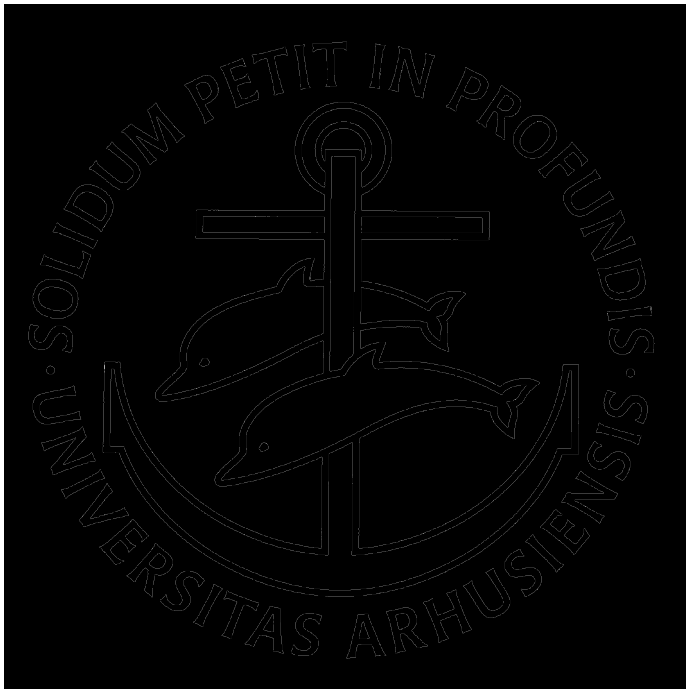
Studies on immune presenting systems with potential of improving vaccines



Ph.D. thesis

By

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Preface

The aim of this section is to account for the initial elaboration of my Ph.D. project as well as elucidate the subsequent paths taken to reach the final goals.

For the past 4 years and 6 months I have been a part of the phage display group at the University of Aarhus. The last four years extensively collaborating with Nemod Biotherapeutics GmbH & Co. KG, Berlin. As I entered the phage display group a number of people undertook experiments with the aim of generating antibodies against differentially expressed cellular antigens. Dr. Kim Bak Jensen was deeply involved in the selection of phage displayed antibodies against keratinocyte specific cell surface antigens, but encountered severe difficulties in the active soluble expression of a number of such selected antibodies. At the same time my colleagues at Nemod Biotherapeutics GmbH & Co. KG were eager to generate immune responses towards anti-idiotypic antibodies potentially mimicking various tumour associated antigens. We therefore initiated a study on a generic approach, which lead to a dramatic increase in antibody performance as well as immunogenecity. The approach enabled the further characterisation of other wise poorly performing antibodies, and the identification of mimicking anti-idiotypic antibodies.

The second part of my Ph.D. training was focused on cellular immunology. The interest in presenting and selecting peptides mimicking tumour associated antigens as well as TCR specific peptides encouraged us to generate a phage displayed human single-chain MHC class I molecule. Prior a number of studies on soluble single-chain MHC class I molecules had been reported, and in particular a phage displayed mouse single-chain MHC class I molecule was reported by Gorochov and co-workers (Le Doussal et al., 2000). The phage display approach was established during my time in the phage display laboratory in Aarhus. The immunological experiments to verify the biological active folding of the single-chain molecule was subsequently conducted at the immunology laboratory in Berlin during the final two years of my Ph.D. training. The stay in Berlin considerably expanded my experience with cellular immunology due to a project on the transfection of a fully functional human dendritic cell line, taking special interest in the $CD8^+$ T cell response generated by dendritic cells transfected with various nucleic acid samples. The single-chain MHC class I and dendritic cell projects were conducted in parallel and did therefore indirectly support each other both theoretically and technically.

The thesis is divided into 5 major sections, 1) introduction, 2) results and discussion, 3) conclusion, 4) a section describing the materials and methods not included in the full-length manuscripts enclosed in appendices and 5) references. The introduction is subdivided in two sections devoted to A) the phage display technology and B) humoral and cellular immunology, thus elaborating on the conceptual background of the result section. The first part will describe the morphology and biology of the filamentous bacteriophage as well as the various fusion systems studied with the phage, with special emphasis on fused antibodies and single-chain MHC molecules. The second part will conduct a tour through the mammalian immune system, drawing special attention to adjuvant mediated vaccine delivery, the receptor and signal molecules involved in T cell activation and finally the biological significance of dendritic cells. Chapters II and III include results, discussion, conclusion and future perspectives for the work presented.

The thesis is terminated by three appendixes containing three manuscripts reporting on the findings of some of the projects presented in this thesis. The data presented in chapter 2.7 regarding a method to genetically modify a fully functional dendritic cell line will be compiled in a manuscript shortly. Below experimental work performed by co-authors is specified for each project.

Chapter 2.1 – Appendix A: Jensen KB[#], Larsen M[#], Pedersen JS, Christensen PA, Álvarez-Vallina L, Goletz S, Clark BFC and Kristensen P. Functional improvement of antibody fragments using a novel phage coat protein III fusion system. Biochem Biophys Res Commun, 2002. 298(4): p. 566-73.

[#] These authors contributed equally to the work.

Kim Bak Jensen performed the studies on L36 and D4.

Jesper Søndergaard Pedersen contributed with the fluorimetric stability measurements on L36 derivatives.

Chapter 2.2 – Appendix B: Larsen M[#], Jensen KB[#], Suárez E, Christensen PA, Sanz L, Löffler A, Ravn P, Clark BFC, Goletz S, Álvarez-Vallina L and Kristensen P. Functionally Fused Antibodies – a novel adjuvant fusion system. Journal of Immunology, under revision.
[#] These authors contributed equally to the work.

Kim Bak Jensen prepared L36 for immunisation studies.
Peter Astrup Christensen compiled the data on PACA17.
Laura Sanz contributed to the anti-idiotypic study on sera from L36 immunised mice.

Chapter 2.3 – Appendix C: Suárez E, Larsen M, Jensen KB, Sanz L, Kristensen P and Álvarez-Vallina L. Filamentous bacteriophage coat protein III domain I in DNA vaccines promotes a Th1-type dominated immune response. Journal of Immunology, under revision.

Eduardo Suárez generated and verified the DNA vaccines. Subsequently he immunised the mice and contributed to the analysis of the sera for anti-idiotypic antibodies.
Kim Bak Jensen aided to the generation of the DNA vaccines.
Laura Sanz contributed to the detection of anti-idiotypic antibodies.

A patent was filed on the novel ideas and results presented in Appendix A and B.

Appendix B and C are thought to be published back-to-back.

University of Aarhus
March 2004

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1 Introduction

1.1 The filamentous bacteriophage

Bacterial vira were discovered independently by Twort (1915) in England and d'Herelle (1917) in France (Lipton and Weissbach, 1969). The infectious life cycles of the bacteriophages were elucidated through the 1920s, although proof of the particle nature of the vira was first established in 1940 with the first electron microscopy pictures (Ruska, 1940). The initial interest was evoked by the anti-bacterial function of lytic bacteriophages, which was clinically employed world wide. The invention of antibiotics made the Western world abandon bacteriophage therapy, whereas primarily the Soviet Union for political and economical reasons remained faithful to bacteriophage therapy (reviewed by Summers (Summers, 2001) and Stone (Stone, 2002)). After the 2nd World War bacteriophage biology and morphology were intensively studied leading to classification of bacteriophages according to receptors crucial for infection, host range as well as life cycle and genomic characteristics (Lipton and Weissbach, 1969; Marvin and Hohn, 1969). This information has led to the extensive use of in particular the non-lytic filamentous bacteriophage in technological research areas, such as cloning and sequencing (Smith, 1988). The latest major breakthrough for the filamentous bacteriophage research happened in 1985, when George P. Smith reported on the incorporation of a genetically engineered chimeric protein integrated in the phage particle, leading to display of the non-phage protein fragment (Smith, 1985). Based on this discovery research up through the 1990s developed a biotechnological tool known as phage display technology. The potentials and limitations of the technology are tightly connected to the biology of the filamentous bacteriophage.

1.1.1 Biology of the filamentous bacteriophage

Phage are the most abundant biological entities on the planet, represented with approximately 10^{10} phage per litre of surface seawater and a total of 10^{31} phage in the biosphere (Bergh et al., 1989). Despite their great abundance very little is known about their diversity, primarily because the historical characterisation of phage was based on host range and biophysical properties, such as size and shape of the phage particle, resistance to temperature and organic solvents as well as size and type of the genome (single-stranded DNA (ssDNA), double-stranded DNA (dsDNA) or RNA). Recently, phage taxonomy with a genetic approach has been initiated (Rohwer and Edwards, 2002). Phage display technology employs the M13 filamentous bacteriophage, which is member of a large class of non-lytic ssDNA vira (Inoviridae, Inovirus) (Rohwer and Edwards, 2002; Salivar et al.,

1964). M13 phage specifically infect F^+ *E. coli* through interaction with F-pilus. For simplicity, the use of the phrase phage refers to M13 filamentous bacteriophage through out this thesis.

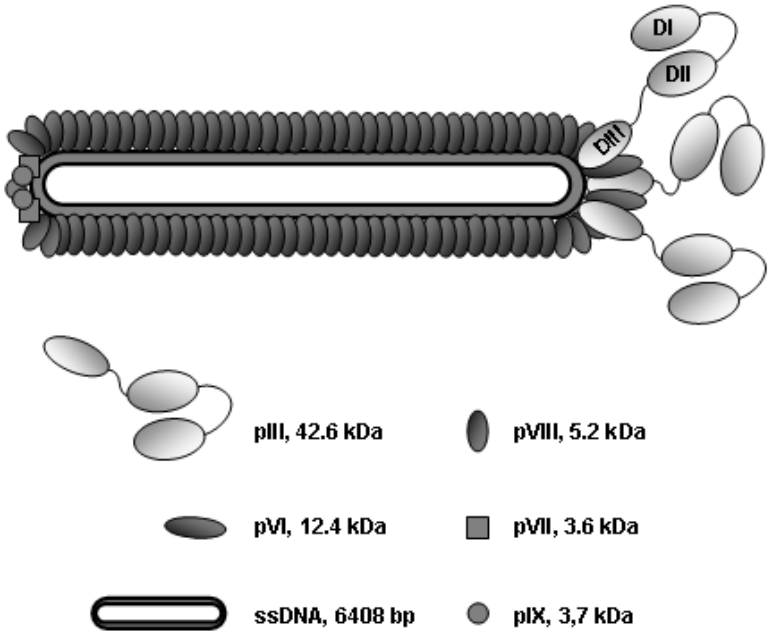


Figure 1 – Phage morphology. Schematic drawing of the phage particle, showing position and size of the capsid proteins pIII, pVI, pVIII, pVII, pIX.

1.1.1.1 Morphology

The phage is a rod shaped particle measuring approximately 900nm in length and 6,5nm in diameter. The circular ssDNA genome consist of 6407 nucleotides, encoding eleven proteins involved in the phage lifecycle (van Wezenbeek et al., 1980). Five are structural, three are needed for DNA replication and three are necessary for phage assembly (figure 1 and table 1).

Gene product	MW/kDa	Function	Localisation
I	39.5	Assembly	*E. coli*, inner membrane
II	46.3	DNA synthesis	*E. coli*, cytoplasm
III	42.6	Minor coat protein – infection	Phage tip
IV	45.8	Assembly	*E. coli*, outer membrane
V	9.7	Packaging of phage genome	*E. coli*, cytoplasm
VI	12.4	Coat protein	Phage tip
VII	3.6	Coat protein	Phage tip
VIII	5.2	Major coat protein	Phage capsid
IX	3.7	Coat protein	Phage tip
X	12.7	DNA synthesis	*E. coli*, cytoplasm
XI	12.4	Assembly	*E. coli*, inner membrane

Table 1 - Filamentous bacteriophage proteins. The size, function and localisation of each of the 11 phage proteins encoded by the phage genome. MW, Molecular Weight and kDa, kilo Dalton.

The wall of the phage is made up of approximately 2800 copies of the major coat protein VIII depending on the size of the genome. Protein VII and protein XI are present at one end and protein III and protein VI at the other end, terminating the rod shaped phage, represented by approximately 5 copies each (Endemann and Model, 1995; Webster, 1996). The exact copy number of the four terminating proteins has not been determined, but the five-fold rotary symmetry of protein VIII in the phage particle suggests that the copy number is five (Marvin, 1998).

1.1.1.2 Lifecycle

Phage infection is initiated when a phage encounters an F-pilus expressed on male *E. coli*. The recognition of F-pilus is mediated by the minor coat protein III, which can be divided into three structural domains, DI (residue 1-66), DII (residues 86-216) and DIII (residues 257-377), which are separated by glycine rich linkers. The protein is initiated with an 18 a.a. signal peptide and terminated with a membrane anchor (378-406) (numbering according to Holliger and Riechmann (Holliger and Riechmann, 1997)). The NMR structure of DI (Holliger and Riechmann, 1997) and the crystal structure of DI-DII (Holliger et al., 1999; Lubkowski et al., 1998) have been solved, and the latter suggests that DI and DII interact prior to infection. DIII anchors protein III in the phage particle, whereas DI and DII are responsible for the infection mechanism (Crissman and Smith, 1984; Holliger and Riechmann, 1997).

The F-pilus is attached to the bacterial inner membrane and protrudes through the outer membrane. The phage internalisation is induced through the interaction between DII and the F-pilus, thereby abolishing the original interaction between DI and DII. The F-pilus is thought to respond by retraction due to depolymerisation of the pilin subunits (figure 2.A) (Hultgren et al., 1991; Maneewannakul et al., 1992). This brings DI in proximity of the secondary receptor - the TolA of the Tol ("tolerant") complex (Deng et al., 1999; Riechmann and Holliger, 1997; Webster, 1991). In its activated state TolA spans the periplasmic space with the N-terminal incorporated into the bacterial inner membrane and the C-terminal binding in an energy dependent manner to PAL, another component of the Tol complex associated with the bacterial outer membrane (Cascales et al., 2000). Subsequently, the phage genome is internalised by directional unwrapping of the phage particle dependent on the Tol complex. Synchronously, the phage capsid depolymerises and stripped of phage capsid proteins are inserted into the bacterial inner membrane (figure 2.B) (Click and Webster, 1997; Click and Webster, 1998; Smilowitz, 1974). The F-pilus is not a necessity for

infection, as F^- *E. coli* are infected with an efficiency of 6-7 orders of magnitude lower than F^+ *E. coli*. This pilus independent infection is enhanced by two orders of magnitude in the presence of Ca^{2+} and further augmented with a DII deletion phage (Riechmann and Holliger, 1997; Russel et al., 1988).

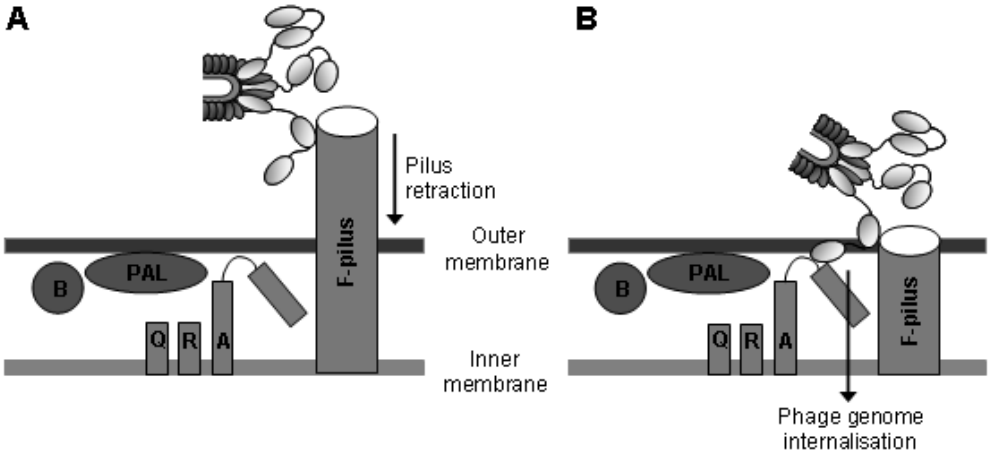


Figure 2 – Phage infection pathway. Schematic representation of the interactions between the phage particle and the bacterial receptors, F-pilus and TolA, leading to phage internalisation. A) DII of protein III interacts with the F-pilus and the F-pilus retracts due to depolymerisation. B) The interaction between DII and the F-pilus abolish the DI-DII interaction, whereby DI is ready to interact with the secondary receptor, TolA. The TolAQR-complex subsequently mediates internalisation of the phage genome (Riechmann and Holliger, 1997).

When the single-stranded phage genome (+ strand) has entered the bacteria, it is converted into replicative double stranded DNA (dsDNA) through the activity of host enzymes. Subsequently, transcription and translation of phage proteins are initiated from the dsDNA. The phage genome is regenerated via a rolling circle mechanism initiated by specific nicking of the dsDNA (+) strand by the pII, one of the first genes to be transcribed. An internal start site in the 3' end of gene II encodes pX, which therefore has the same sequence as the C-terminal third of pII, and is thought to regulate pII (Fulford and Model, 1984). The production of phage genomes is regulated by the levels of ssDNA bound to dimers of pV, host enzymes and RNA (-) strand primers. The pV binding to ssDNA further protect the ssDNA from conversion into dsDNA, and induces a rod-like structure of the phage genome, which is important for subsequent phage assembly (Fulford and Model, 1988a; Fulford and Model, 1988b). The replicative form is found in the bacteria with a copy number of approximately 15, and surplus of ssDNA is targeted to the membrane for phage assembly (Kay et al., 1996). The assembly process is mediated through the action of pIV in the outer membrane together with pI and pXI in the inner membrane that in collaboration form a pore spanning the

periplasmic space (figure 3.A) (Linderoth et al., 1997; Rapoza and Webster, 1995; Russel et al., 1997).

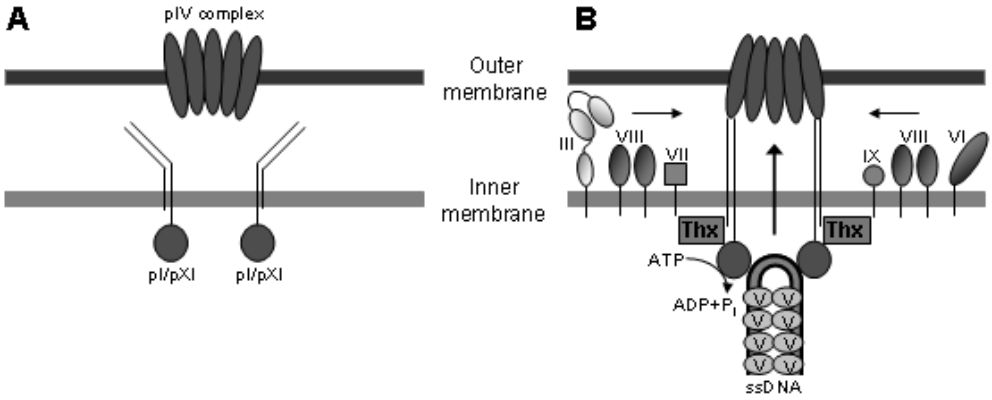


Figure 3 – Phage assembly and export. Schematic drawing of A) the localisation of the assembly proteins, consisting of homo multimeric pIV in the outer membrane and pI/XI in the inner membrane. B) The assembly complex spanning the periplasmic space of the infected *E. coli* is formed due to an energy dependent conformational change of pI/pXI, which is induced upon encounter of the ssDNA, thioredoxin (Thx) and the cytoplasmic domain of pI. The DNA binding pV is released with an adenosine-5'-triphosphate (ATP) dependent mechanism, thereby enabling the assembly of capsid proteins on the ssDNA. Initially, pVII and pIX attaches to the packing signal at the tip of the phage particle. Subsequently, the pVIII containing phage capsid is generated, and the phage is finally terminated by the addition of capping proteins, pIII and pVI (Rakonjac et al., 1999; Russel et al., 1997).

