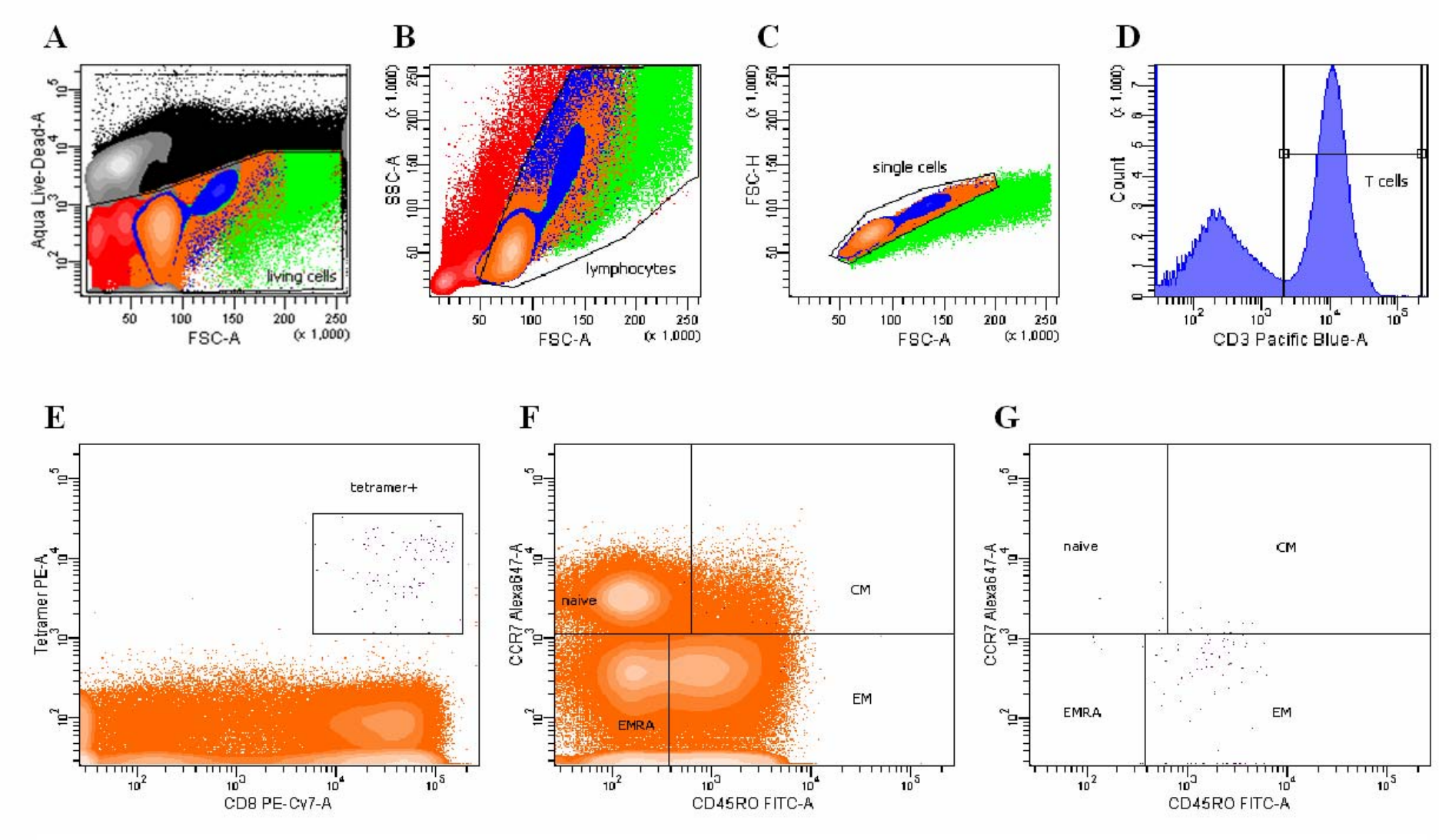


Figure 3.19: Characterization of LLDQLIEEV-specific precursor T cells from HLA-A*02-positive donor #1026. **A:** FSC/Live-Dead plot showing the living cell gate. **B:** FSC/SSC plot showing the lymphocyte gate among living cells. **C:** FSC-Area/FSC-Height plot showing the single cell gate among lymphocytes. **D:** CD3 histogram showing the T cell gate among single cells. **E:** CD8/tetramer plot of T cells. **F:** CD45RO/CCR7 plot of all T cells showing the gating for the different subtypes (naïve, central memory, effector memory and EMRA). **G:** CD45RO/CCR7 plot of tetramer-positive cells.

In donors #793 and #1016 central memory cells predominate while the reverse is true for donor #1026. In donor #1054 the number of central and effector memory T cells is about equal.

3.9. Amplification of multifunctional T cells by the expansion protocol

To test the functionality of the specific T cells after application of the expansion protocol, the production of the intracellular cytokines IFN-γ, TNF, and IL-2 by T cells of from stimulated PBMCs was analyzed. Additionally, staining for the presence of the degranulation marker CD107a on the cell surface was performed. Production of each cytokine and degranulation were regarded as single functions. One donor was selected for each immunodominant epitope (Fig. 3.20).