The capsid phage proteins are inserted into the inner membrane, where they are ready for phage assembly (Endemann and Model, 1995). Due to transcriptional regulation pVIII is the most highly expressed phage protein in agreement with its abundance in the phage particle (Beck and Zink, 1981). Phage assembly is a polarised process initiated through binding of a few copies of membrane bound pVII and pIX to a packing signal on the single-stranded phage genome (Russel and Model, 1989; Webster et al., 1981). This is followed by phage protrusion through the pore complex in the bacterial membrane, which is synchronously coupled to an energy requiring replacement of pV with pVIII, which has a positively charged C-terminal ideal for interacting with the negatively charged ssDNA (Russel, 1995). When the last pV is replaced with pVIII the phage is capped with pIII and pVI and the phage is released from the cell (figure 3.B) (Bauer and Smith, 1988; Crissman and Smith, 1984; Rakonjac et al., 1999). The scarcity of capping proteins pIII, pVI, pVII and pXI leads to the production of polyphages, having more than one phage genome per phage particle (Gailus et al., 1994; Lopez and Webster, 1983). Modifications of protein III can also induce polyphages, for instance when DI, DII or DI-DII are deleted (Riechmann and Holliger, 1997). Whether this effect is caused by malfunction of the modified pIII or due to regulatory elements in the deleted gene

fragment, leading to modified expression of genes encoded by the protein III encoding cistron is not known. Infection of bacteria is permanent, and leads to the release of 100-200 phage particles per doubling time (Webster, 1996).

1.1.2 Phage display technology

Due to the replicative phage biology the filamentous bacteriophage has been extensively applied in technological research areas such as cloning and sequencing. For this purpose it is important that genes can be inserted into nonessential regions of the phage genome without changing the phenotype of the phage apart from increasing the length of the phage according to the size of the cloned DNA insert (reviwed by Smith (Smith, 1988)).

In 1985 Smith GP abandoned the previous tradition of inserting DNA into nonessential parts of the phage genome, and inserted a fragment of the EcoRI endonuclease in-frame with the essential gene III. He further showed that the expressed chimeric protein is integrated in the phage particle enabling immunological recognition and enrichment of the EcoRI fragment displaying phage. The fusion phage particles are virtually not affected phenotypically with regard to infectivity and stability, which make it a perfect package linking phenotype and genotype of the fusion molecule (Smith, 1985).

Coat protein	Fused protein	Reference
pIII	Antibody cDNA Peptide	(McCafferty et al., 1990) (Crameri et al., 1994) (Scott and Smith, 1990)
pVI	cDNA	(Hufton et al., 1999; Jespers et al., 1995b)
pVIII	Peptide cDNA	(Felici et al., 1991) (Santini et al., 1998)
pVII/pIX	V_H/V_L Peptide	(Gao et al., 1999) (Gao et al., 2002)

Table 2 - Examples of fusions to the five coat proteins of the filamentous bacteriophage.

The phage particle allows functional integration of fusion variants of each of the five coat proteins. For pIII, pVII, pVIII and pIX N-terminal fusion leads to solvent exposed fusion proteins, whereas pVI enables C-terminal fusion (cf. table 2), which is especially important for cDNA fragments that include stop-codons (Jespers et al., 1995a). Studies by Sidhu and co-workers generated an artificial pVIII with reversed orientation in the phage coat, thus enabling C-terminal display (Fuh et al., 2000; Weiss and Sidhu, 2000). Likewise, Suter and co-workers report on a phage display system

employing the eukaryotic leucine zippers Jun and Fos fused to protein III and a cDNA fragment, respectively. This system exploits the heteromultimerisation of Jun and Fos, and enable C-terminal fusion of cDNA onto the Fos gene (Crameri and Suter, 1993). The potential of such systems await further certification.

1.1.2.1 Display formats

Although display on all coat proteins is feasible most phage display systems employ N-terminal fusion to pIII or pVIII. Fusions to pVIII have primarily involved peptides, since the small size of pVIII entail an upper limitation with regard to the size of the fusion, which has been empirical determined as 8-10 amino acids (Iannolo et al., 1995). A hybrid pVIII fusion system composed of both wild type and fusion pVIII has circumvented this limitation, enabling fusion of polypeptides as large as 86 kDa (Verhaert et al., 1999). Fusions to the much larger pIII are less constrained, which can be partly explained by the structure of the two N-teminal domains of pIII, which shows that the N-terminal is accessible and located in the periphery of the molecule spatially separated from the sites interacting with F-pilus and TolA (figure 4) (Lubkowski et al., 1998). Indeed, a 86 kDa protein was fused to pIII and displayed successfully on the phage particle (Verhaert et al., 1999).

Figure 4 – N-terminal domains of protein III. Structure of the N-terminal domains, DI (red) and DII (blue), of the minor coat protein III as determined by x-ray crystallography (Lubkowski et al., 1998). The structure indicates that the N-terminal is located in the periphery of the structure, which explains why N-terminal fusion does not interfere with the DI-DII interaction as well as pIII's interaction with F-pilus and TolA (Lubkowski et al., 1999).

The genetic information constituting the fusion protein can either be carried by a phage vector or a phagemid. The **phage vector** carry all information for phage production, and genetic fusion can be made to either gene III or gene VIII (type 3 and type 8 vector, respectively) resulting in display on all coat proteins. Alternatively, an extra copy of gene III or gene VIII carrying the fusion (type 33 and type 88 vector, respectively) can be inserted in the genome resulting in fractional coat protein display. A **phagemid** only contains the gene encoding the fusion protein, a selection marker and a

signal for packaging and replication. Therefore the phagemid system is dependent on a supply of the missing phage proteins from a helper phage. The term "rescue" refers to this phenomenon. Genetic fusion to gene III and gene VIII (type 3+3 and type 8+8 vector, respectively) result in variable display dependent on the properties of the fusion protein (display systems are designated according to Kay and co-workers (Armstrong et al., 1996)). The small size of phagemid vectors as well as their exclusively double stranded nature until addition of helper phage ensure easy handling, genetic stability and increases the transformation efficiency compared to phage vectors (Geider et al., 1985), which was also the motivation for their invention in the era, where phage technology comprised cloning and sequencing (reviewed by Kemper and co-workers (Mead and Kemper, 1988)).

The term "display level" describes the percentage of phage displaying a fusion coat protein. Generally, the phagemid system leads to lower display levels than phage vectors. For pIII fusions display levels varies in a fusion molecule dependent manner and is usually in the range from 0.1 - 10% (Goletz et al., 2002; Jensen et al., 2003). Display levels for pVIII fusion phagemids are reported as high as 30% (Greenwood et al., 1991). The display level is primarily influenced by the properties of the fusion protein, such as toxicity and ability to integrate in the phage coat and simultaneously allow for the phage to pass through the assembly complex. The display level has been optimised by modification of parameters like temperature, expression of the fusion protein, leader signals and helper phage (Jestin et al., 2001).

1.1.2.2 Helper phage

A helper phage is traditionally a viable phage that provides all the proteins necessary for phage propagation but with an impaired packaging signal, which favours packaging of phagemids above the helper phage genome (Russel et al., 1986). The dissimilarity of the antibiotic resistance genes encoded by the helper phage and phagemid, respectively, in combination with the impaired packaging signal ensures convenient maintenance of the geno- phenotype linkage. The linkage is however hampered by the relatively high number of non-displaying phage in phagemid systems as illustrated by the display level. Therefore, a number of helper phages have been generated to augment display levels by removal or reduction of the wild type protein III from the helper phage. Such removal has been accomplished by deletion of the full-length gene III in the phage genome and substitute with pIII from plasmids contained in the helper phage producing host cells (Duenas

and Borrebaeck, 1995; Rakonjac et al., 1997) or from the *E. coli* genome of a packaging cell line for production of helper phage (Rondot et al., 2001). This approach is however hampered by low yields of helper phage (Soderlind et al., 1995). A reason for the low helper phage yield could be that deletion of gene III influences transcriptional as well as translational elements for the additional genes transcribed from the polycistron encoding protein III, thus leading to production of polyphages. To circumvent the problem of deleting gene III an alternative helper phage including stop codons in gene III was generated. Infective helper phage are produced in a suppressor host strain, whereas subsequent rescue of phagemids is performed in non-suppressor strains (Baek et al., 2002). Theoretically, these phage systems should produce polyvalent phage exclusively harbouring fusion pIII (100% display level), but due to proteolysis in the DI-DII region of pIII generally less than one full-length fusion pIII is present per phage particle (Larocca et al., 2001; Rondot et al., 2001).

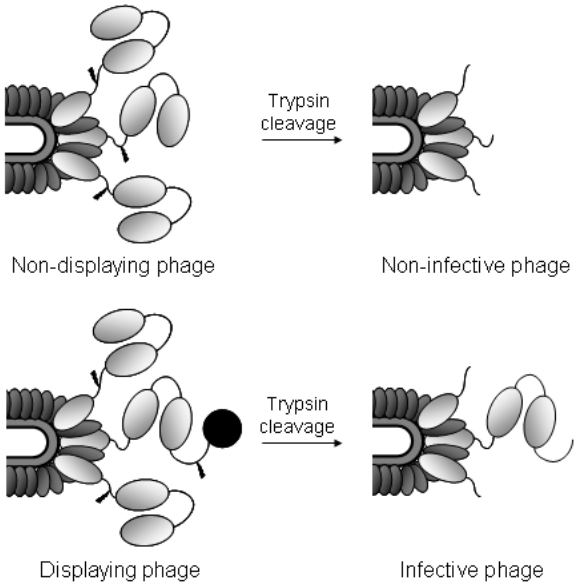


Figure 5 – Principle of the cleavable helper phage with regard to affinity selection. Phagemid systems generally reach approximately 0,1-10% display levels, whereas the remaining phage include only helper phage derived pIII (non-displaying phage). The cleavable helper phage encodes a pIII encoding a trypsin site between DII and DIII. Trypsin treatment therefore inactivates all helper phage derived pIIIs, thus rendering all non-displaying phage non-infective, whereas phage containing phagemid derived fusion pIII are trypsin resistant, thus retaining infectivity. The phagemid derived pIII includes a trypsin site between pIII and the fusion protein, which enables trypsin elution of displaying phage (Goletz et al., 2002; Jensen et al., 2003; Kristensen and Winter, 1998).

The cleavable helper phage (KM13) was originally invented to enable selection for stability (Kristensen and Winter, 1998), but has proven valuable for phage display in general (Goletz et al., 2002; Jensen et al., 2003; Ravn et al., 2000). This helper phage encode a proteolytic cleavage site between DII and DIII, which can be used to inactivate all helper phage derived pIII, but not pIII derived from phagemids. The technique thus renders non-displaying phage non-infective, which imply that the pool of infective phage all display the phagemid protein III fusion. Since the non-displaying group comprises more than 90% of phage in a phagemid system, the cleavable helper phage removes a significant amount of background phage (false positives). The principle is outlined in figure 5.

1.1.2.3 Selection strategies

Cloning of genetic repertoires as insertions to phage coat genes enables the isolation of variants with desired characteristics. The majority of studies applying phage displayed repertoires are aimed at isolation of phage fusion proteins characterised by their ability to bind to a specific binding partner. Such isolation is performed by affinity selection, which is based on the principles of affinity purification. Briefly, phage displaying a repertoire of potential binding proteins are incubated with the target, which is either immobilised on a plastic surface (panning) (Marks et al., 1991b) or attached with an affinity tag (biopanning) (Parmley and Smith, 1989). Next, non-specific phage are removed by stringent wash conditions. Bound phage are finally eluted and propagated in *E. coli* producing a new phage stock enriched for binders to the target. The ability to wash away unspecific binders is crucial for a successful selection, since the phage by nature binds to virtually anything, including plastic surfaces, affinity tags etc., thereby making unspecific binding a major obstacle for phage display. The general solution to this problem has been to perform succeeding rounds of selection, employing the fact that each round of selection enriches the selected phage pool for binders. Application of the novel cleavable helper phage outlined in figure 5 is one approach to remove such background binding. Finally, the selected phage are subjected to affinity screening to verify the specific binding of the target.

Complex selections targeting specific or differentially expressed cell surface molecules or structure dependent epitopes are difficult due to the large number of irrelevant binding sites. Several strategies have been outlined to augment the selection efficiency, such as depletion, competition (Stausbøl Grøn et al., 1996), epitope masking (Ditzel et al., 1995) as well as specific elution (Goletz et al., 2002). However, irrelevant binding sites are often recognised by high affinity binders, which despite the optimised selection strategies are enriched due to the use of multiple selection rounds. This problem is circumvented with the cleavable helper phage since one round of selection is sufficient to isolate specific binders (Goletz et al., 2002; Jensen et al., 2003). The affinity of such potentially low affinity binders can subsequently be improved by affinity maturation (Hawkins et al., 1992; Low et al., 1996; Marks et al., 1992; Schier et al., 1996).

Affinity selection elucidating interactions between cognate protein pairs has also been achieved by rendering the infectivity of the phage particle dependent on the interaction of the protein pair. This is achieved by coupling the protein pair to each their fragment of a bipartite pIII, thereby

reconstituting infection competent pIII. The strategy has been developed in several labs and received acronyms, such as SAP, DIRE and SIP (Duenas and Borrebaeck, 1994; Gramatikoff et al., 1994; Krebber et al., 1995). However, the general observation has been that these methods only work for high affinity interactions.

Selection for other properties than affinity has been described, such as selection for proteolytical stability (Chakravarty et al., 2000; Kristensen and Winter, 1998; Pedersen, 2001; Sieber et al., 1998) and enzymatic activity (Atwell and Wells, 1999; Jestin et al., 1999; Pedersen et al., 1998).

1.1.2.4 Phage displayed repertoires

A large variety of polypeptides have been displayed as repertoires of individual mutants on phage particles. The most widely employed are peptides (Cwirla et al., 1990; Devlin et al., 1990; Felici et al., 1991; Oldenburg et al., 1992; Scott and Smith, 1990) and antibody fragments (Breitling et al., 1991; Kang et al., 1991; McCafferty et al., 1990). Some of the additional molecules that have been displayed as repertoires are human growth hormone (Bass et al., 1990), human plasminogen-activator inhibitor 1 (Pannekoek et al., 1993), DNA-binding zinc fingers (Jamieson et al., 1994; Rebar and Pabo, 1994; Wu et al., 1995), β-lactamase inhibitory protein (Huang et al., 1998), monovalent Fc-binding domain of staphylococcal protein A (Nord et al., 1995), the small GTPase Ras (Wind et al., 1999), barnase (Kristensen and Winter, 1998; Pedersen, 2001), lipases (Danielsen et al., 2001) and many more.

1.1.2.4.1 Peptide repertoires and scaffolds

The peptide repertoires have been employed for elucidating protein interactions, for instance determining target sequence of proteases (Matthews and Wells, 1993; Smith et al., 1995) and kinases (Schmitz et al., 1996), epitopes for antibodies (Cwirla et al., 1990) and ligands for receptors (Goodson et al., 1994) and MHC class II molecules (Hammer et al., 1992) (reviewed by Felici and co-workers (Cortese et al., 1996)). Additionally, peptide agonists and antagonists have been generated from such repertoires, as these are of major interest in the development of small active peptide therapeutics (Zwick et al., 1998).

The peptide repertoires synthesised so far have been 6-40 residues long and displayed on pIII and pVIII of the phage primarily in the type 3 (Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith,

1990) and type 8+8 vector systems (Felici et al., 1991; Luzzago et al., 1993). However, type 3+3 (Ravera et al., 1998) and type 88 (Enshell-Seijffers et al., 2001) vector systems have also been applied.

Randomised peptide repertoires are usually constructed from synthetic oligonucleotides based on codons encoded by either NNK or NNS (N represents an equal mixture of nucleotides G, A, T and C, K represents G or T and S represents G and C). NNK and NNS codons encode all the 20 natural amino acids. No adenine on the third position eliminates all ochre and opal stop codons (UAA and UGA, respectively), but allows amber stop codon to occur with a frequency of 1/32. In this way randomised peptide repertoires can be constructed with a minimum of non-displaying phage due to stop codons. NNK and NNS each represent 32 different nucleotide sequences, thus for a repertoire encoding 6 randomised residues a repertoire size of ~10^9 (32^6) individual clones is needed to cover the entire sequence span. Because of the rapid increase in required repertoire size connected to an increasing number of randomised residues different strategies have been applied to restrict the number of amino acids represented in each position of the peptide. A large reduction can be accomplished by intelligently choosing, which chemical properties the amino acids in each position should have (cf. table 3).

Codon	No. of combinations	Encoded a.a.	Chemical properties
TDS	6	Phe, Tyr, Trp, Cys, Leu,	Mainly aromatic residues
VAS	6	Asp, Glu, Gln, Asn, His, Lys	Charged residues
WSC	4	Ser, Cys, Thr	Hydrophilic residues

Table 3 – Biasing codons according to chemical properties. Restricted codons encoding amino acids with similar chemical properties (D = A/G/T, V = A/C/G, S = G/C, W = A/T).

Peptide repertoires can in this way theoretically be biased towards being good carbohydrate peptide mimics, since it has been observed that peptides mimicking carbohydrates include a significant higher frequency of aromatic residues (Kieber-Emmons et al., 1997; Luo et al., 1998). If the aim of the peptides is to evoke an immune response, it could be advantageous to bias the peptides towards binding to MHC receptors. It is known that the different alleles of MHC receptors preferably display peptides that include certain given residues at specific positions, and since the binding motifs are available (http://wehih.wehi.edu.au/mhcpep/), they can be implemented in the peptide repertoire construction, thereby dramatically reducing the required repertoire size.

The first peptide repertoires constructed were mainly structurally non-constrained (Cwirla et al., 1990), allowing the peptide displayed on the phage to attain different conformations. From thermodynamics it can be reasoned that the entropy of a non-constrained, a constrained and a bound peptide is decreasing in that order ($S_{non\text{-}con.} > S_{con.} > S_{bound}$) generating more order the more constrained it is. Therefore, the entropy difference between the bound state and a constrained peptide is less than between a bound peptide and a non-constrained peptide ($|\Delta S_{non\text{-}con/bound}| > |\Delta S_{con/bound}|$) meaning that less energy is needed to structurally freeze the peptide. The constrained peptides are therefore more likely to bind the target protein. The most widely employed constrain is to introduce a loop formation of the peptide by insertion of flanking cysteins forming a disulfide bridge (Giebel et al., 1995; Luzzago et al., 1993; McConnell et al., 1994; McLafferty et al., 1993; Pierce et al., 1996). Small polypeptide scaffolds displaying β-turn loops have also been described (Cochran et al., 2001; Tramontano et al., 1994), and for some antibodies it has been postulated that the CDR regions are merely constrained peptides using the antibody core structure as scaffold. This postulation is due to the observation that the heavy-chain CDR3 region of anti-idiotypic antibodies (Ab2β) in some cases is exclusively responsible for binding to the primary antibody (Ab1), thus also being the mimic of the original antigen (cf. figure 7) (Westerink et al., 1995).

Peptide display on phage has furthermore been utilised for immunisations (de la Cruz et al., 1988; Greenwood et al., 1991; Minenkova et al., 1993; Yip et al., 2001). The effect of fusion to pVIII is much more prominent than that of pIII, possible due to the closer spacing and higher number of peptides on the particle (Yip et al., 2001), and has led to a specific anti-HIV cytotoxic T cell response in mice (De Berardinis et al., 2000). The phage particle is very attractive as a vehicle for immunisation, as no pathogenic side effects have been associated with it, and other more bacteriovirulent phage types are already applied in human therapy (Carlton, 1999). Moreover, the phage is by itself capable of stimulating the immune system of rodents, which obviates the need for adjuvants (Willis et al., 1993). One study regarding immunisations of monkeys and chimpanzees shows that induction of a similar adjuvant independent immune response against the fusion peptide requires additional adjuvants, although immune responses against the phage itself were prominent (Galfre et al., 1996). Further studies will elucidate if the lacking response in monkeys and chimpanzees is due to the choice of immunogen or an artefact caused by an insignificant number of animals.

1.1.2.4.1.1 Single-chain MHC class I molecules as scaffolds

Trimeric major histocompatibility (MHC) class I complexes comprise a 45 kDa membrane bound human lymphocyte antigen (HLA) subunit, a 12 kDa soluble β2-Microglobuline (β2MG) subunit and a 8-10 a.a. peptide bound in the HLA associated binding groove (York and Rock, 1996). The structurally related trimeric MHC class II complex comprises two membrane bound subunits (a heavy α chain (34 kDa) and a lighter β chain (29kDa)) forming a combined peptide binding groove binding a 13-25 a.a. peptide (Springer et al., 1977). The intrinsic ability to bind peptides and present these to T cells makes MHC molecules natural scaffolds for peptide display, and theoretically they are therefore ideal for the isolation of T cell receptor specific peptide ligands.

A large number of studies confirm the functionality of single-chain MHC molecules isolated from *E. coli*, but in all cases protein is purified from inclusion bodies and refolded *in vitro* (Cohen et al., 2003b; Denkberg et al., 2000; Lone et al., 1998; Mottez et al., 1991; Sylvester-Hvid et al., 1999; Yu et al., 2002). One of the main obstacles in folding and assembly of MHC complexes *in vivo* in the periplasmic space of bacteria is the lack of specific chaperones, such as the transporter associated with antigen processing (TAP), the membrane protein calnexin and BiP (the chaperones needed for folding and assembly of MHC molecules are reviewed by Watts and co-workers (Williams and Watts, 1995) and the biology of the periplasmic space is reviewed by Wulfing and Plückthun (Wulfing and Pluckthun, 1994)). Another obstacle is the lack of glycosylation, which is reported to influence the orchestration of chaperones, serve as quality control for correctly folded MHC molecules and protect the molecule from proteolytic degradation (reviewed by Dwek and co-workers (Rudd et al., 2001)). However, glycosylation of MHC class I is not a prerequisite for T cell recognition (Goldstein and Mescher, 1985). A recent study shows that folding of MHC class II molecules in insect cells is profoundly augmented by coexpression of calreticulin, a 46 kDa soluble endoplasmic reticulum (ER) chaperone with some resemblance to the luminal domain of calnexin (Fourneau et al., 2004). A similar strategy has been reported to work for antibody fragments in bacterial expression systems (Bothmann and Pluckthun, 2000), and might therefore also be valuable for bacterial expression of MHC complexes.

Despite these obstacles phage display of murine major histocompatibility complex (MHC class I) in a single-chain format has been reported (Kurokawa et al., 2002; Le Doussal et al., 2000; Vest Hansen et al., 2001), although in all cases with low display levels. One study using the phage vector

system suggests that the low display level is due to proteolytic degradation (Le Doussal et al., 2000). Theoretically, it could also be due to inability of the Sec mediated protein export system (de Keyzer et al., 2003) to transport the fusion protein across the inner membrane to the periplasmic space, integration in the phage particle and transport through the pI/pIV assembly complex. All three studies, in which phage displayed murine single-chain MHC molecules have been employed, verify that the peptide is truly bound in the peptide binding groove of the MHC complex. The verification is accomplished by the utilisation of antibodies specific for a peptide-MHC complex (Vest Hansen et al., 2001), specific binding to soluble or cell bound peptide specific T cell receptors (Le Doussal et al., 2000) and a novel approach verifying that proteolytically cleaved of peptides are still associated with the single-chain MHC molecule (Kurokawa et al., 2002). None of the phage displayed MHC molecules were capable of inducing a T cell response, although soluble multimeric single-chain MHC molecules have this feature, both with non-covalently (Denkberg et al., 2000; Lone et al., 1998) and covalently coupled peptide (Cohen et al., 2003b). Display of human single-chain MHC complexes has been the aim of the project described in chapter 2.5.

1.1.2.4.2 Antibodies employed in phage display

The human genome project has lead to the identification of , in principle, all genes present in the human genome. However, a bottleneck arises conserning the analysis of the corresponding gene products, since the field of proteomics still rely on low-throughput technologies. The need to analyse these gene product entails a paramount demand for monoclonal antibodies specific for the gene product. Traditionally, antibodies have been generated with the rather labour intensive hybridoma technology (Kohler and Milstein, 1975). However, for more than a decade phage display of antibody fragments has proven to be a strong alternative (Barbas et al., 1991; Clackson et al., 1991; Hoogenboom et al., 1991; Marks et al., 1991b; McCafferty et al., 1990).

1.1.2.4.2.1 Immunoglobulin G and derivatives

The antibody fragments employed in phage display are derived from the general immunoglobulin fold. The immunoglobulin G (IgG) is made up of two heavy chains and two light chains connected via disulfide bridges (Harris et al., 1997). Each chain can be parted in a variable region (light blue), responsible for antigen binding, and a constant region (red) (figure 6), responsible for the communication with the complement system and antigen presenting cells (cf. chapter 1.2.3). The two antigen binding sites (paratopes) are composed of a variable heavy (V_H) chain and a variable

light (V_L) chain. The structure of V_H and V_L, respectively, can be reduced to a so-called immunoglobulin fold, which consist of two β sheets packed against each other. The three loops connecting the strands in the β sheets can vary in length and sequence, and they are therefore called the hypervariable regions or complementarity determining regions (CDRs). The structure of the antigen binding site is determined by the tight packing of the two β barrels from V_H and V_L giving rise to a barrel structure comprising β strands from both variable domains. Binding of the antigen is primarily determined by the CDR loops, which are located in one end of the barrel (Poljak et al., 1973).

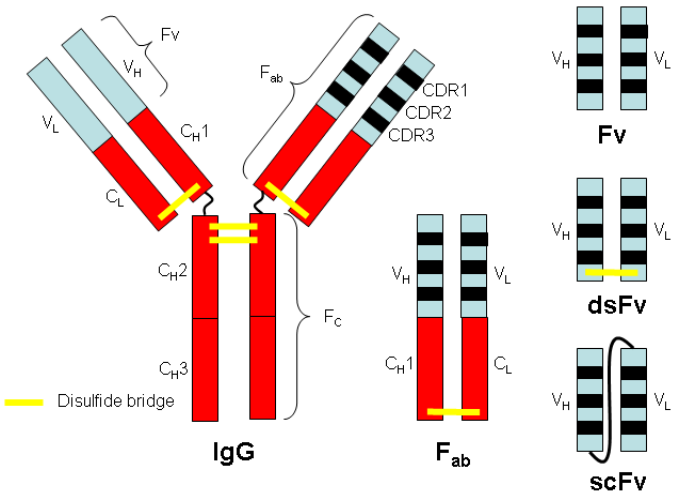


Figure 6 – The immunoglobulin G and derivatives. Schematic drawing of various antibody fragments retaining functionality in regard to antigen binding. The sketch includes intact Immunoglobulin G (IgG), Fragment antigen binding (F_{ab}), Fragment variable (Fv), disulfide stabilised Fragment variable (dsFv) and single-chain Fragment variable (scFv).

Initial antibody diversity *in vivo* is introduced by randomisation of the CDR regions of V_H and V_L by rearrangement at the DNA level. The V_H is generated by rearrangement of the 51 variable heavy chain (V) segments, the 27 different diversity segments (D) and 6 joining segments (J) (Cook and Tomlinson, 1995; Cook et al., 1994; Corbett et al., 1997; Tomlinson et al., 1992), and V_L is similarly generated by rearrangement of the 40 V_κ or 30 V_λ variable light chain (V) segments and the 5 J_κ or 7 J_λ joining (J) segments(Cox et al., 1994; Frippiat et al., 1995; Tomlinson et al., 1995; Williams et al., 1996). The genetic rearrangement mostly influences the CDR3 regions, whereas CDR1 and CDR2 are included in the V segments. Therefore the CDR3 regions are main contributors to the antigen specificity and affinity characterising the primary immune response (Corbett et al., 1997). Hypermutation of all of the CDR regions is accomplished in the subsequent maturation process, which is initiated by signals for class switching (chiefly IgM → IgG) (cf. chapter 1.2.8) (Rada and Milstein, 2001; Tomlinson et al., 1996). The outcome is antibodies of high specificity and affinity.

So far the antigen binding properties of antibodies have been transferred to the phage in either one of two formats, Fab or Fv (cf. figure 6). Dimeric Fab fragments are dependent on the formation of a disulfide bridge between its two constant domains, whereas Fvs are stabilised either with a disulfide-bridge (dsFv) (Brinkmann et al., 1995; Glockshuber et al., 1990) or by a flexible linker covalently coupling the variable heavy and light chain (scFv) (Bird et al., 1988; Huston et al., 1988; McCafferty et al., 1990). The most frequent applied scFv format contains the immunoglobulin V_H domain connected through a 15 amino acids glycine-rich linker to the V_L domain. Both sequence and order of the variable domains as well as linker length and sequence influences the properties of the scFvs (Alfthan et al., 1995; Arndt et al., 1998; Desplancq et al., 1994; Hennecke et al., 1998; Tang et al., 1996; Turner et al., 1997).

1.1.2.4.2.2 Antibody repertoires

The antibody repertoires have been cloned from varying sources and using different strategies. The four most common repertoire types are immune repertoires, naive repertoires, semi-synthetic repertoires and single framework repertoires. The immune and naive repertoires arise from cloning the full length V_H and V_L genes into the scFv format using either immunised or non-immunised (naive) donors, respectively (reviewed by Hoogenboom (Hoogenboom, 1997)).

The semi-synthetic repertoires are constructed according to the fact that antibody binding is primarily due to the diversity of the CDR regions. *In vivo* the greatest sequence diversity is located in the CDR3 regions. Variability induction into CDR3 has therefore gained much attention (Hanes et al., 2000; Knappik et al., 2000). Employing the principles of the semi-synthetic repertoire construction, it is possible to generate a single framework repertoire. This type of repertoire uses the same V_H and V_L gene for all antibodies, only substituting and diversifying the CDR regions. In this way repertoires can be constructed based on a stable scaffold and optimised codon usage, thus increasing the amount of correctly folded and functional antibodies in the repertoire (Kirkham et al., 1999; Pini et al., 1998).

1.1.2.5 Alternatives to phage display technology

Despite the rapid improvements of phage display technology, several obstacles remain, such as the limiting repertoire size of non-cell-free systems and the inability to display some intracellular proteins due to the non-reducing conditions in the periplasmic space of *E. coli*. Generating of alternative systems that couple genotype and phenotype has therefore been given much effort. A

few examples are Ribosome display (Hanes and Pluckthun, 1997; Hanes et al., 2000), mRNA display (Roberts and Szostak, 1997; Wilson et al., 2001), polypeptide-on-plasmid display (Cull et al., 1992; Gates et al., 1996; Speight et al., 2001), oil-water emulsion display (Tawfik and Griffiths, 1998) and bacterial display (Chang et al., 1999; Christmann et al., 2001; Kim et al., 2000b; Lattemann et al., 2000; Wentzel et al., 2001).

1.2 Vaccines

Edward Jenner's revolutionary invention of the small pox vaccine reported in 1798 launched a quest into exploiting the immune system in treatment of diseases. Jenner's invention was to immunise patients with live cow pox, a small pox analogue obtained from cows. However, the use of live pathogen entails the risk that mutated pathogens can cross the species barrier, which in combination with contaminated and poorly prepared vaccines made vaccination a very dangerous treatment in the early years. However, due to the tremendous success of the vaccination approach for treatment of infectious diseases, vaccines against various diseases were continuously investigated. The safety issue was approached by among others Luis Pasteur, employing live attenuated pathogens and whole inactivated organisms that are characterised by their reduced virulence and retained immunogenecity. The expanding knowledge on the molecular and cellular mechanisms constituting the immune system has enabled the design of much less complex vaccines comprising fragments of a pathogen, which are typically characterised by their specificity and immunogenecity. However, to be fully effective the fragments have to be administered in combination with immunomodulatory components, such as incomplete Freund's adjuvant, cytokines or dendritic cells. The immunomodulatory components are characterised by their ability to modify the strength and type of immune response generated by a vaccine. In addition to the development of new vaccine formats, vaccines are also developed for the treatment of non-infectious diseases, such as cancer. One goal is to be able to use vaccinations not only as protection, but also as therapy. The novel vaccines are valuable due to their reduced complexity and reduced risk of side effects. Even though these new vaccine strategies may provide certain advantages, difficulties still remain regarding insufficient immunogenecity compared to traditional vaccine formats and difficulty of producing vaccines that are applicable for all patients.

1.2.1 Fragments of pathogens and mimicry thereof

Recently, several subunit vaccines have been approved for human administration by U.S. Food and Drug Administration (FDA), and a large number of subunit vaccines are in the pipeline, being tested in clinical trials. Two FDA approved examples are the hepatitis B vaccine, Engerix-B™ (GlaxoSmithKline), and the Lyme disease vaccine, LYMErix™ (GlaxoSmithKline). The vaccines consist of Hepatitis B surface antigen produced in yeast and outer surface protein OspA from *Borrelia burgdorferi sensu stricto* produced in *E. coli*, respectively. Both surface antigens are

adsorbed on aluminium hydroxide before administration to augment the induced immune response. The use of surface antigens in vaccine design induces a humoral immune response, which can potentially produce neutralising amounts of anti-pathogen antibodies. To withstand such humoral immune response many pathogens frequently mutate their surface antigens (Veljkovic et al., 2001).

1.2.1.1 Human Immunodeficiency Virus (HIV-1)

HIV-1 is such pathogen. The virus encodes nine open reading frames. Three of these encode the GAG, POL and ENV polyproteins that are proteolytically processed into individual proteins common to all retroviruses. The three POL proteins have enzymatic functions that are important for the viral lifecycle. The four GAG and two ENV derived proteins are structurally components that comprise the core and outer membrane envelope of the virion, respectively. The 6 remaining open reading frames encodes accessory proteins important for the transport of the viral genome to the nucleus (Vpr), transcription of viral genes (Tat), export of non-spliced RNA to cytoplasm (Rev), assembly of the virion (Vpu, Nef) and virion infectivity (Vif) (reviewed by Frankel and Young (Frankel and Young, 1998) and Pomerantz (Pomerantz, 2003)). Despite its large variability the ENV surface proteins have been employed frequently in vaccine studies. The wild-type HIV-1 precursor envelope protein is matured by proteolytical processing of the signal peptide (1-28 a.a.) and further at the C-terminal of the Arg-Glu-Lys-Arg motif. This leads to a separation into an extracellular fragment, gp120 (29-509 a.a.), and a membrane spanning fragment, gp41 (510-854 a.a.) (Fenouillet and Gluckman, 1992). The gp41 is docked in the membrane of the budding virus particle with gp120 non-covalently attached. Gp120 is responsible for the selective infection of $CD4^+$ T cells due to its specific interaction with its primary receptor CD4 on host cells. The membrane fusion that follows the above mentioned gp120 CD4 interaction has been reported to be caused by gp41 (Lawless et al., 1996).

Neutralising antigen responses against gp120 surface antigen have been thoroughly investigated by Dr. Burton (Burton et al., 2004) and applied in the first extensive clinical trials by the company Genentech and subsequently VaxGen (AIDSVAX^{TM}). Their phase I and II trials were positive, showing induction of antibodies capable of neutralising virions of the strain from which the recombinant gp120 originated. However, the phase III trials could not distinguish between vaccinated and placebo treated individuals with regard to HIV-1 infection. This confirms the difficulty associated with effectively directing a humoral immune response against the variable and

highly glycosylated HIV-1 surface antigen, gp120. Several other studies have employed the more conserved GAG and POL derived proteins. Whereas these proteins are inaccessible to the humoral immune response in the natural virion, they represent important targets for cytotoxic $CD8^+$ T cells (Betts et al., 2002). Future studies will reveal whether vaccination strategies inducing both the humoral and the cytotoxic immune response, are beneficial strategies for HIV-1 prevention and treatment. In chapter 2.7 we report on a gp120 encoding DNA vaccine that in addition to the already reported humoral response mediates $CD8^+$ T cell responses *in vitro* due to antigen presentation by dendritic cells.

1.2.1.2 Tumour vaccines

Identifying the best suitable pathogenic fragment for a vaccine is a difficult task. Tumour associated carbohydrate antigens are characterized by their biased presence on tumour cells, but also by a low intrinsic immunogenecity. The reason for the low immunogenecity of carbohydrate antigens may be that they primarily evoke T cell independent immune reactions (reviewed by Lesinski and Westerink (Lesinski and Westerink, 2001)). GalNAc (Tn) antigen and Galβ1,3GalNAc disaccharide Thomsen-Friedenreich (TF) antigen, which are exposed unilaterally on several carcinomas due to incomplete glycosylation (Grant et al., 1998; Springer, 1997; Taylor-Papadimitriou et al., 1999). Tn and TF are O-linked carbohydrate core structures, first described to be present on desialylated glycophorin A, the carrier of the MN blood group (Springer et al., 1985). Additionally TF and Tn are found on MUC1 (Cao et al., 1997), which is a highly glycosylated membrane bound protein, consisting of an extracellular domain made up of 20 amino acid tandem repeats, each containing 5 potential O-glycosylation sites. MUC1 is shed to the blood stream, where it is bound and internalised by mannose receptors on antigen presenting cells (APCs). However, internalisation leads to no presentation. Two reasons have been suggested for this phenomenon; 1) the huge glycosylation of MUC1 renders the interaction with the mannose receptors so tight that it is never released from the early endosome, and thus never processed for MHC class II presentation (Hiltbold et al., 2000), and 2) the O-glycosylations mask the proteolytic cleavage sites, thus rendering the protein inert to the antigen processing machinery in the endosomes (Hanisch et al., 2003).

One strategy to circumvent these problems is the generation of molecular mimics of tumour associated antigens (TAAs) (reviewed by Kieber-Emmons (Kieber-Emmons, 1998)). The traditional drug discovery technologies applied for the generation of such mimics are synthetic

chemistry and combinatorial chemistry (reviewed by Kieber-Emmons T (Kieber-Emmons et al., 1997)). More recently mimicry of various molecular structures has been generated with peptides and anti-idiotypic antibodies (reviewed by McKenzie and co-workers (Apostolopoulos et al., 1999) and Granoff and co-workers (Moe et al., 1999)). Phage display technology has so far been the most successful approach for the generation of peptides mimicking various disease-associated carbohydrates, such as Lewis A (Fukuda et al., 2000), glycosphingolipids (Taki et al., 1997), carbohydrates on *Neisseria meningitidis* (Bambos et al., 2000; Grothaus et al., 2000) and *Shigella flexneri* (Phalipon et al., 1997) as well as Lipopolysaccharide (De Bolle et al., 1999). Typically, the peptides are affinity selected for their ability to bind to carbohydrate specific antibodies. Several studies further verify the ability of such potential mimotopes to evoke carbohydrate specific T cell dependent immune responses both as phage vaccines (De Bolle et al., 1999), peptide vaccines (Agadjanyan et al., 1997; Apostolopoulos et al., 2000; Kieber-Emmons et al., 1999) and DNA vaccines (Kieber-Emmons et al., 2000; Lesinski et al., 2001).

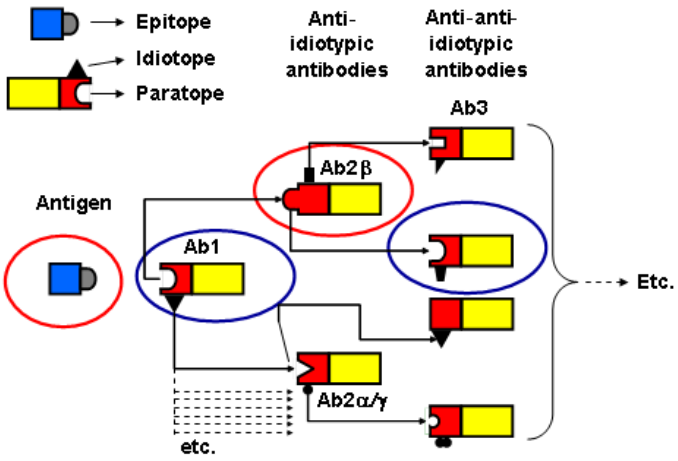


Figure 7 – Jerne's "Idiotypic network hypothesis". A schematic drawing of the immune response towards an antigen and the subsequent immune response against the idiotopes as well as paratopes of the induced immunoglobulins. The immunoglobulin(s) in the first line of defence (Ab1) bind(s) the epitope(s) on the antigen. The next line of defence (Ab2) binds with its idiotopes or paratope to the idiotopes (Ab2α/γ) or paratopes (Ab2β) of the Ab1 antibodies. Ab2α and Ab2γ bind in an antigen noncompetitive (Ab2α) or competitive (Ab2γ) manner to Ab1. Ab2 antibodies are designated anti-idiotypic antibodies. The next generation of antibodies (Ab3) are called anti-anti-idiotypic antibodies etc. Molecules that partly resemble each other are indicated with circles. See text for further explanation. The figure was adapted from Lesinski and Westerink (Lesinski and Westerink, 2001).