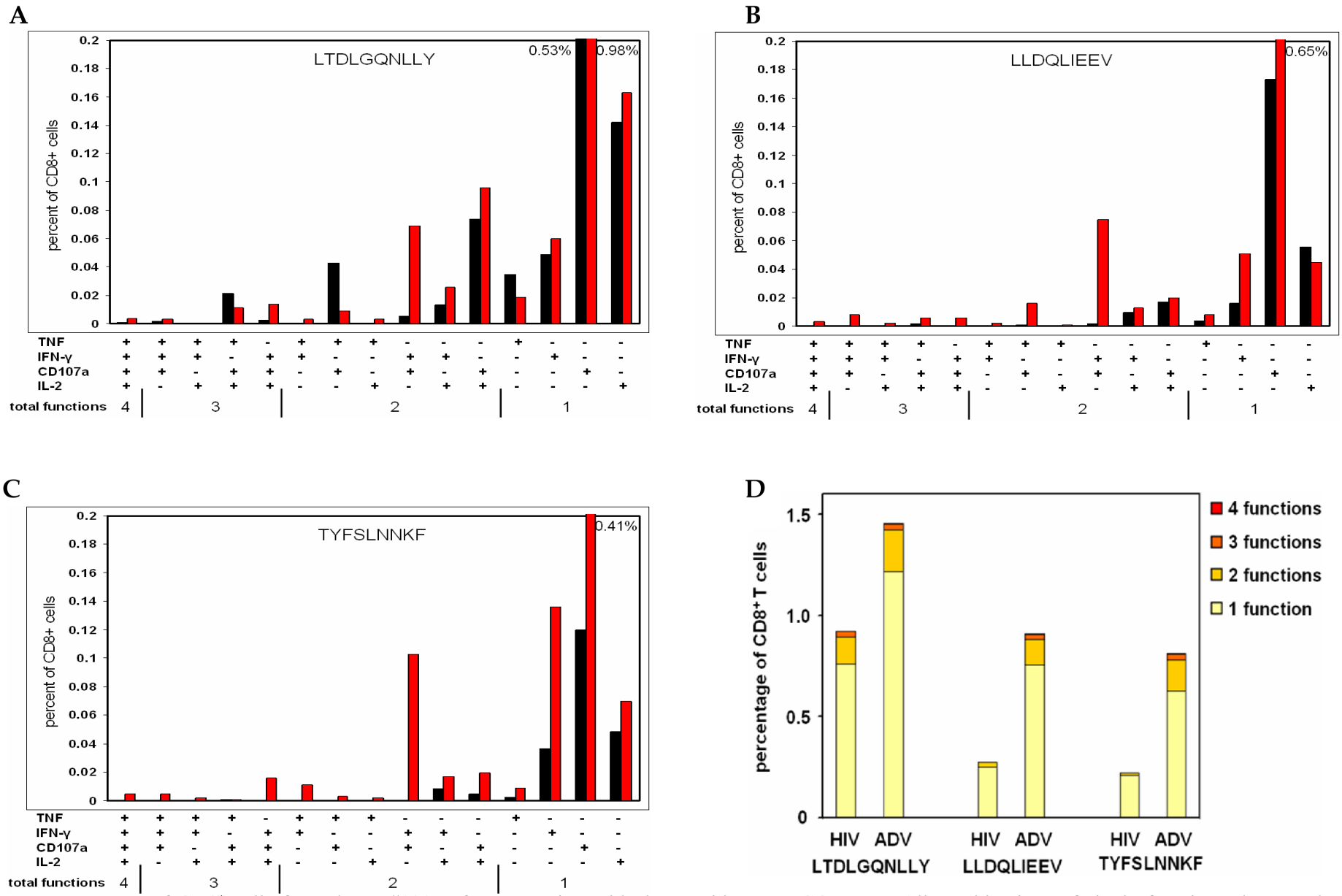


Figure 3.20: A: Response of $CD8^+$ cells from donor #1005 after expansion with the peptide LTDLGQNLLY. All combinations of single functions shown. Black bars: Stimulation with HIV control peptide. Red bars: Stimulation with adenoviral peptide. **B:** Response of $CD8^+$ cells from donor #1113 after expansion with the peptide LLDQLIEEV. All combinations of single functions shown. Black bars: Stimulation with HIV control peptide. Red bars: Stimulation with adenoviral peptide. **C:** Response of $CD8^+$ cells from donor #1281 after expansion with the peptide TYFSLNNKF. All combinations of single functions shown. Black bars: Stimulation with HIV control peptide. Red bars: Stimulation with adenoviral peptide. **D:** Summary of A-C with only the number of functions observed shown.

Increased numbers of mono- and multifunctional $CD8^+$ T cells can be observed in all donors compared to the HIV control. The large background of monofunctional cells after stimulation with HIV peptide, especially in donor #1005, is largely due to unspecific background staining of CD107a. For all peptides, IFN-γ secretion was the most characteristic function of the specific response.

3.10. Cytotoxicity assays

For further testing of the functionality of the adenovirus-specific T cells obtained by the expansion protocol, cytotoxicity assays were performed with peptide-loaded T2 cells as targets. T2 cells express only HLA-A*02 as MHC class I molecules[336] and lack the peptide transporter TAP[337;338], allowing to load their class I molecules efficiently from outside of the cell. Obtaining the necessary adenovirus-specific effector cells was difficult because of the large number of them needed for the cytotoxicity assays. PBMCs from a donor (#1114) who was known to respond to the HLA-A*02-restricted peptide LLDQLIEEV were obtained in large amounts by leukapheresis. After application of the expansion protocol, about 2% tetramer-positive T cells were found among the PBMCs (Fig. 3.21A). MACS purification with tetramers was now applied to isolate the specific T cells as pure as possible in order to avoid unspecific reactions in the cytotoxicity assays. After purification, nearly 90% of the cells in the sample were tetramer-specific (Fig. 3.21B).

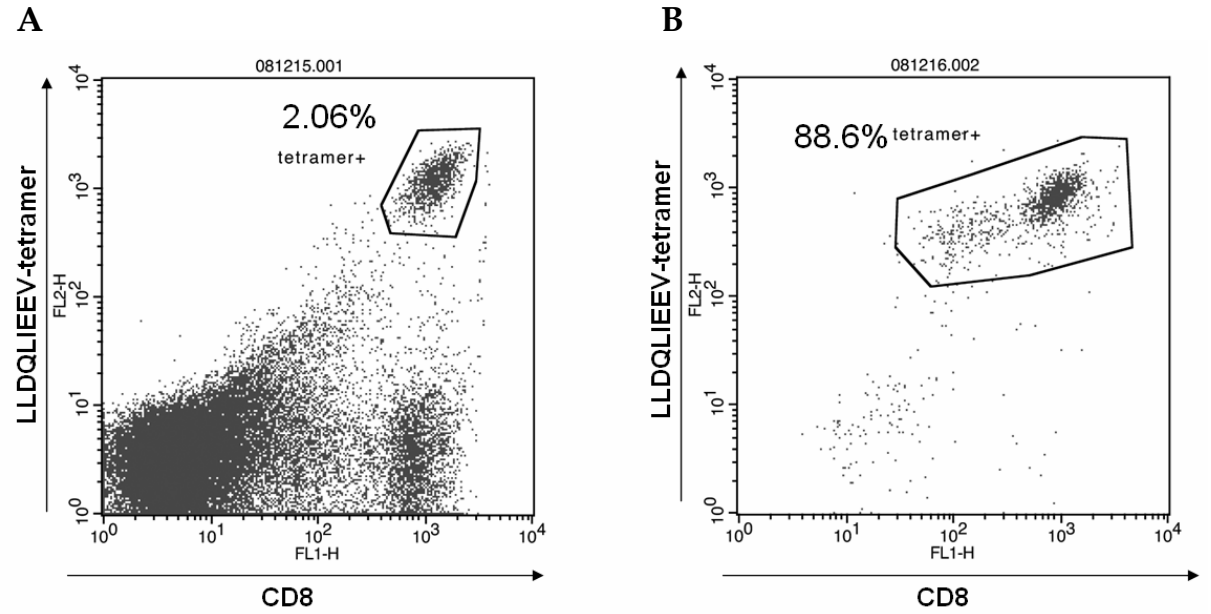


Figure 3.21: MACS purification of LLDQLIEEV-specific T cells from expanded PBMCs of donor #1114. **A:** Before purification. **B:** After purification.

With the purified T cells, cytotoxicity assays by fluorolysis and by chromium release were carried out. Cells were loaded with two different peptide concentrations, 1 µg/ml or 10 µg/ml. Cells loaded with 10 µg/ml HIV peptide served as controls. Target and effector cells were incubated for 24 hours.

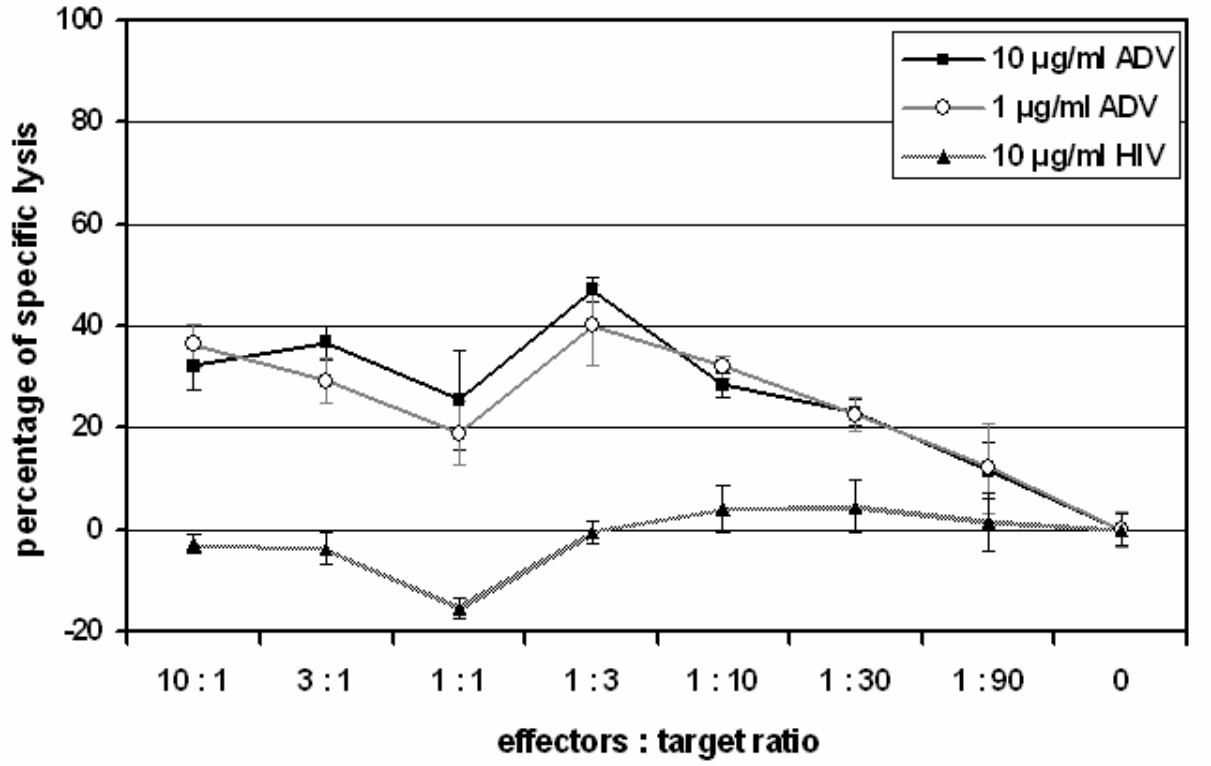


Figure 3.22: Chromium release assay with peptide-loaded T2 cells as targets and purified LLDQLIEEV-specific T cells as effectors.