The idiotypic network theory developed by Jerne in 1974 suggests that the variable idiotopes of antibodies can be immunogenic, and thereby induce anti-idiotypic antibodies (Ab2), characterised by their ability to bind the primary antibody (Ab1) (Jerne, 1974). Anti-idiotypic antibodies can be divided in three groups; 1) antibodies binding Ab1 outside the antigen binding site (Ab2α), 2) antibodies binding Ab1 in an Ab1 antigen competitive manner (Ab2γ) and 3) antibodies binding the Ab1 antigen binding site and act as an internal image of the antigen bound by Ab1 (Ab2β) (figure 7) (Tripathi et al., 1998). The concept of the internal image does not imply that the Ab2β molecule

carries a structure resembling the entire antigenic site. Rather, it represents an image of a particular epitope and perhaps only of the contact residues within the epitope seen by the Ab1 (Fields et al., 1995). Ab2α have been proposed to multimerise Ab1 antibodies, and thereby increase the affinity of the Ab1-antigen interaction to avoid pathogenic escape (Denisova et al., 2000). The network theory propose a complex regulation of B cell proliferation and apoptosis by the complement system (Mitra-Kaushik et al., 2001a) and T cell responses against B cells presenting the specific idiotypes of the respective B cell receptors on MHC class I and II complexes (Mitra-Kaushik et al., 2001b).

Isolation of anti-idiotypic antibodies (Ab2β), has been successfully accomplished using conventional hybridoma technology (Bhattacharya-Chatterjee et al., 1990) as well as phage display technology (Beninati et al., 2004; Goletz et al., 2002; Vogel et al., 1994). B cell lymphoma therapy has employed several anti-idiotypic antibodies for tumour targeting (Cohen et al., 2003c; Spellerberg et al., 1997). However, the network theory also suggests that anti-anti-idiotypic antibodies (Ab3) can be generated against Ab2β, which potentially leads to antibodies against the original antigen mimicked by the Ab2β. The potential of anti-idiotypic antibody vaccines have been thoroughly explored in animal studies and clinical trials (reviewed by Foon and co-workers (Bhattacharya-Chatterjee et al., 2000) and Granoff and co-workers (Moe et al., 1999)). One study, employing a CD55 mimicking anti-idiotypic antibody for treating colorectal carcinomas, successfully established humoral immune response and T cell infiltration in a significant number of patients. CD55 protects eukaryotic cells from complement mediated lysis (Rudd et al., 2001) and is often overexpressed on tumour cells (Durrant et al., 2000). Whereas peptide mimics are easier to produce and more stable than anti-idiotypic antibodies the latter benefit from their intrinsic ability to bind Fc-receptors on APCs. This was illustrated in a study, employing an anti-idiotypic antibody mimicking the carcinoembryonic antigen (CEA) pulsed onto bone marrow derived dendritic cells (Saha et al., 2003).

1.2.2 Vaccine formats and Administration routes

Peptide mimics and anti-idiotypic antibodies are generally administered as protein and DNA vaccines combined with a carrier and an adjuvant. Different alternatives to classical adjuvants, such as incomplete Freund's adjuvant (IFA) have been explored, including fusion of protein antigens like scFv or peptides to immuno-stimulatory cytokines, such as GM-CSF, IFN-γ, IL-1β, IL-2 and IL-12 (Batova et al., 1999; Hakim et al., 1996; McCormick et al., 2001; Penichet and Morrison, 2001).

Also a few reports on antigens fused to heat-shock proteins (HSP) and pathogenic proteins are available: HSP65 (Anthony et al., 1999), HSP70 (Rico et al., 1998), HSP71 (Anthony et al., 1999), tetanus toxin (Spellerberg et al., 1997), serum albumin-binding region of streptococcal protein G (Sjolander et al., 1997), Hepatitis B virus core antigen (Schodel et al., 1996), *E. coli* heat-labile enterotoxin B subunit (Schodel et al., 1990) and B-subunit of cholera toxin (Lebens et al., 2003). Adjuvants are typically able to bias the immune response in specific directions, such as humoral or cytotoxic immune response (several adjuvants are reviewed by Dalgleish and co-workers (Dredge et al., 2002), discussed in chapter 2.2 and appendix B).

The carrier molecules are supposed to deliver the vaccine specifically to target cells or tissue, such as APCs (reviewed by Hovgaard and co-workers (Foged et al., 2002)) as well as mucosal surfaces (reviewed by Singh and O'Hagan (Singh and O'Hagan, 1999) and Singh and co-workers (O'Hagan et al., 2001)). Multivalent display in vaccination strategies as a beneficial carrier property has been investigated in several studies. These works have primarily focused on display of antigens on cell surfaces (de Ines et al., 2000; Paul et al., 2000; Rode et al., 1999) and virus particles (Jiang et al., 1997; McInerney et al., 1999; Mottershead et al., 2000), although a few fusion protein systems have been investigated, such as the pentameric cholera toxin B subunit (Liljeqvist et al., 1997) as well as the closely related pentameric E. coli heat-labile enterotoxin B subunit (Schodel et al., 1990). The two latter examples both perform well in oral administration, although complete or incomplete Freunds adjuvant was added to evoke the immune response. Other multivalent display systems have been applied in immunizations without directly investigating the possible beneficial effect of multimerisation e.g. dimeric IFN-γ (McCormick et al., 2001) and dimeric glutathione S-transferase (Anthony et al., 1999; Yip et al., 2001).

Peptide and anti-idiotypic antibody vaccines are typically administered intravenously (i.v.), intramuscular (i.m.), intraperitoneal (i.p.), subcutaneously (s.c.) or intradermally (i.d.), according to antigen, vaccine format and the desired type of immune response. Technically, parental administration can be performed by injection, gene gun and electroporation. Mucosal administration routes, such as oral, nasal, pulmonal, vaginal and rectal administration routes, resembles the infective path of naturally occurring pathogens and lead to both mucosal and parental immunity, but problems regarding stability, efficient uptake and tolerance still need to be optimised (reviewed by Pamonsinlapatham and co-workers (Bouvet et al., 2002)). Future vaccines may enable the use of

such mucosal administration routes, which would render vaccination needle-free and thus much more convenient especially in developing countries.

Vaccines are largely dependent on the immunologic competence of the cells at the injection site. The skin has a large immunological potential due to antigen presenting Langerhans cells (LC) comprising 1% of the cell population and 20% of the cell surface area. In some studies LCs are targeted by s.c. or i.d. administration of protein, whereas other studies target APCs in draining peripheral lymph nodes, mesenteric lymph nodes and Peyer's patches by i.m. administration (Bouvet et al., 2002). DNA vaccines can be administered through the same routes as protein vaccines. However, compared to protein vaccines the pathway leading to antigen presentation on APCs differs. One study suggest that targeting APCs directly is important for i.p. and i.v. administration of DNA vaccines, whereas antigen presentation through s.c., i.m. and i.d. administration is suggested to be mediated by expression and secretion of the encoded antigen in by-stander cells, which is subsequently taken up and presented by APCs (Shen et al., 2002). Most parentally administered rodent DNA vaccine studies have been performed by i.m. administration, where the by-stander cells are muscle cells (Alpar and Bramwell, 2002). It has been shown that muscle cells are particularly sensitive to DNA transfection (Fu et al., 1997; Wolff et al., 1990), which is probably due to the fact that muscle cells comprise multiple targetable nuclei per cell (Dowty et al., 1995). The antigen is then expressed and released by secretion or cell death, which lead to antigen presentation on APCs. The theory of muscle cell mediated antigen presentation as opposed to direct targeting of APCs has however been questioned and still needs final verification (Torres et al., 1997). It is further believed that antigen uptake by APCs is enhanced by tissue damage due to recruitment and activation of APCs (Davis et al., 1993; Ertl and Xiang, 1996).

The general aim of most vaccines is to induce or augment antigen presentation by APCs. This step can be by-passed by dendritic cell therapy, which employs *in vitro* pulsing of antigens onto APCs and subsequent administration (reviewed by Morse and Lyerly (Morse and Lyerly, 2002)). The administration route for DC therapy is very much dependent on the DC differentiation stage, which is directly correlated with the homing receptors expressed on the cells. The DCs can generally be administered to the location, from where they were originally isolated. At least if the *in vitro* manipulations do not include any additional differentiation and maturation steps (Onaitis et al., 2002).

1.2.3 Uptake by professional antigen presenting cells (APCs)

Antigen presentation by professional APCs, such as macrophages, B cells and dendritic cells, triggers the activation of the adaptive immune response. To be presented antigens however have to be obtained from the extracellular environment by internalisation. Vaccine or pathogen derived antigens can be internalised by professional APCs through various pathways, some of which are 1) Complement receptors on professional APCs recognising products of the complement present on opsonised antigen particles, 2) Mannose receptors on professional APCs recognising mannose and fucose exposed on many pathogens (Aderem and Underhill, 1999), 3) Fc receptors on professional APCs recognising IgG (Fcγ-receptor) or IgE (Fcε-receptor) immuno complexed antigen, (4) Toll like receptors for recognition of pathogen patterns (Schjetne et al., 2003) and 5) Receptor independent phago- and pinocytosis (Day et al., 1997; Norbury et al., 1997; Sallusto et al., 1995).

1.2.3.1 Complement mediated antigen uptake

The innate immune system has evolved highly specific proteins that recognise and opsonise pathogenic ligands, such as mannan binding lectin (MBL), proteins of the complement system and natural antibodies. The mannan binding lectin is recognised and internalised via the C1q receptor on professional APCs (Ghiran et al., 2000). Complement is divided into a classical pathway, which tags IgM antibodies with C1q, and an alternative pathway, which attaches C3b non-specifically but covalently to the carbohydrate surface of pathogens. The tagged pathogen can either be lysed by the membrane attack complex (C5-C9) or be bound to complement receptors (CRs), CR1/CD35 (C3b, iC3b, C3d and C4b and C4d), CR2/CD21 (iC3b, C3d and C4d)), CR3/CD11b (iC3b) and CR4/CD11c (iC3b), on APCs (Aderem and Underhill, 1999; Carroll, 1998). However, phagocytosis is not initiated without the presence of additional stimuli, such as tumour necrosis factor alpha (TNF-α) and granulocyte/macrophage colony-stimulating factor (GM-CSF) (Wright and Griffin, 1985).

1.2.3.2 Fcγ receptor mediated antigen uptake

There are two classes of Fcγ receptors (FcγR), those that mediate phagocytosis and those inhibiting phagocytosis. FcγRI (CD64), FcγRIIA (CD32 – absent in mice) and FcγRIII (CD16) mediate the uptake of immune complexes, whereas FcγRIIB inhibit phagocytosis due to a lacking immunoglobulin gene family tyrosine activation motif (ITAM) in the cytoplasmic tail compared to FcγRIIA. FcγRI and FcγRIII also lack ITAMs on their cytoplasmic tails. For proper expression and

signalling, these two receptors therefore must interact with small transmembrane protein dimers that contain the ITAMs. Ligation of IgG complexed antigen and Fcγ receptors lead to cross-linking of ITAMs and subsequently tyrosine phosphorylation, which initiates the downstream cell signalling. Contrary to the complement mediated phagocytosis, Fcγ receptor mediated antigen uptake need no further signalling to initiate phagocytosis and induce release of several inflammatory mediators, such as reactive oxygen intermediates (Wright and Silverstein, 1983) and arachidonic acid metabolites (Aderem et al., 1985). The FcγR mediated antigen uptake is reviewed by Aderem and Underhill (Aderem and Underhill, 1999).

1.2.4 Antigen loading on MHC Class II molecules

MHC class II antigen presentation is restricted to professional antigen presenting cells, such as B cells, macrophages and DCs. However, a few non-classical APCs, such as T cells and cytokine activated keratinocytes or eosinophils also acquire MHC class II presentation under certain circumstances, although their functionality seems rather distinct compared to profession APCs (Pichler and Wyss-Coray, 1994), The extracellular derived antigens are primarily destined to MHC class II presentation (figure 8). Newly translated heterodimer comprising α and β transmembrane subunits are located in the ER, where it is stabilised by binding to the invariant chain (Ii). A preassembled trimeric Ii first binds three α chains, and the heterodimeric a/β complex is subsequently formed by addition of three β chains, which results in a nonameric $(Ii\alpha\beta)_3$ complex (Anderson and Cresswell, 1994). The heterodimer binds a region of Ii (amino acids 82-102) designated class II associated invariant chain peptides (CLIP), which includes a number of nested peptides with affinity to MHC class II molecules (reviewed by Williams and Watts (Williams and Watts, 1995)). A cytoplasmic di-leucine like motif located in the cytoplasmic tail of Ii targets the nonameric complex for subsequent transport to late endosomes via the golgi compartments. The late endosomes are the point of rally for extracellular antigens and $(Ii\alpha\beta)_3$.

The extracellular antigens are internalised into early endosomes. The transport of antigen from early to late endosomes is a highly compartmentalised process, which separates antigen release from cell surface receptors, proteolytic antigen processing and antigen loading on MHC class II molecules. This enables recycling of cell surface receptors as well as differential processing of antigens according to their route of internalisation (reviewed by Watts (Watts, 2001)).

In the late endosomes proteolytic processing of Ii renders the $(Ii\alpha\beta)_3$ complex ready for peptide loading. The processing leaves the MHC class II molecules with various CLIP derived peptides. These peptides are eluted by the acidic environment in the late endosomes, but the heterodimers HLA-DM (human) and H-2M (mice) play an important role as a sink for the released CLIP derived peptides, which removes them from the pool of antigenic peptides competing for binding to the unloaded MHC class II molecules (Alfonso and Karlsson, 2000; Watts, 2001). The loaded MHC class II complex is subsequently transported to the cell surface, and remaining antigen is transported to lysosomes for final degradation.

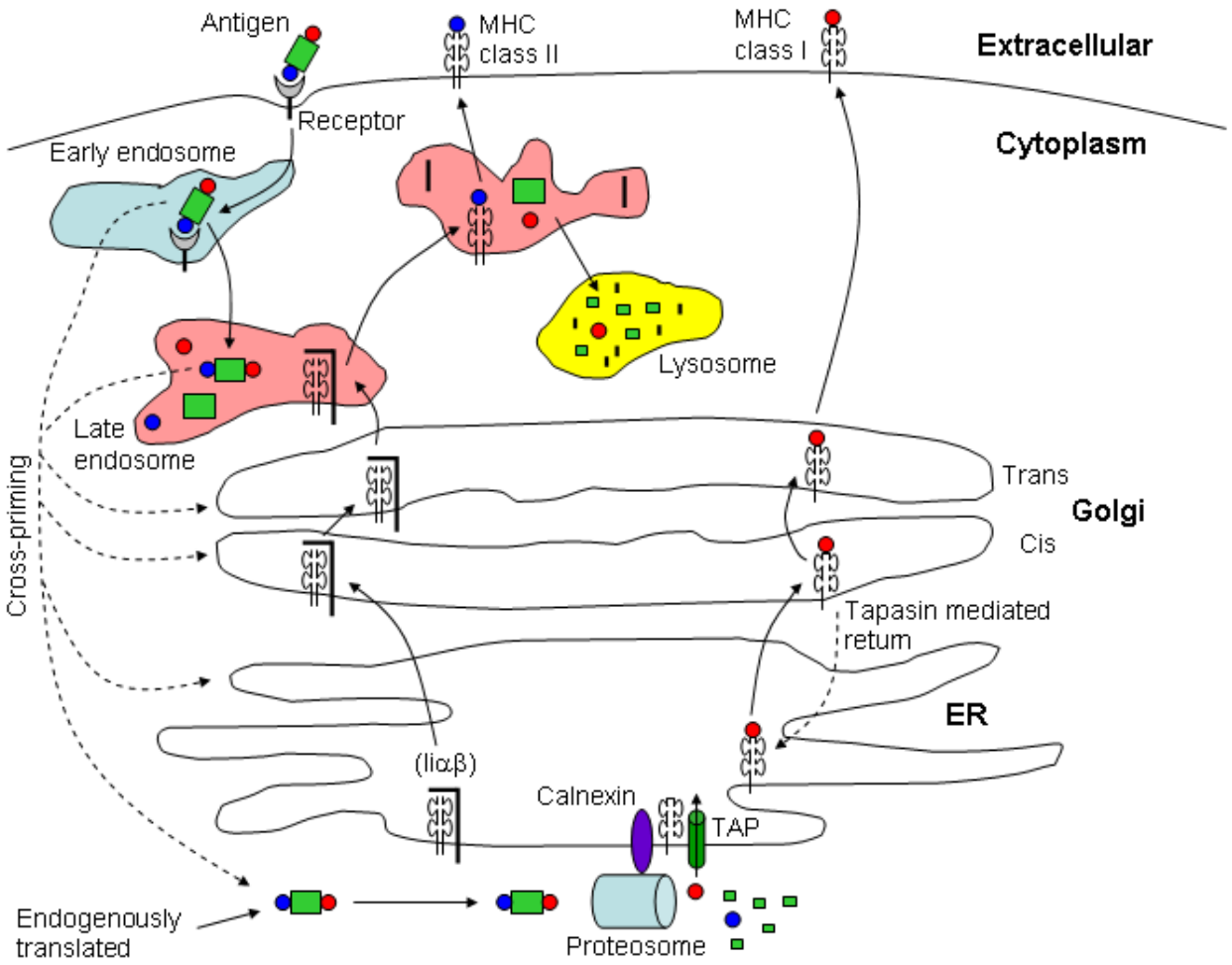


Figure 8 – MHC class I and II antigen presentation. A schematic drawing of the various pathways feasible for presentation of both extracellular and intracellular derived antigens. The various compartments and their role in antigen processing and presentation are indicated. The chaperone molecules, Ia, TAP and calnexin are presented, however tapasin as well as other chaperones are excluded. The tapasin mediated return of nonoptimally folded MHC class I molecules from golgi is displayed. The multi-subunit proteosome complex is for convenience depicted as one box. The routes of cross-priming, which is primarily active in professional APCs, are indicated. Abbreviations: Invariant chain (Ii), Transporter associated with antigen processing (TAP) and Endoplasmic reticulum (ER).

1.2.5 Antigen loading on MHC Class I molecules

MHC class I antigen presentation occurs predominantly with self antigens derived from cytosolically degraded proteins. However, for professional APCs also extracellular derived antigens in the endosomal compartments can gain access to the MHC class I antigen presentation pathway, in particular for DCs (Norbury et al., 1997; Rodriguez et al., 1999). The latter pathway is designated cross-priming as described by Bevan (Bevan, 1976a; Bevan, 1976b). These extracellular derived antigens can gain access to the cytoplasm, and thus the standard MHC class I loading pathway (Norbury et al., 1997; Rodriguez et al., 1999). Alternatively, they can target MHC class I molecules on the cell surface, phagocytosed MHC class I molecules or the newly synthesised MHC class I molecules in golgi and ER (figure 8) (Chefalo and Harding, 2001; Watts, 2001).

The classical MHC class I loading pathway obtain antigens that have been processed by the proteosome in the cytoplasm and subsequently by N-terminal exopeptidases located in both cytoplasm and ER (Rock and Goldberg, 1999; Yewdell and Bennink, 2001). Interestingly, some HIV-1 associated antigens can be trimmed extracellularly for MHC class I presentation (Nakagawa et al., 2000; Polakova and Russ, 2001). The transporter of antigen presentation (TAP) is an ER integral protein that potentially in cooperation with the trans-membrane chaperone calnexin (Realini et al., 1994) protects cytoplasmic antigens from further degradation and transport them into the ER lumen (Townsend et al., 1989), where they associate with the TAP associated heterodimer composed of glycosylated HLA and β2-Microglobulin (β2MG). As the ternary complex (HLA-β2MG-peptide) has formed it dissociates from TAP, and export through golgi to the cell surface is initiated. The HLA-β2MG heterodimer is relatively unstable, which secures that no unloaded MHC class I molecules are presented on the cell surface, thus avoiding non-specific targeting by $CD8^+$ cytotoxic T lymphocytes (CTLs). If nonoptimally loaded MHC class I molecules unintentionally are exported to golgi, the chaperone tapasin mediates the recycling of the MHC class I molecule back to ER (Paulsson et al., 2002).

Not all peptides can bind to the HLA-β2MG heterodimer. In particular certain essential HLA allele specific anchor residues have been observed; typically position 2 and the C-terminal residue of the 8-10 a.a. peptides bound by MHC class I (Stern and Wiley, 1994). Large numbers of MHC class I presented antigens have been analysed by x-ray crystallography, Edman sequencing, phage display and lately by elution and subsequent mass spectrometric analysis (reviewed by Hammer (Hammer,

1995)and de Jong (de Jong, 1998). The large amount of data obtained has been gathered in a database, from which consensus sequences of the anchor residues can be deducted (Brusic et al., 1998).

Interestingly, much of the MHC class I presentation machinery is inducible by IFN-γ, including certain immunoproteosomes, such as LMP2, LMP7 (Monaco and McDevitt, 1986) and MECL1 (Hisamatsu et al., 1996). Additionally, N-terminal exopeptidase activity performed by the cytosolic leucine aminopeptidase is IFN-γ induceble (Beninga et al., 1998). IFN-γ secreted from $CD8^+$ CTLs and DCs are therefore of great importance for the up-regulation of MHC class I molecules on surrounding tissue, for instance in tumours that often trigger immune escape via down-regulation of surface presented MHC class I molecules (Ting and Baldwin, 1993).