Figure 3.22 shows the results of the chromium release assay and Figure 3.23 of the fluorolysis assay.

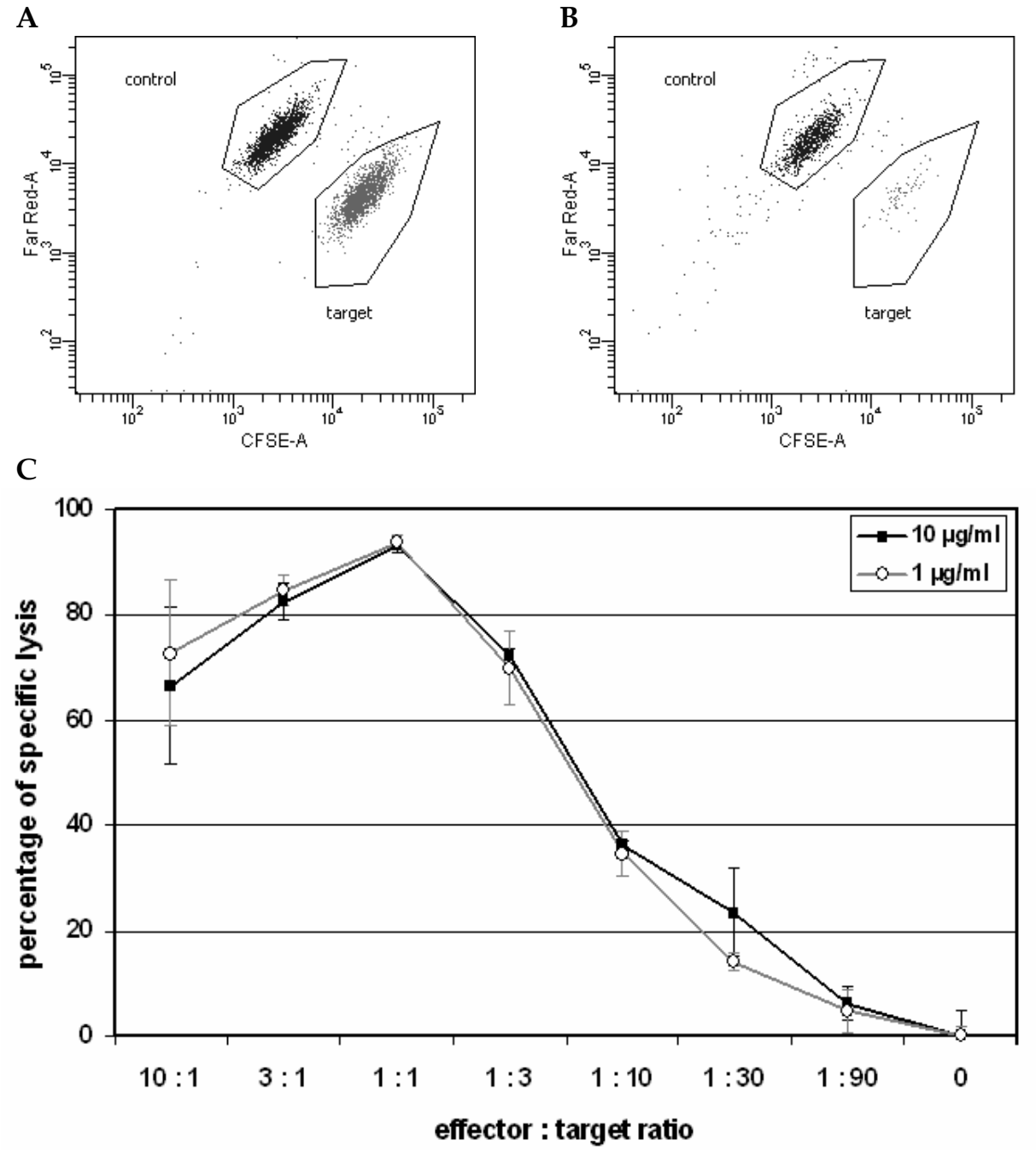


Figure 3.23: Fluorolysis assay with peptide-loaded T2 cells as targets and purified LLDQLIEEV-specific T cells as effectors. **A:** Example of one well with T2 cell populations loaded with 10 µg/ml adenoviral target or HIV control peptides. No effectors were present in this well. **B:** Same as A, but with an effector : target ratio of 1 : 1. **C:** Diagram showing the specific lysis for the different effector : target ratios.

In neither assay is there any great difference between the two peptide concentrations used for peptide loading, probably because 1 μg/ml is already an unphysiologically high concentration that is sufficient to achieve saturation. While in the chromium release assay a specific lysis of about 40% can be achieved with the highest effector : target ratios, in the fluorolysis assay the highest lysis values are nearly 100% and start to drop again for very high effector numbers. The reason for this is that in the fluorolysis assay, a significant lysis of the control cells can be observed that becomes nearly as strong as that of the target cells for high effector cell numbers. Neither reducing the incubation time from 24 h to 6 h nor the use of unloaded cells instead of cells loaded with HIV peptide as control cells improved this. Thus the results of the chromium release assay are likely to be more reliable, even though this method has the serious drawbacks of requiring the handling of radioactive substances during the experiment and also much higher target cell numbers for evaluation, which necessitates higher effector cell numbers in turn to achieve the same effector : target ratios. Nevertheless, it could be shown that the $CD8^+$ T cells amplified by the expansion protocol are indeed capable of lysing cells that present their cognate peptides.

4. DISCUSSION

Since an increased risk of adenoviral infection in immunocompromised patients has been shown to correlate with low numbers of endogenous T cells[114;339], efforts have been made over the past years to identify MHC class I-restricted as well as MHC class II-restricted epitopes[279;281-287]. While progress has been made with class II epitopes, the number of published class I epitopes has been rather low. Also, most of the studies have focused on hexon, the main capsid protein of the virion, and thus the bulk of the known class II epitopes and all class I epitopes are derived from this source, with only a handful of class II epitopes from other proteins described[284].

4.1. Identifying and assessing the immunodominance of adenoviral epitopes by reverse immunology

Apart from the scarcity of known $CD8^+$ T cell epitopes, another hindrance for the application of epitope information in diagnosis or immunotherapy is the unknown immunodominance of the epitopes discovered. Generally, epitope identification has been carried out by analyzing the specificities of single T cell lines or clones. While this approach doubtlessly allows the identification of epitopes, it does not permit conclusions whether the identified peptides are immunodominant on their respective HLA allotypes or play any important role at all in the cellular immune response against the virus. Data reliably identifying the immunodominant epitopes on widespread HLA alleles would be very helpful for the establishment of adoptive transfer strategies. The results of this work suggest that mapping adenoviral CTL epitopes by the strategy of reverse immunology[340;341] is a feasible approach to this end. Instead of determining the TCR specificities of single CTL lines or clones, possible epitope candidates are first predicted by an algorithm (SYFPEITHI[191] in

our case) before searching for CTL responses to the corresponding peptides in healthy blood donors. In this way, new epitopes cannot only be simply identified, but their relevance and immunodominance can be assessed by the number of donors they have elicited responses in.

As a matter of course, this strategy hinges on the presence of adenovirus-specific memory T cells in healthy blood donors, since these are to be analyzed. Adenoviruses, however, are very widespread pathogens and the frequency of infections in children[94;95] makes it seem likely that a large part of the population has encountered the disease. The epitope screening in this work was performed without any kind of confirmation of previous infections, and even though only peptides from two adenoviral strains have been tested, a large majority of donors has responded to at least one peptide. 68 out of 73 HLA-A*01-positive donors (93%) have responded to the peptide LTDLGQNLLY in the ELISPOT screening. This figure is in good agreement with the total of 159 out of 174 donors (91%) used in the screening of peptides predicted or published to one of their HLA allotypes that have shown a response. The true figure of donors with a previous adenoviral infection is likely to be higher, since not all donors were tested for the same number of peptides and it is conceivable that some donors would have responded only to peptides not included in this study. The high prevalence of adenoviral infections is in any case confirmed by these results.

The results also show that the ELISPOT screening, which allows a comparably high throughput of peptides to be tested, needs to be backed up by more specific assays, such as tetramer or cytokine staining. Several peptides that had been predicted with high SYFPEITHI scores for an MHC allele and elicited frequent responses turned out to be MHC class II epitopes or at least parts of class II epitopes. Examples are the peptides YTYSGSIPY (predicted for HLA-A*01), TLLYVLFEV (predicted for HLA-A*02), or NYIAFRDNFI (predicted for HLA-A*24).

4.2. Comparison of ELISPOT, tetramer staining, and cytokine staining results

Since the IFN-γ ELISPOT screening and the intracellular staining for IFN-γ measure the same parameter, donors should respond the same way in both assays. Figure 4.1A shows an analysis of the responses for donors where both an ELISPOT and intracellular IFN-γ staining were performed.

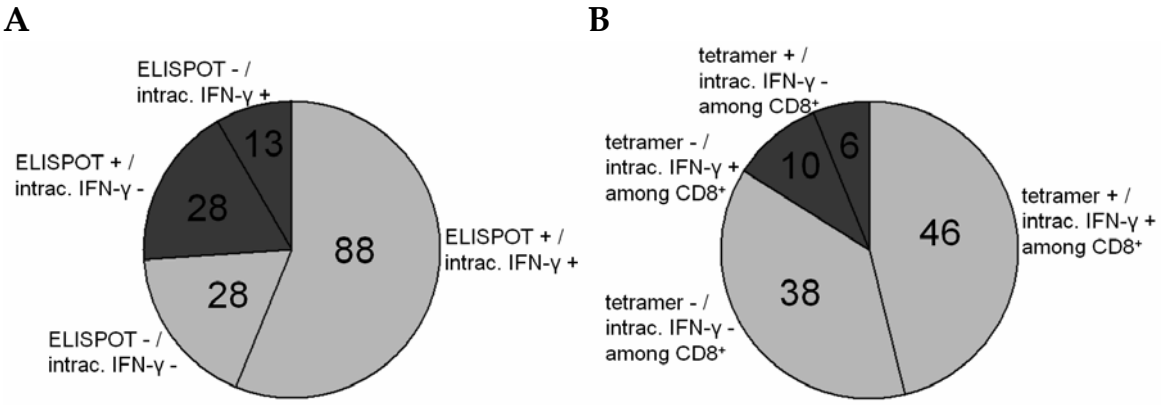


Figure 4.1: A: Comparison of responses in IFN-γ ELISPOT assays and intracellular IFN-γ staining where both assays have been performed for the same donor-peptide-pair. Light gray marks corresponding results in both assays, dark gray divergent results. **B:** Comparison of the presence of tetramer-positive cells and responses by $CD8^+$ cells in intracellular IFN-γ assays. Light gray marks corresponding results in both assays, dark gray divergent results.

Nevertheless, corresponding results were observed in only about 74% of all peptide-donor-pairs where both assays had been performed. In 18% of the cases, responses in ELISPOT were found that could not be reproduced in intracellular staining, while the opposite was the case in 8% of the pairs. These findings are probably partly due to differences in the assay time. In the ELISPOT experiments, cells were incubated 24 h in the presence of peptide, while in the intracellular cytokine stainings the incubation time was only 6 h. Slower responses might thus have been missed. Moreover, intracellular staining was performed after the ELISPOT screening with promising epitope candidates. Thus, the cells used in the experiments were derived from separate cell expansions that did not necessarily yield the same results. The low number of precursor cells in PBMCs can be expected to lead to random effects in the

efficiency of expansion, because of contingently different numbers of precursors in different batches. Also, it cannot be taken for granted that every precursor cell meets a suitable APC in the expansion culture. In some cases where several cytokine staining assays from different stimulations with the same donor-peptide-pair have been carried out, the results did differ as well (e.g. donor #1180 with the peptide TYFSLNNKF in tables 3.34 and 3.35). Of course, both unspecific staining in cytokine staining assays as well as random spots in the ELISPOT screening are likely sources of error, too.

Figure 4.1B compares the presence of tetramer-positive cells and IFN-γ responses in $CD8^+$ cells in the same manner. Functional and specific cytotoxic T cells should at least in part be both tetramer-positive and cytokine producers. However, this does not necessarily mean the production of IFN-γ, and monofunctional pure killer cells that do not produce cytokines can also occur. Cytokine production by $CD4^+$ T cells was excluded in this comparison, since it is canonically mediated by MHC class II molecules and thus does not suppose a tetramer-positive population. For these two parameters, 84% of the donor-peptide-pairs showed corresponding results. 10% had IFN-γ producing $CD8^+$ cells without a tetramer-positive population, and 6% had tetramer-positive T cells but were negative in IFN-γ staining. In these assays, however, tetramer and cytokine staining was always performed with the same batch of expanded cells, so that differences arising from the expansion conditions can be excluded.

In four of the 10 cases where tetramer-staining was absent but cytokine production observed, the fraction of cytokine producing cells in the $CD8^+$ population is barely above the threshold of 0.1% and may be due to background staining. In the other six cases (one for TLLYVLFEV, three for SMPNLVPEV, and two for LLDQLIEEV) the cytokine responses are substantial and might be mediated not by HLA-A*02 but

another HLA allotype. In this case, staining with A*02-tetramer would yield no results.

In six cases, a tetramer-positive population was present without IFN-γ production. This happened twice each with the peptides LTDLGQNLLY, SMPNLVPEV, and TYFSLNNKF. Additionally, seven donors had cells that could be stained by an A*02-ILRRPTSPV-tetramer, but no responses in the ELISPOT screening were ever observed. Most of these populations were quite large, so that unspecific tetramer staining can be excluded. They could consist of CTLs that produce no cytokines or other ones than IFN-γ. In the cases where staining for TNF in $CD8^+$ cells has also been performed, however, although cells producing only TNF occurred frequently, there were no instances of tetramer-positive cells accompanierd only by a TNF response but no IFN-γ response. Rather, the TNF responses either occur together with IFN-γ responses, or they are found to be mostly weak and without concomitant tetramer staining, suggesting background staining and a false positive result. Thus the impact of populations consisting IFN-γ-negative functional CTLs on the data is difficult to assess.

It has been long known that dysfunctional T cells can also be stained with tetramers[342], and the above-mentioned populations could be made up of such cells as well. Accidents during the expansion culture could be responsible for the induction of a tolerant or anergic phenotype. Furthermore, the induction of $CD8^+$ T regulatory cells by viral infection has been described[343], and persistent infections are thought to be especially favorable to this process[344]. It is interesting that cells specific for the peptide ILRRPTSPV seem to be very frequently affected by this phenomenon. Indeed, since it elicited no responses in the ELISPOT screening at all, the tetramer-specific populations were discovered only because the respective tetramer happened to be already in stock in the lab. Thus it cannot be excluded that other peptides induce such anergic or dysfunctional populations as well. ILRRPTSPV

is derived from the protein E1A, which is not a part of the virion but is a regulator that is among the first proteins transcribed after adenoviral infection. Although persistent infection by adenoviruses is yet poorly understood, it has been shown over the last years that it is characterized by a latent presence of viral DNA rarely accompanied by infectious particles[122;123]. From this it could be inferred that while MHC ligands from virion components such as hexon protein are rarely displayed during persistent infection, ligands from intracellular regulatory proteins might be so more frequently, which could increase their potential for the induction of anergic or regulatory T cells. On the other hand, on HLA-A*02 the immunodominant epitope is derived from E1A (see 4.4.), showing that epitopes from this protein nevertheless play an important role in the anti-adenoviral immune response.