1.2.6 Mobility of dendritic cells.

The remaining introduction will focus on dendritic cells and their role in antigen presentation towards naïve T cells as well as B cells. Generally speaking DCs represent three biologically distinct cell types, precursor DCs, immature DCs (iDCs) and mature DCs (mDCs) residing distinct tissues (figure 9). Antigen uptake is performed by iDCs present in the peripheral inflammatory sites. These iDCs origin from bone marrow derived precursor cells circulating in the blood, which are recruited to specific tissues due to tissue secreted chemokines. Cell migration into the tissue is mediated by a number of endothelial receptors and their corresponding receptor on the DC, such as endothelial ICAM-1 binding to CD11a/CD18 on DCs (reviewed by Reid and co-workers (Robinson et al., 1998)).

To increase antigen uptake recruitment of DCs to the site of inflammation is important. Pathogen derived compounds, such as lipopolysaccharide (LPS) or CpG DNA, bind to Toll-like receptors on tissue macrophages, which induces secretion of danger signalling chemokines that attract iDCs in the surrounding tissue. Upon antigen encounter iDCs are capable of producing chemokines, which recruit scavenger cells, such as neutrophils, to the site of inflammation (reviewed by McColl (McColl, 2002)). Although antigen is internalised at the iDC differentiation stage as described above (cf. chapter 1.2.3) efficient antigen presentation requires not only antigen processing and presentation on MHC molecules, but also relocation to lymphoid organs containing effector cells, such as T and B cells. To leave the site of inflammation the iDCs have to replace their homing

receptors with receptors targeting the lymphoid organs. This process is included in the maturation process of DCs, where the inflammatory chemokine receptors, such as CCR1, CCR2, CCR5 and CCR6, are downregulated and CCR7 upregulated, which enables the mDC to respond to the CCR7 ligands CCL19 (MIP-3β) and CCL21 (SLC) (Saeki et al., 1999) that are constitutively secreted from T cell zones in draining lymph nodes (McColl, 2002). Upon migration to the lymphoid organs, antigen presentation on surface displayed MHC class II molecules is severely upregulated on mDCs (Sallusto et al., 1995). Expression of costimulatory molecules important for efficient presentation of antigens to naïve T cells is similarly enhanced (Robinson et al., 1998), and antigen presentation is further improved due to upregulation of chemokines, which attract T and B lymphocytes, such as CCL17 (TARC), CCL18 (DC-CK1), CCL19 (MIP-3β) and CCL22 (MDC) (reviewed by McColl (McColl, 2002)).

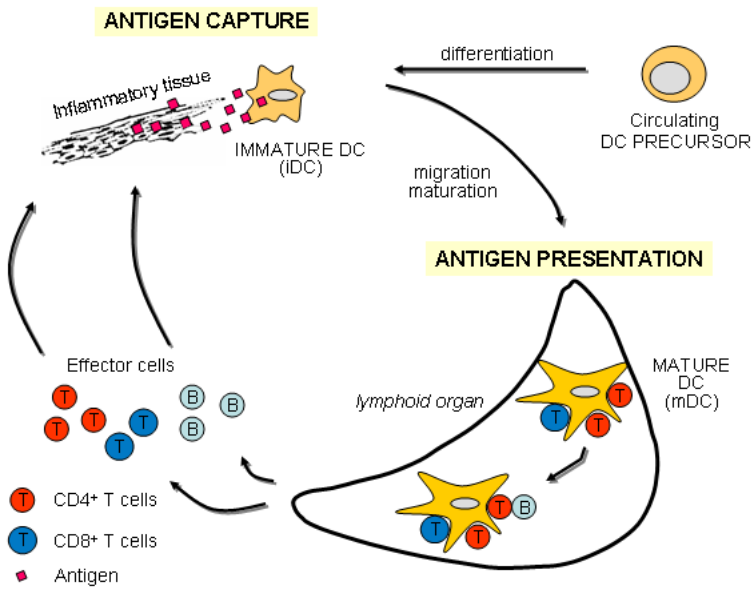


Figure 9 – Dendritic cell mobility corresponding to differentiation stage and functionality. Circulating DC precursor cells are recruited to inflammatory tissue via inflammatory chemokines and cytokines inducing differentiation of the precursor cells into iDCs. The iDCs take up antigens in the inflammatory tissue and process it for presentation. After antigen capture the final differentiation of iDCs into mDCs occurs. This induces homing of maturating DCs to lymphoid organs, where the fully matured DC effectively present the captured antigen to $CD4^+$ T cells (red) and $CD8^+$ T cells (blue). Subsequently activated $CD4^+$ T cells activate corresponding B cells and both activated T and B cells become mobile and home to inflammatory tissue to perform their effector function. Read text for further information. Adapted from Palucka and co-workers (Banchereau et al., 2000).

1.2.7 DC stimulation of T cells

As described in chapter 1.2.4-5 exogenous derived antigens can be presented on both MHC class II and class I molecules, although the importance of MHC class I presentation by DCs is still being discussed (reviewed by Melief (Melief, 2003)). The presented antigens are recognised by antigen specific T cell receptors (TCRs) expressed on T cells (MHC/TCR recognition is structurally reviewed by Rudolph and Wilson (Rudolph and Wilson, 2002)). Morphologically the DC T cell encounter can be divided in three phases, 1) short encounters with highly mobile T cells that

gradually decrease their mobility upon encounter, 2) a long-lasting ligation, which induce T cell cytokine secretion and 3) rapid proliferation and increased mobility (Mempel et al., 2004). In the highly mobile phase mDCs are capable of scanning 500 different T cells per hour, and during the long-lasting ligation each mDC can maintain interaction with 10 T cells (Bousso and Robey, 2003).

The T cells distinguish between MHC class I and class II through the coreceptors CD8 and CD4, respectively (figure 10). These two receptors are capable of binding peptide-independent to MHC complexes on APCs, which, combined with the peptide specific recognition through the TCR, augment the functional affinity between APCs and T cells (Choi et al., 2003; Garcia et al., 1996). The TCR is colocalised with CD3, which is partly responsible for the signal transduction in T cells upon receptor ligation (Lewis, 2001). However, T cells can commit to two fates upon primary receptor ligation. 1) T cell activation and proliferation, which is dependent on coreceptors and cytokines and 2) T cell anergy in the absence of such costimulatory molecules (Gimmi et al., 1993; Williams et al., 1992). In addition to CD4 and CD8 a very large number of coreceptors taking part in the interaction between T cells and APCs have been identified (reviewed by Lipsky and co-workers (Geppert et al., 1990)), such as Flt3R vs. Flt3L (Mosca et al., 2002), CD2 vs. CD58 (LFA-3), CD11a (LFA-1) vs. ICAM-1,2,3, CD28 vs. CD80 and CD86, CD152 (CTLA-4) vs. CD80 and CD86 and CD154 (CD40-ligand (CD40L)) vs. CD40 (table 4 and further discussed in chapter 2.5.7.4).

T cell coreceptor	APC coreceptor	Description	Reference
CD2	CD58	Mediate cell-cell contact preceding TCR/MHC contact. Maintain the correct cell-cell distance for optimal antigen recognition.	Reviewed by Wang and Reinherz (Wang and Reinherz, 2000)
CD11a/CD18	ICAM-1,2,3	Cell adhesion	Reviewed by Lipsky (Geppert et al., 1990)
CD28	CD80 and CD86	Costimulation inducing T cell activation potentially through a separate pathway than TCR/CD3-MHC interaction.	(June et al., 1989) and reviewed by Thompson (June et al., 1990)
CD152	CD80 and CD86	Inhibitor of activation via binding of CD80 and CD86 with higher affinity than CD28. Induced upon T cell activation	(Gimmi et al., 1993)
CD154	CD40	Induce cytokine production and activation of various immunologically relevant cell types.	reviewed by van Kooten and Banchereau (van Kooten and Banchereau, 2000)
ICOS	B7H	Replace CD28 upon activation of T cells. Receptors are homologous but not cross-reactive.	(Dong et al., 2001; McAdam et al., 2001; Tafuri et al., 2001)

Table 4 - An extracted number of important costimulatory molecules for immunological cell-cell interaction.

Cytokines represent an additional signal mediating proliferation of naïve T cells. Signalling through the TCR and CD28 induce IL-2 secretion from T cells as well as increase the level of surface expressed CD25 (IL-2R). IL-2 exhibits autocrine functionality, which augment T cell proliferation. Moreover, IL-1 and IL-12 also mediate T cell activation for $CD4^+$ and $CD8^+$ T cells, respectively (Curtsinger et al., 1999). IL-2 and IL-12 furthermore up-regulate the expression of both IL-2R and IL12-R (Valenzuela et al., 2002). Such cross-regulation might be important for DC activation of naïve $CD8^+$ T cells, since IL-12 is secreted by dendritic cells devoted to type 1 immune responses (DC1).

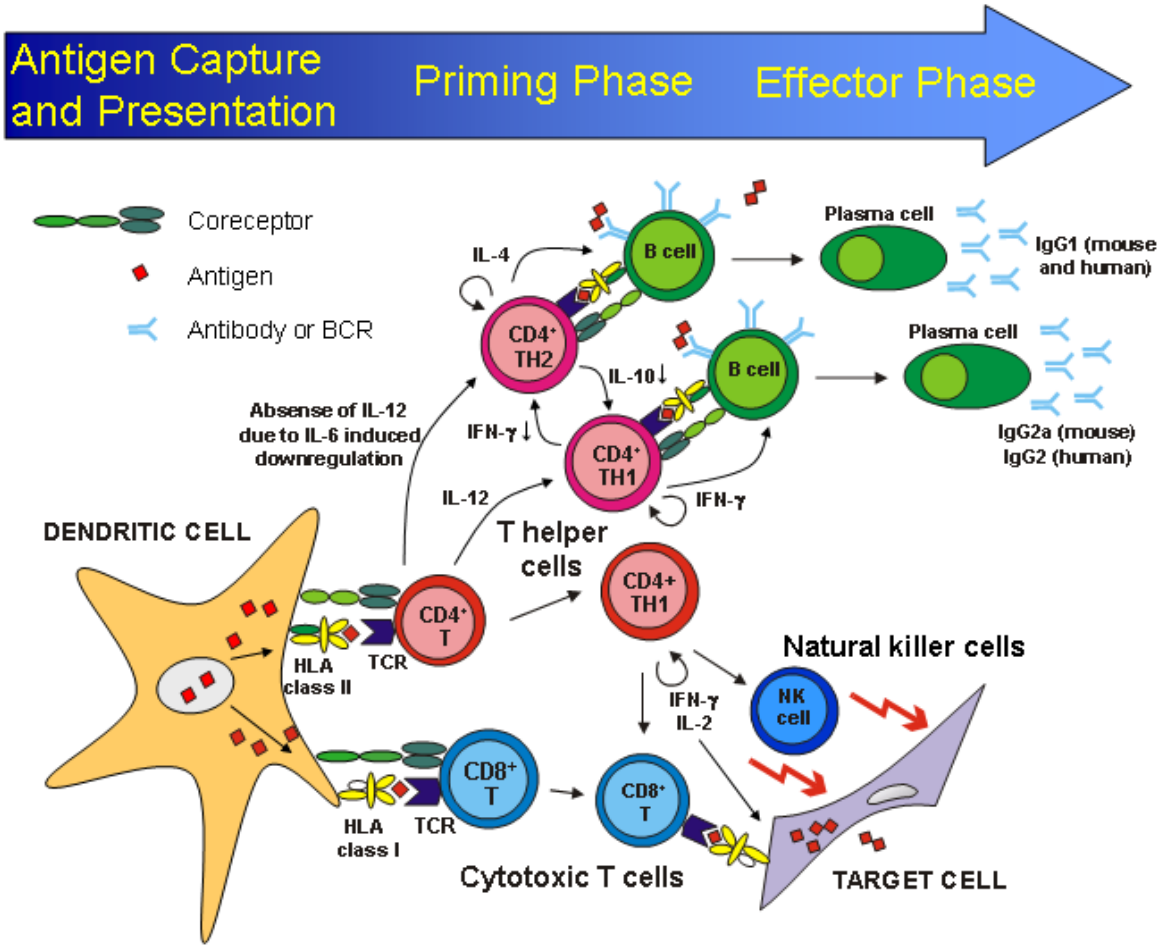


Figure 10 – Activation of naïve $CD4^+$ and $CD8^+$ T cells by DCs. Mature dendritic cells are capable of presenting exogenous antigens on both MHC class I (cross-priming) and MHC class II in combination with appropriate coreceptors, which enable activation of both naïve $CD4^+$ and naïve $CD8^+$ T cells. The activated $CD4^+$ T cells subsequently differentiate into Th1 or Th2 cells, in response to respectively IL-12 and lack of IL-12 descended from the DC. Both Th1 and Th2 cells are capable of activating B cells, but their cytokine patterns induce various class switching. The Th1 secreted cytokines, IFN-γ and IL-2, have a positive stimulating effect on $CD8^+$ T cells and IFN-γ further induce antigen presentation on potential target cells.

1.2.8 Type 1 and type 2 immune responses

Immune responses have been classified in two categories primarily due to the cytokine secretion profile of two subsets of $CD4^+$ T cells, designated Th1 and Th2 (figure 10). $CD4^+$ T cells commit to the Th1 and Th2 differentiation pathway upon stimulation with DC derived cytokines IL-12 and IL-6, respectively. IL-6 seems to function by impeding IL-12 production (Dodge et al., 2003), which suggests that Th2 might be the default differentiation pathway, whereas Th1 needs extra signalling (Moser and Murphy, 2000). Th1 cells secrete primarily IFN-γ, IL-2, IL-12 and IL-18, which is beneficial for the induction of $CD8^+$ CTL response, which is particularly important in the struggle against virally infected cells or tumour cells (reviewed by Dalgleish and co-workers (Dredge et al., 2002)). Th2 cells secrete primarily IL-4, IL-5 and IL-10, which seems to have special beneficial properties for induction of humoral response and downregulation of $CD8^+$ CTL activity, which is potentially important to avoid autoimmune diseases (Kidd, 2003).

1.2.9 B cell activation – role of antigen, $CD4^+$ T cells and follicular DCs

When antigen enters the body by pathogenic invasion or by vaccination a fraction of the antigen is taken up by APCs and subsequently presented on MHC molecules (cf. chapter 1.2.3). In addition antigen enters the bloodstream and circulates in the body. Resting B cells in lymphoid organs express a surface bound B cell receptor (BCR), which upon interaction with its cognate antigen induce secretion of soluble immunoglobulin. The antigen-BCR interaction can be improved through the innate immune system. B cells express CD21 (complement receptor 2), which binds complement protein C3d tagged antigens, which again leads to increased functional affinity as well as colocalisation of antigen and BCR (reviewed by Caroll (Carroll, 1998)). The primary secreted immunoglobulins are IgM and IgD; IgD being a result of alternative RNA processing. As described in chapter 1.1.2.4.2 a T cell dependent affinity maturation of the primary immunoglobulins can give rise to secondary antibodies, IgG, IgA and IgE, with high affinity and specificity towards the antigen. The class switches occur by irreversible DNA recombination and are primarily mediated through CD40-CD40L signalling by activated $CD4^+$ $CD40L^+$ T cells (McAdam et al., 2001). The class switch is furthermore governed by the $CD4^+$ T cell cytokine profile, thus IL-4 (type 2 cytokine) induce class switching to IgG1 and IgE, whereas IFN-γ (type 1 cytokine) induce class switching to IgG2a and IgG3 (mouse IgG subclasses are indicated) (depicted in figure 10, discussed in Appendix B and reviewed by Snapper and Mond (Snapper and Mond, 1993) as well as Stavnezer (Stavnezer, 1996)).

B cell activation and antibody secretion is believed to be regulated through a feedback mechanism, which is believed to involve the cross-linking of the BCR and the low-affinity IgG FcγRIIB in the presence of immunoglobulins (Kato et al., 2002). The receptor can only bind IgG molecules in the form of an immune complex, whereas monomers cannot bind. A special subfamily of dendritic cells has evolved to regulate this feedback mechanism, follicular dendritic cells (FDCs). They reside in follicles in the lymphoid organs in proximity of active B cells, and are able to bind complement, mannose and antibody coupled antigens on its surface for sustained antigen presentation to proximal B cells. Interestingly, these cells do not internalise the antigen for MHC presentation similar to other dendritic cells. The FDCs express high levels of FcγRIIB, which binds IgG molecules bound to antigen, thus bypassing the inhibitory effect of feedback regulation on B cells (reviewed by Qin (Tew et al., 2001) and Heyman (Heyman, 2003)). Following sufficient activation B cell plasmablasts home to the bone marrow, where they reside as fully maturated antibody secreting plasma cells (Tew et al., 1992).

1.2.10 $CD8^+$ CTL stimulation by $CD4^+$ T cells

It is generally believed that cytotoxicity is related to type 1 immune responses. The Th1 secreted IFN-γ serves both as an activator of CTLs and natural killer cells (NK cells), but simultaneously IFN-γ also induces antigen presentation on potential target cells, thereby rendering the target cells susceptible to CTL killing (cf. chapter 1.2.8). The mobility of the activated $CD4^+$ T cells also differs according to the type of immune response. Type 1 cytokines induce homing signals that allows Th1 infiltration of e.g. tumour tissue, whereas type 2 cytokines induce homing of Th2 cells to the periphery of such tumour tissue, where it induces necrosis of the tumour, possibly through release of superoxide and nitric oxide from activated macrophages and eosinophils (Nishimura et al., 1999). In the type 1 immune response $CD8^+$ CTLs will infiltrate the tumour and kill target cells upon encounter with MHC class I presented specific antigen. The CTL response is further regulated by TCR-mediated internalisation of the MHC class I presenting antigen complexes, which render the CTLs susceptible to themselves (Huang et al., 1999).

1.2.11 $CD8^+$ CTL – killing mechanisms

CTL mediated killing is accomplished through two separate pathways; 1) Granules containing proteolytic granzymes and perforin fuse with the target cell, and perforin establish a pore in the target membrane (homologous to the complement derived membrane attack complex), which grant

granzymes access to the target cell cytoplasm, where they induce apoptosis and 2) FasL on CTL cells interact with Fas on target cells, which thereby induce caspase mediated apoptosis signals (Russell and Ley, 2002). Extensive cellular dynamics are involved in the attack with granules. In NK cells it can be divided in three phases; 1) Maintenance of a tight cell-cell interface, 2) The lymphocyte microtubule organising center (MTOC) orients toward the interface and 3) accumulation of actin filaments at the interface creating an effective infrastructure, which enable rapid exocytosis of granules. The same three parameters are important for CTLs, however, the cytoskeletal polarisation was instantaneous. The stepwise nature for NK cell effector function suggests that the low specificity of NK cells is compensated with a hesitating nature leaving room for second thoughts (Kuhn and Poenie, 2002; Wulfing et al., 2003).

1.2.12 Dendritic cell therapy

In recent years dendritic cell research has become the hottest area in immunotherapy. Dendritic cells are nature's top-tuned adjuvant, the adjuvant to rule them all! They are able to activate naïve $CD4^+$ T cells, naïve $CD8^+$ T cells as well as a NK cells, and their cytokine pattern enables directional induction of type 1 or type 2 immune response according to the disease of interest. Targeting DCs *in vivo* is already a major field of interest (reviewed by Hovgaard and co-workers (Foged et al., 2002)), but also *in vitro* manipulations succeeded by readministration of DCs isolated from patient's own blood has been accomplished. The first dendritic cells were isolated and characterised from murine spleen and lymph nodes (Steinman and Cohn, 1973). A decade later human dendritic cells from peripheral blood were isolated (Van Voorhis et al., 1982). However, the very low numbers of DCs present in blood detained the invasion of the field.