4.3. Immunodominant epitopes found for all three allotypes

For all three allotypes, the study has identified one immunodominant epitope, meaning that more than half of the tested HLA-matched donors were responders in ELISPOT. Figure 4.2 summarizes the results of the ELISPOT screening for HLA-matched and unmatched donors.

From the HLA-A*01 candidates, (L)TDLGQNLLY in both length variants elicited a large number of responses, the longer version in more than 90% of tested HLA-matched donors, making it arguably the major adenoviral CTL target in HLA-A*01 positive individuals. The shorter version of the peptide has already been published as an epitope[279]. Most other peptides predicted as epitopes elicited only sporadic, if any, responses in ELISPOT assays, while the responses to YTYSGSIPY were not specific for HLA-A*01. Most likely the responses were mediated by $CD4^+$ T cells, as the peptide is part of a published $CD4^+$ T cell epitope[282].

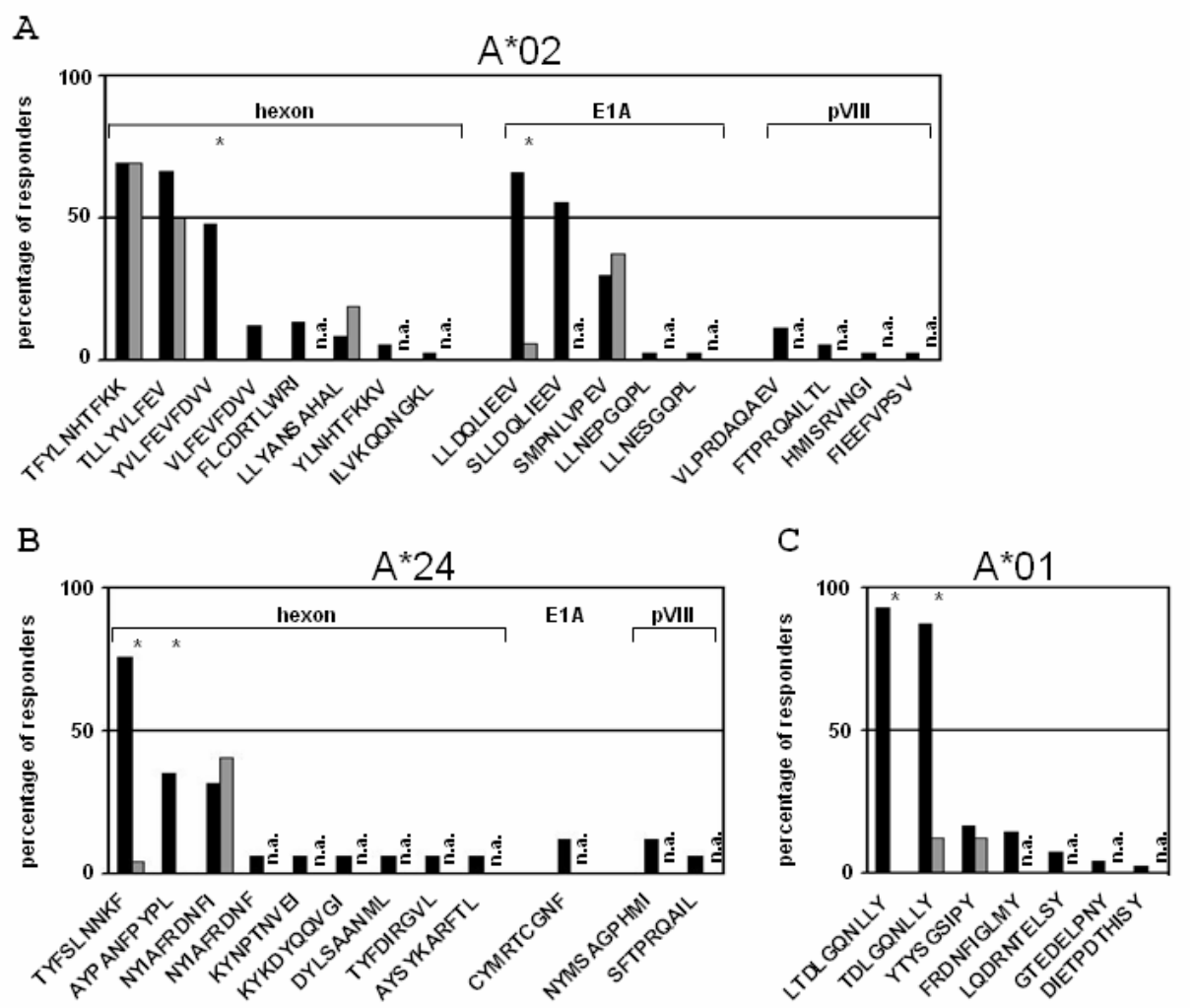


Figure 4.2: Percentage of donors responding to stimulation with peptide in ELISPOT assays after presensitization. Peptides predicted for HLA-A*02 (**A**), HLA-A*24 (**B**), and HLA-A*01 (**C**) with their proteins of origin (all hexon for HLA-A*01). Black bars: donors positive for the respective allele. Grey bars: donors negative for the respective allele. Significant differences are indicated ($p<0.05$, Fisher's exact test).

On HLA-A*24, an immunodominant epitope is the hexon-derived peptide TYFSLNNKF, already published[279]. Responses are less frequent than to LTDLGQNLLY, however. A possible reason could be that the A*01-restricted epitope is more highly conserved in different adenoviral strains. TYFSLNNKF and LLDQLIEEV are found only in the four species C strains (Ad1, 2, 5, and 6), while LTDLGQNLLY is additionally present in five of 11 species B strains as well (Ad7, 11, 16, 21, and 50). Furthermore, it cannot be excluded that the distribution of HLA subtypes in the tested donors also plays a role. Only low-resolution HLA typing of the donors was employed, and it is conceivable that some of the identified epitopes

are presented efficiently only on certain subtypes of an allotype. This effect is probably not large, however. No studies of subtype distribution for German populations exist, but studies of other Western European populations show that the overwhelming part of HLA-A*01, -A*02, and A*24 allele frequencies are made up of the subtypes A*01:01, A*02:01, and A*24:02, respectively (www.allelefrequencies.net). These are also the subtypes the epitope prediction has been carried out for.

An additional novel epitope, AYPANFPYPL, could be identified, also derived from hexon and eliciting responses by $CD8^+$ T cells in about 28% of tested donors. Responses to the predicted candidate NYIAFRDNFI have turned out not to be HLA-A*24 specific, but rather to be effected by $CD4^+$ T cells. This peptide is also part of an already published $CD4^+$ T cell epitope[282]. Several other sporadic responses were detected with ELISPOT assays, but were rather marginal in comparison.

In the case of HLA-A*02, a novel and immunodominant epitope was identified with LLDQLIEEV and its length variant SLLDQLIEEV. SMPNLVPEV is another novel epitope at least partly restricted to HLA-A*02, but it triggers clearly also responses by $CD4^+$ T cells in some donors as well as $CD8^+$ T cell responses not restricted to HLA-A*02. Statistical analysis of the ELISPOT screening results revealed that responses among donors who were positive for two other HLA allotypes were significantly more frequent than those among other donors: 10 of 20 HLA-A*03-positive donors responded, while only 13 of 57 A*03-negative ones did ($p=0.025$), and 16 of 30 HLA-B*07-positive donors showed responses, while among the B*07-negative ones the ratio was 7 of 47 ($p=0.0028$). The three donors that showed substantial responses in intracellular cytokine staining without having a tetramer-positive population (#970, #1324, and #1352) are all B*07-positive. These findings are surprising because the SYFPEITHI scores for the peptide on both HLA-A*03 (score 2) and HLA-B*07:02 (score 8) are very low. Nevertheless, SMPNLVPEV could be an

unusual epitope on one of these allotypes, additionally to being also an infrequent epitope on MHC class II.

Furthermore, the published peptide LLYANSAHAL[287] could be confirmed as an A*02-epitope in some cases, but is also eliciting responses in $CD4^+$ T cells. The results for the putative HLA-A*02 epitope TFYLNHTFKK demonstrate that intracellular cytokine staining combined with staining of surface molecules is especially important in the validation of $CD8^+$ T cell epitopes and distinguishing responses from those mediated by $CD4^+$ T cells.

4.4. The HLA-A*02-mediated response is directed mainly against E1A

The most interesting finding of this study is the identification of MHC class I epitopes from the protein E1A. Actually on the very widespread allotype HLA-A*02 the immune response is directed mainly against an epitope from this protein, (S)LLDQLIEEV. The discovery of CTL epitopes from E1A is especially interesting since this protein has also been described as a source of immunodominant epitopes from the human adenovirus type 5 in mice[274] and rats[275]. However, no epitopes derived from this protein have hitherto been found in humans. Epitopes from E1A might prove to be especially valuable to strategies of adoptive transfer because this protein is a regulatory factor expressed early on in the infection cycle of adenoviruses, in contrast to the structural protein hexon, which is typical for the late stage of infection. E1A-derived peptides would therefore arguably be found earlier on the cell surface and render the cell susceptible to faster killing after infection. On the other hand, the identification cells specific for SMPNLVPEV and ILRRPTSPV by tetramer, both also E1A-derived, that did not produce cytokines suggests that the above-mentioned problem of tolerance induction might apply especially to epitopes

from non-capsid proteins. Memory T cells present in PBMCs after long persistent infection could be tolerant or dysfunctional and possibly need special expansion conditions to restore their cytolytic activity.

The peptides from protein pVIII rarely elicited responses in the ELISPOT screening with matched donors and were not included in the later experiments.

4.5. Responses mediated by different HLA alleles do not interfere with each other

It was also analyzed whether immune responses restricted by a certain HLA allotype interfere with responses restricted by other allotypes. Immunodomination, meaning the suppression of weak responses by immunodominant ones, has first been described more than 30 years ago[345;346]. It has been observed for $CD8^+$ T cell responses to a variety of targets, including tumor antigens[347], minor H antigens[348], and viruses[349;350]. In some cases, even complex hierarchies of domination could be shown to exist[351]. On the other hand, previous work by this group had found no clear hierarchy, but rather codominance of epitopes in cytomegalovirus memory responses[331]. It has also been reported that for some viral infections immunodomination is limited to primary responses and does not affect the memory T cell pool[352;353].

For adenoviral responses mediated by the three allotypes examined here, exactly one immunodominant response for each allotype could be identified, implying the possibility of immunodomination over responses mediated by the same allotype. To address this question for different HLA molecules, donors who responded to an immunodominant peptide restricted by one allotype were checked whether they showed any significant differences in the response frequency to another

immunodominant peptide restricted by a different allotype, compared with donors who did not have the first response (Figure 4.3).

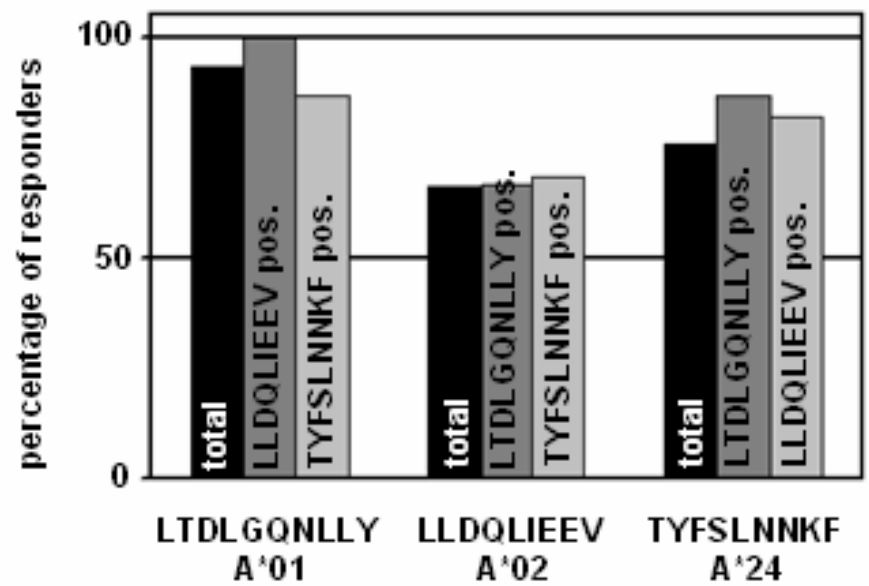


Figure 4.3: Percentage of all donors with indicated HLA type responding to respective peptide compared to donors also responding to immunodominant peptides of other alleles.

No differences could be detected with any of the combinations of the three immunodominant epitopes LTDLGQNLLY (A*01), LLDQLIEEV (A*02), and TYFSLNNKF (A*24). Thus it can be concluded that T cell responses mediated by adenoviral epitopes on different allotypes do not interfere with each other and codominance of such epitopes is a characteristic phenomenon in adenovirus-specific memory responses.

5. SUMMARY

Human adenovirus infections are a serious threat to immunocompromised individuals, especially stem cell transplantation patients, and a considerable cause of mortality. Although the T cell immune response seems to be essential for the control and clearance of the disease and correlates with a positive course of disease, few adenoviral CD8 T cell epitopes have been identified and their clinical relevance has remained largely unknown.

This study was therefore aimed at applying the strategy of reverse immunology and mapping the cytotoxic T cell epitopes from adenovirus for three important HLA allotypes. The main focus was the identification of immunodominant epitopes that elicit T cell responses in most infected hosts of the appropriate HLA genotype. Therefore, the SYFPEITHI algorithm was applied to predict likely epitopes from the primary sequences of three proteins (hexon, pVIII, E1A) of two viral strains (Ad2, Ad5). Candidate epitopes were synthesized as peptides and the PBMCs of healthy donors examined for memory T cell responses specific to them. For this purpose specific T cells were amplified before testing in order to recognize weak responses difficult to detect *ex vivo*. The HLA restriction of the identified epitopes was verified by testing donors with and without the supposed allotype and by tetramer staining. Specific cell populations were further characterized phenotypically and functionally.

For each of the three allotypes one immunodominant epitope could be identified, i.e. LTDLGQNLLY for HLA-A*01, LLDQLIEEV for HLA-A*02, and TYFSLNNKF for HLA-A*24. Furthermore, it became apparent that the T cell response mediated by HLA-A*02 is directed mainly against the protein E1A. The epitopes from E1A described here are the first to be described that are presented on MHC class I and not derived from the hexon protein. It could also be shown that no hierarchy amongst $CD8^+$ T cell epitopes restricted by different HLA allotypes exists, but parallel, co-dominant responses are detectable. These results may prove to be useful in the effort to establish adoptive transfer strategies of adenovirus-specific T cells for the treatment of immunocompromised patients.

6. ZUSAMMENFASSUNG

Infektionen mit humanpathogenen Adenoviren stellen eine ernste Bedrohung für Menschen mit einem geschwächten Immunsystem dar, wie z.B. Patienten nach einer Stammzelltransplantation, und sind eine zahlenmäßig relevante Todesursache. Obwohl die T-Zell-Immunantwort für die Eindämmung und Beseitigung der Infektion offenbar essentiell ist und mit einem positiven Krankheitsverlauf korreliert, sind bisher nur wenige adenovirale CD8-T-Zell-Epitope identifiziert worden, und ihre klinische Relevanz ist größtenteils unbekannt.