The various cellular members of the immune system all derive from one pluripotent stem cell (PPSC) (figure 11). Two $CD34^+$ DC progenitor cells designated lymphoid and myeloid both origin from PPSCs. Stimulation with appropriate cytokines will differentiate these cells into monocyte precursors with various phenotypic markers according to their origin and cytokine environment. Further proliferation and differentiation produce immature dendritic cells that are able to undergo final maturation upon encounter with CD40L and various inflammatory or pathogenic compounds, such as TNF-α, LPS and CpG (Banchereau et al., 2000; Robinson et al., 1998; Vandenabeele and Wu, 1999). From figure 11 it is clear that macrophages and dendritic cells are closely related, since exchanging GM-CSF with M-CSF during differentiation of monocyte precursors commit the cells

to become macrophages instead of dendritic cells. Both myeloid and lymphoid precursor cells are dependent on endogenous IL-6 production to differentiate into mature DCs, but a difference between lymphoid and myeloid derived DCs is the augmented secretion of IL-12 by lymphoid DCs vs. myeloid DCs (Brasel et al., 2000). IL-12 furthermore induces IFN-γ expression in lymphoid DCs but not in myloid cells (Banchereau et al., 2000).

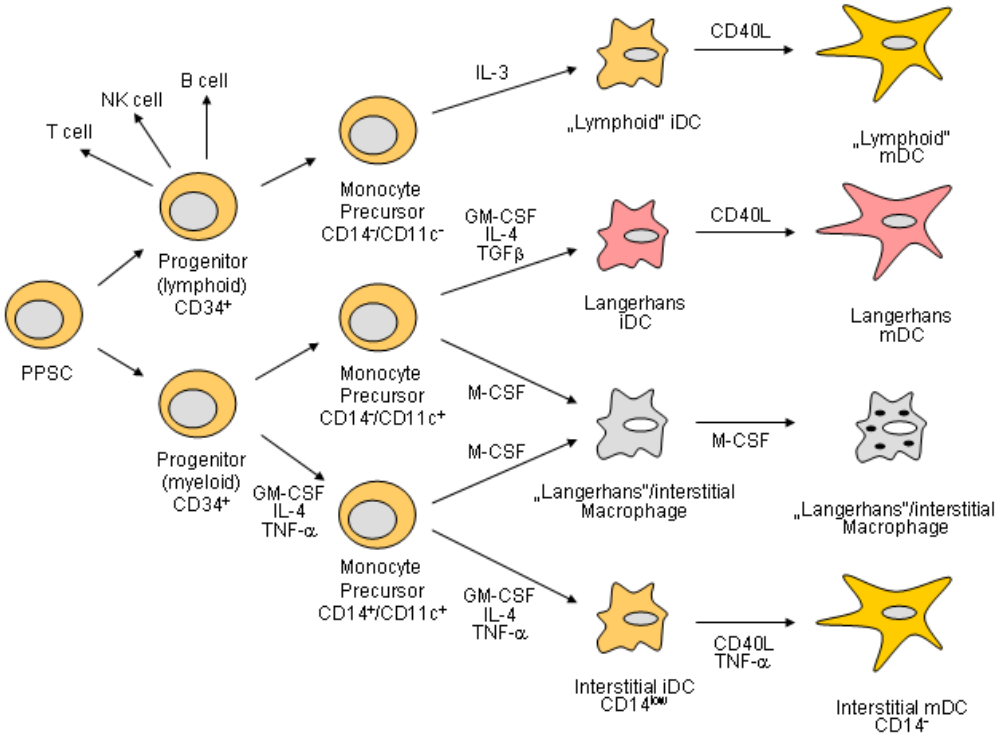


Figure 11 – Origin and differentiation of dendritic cells. The pluripotent stem cells (PPSC), from which dendritic cells descend, is depicted with the potential off spring when exposed to cytokines inducing differentiation into dendritic cells via a number of intermediate differentiated PPSCs. Read text for further explanation. The indicated maturation cocktails are examples, not a complete list of maturation conditions. Abbreviation: Granolucyte/Macrophage-Colony Stimulating Factor (GM-CSF), Macrophage-Colony Stimulating Factor (M-CSF), Tumour Growth Factor β (TGF-β) and Tumour Necrosis Factor α (TNF-α). (Banchereau et al., 2000; Robinson et al., 1998; Vandenabeele and Wu, 1999).

In the early 1990s the first method of *ex vivo* generation of DCs from murine bone marrow was developed. Shortly after, similar techniques were established for the generation of human DCs. DCs can be generated from $CD34^+$ precursors and $CD14^+$ monocyte precursors. The differentiation of precursor cells into immature DCs (iDCs) is mediated by cytokines such as GM-CSF, IL-4 and TNF-α (Robinson et al., 1998). The technique has later been optimised for clinical applications (Thurner et al., 1999). Elevated levels of TNF-α (Masterson et al., 2002) as well as signalling through the CD40/CD40L receptor-ligand pair (Rosenzwajg et al., 2002) induce the final maturation of the iDCs into mature DCs (mDCs).

Despite the improved technology in DC generation from human precursor cells, it remains problematic to generate large amounts of dendritic cells of required quality using precursor cells from human donors. The isolation of the $CD34^+$ human acute myeloid leukemia cell line MUTZ-3 seems to solve this problem (Hu et al., 1996). MUTZ-3 cells behave as an immortalised equivalent of $CD34^+$ DC precursors. Upon stimulation with specific cytokine cocktails, they acquire a phenotype consistent with either interstitial- or Langerhans-like DCs and upon maturation, they express CD83. MUTZ-3 DC has the full range of features covering functional antigen processing, presentation and T cell activation. (Masterson et al., 2002) The optimised human dendritic cell line, NemodDC, derived from the MUTZ-3 cell line, has additional beneficial features like improved proliferation rates, serum-free cell culture conditions and the option to freeze and thaw immature and mature DCs without losing their phenotypic and functional characteristics (Unpublished results, Baumeister H). These features demonstrate the unique suitability of NemodDCs as an unlimited source of $CD34^+$ DC progenitors for the study of dendritic cell biology.

2 Results and discussion

2.1 "Functional improvement of antibody fragments using a novel phage coat protein III fusion system." – summary of appendix A and additional data

Expression and purification of eukaryotic derived recombinant proteins in different expression systems has been of major importance in recent years. Bacterial expression systems have been widely employed, due to factors like easy handling, rapid growth and low costs, which seem to overcome shortcomings, such as protein folding and post translational modifications. In recent year's reports on phage display derived single chain fragment variables (scFvs) have risen, and so has the interest in expression of such recombinant antibodies. It has often been observed that phage display derived scFvs losses functionality when expressed as soluble protein, although they are actively expressed when fused to the minor coat protein III of the filamentous bacteriophage.

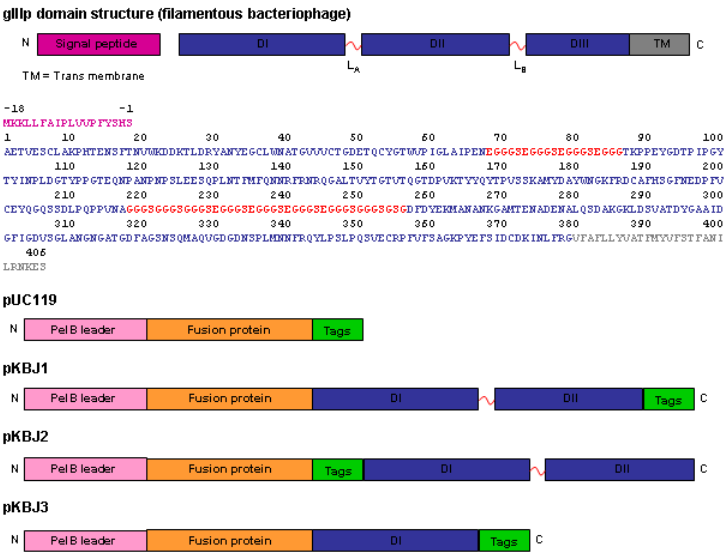


Figure 12 – FuncFAb gene construction. The domain structure and protein sequence of the protein III from the filamentous bacteriophage is shown (van Wezenbeek and Schoenmakers, 1979; van Wezenbeek et al., 1980). Relevant fragments are highlighted with colour codes - the signal peptide (purple), three extracellular domains (blue) and the transmembrane region (grey). The standard non-fused scFv as obtained from the pUC119 plasmid and the three FuncFAb fragments consisting of a PelB leader (light purple) (Lei et al., 1987), a scFv (brown) and either the first or the first two extracellular domains of protein III (blue) with tags (green) added at desired positions are depicted in block diagrams. The tag consists of the c-Myc epitope for immunodetection and a Hexa-His tag for purification. The amino (N) and carboxy (C) terminals are indicated. The original signal peptide from the filamentous bacteriophage was replaced with the PelB leader from pUC119 cloning plasmid and the derived pHEN2 phagemid (Krebs et al., 1998), was employed as founder plasmid for the three pKBJ plasmids.

We observed that some functional phage displayed scFv completely lost functionality when expressed as soluble scFv. We therefore rationalised that the loss of function observed by detaching the scFv from its fusion partner, the filamentous bacteriophage coat protein III, could be reversed by reattaching the scFv to fragments of protein III. Protein III consist of three domains linked with

glycine-rich polypeptides. The C-terminal domain (DIII) is partly integrated in the phage capsid, and it is therefore difficult to predict, which fragment(s) of DIII would fold natively. Moreover, DIII has in previous studies been observed as toxic to *E. coli* (Peter Kristensen, personal communication). For these reasons only the two N-terminal domains (DI and DII) were employed as fusion partners for the scFvs. We thus constructed three expression plasmids, two fusing the scFv with both DI and DII (pKBJ1 and pKBJ2) and one fusing the scFv with DI alone (pKBJ3) (figure 12). Although structural data are available for the N-terminal domains of protein III (Holliger and Riechmann, 1997; Lubkowski et al., 1998), no structure has been determined showing the possible interactions between a fused scFv and the phage coat protein III. It was therefore not possible to further narrow down the requisite portion of protein III by rational design. The fusion system was designated FuncFAb – **Func**tionally **F**used **A**nti**b**ody.

We investigated the effect of FuncFAb on three scFvs – D4 (Jensen et al., 2003), L36 (Sanz et al., 2001) and R5 (Steffen Goletz, Unpublished results). D4 recognises fibronectin and L36 binds antagonistically a conformational epitope in the middle part of the rod-like coiled-coil portion of Laminin-I, which is responsible for binding to the α2β1 integrin on the surface of endothelial cells during angiogenesis (Sanz et al., 2003). R5 is an anti-idiotypic antibody binding in a competitive manner to the antigen combining site of the murine IgG1 antibody A76-A/C7 (Karsten et al., 1998; Price et al., 1998). A76-A/C7 recognises a MUC-1 conformational peptide epitope of the immunodominant tandem repeat region of MUC-1, which is induced by a Tn or Thomsen-Friedenreich glycosylation of the DTR motif.

D4 was completely inactive when expressed as non-fused scFv, but the functionality was restored when fused to DI-DII and activity was further increased when only fused to DI. R5 and L36 were both active as non-fused scFv. For R5 but not L36 activity was improved when fused to DI-DII, whereas fusion to DI improved functionality for both R5 and L36. Subsequently 9 out of 10 non-active scFvs were reactivated using the FuncFAb system, further establishing the value of the FuncFAb system (data not shown).

2.1.1 Stability of the FuncFAb proteins

The molecular explanation for the increased functionaility observed in the FuncFAb system was investigated further, with particular focus on stability, solubility and affinity. Purified L36 and R5

protein as non-fused scFv as well as in the two FuncFAb formats pKBJ1 and pKBJ3, were tested for stability by incubating samples of the protein at room temperature, 30°C and 37°C for up to 4 days, followed by ELISA analysis, allowing evaluation of possible loss of reactivity. The constructs showed no change in activity over this period of time. D4 was also analysed and showed a decreased activity when subjected to 30°C and a complete loss of function after incubation at 37°C. Since the non-fused D4 scFv was not active no comparison could be made and thus no conclusion in regard to stability changes by employing the FuncFAb system could be drawn (data not shown). After incubation at the elevated temperature the samples were stored at 4°C until ELISA was performed, and it could thus be argued that we are merely measuring the ability of the constructs to refold into native conformation.

Next L36 in the four formats (figure 13) was subjected to guanidinium chloride induced unfolding, and the guanidinium chloride concentration needed to reach half denaturation was measured by steady state fluorescence spectroscopy. All but the domain I fused scFv showed equal stability being half denatured at around 2.7M guanidinium chloride, whereas the domain I fusion was slightly less stabile being half denatured at 2.4M guanidinium chloride.

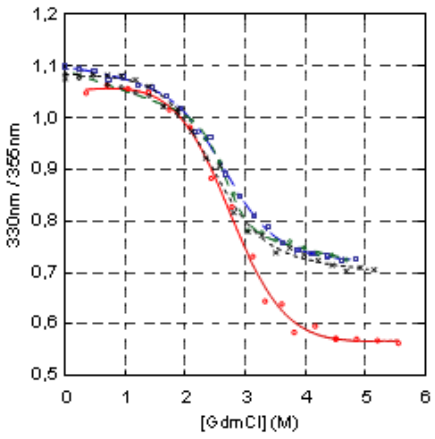


Figure 13 – Stability measurements of antibody clone L36 and derivatives. Guanidinium chloride induced unfolding measured by steady state fluorescence spectroscopy of L36 expressed from pUC119 (red circle), pKBJ1 (blue box), pKBJ2 (green diamonds) and pKBJ3 (black cross).

The solubility of the different constructs was measured by SDS-PAGE analysis of soluble and insoluble protein fractions from the different constructs. The ratios between soluble and insoluble protein for the different FuncFAb constructs as well as non-fused scFv were all similar, indicating that the increased activity was not due to increased solubility (Personal communication Kim Bak Jensen).

2.1.2 Hypothesis on the folding capacity of the FuncFAb system

Since the FuncFAb system did not seem to alter the stability of the protein, one could speculate, whether it could have a positive effect on the folding of the scFv due to a chaperone-like activity. Chaperones are believed to stabilise hydrophobic patches exposed at stages of intermediate folding. The stabilisation is accomplished through the binding of a chaperone to the unfolded hydrophobic patches thereby allowing the protein to fold into a native water soluble form (Caramelo et al., 2003). The crystal structure of the two N-terminal domains of protein III shows that DI and DII interact (Lubkowski et al., 1998). The dynamics of DI and DII interactions have been further explored by Schmid and co-workers, who found that the folding of protein III is an ordered process (Martin and Schmid, 2003b). First, DI folds within few milliseconds forming a platform for the folding of the less stable DII, and finally the DI docks into the larger DII, thereby terminating the folding approximately five hours after the initial folding of DI (Martin and Schmid, 2003a). The exposed DI binding site could be a site of interaction for hydrophobic exposed regions and thus potentially induce chaperone-like activity. If so the very slow docking kinetics would allow DI to perform folding improvement on the fused scFv, at least until the DII finish it's folding and docks with DI. This argument is supported by the large number of aliphatic amino acids in the DI binding site reported to point into the central cavity of the horseshoe shape formed by DI-DII interaction (Deng and Perham, 2002). In the DI fusion system the DI binding site has no DII to dock with, therefore increasing the time span of a potential chaperone-like activity. Alternatively, the FuncFAb system could orchestrate bacterial chaperones that are not summoned by non-fused scFvs.

2.1.3 Multimerisation of FuncFAb proteins

Next, we investigated whether the improved affinity was due to multimerisation of the FuncFAb constructs. Multimerisation was analysed by gel filtration, native gels and non-reducing SDS-gels. The preparative TSK-gel G3000 SW HPLC gel filtration column enabled the separation of monomers, dimers and higher order multimers. We did indeed observe multimerisation for both DI-DII fusions as well as DI-fusions (see Appendix A for more details). Briefly, no clear correlation between multimerisation and activity of the tested scFvs emerged. However one observation was identical for all tested scFvs; namely that DI fused scFvs seem to posses multimerisation abilities that retain the binding activity of the fused scFv. To resolve multimers larger than dimers, IMAC purified protein was analysed on a TSK-gel G4000 SW gel filtration column with a theoretical cut-of of 7 MDa. It was a general problem for this experiment that the proteins aggregated on the pre-

column, and this seemed to affect the overall output as well, since the protein eluted in the void volume fraction. This implies that the protein forms structures larger than 100-200mers. Such structures are very unlikely to form in an ordered and active manner, and it therefore suggests that what we observe is aggregated protein. Peaks were however observed in the resolved area around the 200 kDa marker especially for the R5 scFv fused to DI (figure 14).

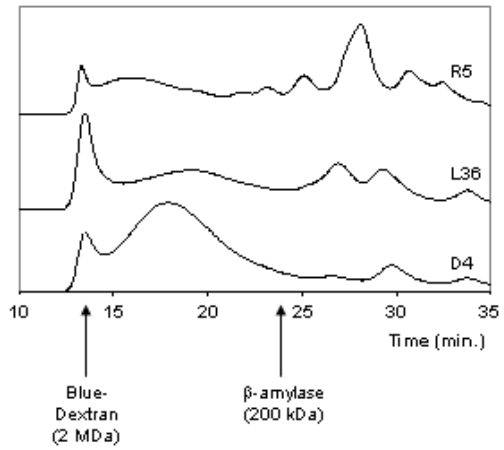


Figure 14 – Gel filtration analysis of R5, D4 and L36 scFvs fused to DI of protein III. Three scFv-DI fusion proteins were purified by IMAC and analysed for multimerisation on a GX4000 SW gel filtration column. The light absorbance at 254nm of eluted protein was detected and signals were correlated with the elution time measured from time of protein injection in minutes. Molecular weight markers Blue dextran (2 MDa) and β-amylase (200 kDa) are marked.

To analyse the multimerisation further we turned to iso-electric focusing of native R5 scFv fused to DI on a 0,8% agarose gel (Kim et al., 2000a). The experiment confirmed that the protein was positively charged (pI = 5,39) moving towards the cathode but it did not show distinct bands merely a smear of protein (data not shown). Finally, we analysed the influence of disulfide bridge formation on the multimerisation using a standard SDS-PAGE approach, but loading the R5 scFv fused to domain I in a loading buffer not containing any reducing agent (- DTT) and without boiling. A control sample including DTT was included. The SDS-PAGE was subsequently blotted to a nitrocellulose membrane allowing for immunodetection of cMyc tagged protein. The western blot shows a range of high molecular weight bands (above 116 kDa) being much more intense in the lane without DTT, indicating that the two intradomain disulfide bridges in DI (Deng and Perham, 2002) are at least partly responsible for the multimerisation (figure 15). The two disulfide bridges in the scFv are more unlikely to take part in the multimerisation, since they are important for the stabilisation of the V_H and V_L domains respectively (Ramm et al., 1999), although disulfide free scFvs have been reported (Worn and Pluckthun, 1998). No free cysteines are present in neither DI nor the scFv.

Theoretically, the observed multimerisation is no surprise, since DI and DII have been shown to interact with each other. This at least explains multimerisation for DI-DII fusion proteins. DI has so

far not been shown to form homomultimers, but also multimerisation of DI fusion proteins is explainable, because the construct includes the linker connecting DI and DII (L1, cf. figure 4), which has been observed to play a prominent part in the DI-DII interaction (Holliger et al., 1999; Lubkowski et al., 1999). Alternatively, multimerisation could be induced by the hydrophobic binding site exposed by DI, which will be unfavourable to dissolve in water. It can thus be argued that a thermodynamically more stable format can be reached by homomultimerising with other DI molecules with equally exposed binding sites.

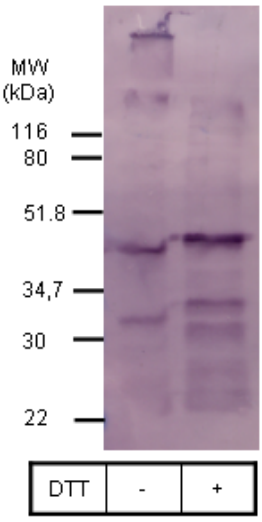


Figure 15 – The importance of disulfide bridges for multimerisation of scFv-DI fusion proteins. A sample of IMAC purified scFv-DI fusion protein was analysed by western blotting. The protein was loaded on a 12% SDS-gel in a reducing loading buffer containing 1mM DTT and a non-reducing loading-buffer with no DTT or β-Mercaptoethanol added and after blotting the protein was detected by anti-cMyc antibody (9E10) (Evan et al., 1985). A molecular weight marker is depicted as reference. The blot is representative of two independent experiments.

2.1.4 Additional results regarding the FuncFAb system.

The multimerisation of FuncFAb proteins inspired us to investigate whether FuncFAb constructs could be rescued by a traditional full length protein III helper phage and three derived phage with deletions in protein III. The terminology “rescue of a phagemid construct” traditionally involves the insertion of a phagemid encoded protein III in a phage coat alongside with wild-type protein III from the helper phage. On the contrary, the rescue of a FunFAb plasmid would involve multimerisation of FuncFAb protein with protein III from a helper phage. The rescue with a full length protein III helper phage (KM13) was analysed by phage ELISA detection of R5 scFv binding to its antigen A/C7, but no effect was observed (data not shown). Since the N-terminal domains of protein III seem to induce multimerisation we turned our interest towards three deletion mutant phage vectors (Riechmann and Holliger, 1997), a DI deletion (fd-DII-DIII), a DII deletion (fd-DI-DIII) and a DI-DII deletion (fd-DIII). Since DI and DII are responsible for the phage infection of *E. coli* these deletion phages can not effectively infect *E. coli* transfected with the FuncFAb plasmids. We therefore made double transfected clones that were selected due to their double resistance (ampicillin and tetracycline). One clone did not grow (R5-DI in combination with fd-DIII), whereas

several of the other clones did not give a signal in neither PCR (figure 16.A) nor western blotting (figure 16.B) of the FuncFAb gene. The clone combining R5-DI and fd-DII-DIII seemed to recombine generating a PCR fragment, which was similar to the R5-DI-DII-DIII fusion gene. Protein expression was undetectable for this clone. R5 scFv-DI-DII did indeed associate with the DI deleted phage resulting in binding to A/C7 in phage ELISA. As expected the result indicated that non-fused scFv cannot be displayed on neither the DI deleted phage nor the DI-DII deleted phage (figure 16.C). Previously, the deletion phages have been shown to induce polyphage production, which decrease the amount of phage produced by the individual clones, thus rendering analysis difficult (Riechmann and Holliger, 1997). The observed recombination can possibly be explained by the fact that the R5-DI gene entails a part of the linker connecting DI and DII. Therefore, there are overlapping genetic regions in the DI deletion phage and the R5-DI FuncFAb gene, which potentially could recombine to a full-length R5-DI-DII-DIII phagemid gene.

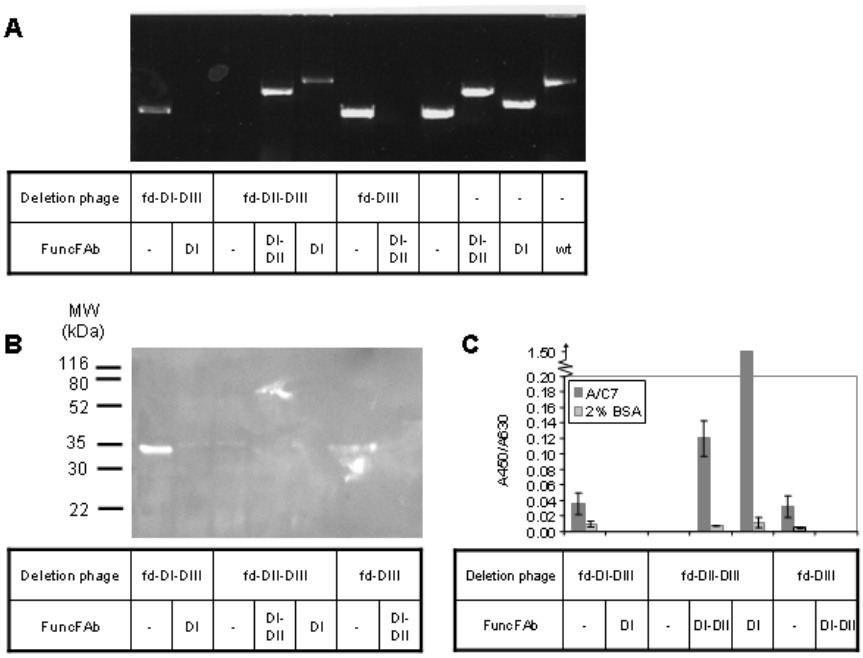


Figure 16 – Rescue of FuncFAb plasmids with a modified M13 helper phage including pIII deletions. Clones containing two plasmids, where the first encodes R5 scFv non-fused or genetically fused to either the first or the first two domains of the gIIIp, and the second encodes one of the three deletion phages - DI deletion (fd-DII-DIII), DII deletion (fd-DI-DIII) and DI-DII deletion (fd-DIII) (Riechmann and Holliger, 1997) were grown to OD(650nm)~0,5. Glucose containing medium was removed and 1mM IPTG was added to the new medium and over night phage production at 18°C was initiated. Presence of the FuncFAb genes (A) and expressed protein (B) was analysed with PCR including pure vector as controls and by Western Blotting, respectively. (C) The generated phages were tested for non-covalently displayed R5 protein in a phage ELISA recognising the major phage coat protein VIII. Only cultures where the FuncFAb gene could be detected were analysed. The clone encoding R5-DI in combination with fd-DIII did not grow, and was therefore omitted.

Due to the easy expression and purification of large quantities of pure protein using the FuncFAb system, we applied the R5 scFv fused to DI in a crystallisation study using the Hampton crystal screen kits (Jancarik et al., 1991), whereby 96 different crystallisation conditions were tested. The crystal structure would have been the final evidence for or against that a scFv interacts with the N-terminal domains of protein III in the FuncFAb system. Unfortunately no protein crystals were detected and further optimisation was discarded, since the non-homogeneous mixture of multimers observed by gel filtration was expected to disrupt the possible crystallisation.

We also investigated whether the FuncFAb system could reactivate scFvs, which were not selected by phage display. The scFv clone with a standard 18mer glycine-rich linker established from the Thomsen-Friedenreich (TF) specific monoclonal hybridoma antibody A78-G/A7 (Karsten et al., 1995) is merely active. A shortening of the linker gave rise to various multibodies and increased activity ((Ravn et al.), ready for submission). Due to these characteristics the scFv with an 18mer linker was analysed in the FuncFAb system. Purified scFv fusion protein was tested for activity in ELISA, but the FuncFAb fused scFvs were as active as the non-fused scFv (figure 17). This suggests but does not prove that the FuncFAb system works for proteins selected by phage display, due to the fact that proteins displayed on a phage are not solely selected for their intrinsic properties but merely for the properties they have as an entity with their phage fusion partner.

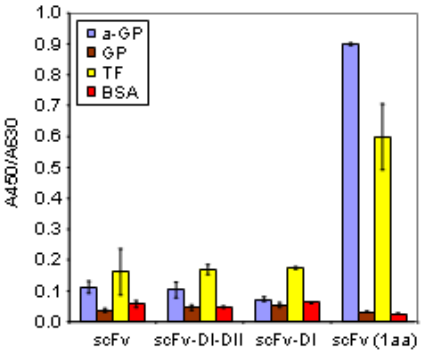


Figure 17 – Activity of G/A7 scFv derived from the G/A7 mouse hybridoma employing the FuncFAb system. The G/A7 scFv with a standard 18mer glycine rich linker was expressed as non-fused scFv (scFv), or fused to the N-terminal domains of protein III from the filamentous bacteriophage (scFv-DI-DII and scFv-DI). As positive control a G/A7 scFv with a 1mer linker was applied. The proteins were analysed in ELISA for their ability to bind neuraminidase treated glycophorin A (asialoglycophorin A, a-GP), glycophorin A (GP), the Thomsen-Friedenreich disaccharide PAA conjugate (TF) and non-coated negative control (BSA). Glycophorin A and BSA were included as negative controls, since they do not present any TF epitopes.

2.2 "Functionally Fused Antibodies – a novel adjuvant fusion system." – summary of appendix B

Inspired by the multimerisation of DI fused scFvs the potential heteromultimerisation of scFvs fused to DI was analysed. The *in vivo* heteromultimerisation of R5 scFv fused to DI and D4 scFv fused to DI was investigated in double transfected *E. coli*, with R5 fused to DI in a chloramphenicol resistant plasmid and D4 fused to DI in the standard pKBJ3 ampicillin resistant plasmid. Protein expression and purification showed similar yield, purity and multimerisation as R5 and D4 scFv fused to DI did separately. The protein was tested for the presence of active heteromultimer in a sandwich ELISA setup. Fibronectin or the A/C7 mouse antibody was coated to the wells and sample was added. Bound protein was then detected by the opposite antigen than the coated antigen and a secondary HRP conjugated antibody was finally added. The sandwich ELISAs confirmed the presence of active heteromultimer (cf. Appendix B).

Such bispecific antibodies could potentially be used for targeting antigens to dendritic cells, e.g. by introducing a CD40 scFv as the one partner in the heteromultimer (Barr et al., 2003). Alternatively it could be used to improve the angiogenesis inhibiting effect of L36 (Sanz et al., 2001) by improving the tumour specificity of the treatment. A similar study showed improved therapeutic effect towards a B-cell lymphoma combining an anti-idiotypic antibody recognising the B-cell idiotope and an antibody, which inhibits homing due to the LFA-1 adhesion molecule. (Cohen et al., 2003c).

2.2.1 Determination of the immunogenic nature of the FuncFAb system

The therapeutic applications for the FuncFAb system are very dependent on the immunogenic properties of the system. If FuncFAb was non-immunogenic they could be applied in a range of targeting applications, using several rounds of administration. Opposite if FuncFAb was immunogenic they could prove valuable as carrier and adjuvant in immunisation strategies. The immunogenic nature of the N-terminal domains of protein III was therefore further investigated.

Immunisations of mice and rats with the filamentous bacteriophage particle as carrier induces immune response against polypeptides fused to the phage coat protein III (de la Cruz et al., 1988) and protein VIII (Felici et al., 1993; Greenwood et al., 1991; Minenkova et al., 1993; Willis et al.,

1993; Yip et al., 2001) without the need for supplementary adjuvants like incomplete Freund's adjuvant (IFA). Aside from the whole phage particle also protein III alone is immunogenic (Minenkova et al., 1993; Tsunetsugu-Yokota et al., 1991). In our study, we analyse N-terminal fragments of protein III for their immunogenic properties. More precisely, we demonstrate the immunogenecity of the FuncFAb system.

Two scFvs were applied in the immunisation study – L36 and PACA17. PACA17 (selected as described in (Goletz et al., 2002)) bind in a competitive manner to the antigen combining site of the murine IgM antibody A46-B/B10, which recognizes the H type 2 trisaccharide (Fucα1-2Galß1-4GlcNAcß1) (Karsten et al., 1988). Thus PACA17 is an anti-idiotypic antibody, which potentially mimics the carbohydrate moiety recognised by the hybridoma antibody A46-B/B10. To prove the authenticity of the mimic, an immune response directed against the anti-idiotypic antibody, should also bind the carbohydrate moiety.

The two scFvs were purified as soluble non-fused protein as well as fused to DI. In the case of L36 also DI-DII fused scFv was prepared. All proteins were administered to groups of three mice, supplemented with either IFA, soluble DI or without supplements. Each mouse was immunised on day 0 and 14. Pre-immune sera and sera from day 28 was collected and analysed for the presence of IgG1 and IgG2a antibodies against non-fused L36 scFv (figure 18.A) and non-fused PACA17 scFv (figure 18.B). Our results show that an IgG immune response is generated towards the nearly non-immunogenic scFvs, when coadministered with IFA and soluble DI. Administration of scFv fused to DI likewise induces an IgG response towards the scFv. The soluble DI was only employed in the PACA17 study. Whereas the DI fusion proteins seemed to be slightly less immunogenic than non-fused scFvs administered with IFA, the non-fused PACA17 administered with an equal molar amount of soluble DI seemed to be slightly more immunogenic than the IFA mixture. One explanation for the augmented immune response towards non-fused PACA17 administered with soluble DI is that both PACA17 and DI carries the potentially immunogenic cMyc tag. When administered the mixture therefore carries twice as many cMyc tags in comparison to the fusion protein (Personal Communication, Peter Astrup Christensen). Whether soluble DI is a more potent adjuvant compared to fused DI, or whether the augmented immune response is simply an artifact of the additional cMyc tags remains to be elucidated, but the intrinsic immunogenecity of DI seems

indeed to confer adjuvant properties to the FuncFAb system. The DI-DII fused L36 scFv induced a scFv specific immune response comparable to the DI fusions.

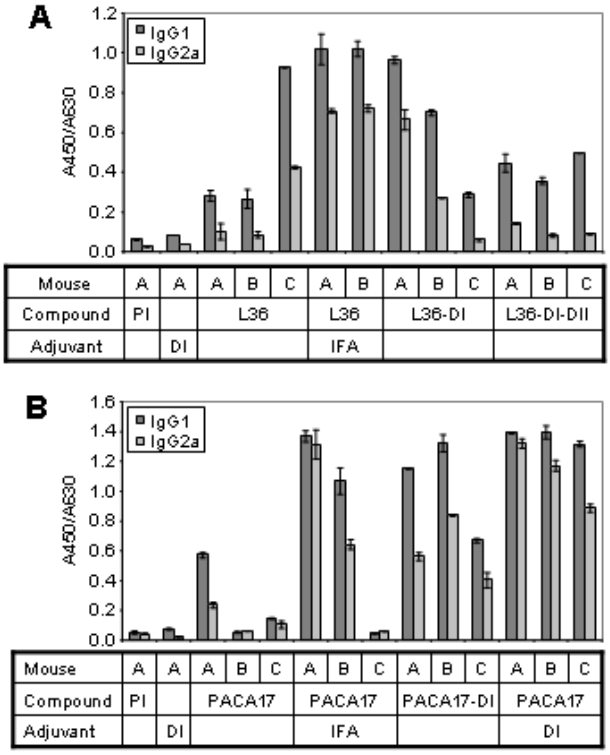


Figure 18 – Immune response generated by mouse immunisations with the FuncFab proteins. The bar diagrams show the immune response for different mice immunized with L36 scFv and its derivatives (A) and PACA17 scFv and its derivatives (B). The immune response was for each mouse determined specifically against the non-fused scFv from which the immunized protein originated. The immune responses were furthermore analyzed for their IgG1 and IgG2a subclass distribution. As controls pre-immune sera and sera from mice immunized with DI are tested for binding to the scFvs. All pre-immune sera as well as sera from DI immunized mice show equally low signal intensity respectively and each is therefore only represented with sera from one mouse. The error bars represent the standard deviation. Abbreviation: Domain I (DI), Domain II (DII), Pre-immune sera (PI) and Incomplete Freund's Adjuvant (IFA).

The adjuvant properties of the FuncFAb system holds great promise for vaccines based on phage display selected antigens, such as peptides (Fukuda et al., 2000) and anti-idiotypic antibodies (Reinartz et al., 2000; Tripathi et al., 1999) mimicking complex antigens like tumour associated carbohydrates (Goletz et al., 2002), glycolipids (De Bolle et al., 1999), phosphorylcholine (Binder et al., 2003) etc. As described in chapter 2.1 phage display selected polypeptides occasionally demand the presence of the N-terminal domains of protein III to retain activity (Jensen et al., 2002). In particular such polypeptides benefit from the adjuvant properties of the FuncFAb system, since they would be presented to the immune system in a correct conformation in contrast to traditional adjuvant systems. For conformational dependent mimotopes, this is a prerequisite for a successful vaccination.

2.2.2 Analysis of the mimicking properties of PACA17 and R5

Sera from mice immunised with the anti-idiotypic scFv PACA17 were tested againt a panel of carbohydrates, all structurally related to the H-type 2 trisaccharide (figure 19). Binding of PACA17 sera to Fucose, H-type disaccharide, H-type 2 trisaccharide, LeY and Le-b shows recognition of

primarily H-type disaccharide and Fucose but also H-type 2 trisaccharide. The response is solely IgM (figure 20.A) except for two mice immunised with either PACA17 scFv fused to DI or supplemented with soluble DI showing IgG response (figure 20.B) towards Fucose and H-type disaccharide but not H-type 2 trisaccharide. The data suggest that PACA17 is mimicking parts of or the whole H-type 2 trisaccharide, thereby enabling recognition of one or more sugar moieties contained in the trimeric structure. Our observations show clear IgG response against the PACA17 scFv (figure 18.B). The fact that no class switch occurs from IgM to IgG for binding to the sugar moieties could simply be due to affinity. The class switch of multivalent IgM antibodies into divalent IgG antibodies decreases the avidity effect below a critical level, where IgG antibodies are undetectable. This is further supported by the fact that the monoclonal A46-B/B10 antibody, against which PACA17 was selected, is an IgM antibody, thus suggesting that class-shift is difficult to induce for the H-type 2 trisaccharide antigen.

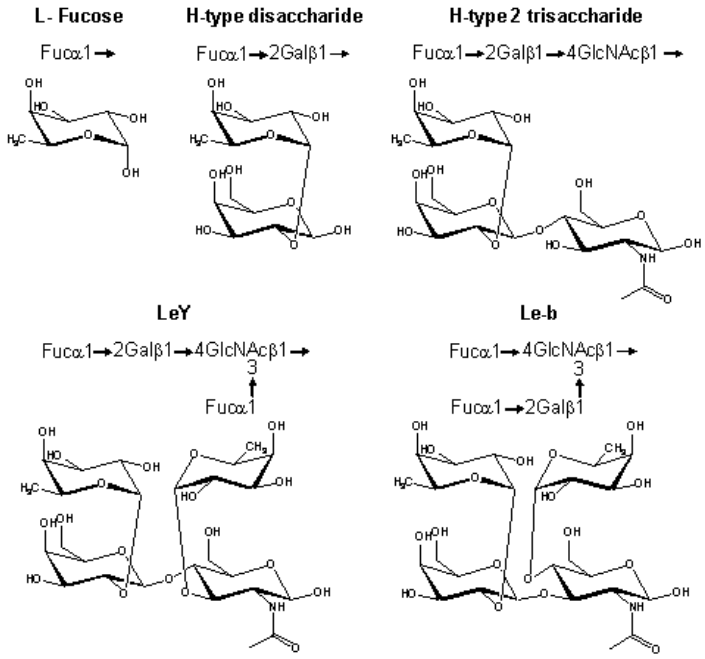


Figure 19 – Schematic structures of L-Fucose, H-type disaccharide, H-type 2 trisaccharide, LeY and Le-b.

The anti-idiotypic R5 scFv (cf. chapter 2.1) was also tested for mimicking the paratope of the corresponding monoclonal antibody A76-A/C7. Sera from three mice immunized with R5 scFv supplemented with IFA were analysed for binding to a 30aa peptide including PDTR repeated motifs with or without TF glycosylation on the threonine. The ELISA experiment showed no recognition of the PDTR sequence independent of glycosylation with the R5 sera (Anja Löffler, Unpublished results).

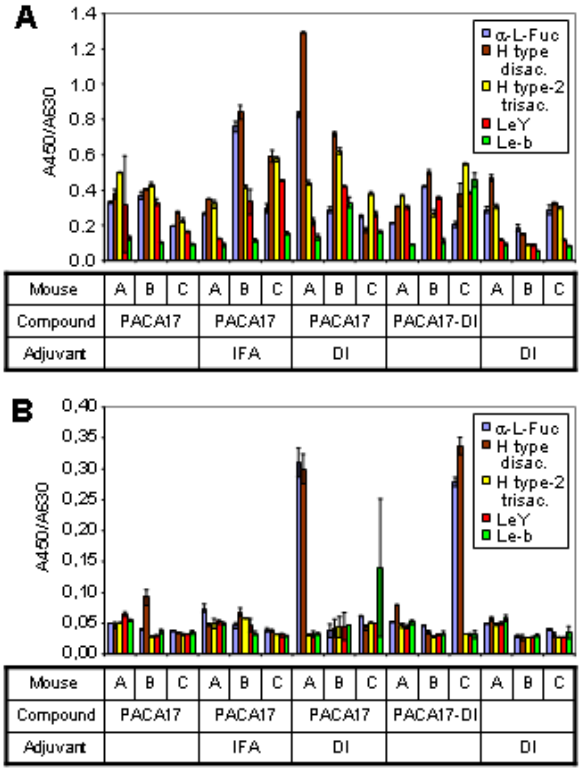
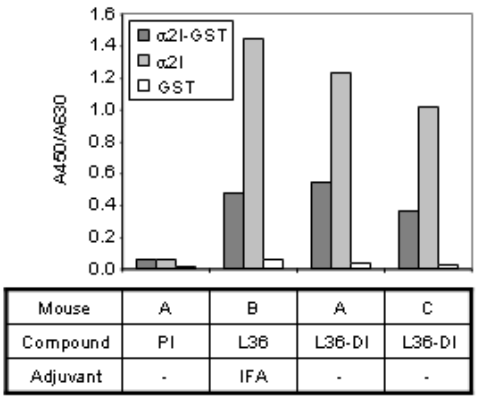


Figure 20 – Anti-idiotypic response against the PACA17 scFv. The bar diagrams show the IgM (A) and IgG (B) humoral immune response for mice immunized with PACA17 scFv supplemented with IFA, DI or without supplements as well as PACA17 scFv fused to DI. The immune response was for each mouse determined specifically against the three related carbohydrate structures, α-L Fucose (α-L-Fuc), H type disaccharide (H type disac.) and H type-2 trisaccharide (H type-2 trisac.) as well as the two unrelated negative controls LeY and Le-b. As a further negative control sera from mice immunised with DI alone were included. The error bars represent the standard deviation. Abbreviation: Domain I (DI), Incomplete Freund's Adjuvant (IFA).