Deshalb sollte in dieser Arbeit nach dem Ansatz der reversen Immunologie eine Kartierung adenoviraler zytotoxischer T-Zell-Epitope für drei wichtige HLA-Allotypen erfolgen. Der Schwerpunkt lag hierbei insbesondere auf der Identifizierung immundominanter Epitope, die in den meisten Infizierten mit passendem HLA-Genotyp eine T-Zellantwort induzieren. Dazu wurden aus den Primärsequenzen dreier Proteine (Hexon, pVIII, E1A) aus zwei Virenstämmen (Ad2, Ad5) mit dem SYFPEITHI-Algorithmus wahrscheinliche Epitope vorhergesagt und als Peptide synthetisiert. PBMCs gesunder Spender wurden anschließend daraufhin untersucht, ob sie Gedächtniszellen enthielten, die gegen die Peptide spezifisch waren. Hierzu wurden spezifische T-Zellen im Vorfeld amplifiziert, um *ex vivo* schwer nachweisbare schwache Antworten erkennen zu können. Die HLA-Restriktion der identifizierten Epitope wurde über den Vergleich der Antworthäufigkeiten von Spendern mit und ohne den vermuteten Allotyp sowie Tetramerfärbungen überprüft. Adenovirusspezifische Zellpopulationen wurden alsdann phänotypisch und funktionell weiter charakterisiert.

Für alle drei Allotypen konnte jeweils ein zytotoxisches T-Zell-Epitop als immundominant identifiziert werden, nämlich LTDLGQNLLY für HLA-A*01, LLDQLIEEV für HLA-A*02 und TYFSLNNKF für HLA-A*24. Des Weiteren wurde deutlich, dass die über HLA-A*02 vermittelte T-Zellantwort hauptsächlich gegen das Protein E1A gerichtet ist. Die hier beschriebenen Epitope aus E1A stellen somit die ersten auf MHC Klasse I präsentierten dar, die nicht aus dem Hexon-Protein stammen. Weiterhin konnte gezeigt werden, dass zwischen adenoviralen Epitopen mit unterschiedlicher HLA-Restriktion keine Hierarchie besteht, sondern parallele kodominante Antworten auftreten. Diese Ergebnisse könnten sich nützlich erweisen bei den Bemühungen, adoptive Transferstrategien für immungeschwächte Patienten zu entwickeln.

7. ABBREVIATIONS

4-1BBL		4-1BB ligand
AdPol		adenoviral DNA polymerase
Adv		adenovirus
AIDS		acquired immunodeficiency syndrome
AIRE		autoimmune regulator
APC		antigen presenting cell
	or	allophycocyanin
ARD		acute respiratory disease
ATP		adenosine triphosphate
ATPase		adenosine triphosphatase
Bak		Bcl-2-homologous antagonist killer
Bax		Bcl-2-associated X protein
BCIP		5-bromo-4-chloro-3-indolyl phosphate
Bcl-2		B cell lymphoma 2
BHS		biotin holoenzyme synthetase
BMT		bone marrow transplantation
BSA		bovine serum albumine
CAR		coxsackievirus-adenovirus receptor
CD		cluster of differentiation
CD40L		CD40 ligand
CDK		cyclin-dependent kinase
CDR		complementarity determining region
CFSE		carboxyfluorescein succinimidyl ester
CLIP		class-II-associated invariant chain peptide
CTL		cytotoxic T lymphocyte
CTLA-4		CTL antigen 4
DBP		ssDNA binding protein
DC		dendritic cell
dCMP		deoxycytidine monophosphate
DC-SIGN		dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
ddH_2O		double-distilled water
DMSO		dimethylsulfoxide
DNA		deoxyribonucleic acid
DNase		deoxyribonuclease
dsDNA		double-stranded DNA
DTT		dithiothreitol
EBV		Epstein-Barr virus
EDTA		ethylenediamin tetraacetate
eIF4E		eukaryotic translation initiation factor 4E

EKC	epidemic keratoconjunctivitis
ELISA	enzyme-linked sandwich assay
ELISPOT	enzyme-linked immunospot
ER	endoplasmic reticulum
ERAAP	ER aminopeptidase associated with antigen processing
ERp57	ER protein 57
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Fmoc	fluorenylmethoxycarbonyl
FoxP3	forkhead box protein P3
FPLC	fast protein liquid chromatography
FRET	fluorescence resonance energy transfer
FSC	forward scatter
GFP	green fluorescent protein
GITR	glucocorticoid-induced TNF receptor
HEK 293	human embryonic kidney cell line 293
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
ICAM	inter-cellular adhesion molecule
ICOS	inducible co-stimulator
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
IPTG	isopropylthiogalactoside
ITAM	immunoreceptor tyrosine-based activatory motif
LAMP-1	lysosomal-associated membrane protein 1
LFA-1	lymphocyte function-associated antigen 1
LICOS	ligand of ICOS
MALDI	matrix-assisted laser desorption/ionization
MHC	major histocompatibility complex
miRNA	microRNA
mRNA	messenger RNA
MRN complex	MRE11-RAD50-NBS1 complex
NBT	nitroblue tetrazolium chloride
NF1	neurofibromin 1
NMWL	nominal molecular weight limit
Oct-1	octamer-binding protein 1

OD	optical density
PB	Pacific Blue
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	R-phycoerythrin
PerCP	peridinium chlorophyll
pH	*potentia hydrogenii*
PHA	phytohemagglutinin
PI	propidium iodide
PKR	protein kinase R
PLC	peptide loading complex
PMA	phorbol myristate acetate
PMSF	phenylmethylsulfonylfluoride
pTP	pre-terminal protein
RID complex	receptor internalization and degradation complex
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
rpm	revolutions per minute
SSC	side scatter
ssDNA	single-stranded DNA
TAP	transporter associated with antigen processing
TBS	Tris buffered saline
TCM	T cell medium
TCR	T cell receptor
TGF	tumor growth factor
T_{CM}	T central memory cell
T_{EM}	T effector memory cell
T_H	T helper cell
TNF	tumor necrosis factor
TP	terminal protein
TRAIL	TNF-related apoptosis-inducing ligand
T_{reg}	T regulatory cell
Tris	Tris(hydroxymethyl)aminomethane
TSB	tetramer staining buffer
UV	ultraviolet

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9. APPENDIX

Low-resolution HLA typing of all blood donors employed in the study for the HLA-A and -B *loci*. When only one allele is given for a *locus*, the donor was homozygous.

donor #	**HLA alleles**			
552	A*01	A*24	B*08	B*60
553	A*02	A*23	B*27	B*44
555	A*01	A*24	B*08	B*35
556	A*03	A*24	B*07	B*61
558	A*02	A*24	B*57	B*62
560	A*02	A*03	B*60	B*62
562	A*02	A*03	B*07	B*13
564	A*02		B*60	B*62
570	A*01	A*02	B*49	B*62
574	A*24	A*26	B*07	B*44
576	A*02	A*26	B*38	B*57
589	A*02	A*24	B*35	B*73
602	A*02	A*24	B*51	B*61
623	A*01	A*24	B*08	B*44
634	A*01	A*24	B*35	B*63
654	A*02	A*24	B*35	B*39
752	A*01	A*02	B*57	
757	A*02	A*24	B*27	B*47
759	A*02	A*03	B*07	B*62
765	A*02		B*03	B*44
768	A*02	A*03	B*07	B*39
771	A*02	A*24	B*27	B*62
773	A*02	A*24	B*35	B*61
776	A*01	A*02	B*37	B*51
778	A*02	A*03	B*07	B*53
779	A*02	A*03	B*08	B*62
780	A*01	A*02	B*08	B*44
781	A*02	A*29	B*35	B*45
784	A*02	A*03	B*13	B*44
785	A*02	A*32	B*44	B*58
788	A*01	A*02	B*08	B*13
789	A*01	A*02	B*55	B*62
790	A*03	A*24	B*07	B*62
791	A*02	A*03	B*35	
793	A*02	A*29	B*08	B*49
794	A*02	A*24	B*07	B*55
797	A*01	A*02	B*08	B*13
800	A*02	A*66	B*13	B*41
801	A*02		B*44	B*62
804	A*02	A*26	B*27	B*62
806	A*01	A*24	B*07	B*08
813	A*02	A*24	B*35	B*57
815	A*01	A*02	B*35	B*60
829	A*02	A*24	B*44	B*62
847	A*01	A*24	B*27	B*44
866	A*01	A*24	B*08	
867	A*01	A*24	B*27	B*50
878	A*01	A*24	B*47	B*62
880	A*02	A*24	B*27	B*62
882	A*02	A*24	B*07	B*55

888	A*01	A*24	B*07	B*08
904	A*01	A*24	B*37	B*55
911	A*01	A*24	B*07	B*52
940	A*01	A*24	B*08	B*50
945	A*01	A*24	B*18	B*57
951	A*02	A*24	B*27	B*56
958	A*02	A*03	B*07	B*45
960	A*02		B*37	B*44
961	A*01	A*24	B*08	B*49
968	A*02		B*27	B*50
970	A*01	A*02	B*07	B*57
973	A*02	A*24	B*35	B*51
983	A*02	A*23	B*13	B*44
984	A*01	A*02	B*62	B*63
988	A*01	A*02	B*37	B*62
989	A*02	A*03	B*18	B*51
991	A*01	A*03	B*08	B*37
992	A*01	A*03	B*56	B*62
995	A*01	A*03	B*07	B*13
998	A*01	A*24	B*13	B*51
999	A*02	A*24	B*27	B*62
1002	A*01	A*25	B*44	B*57
1005	A*01	A*03	B*07	B*08
1006	A*01	A*02	B*35	B*51
1007	A*01	A*24	B*08	B*44
1008	A*02	A*24	B*51	B*64
1009	A*02	A*29	B*44	
1010	A*02	A*24	B*07	B*44
1011	A*01	A*02	B*08	B*49
1012	A*01	A*24	B*13	B*41
1013	A*02	A*23	B*44	B*58
1014	A*01	A*03	B*07	B*08
1016	A*02	A*11	B*55	B*62
1017	A*01		B*08	B*57
1021	A*01	A*24	B*08	B*55
1022	A*02	A*24	B*35	B*55
1025	A*01	A*28	B*08	B*44
1026	A*02	A*28	B*44	B*60
1030	A*01	A*26	B*07	B*39
1033	A*02	A*26	B*08	B*51
1038	A*01	A*03	B*07	B*57
1040	A*01	A*24	B*08	B*44
1043	A*01	A*33	B*08	B*14
1045	A*02		B*27	B*44
1047	A*24	A*31	B*07	B*62
1049	A*03	A*24	B*50	B*62
1050	A*01	A*23	B*44	B*60
1051	A*02		B*62	
1052	A*02	A*24	B*18	B*50
1054	A*01	A*23	B*44	B*57
1055	A*02		B*07	B*08
1057	A*02	A*03	B*07	B*18
1058	A*02	A*23	B*49	B*51
1061	A*02	A*03	B*07	B*64
1062	A*01	A*03	B*07	B*37
1065	A*02	A*32	B*51	B*62
1066	A*02	A*03	B*35	B*44
1067	A*01	A*02	B*57	B*58
1068	A*01	A*03	B*07	B*08

1069	A*01		B*07	B*18
1071	A*02	A*24	B*07	B*27
1072	A*01	A*02	B*08	B*44
1073	A*02	A*24	B*07	B*55
1075	A*01	A*03	B*07	B*62
1078	A*01	A*26	B*07	B*62
1082	A*02	A*24	B*18	B*27
1083	A*01	A*03	B*08	B*58
1085	A*01	A*02	B*08	B*51
1086	A*01	A*32	B*13	B*60
1093	A*01	A*02	B*37	B*62
1098	A*01	A*02	B*07	B*37
1099	A*01	A*02	B*07	B*08
1101	A*24	A*32	B*07	B*35
1102	A*01	A*02	B*08	B*41
1106	A*01	A*02	B*08	B*35
1107	A*02	A*03	B*44	B*60
1108	A*02	A*28	B*07	B*65
1109	A*02	A*11	B*35	B*44
1110	A*01	A*03	B*08	B*38
1111	A*01	A*30	B*18	B*57
1113	A*02		B*13	B*60
1114	A*02	A*30	B*13	B*44
1115	A*01	A*33	B*14	B*62
1117	A*02	A*24	B*27	B*62
1118	A*02	A*24	B*08	B*57
1119	A*01	A*02	B*08	B*44
1122	A*02		B*07	B*08
1123	A*01		B*08	B*35
1124	A*02	A*11	B*07	B*61
1125	A*02	A*03	B*07	B*50
1129	A*02	A*03	B*07	B*62
1130	A*01	A*02	B*07	B*35
1132	A*01	A*32	B*44	B*49
1133	A*01	A*03	B*07	B*08
1135	A*02	A*32	B*07	B*44
1136	A*01		B*08	B*37
1137	A*02	A*30	B*08	B*13
1138	A*01	A*02	B*08	B*44
1139	A*02	A*32	B*44	
1140	A*02	A*26	B*07	B*62
1141	A*01	A*02	B*13	B*61
1142	A*03	A*32	B*07	B*44
1143	A*01	A*03	B*08	B*63
1144	A*01	A*02	B*07	B*08
1150	A*01	A*03	B*07	B*08
1151	A*01	A*03	B*07	B*18
1153	A*03	A*29	B*07	B*45
1154	A*01	A*02	B*07	B*08
1155	A*01	A*02	B*07	B*57
1156	A*02	A*24	B*07	B*60
1160	A*02		B*13	B*14
1162	A*01	A*11	B*07	B*57
1163	A*02	A*31	B*37	B*44
1164	A*03		B*07	B*62
1165	A*01		B*57	B*62
1166	A*02	A*26	B*38	B*60
1169	A*02	A*24	B*07	B*35
1170	A*11	A*24	B*07	B*60

1171	A*01	A*02	B*07	B*08
1173	A*02	A*24	B*35	B*39
1174	A*02	A*32	B*38	B*55
1176	A*02	A*03	B*07	B*44
1180	A*03	A*24	B*07	B*44
1181	A*03	A*28	B*07	B*37
1184	A*02	A*28	B*35	B*44
1187	A*02	A*26	B*44	B*62
1190	A*02	A*24	B*08	B*62
1191	A*02	A*26	B*07	
1192	A*02	A*11	B*08	B*61
1198	A*02	A*28	B*14	B*27
1201	A*02	A*29	B*44	
1238	A*02	A*24	B*51	B*56
1241	A*01	A*03	B*47	B*57
1246	A*01	A*03	B*08	B*60
1250	A*03	A*32	B*07	B*38
1254	A*24	A*31	B*07	B*44
1265	A*03	A*31	B*44	B*62
1271	A*01	A*03	B*07	B*57
1282	A*02	A*24	B*27	B*51
1292	A*02	A*24	B*07	B*18
1293	A*02	A*24	B*44	B*57
1295	A*01	A*24	B*07	B*18
1296	A*03	A*32	B*07	B*44
1320	A*02	A*24	B*07	B*57
1321	A*03	A*26	B*07	B*44
1324	A*03	A*32	B*07	B*55
1325	A*03	A*27	B*07	B*39
1328	A*01		B*55	B*60
1331	A*01	A*24	B*08	B*62
1332	A*01	A*24	B*08	B*62
1334	A*01	A*03	B*07	B*35
1336	A*01	A*02	B*57	B*60
1337	A*02	A*03	B*07	B*44
1338	A*01		B*08	B*37
1340	A*02	A*03	B*07	B*58
1341	A*03	A*24	B*07	B*14
1342	A*02	A*11	B*07	B*51
1343	A*01	A*11	B*08	B*18
1344	A*02		B*61	
1347	A*03	A*28	B*07	B*51
1348	A*02	A*24	B*44	B*60
1349	A*01	A*30	B*08	B*14
1350	A*01	A*02	B*35	B*55
1351	A*01	A*23	B*08	B*44
1352	A*03	A*25	B*07	B*08
1354	A*01	A*24	B*13	B*53
1355	A*01	A*24	B*44	B*57
1356	A*03	A*11	B*07	B*44
1357	A*02	A*25	B*35	B*44
1363	A*01	A*24	B*07	B*18
1369	A*01	A*24	B*07	B*52
1401	A*01	A*24	B*07	B*62
1425	A*02	A*24	B*44	B*60
1430	A*02	A*24	B*37	B*44
1487	A*02	A*24	B*18	B*50

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