The difficulty of generating Ab2β anti-idiotypic antibodies that are mimics of the paratope from the corresponding antibody is an intrinsic feature of Jerne's network theory (cf. chapter 1.2.1.2 and figure 7). The theory suggests that anti-idiotypic antibodies recognise the antigen binding site (the paratope) and a large range of other potential binding sites on the primary antibody (Ab1). Therefore it is expected that the majority of anti-idiotypic antibodies are Ab2α, which are not binding the paratope of Ab1, and are thus not structural mimics of the original antigen. The anti-idiotypic antibody selection method employed in our work (Goletz et al., 2002) seeks to favour anti-idotypic antibodies binding the antibody binding site of the primary antibody (Ab2β and Ab2γ). The strategy is to elute the phage displayed scFvs with the original antigen they are supposed to mimic. Due to sterical reasons anti-idiotypic scFvs binding in the vicinity of the paratope (Ab2γ) are eluted as well, and a rather large number of anti-idiotypic scFvs not having mimicking properties are therefore also enriched. It is therefore not surprising that we had minimal success with the mimicking properties of R5 and PACA17. At the same time it suggests that the FuncFab adjuvant system combined with the improved selection method (Goletz et al., 2002) would be a rational way to establish and identify Ab2β anti-idiotypic antibodies, although screening of several anti-idiotypic antibodies seems necessary.

2.2.3 Analysing the antagonistic effect of L36

The FuncFAb immunisation protocol enables analysis of the mechanisms behind polypeptides that are selected as agonists or antagonists for a biological pathway. Immunisaition with such polypeptide will potentially lead to the production of antibodies mimicking the binding site on the target protein, and potentially even antibodies binding the biological binding partner. The L36 scFv is such an example.



Mouse	A	B	A	C
Compound	PI	L36	L36-DI	L36-DI
Adjuvant	-	IFA	-	-

Figure 21 – Laminin-I mimicking anti-idiotypic antibodies recognising the recombinant human integrin - α2 I-domain. Serum from mice immunised with either L36 scFv non-fused (L36) supplemented with incomplete Freund's Adjuvant (IFA) or L36 scFv fused to domain I of filamentous bacteriophage coat protein III (L36-DI) was tested for components binding to the human α2 I-domain integrin. The recombinant α2 I-domain was coated in ELISA wells fused or non-fused to GST and non-fused GST was coated as negative control. The pre-existence of α2 I-domain antibodies in mice sera was tested with pre-immune (PI) sera.

Sera from mice immunised with L36 derivatives were first tested for the presence of anti-idiotypic antibodies by testing the sera's ability to block binding of L36 to Laminin-I. The experiment showed that this was indeed the case and that the efficiency was proportional with the overall IgG immune response. To demonstrate the presence of Laminin-I mimotopes, the sera was analysed for binding to a polyclonal Laminin-I antibody. This showed that mimotopes were present in some but not all mice. The data further suggest that the successful induction of mimicking anti-idiotypic antibodies is not due to the strength of the immune response but merely the characteristic of the B-cell population responsible for the humoral immune response in the mice. We could thus conclude that in some mice Laminin-I mimicking anti-idiotypic antibodies were induced, and we hypothesised that such mimics would resemble the Laminin-I epitope recognised by L36. L36 inhibits angiogenesis by blocking the interaction between Laminin-I and the α2 integrin subunit from α2β1 integrin (Sanz et al., 2003). Therefore we decided to test whether the Laminin-I mimics present in the mouse sera could bind to the α2 integrin subunit, as was shown for the L36 derived Laminin-I peptide mimic, KHA (Martin and Schmid, 2003b). The binding was analysed by coating recombinant human integrin α2 I-domain fused as well as non-fused to a GST tag. GST tag alone was included as negative control. The α2 I-domain refers to the fact that the α2 subunit belongs to a subset of α integrin chains having an approximately 200 residue insertion near the N-terminal often

referred to as the I (inserted) domain. The ELISA confirmed the binding to the α2 I-domain with serum derived antibodies mimicking the Laminin-I binding site (figure 21), and our study therefore nicely confirms the previous data from Álvarez-Vallina and co-workers showing that L36 is indeed a molecular mimic of the α2β1 integrin.

2.3 "Filamentous bacteteriophage coat protein III domain I in DNA vaccines promotes a Th1-type dominated immune response" – summary of Appendix C.

The L36 scFv non-fused or fused to DI was subcloned into the eukaryotic expression plasmid pCR3.1 and the plasmids were analysed for their ability to induce expression and secretion of scFv derivatives from lipofectamine transfected human embryonic kidney 293T cells. Supernatant from the cells were analysed in western blot for the presence of scFv derivatives, and in ELISA for binding of Laminin-I with active L36 scFvs. The experiment showed presence of both active L36 scFv and active L36 fused to DI, whereas the empty plasmid showed no sign of expressed protein.

The three plasmids where subsequently applied in an immunisation study and L36 scFv protein mixed with IFA was included as positive control. The immunisation with protein and DNA vaccines was administered i.p. and i.m., respectively. Sera from immunised mice were subsequently tested for the presence of IgG1 and IgG2a antibodies raised against L36 scFv. The sera from mice immunised with L36 scFv encoding pCR3.1 supplemented with IFA (but not without IFA) and L36 scFv fused to DI encoding pCR3.1 were indeed containing such antibodies. Another ELISA of sera recognition of a polyclonal Laminin-I antibody suggested that anti-idiotypic antibodies mimicking Laminin-I were also present. In contrast to protein immunisations the IgG1 and IgG2a ratio was interestingly reversed, making IgG2a higher expressed than IgG1, thus suggesting that the immune response is Th1 mediated.

2.4 Concluding remarks on the FuncFAb system

The FuncFAb system has proven to be a very strong tool for re-establishing protein functionality, in particular for inactive scFvs selected by phage display. We propose that the activity of a scFv can improve tremendously by fusion to the N-terminal domains of protein III, and further that fusion to DI of protein III can induce multimerisation leading to even better affinities due to an avidity effect. The stability is unaltered by fusion to DI or DI-DII. The observed multimerisation and chaperone theory inspired us to investigate if scFvs fused or non-fused to the N-terminal domains of protein III could bind non-covalently to the wild-type protein III or deletion mutants thereof. Interestingly we showed that non-fused scFvs could indeed bind to protein III when either DI or DII were deleted. Also scFv fused to DI could bind the DII deleted protein III.

Recently an EST fusion system to DI was published enabling easy purification of denaturated protein and subsequent refolding for use in phage selections. The low pI of DI improves the coating of protein on plastic surfaces, and the subsequent biopanning would not lead to selection of scFvs binding the DI fusion tag, since DI binding scFvs would bind other DIs present on the phage in the pool, and would thus not be selected (Frisch et al., 2003). This principle could be expanded to phage selection with single-framework libraries, where purified and coated DI fused scFvs could be used as selection targets for the isolation of anti-idiotypic antibodies. Antibodies not binding the antibody combining site would bind other phage displaying both DI and the scFv framework. This strategy might improve generation of anti-idiotypic antibodies by phage display compared to phage display isolated anti-idiotypic antibodies against hybridoma antibodies (Goletz et al., 2002).

The adjuvant effect obtained using the FuncFAb system is both observed for protein immunisations (i.p.) and DNA immunisations (i.m.). The immune responses seem to induce a skewed IgG1/IgG2a humoral response with more IgG1 than IgG2a for the protein immunisation, and reversed for the DNA immunisation. The general interpretation of the IgG1/IgG2a response suggests that FuncFAb protein immunisations preferable lead to Th2 response, whereas FuncFAb DNA immunisations primarily lead to Th1 response. The Th1 type response is believed to mediate CTL response, which is preferred in several vaccination strategies e.g. against carcinomas (Dredge et al., 2002). As such it is difficult to choose a vaccination strategy due to theoretical contemplations, but the FuncFAb adjuvant system enables the easy analysis of the effect of both Th1 and Th2 mediated immune responses. Many studies have shown the same vaccine administration and format dependent

Th1/Th2 response, but also opposite examples are reported; like Th2 inducing DNA vaccination (Serezani et al., 2002). Since our results suggest that there is no need for additional adjuvants, the FuncFAb adjuvant system might have immense impact on future vaccine strategies for humans. That the FuncFAb adjuvant system can be administered to humans without hazardous side effects is supported by the administration of filamentous bacteriophage in immunisations of monkeys and chimpaneez (Galfre et al., 1996) as well as the successful use of other strains of bacteriophage for human therapy (reviewed by Adhya and co-workers (Merril et al., 2003) and Summers (Summers, 2001)).

2.5 Generation of a single-chain MHC phage display system

As described in chapter 1.1.2.4.1.1 the MHC class I receptor is a heterodimer composed of the human leukocyte antigen (HLA), a 45 kDa integral membrane glycoprotein, associated with the 12 kDa soluble β2-microglobulin (β2MG). In 1983 a full length cDNA clone was obtained for the family of HLA homologous proteins in mice – H-2Kd (Lalanne et al., 1983). Thereby, it was clarified that the open reading frame of the H-2 family encoded a 21 amino acids long N-terminal signal peptide followed by the three extra cellular domains (α1, α2 and α3). The trans-membrane domain integrating the HLA protein in the cell membrane is defined as the protein sequence from Val285 to Met309 and the succeeding last 38 C-terminal amino acids are located in the cytoplasm. The domain structure of H-2 is visualised in figure 22 and can be directly transferred to the human homologous proteins – HLAs.

Figure 22 – scMHC gene construction. (A) The primary protein sequence for the homologous proteins H-2K (mouse) and HLA-A2 (human) are aligned and relevant sequences are highlighted – signal peptide (purple), transmembrane domain (green) and start and stop sequence (underlined) for the HLA extracellular fragment applied in the single-chain MHC constructs in earlier studies (H-2K) (Le Doussal et al., 2000) and the present study (HLA-A2). (B) The same primary sequence is depicted as a block diagram with the extracellular and cytoplasmic domains in cyan. Further the single-chain MHC constructs analysed in this study are depicted as block diagrams with peptide (yellow) and β2-MG (light brown).

A single-chain variant of the MHC class I molecule can be constructed in two ways as indicated in figure 22. Either the HLA protein is fused to the N-terminal of β2MG (scMHC HLA-linker-β2MG) or the β2MG is fused to the N-terminal of HLA (scMHC β2MG-linker-HLA). To display the fusion protein on the phage the C-terminal of the fusion protein is genetically fused to the N-terminal of the gIIIp. Both designs are functional as fusion proteins (Denkberg et al., 2000; Godeau et al., 1992; Lee et al., 1996; Lone et al., 1998; Mage et al., 1992; Mottez et al., 1991; Sylvester-Hvid et al., 1999; Toshitani et al., 1996) and for the mouse MHC class I genes peptide-H-2K^{d}-β2MG and peptide-β2MG-H-2K^{k} single-chain constructs have been displayed in phage display systems, although activation of T cells have not been observed (Le Doussal et al., 2000; Vest Hansen et al., 2001). Kato and co-workers reported the use of a phage displayed peptide-HLA-β2MG construct employing the HLA-B*5101 allele as well as a novel technique to proteolytically determine the presence of bound peptide in the MHC Class I molecule. This technique was however only employed for the mouse peptide-H-2D^{b}-β2MG construct (Kurokawa et al., 2002). In the work presented here both types of constructs described above have been made for the human MHC class I molecule and tested for display and ability to bind as well as activate a specific T cell line.

Alternatively, a functional MHC class I molecule could be designed resembling the F_{ab} fragment phage display, where the one fragment is attached to the phage particle and the other expressed as a soluble protein. This system has been made to work on the protein level covalently fusing a peptide to the N-terminal of the β2MG fragment (Tafuro et al., 2001; White et al., 1999). Advantages entailed in the single-chain format employed in this study are that the protein subunits are always present in molar equal amounts, and the affinity towards a target is not as affected by the irreversible dissociation of the non-fused subunit. The folding of the single-chain molecule is of course important as well, but the unfolding of the protein can potentially be reversible, since the subunits stay in proximity of each other. The folding of MHC complexes *in vivo* is an extremely complex process involving a number of chaperones (reviewed by Wang and co-workers (Paulsson and Wang, 2003)). The functional folding of single-chain MHC molecules in *E. coli* therefore theoretically could involve tremendous obstacles. Another factor having immense influence on the stability of a single-chain molecule is the linkers connecting the subunits.

2.5.1 Linker Design

Designing linkers for a fusion molecule is a comprehensive work, which should cover research into primarily three areas. 1) Three-dimensional computer models (e.g. crystal- or NMR structures), 2) linkers employed in prior studies and 3) experimental screening of potential linkers.

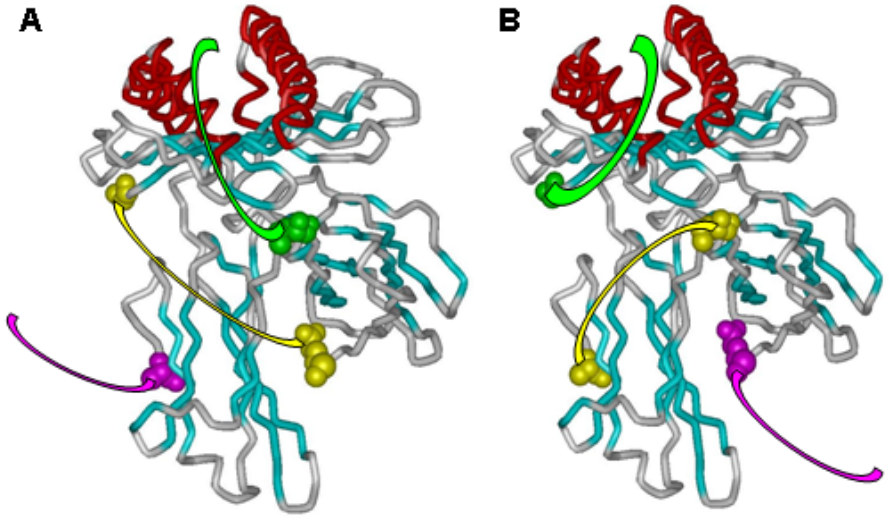


Figure 23 – ScMHC structural model. (A) Structural model showing the hypothetical linkers used for the construction of scMHC β2MG-HLA. (B) The same drawing for the scMHC HLA-β2MG construct. The green linker corresponds to L1, the yellow linker to L2 and the purple linker indicates where the bacteriophage gIIIp is attached. Structural data originate from the 3HLA structure (Saper et al., 1991).

The theoretical analysis of intra molecular distances within the MHC molecule is presented in table 5. The analysis has been performed on the two crystal structures 1HHG (Madden et al., 1993) and 3HLA (Saper et al., 1991) with and without a nonameric peptide bound in the groove respectively. The terms L1 and L2 refers to the linker connecting the peptide to the MHC class I fusion protein and the linker between HLA and β2MG, respectively (as shown in figure 22). A modified structure showing the linkers and docked peptide is shown in figure 23.

Molecular reference	**1HHG**		**3HLA**
Linker	**L1**	**L2**	**L2**
scMHC HLA-β2MG			
Straight line distance	35Å /9 a.a.	51Å /14 a.a.	41Å / 11 a.a.
Semicircle distance	56Å / 15 a.a.	80Å /21 a.a.	65Å / 17 a.a.
scMHC β2MG-HLA			
Straight line distance	20Å / 5 a.a.	30Å / 8 a.a.	32Å / 8 a.a.
Semicircle distance	31Å /8 a.a.	48Å / 13 a.a.	50Å /13 a.a.

Table 5 - Schematic overview of intramolecular distances within the extracellular domains of the MHC molecule. Distances are given in Å and recalculated into number of amino acids spanning such a distance using the fact that the longest distance a peptide can span is #[a.a.]×3,8Å/a.a. (Creighton, 1993). The distances measured on 3HLA indicate that the MHC molecule not binding a peptide has the same approximate distances as 1HHG with a peptide bound for the scMHC β2MG-HLA, whereas the distances differs quite a lot for the scMHC HLA-β2MG.

A list of linkers, used in previous studies investigating single-chain constructs of MHC class I molecules, is presented in table 6. This list includes studies of single-chain molecules with and without covalently fused peptide.

Length	Linker sequence	Note	Reference
6 (L1) 10 (L1) 13 (L2)	GGGSGG $(GGGS)_2GG$ $GGG(SGGGG)_2$	Fusion: Peptide-linker- β2MG Fusion: Peptide-linker- β2MG Fusion: β2MG-linker-HC-K^k	(Vest Hansen et al., 2001)
10 (L1) 15 (L2)	GGPGGGSGGG $GGIGS(GGGGS)_2$	Fusion: Peptide-linker-H-$2K^d$ Fusion: H-$2K^d$-linker- β2MG	(Le Doussal et al., 2000)
8 (L1) 12 (L1)	$(GGGS)_2$ $(GGGS)_3$	Fusion: Peptide-linker- β2MG	(Uger and Barber, 1998)
13 (L1)	$GGGGS(GGGS)_2$	Fusion: Peptide-linker- β2MG	(White et al., 1999)
15 (L1)	$GGGG(GGS)_3GG$	Fusion: Peptide-linker- β2MG	(Tafuro et al., 2001)
15 (L1)	$GGGG(GGS)_3GG$	Fusion: Peptide-linker- β2MG	(Tafuro et al., 2001)
15 (L2)	$(GGGGS)_3$	Fusion: β2MG-linker-H-$2D^d$	(Mage et al., 1992)
15 (L2)	$(GGGGS)_3$	Fusion: β2MG-linker-HLA-A2.1	(Lee et al., 1996)
15 (L2)	$(GGGGS)_3$	Fusion: β2MG-linker-HLA-A2	(Toshitani et al., 1996)
15 (L2)	$(GGGGS)_3$	Fusion: β2MG-linker-HLA-A2	(Denkberg et al., 2000)
10 (L1) 13 (L2)	$(GGGS)_2GG$ $GGG(SGGGG)_2$	Fusion: Peptide-linker-H-K^k Fusion: H-K^k-linker-β2MG	(Sylvester-Hvid et al., 1999)
2 (L2) 10 (L2) 13 (L2) 15 (L2) 17 (L2) 19 (L2) 21 (L2)	GG GGIGSGGGGS GGIGSGGSGGGGS $GGIGS(GGGGS)_2$ $GGIGSGS(GGGGS)_2$ $GGIGSGGGS(GGGGS)_2$ $GGIGSGGGGGS(GGGGS)_2$	Fusion: H-K^d-linker-β2MG All linkers work, but it is suggested that free monkey β2MG (COS-1 cells) can bind to the fusion protein in the case of the 2 a.a. linker.	(Mottez et al., 1991)
15 (L2)	$GGIGS(GGGGS)_2$	Fusion: H-$2K^d$-linker-β2MG	(Godeau et al., 1992)

Table 6 - Different linkers utilised for construction of single-chain MHC molecules. In the first column linker length and linker type (L1 or L2) is stated. Linker type refers to the terms introduced in figure 22.

From the data introduced above it was decided to use the standard 15 amino acids glycine-rich linker as L2. This linker is also utilised for scFv, and as such a firmly established linker. It was further decided to test a 10 amino acids and a 15 amino acids glycine-rich linker as the L1 linker. The same linkers were used for both constructions of the scMHC genes (figure 22).

The peptide genetically fused to the N-terminal of the constructed scMHC genes can principally be any peptide and the exchange of peptides or synthesis of peptide repertoires can easily be performed using the NcoI and XhoI restriction sites. In the model system the MART-$1_{(27-35)}$ peptide (AAGIGILTV) was chosen because of its ability to bind and activate a T cell receptor expressing $CD3^+/CD8^+$ Jurkat cell line, when presented on MHC class I molecules. The DNA and protein sequence for the different constructs can be found in appendix E.

The $CD3^+/CD8^+$ Jurkat T cell line is transfected with a T cell receptor specific for the HLA-A2 presented MART-$1_{(27-35)}$ peptide. We therefore established the scMHC construct composed of the HLA-A*02011 allele. This constellation was chosen to establish proof of principle, but since an HLA-A3 donor was found we also established a single-chain construct based on this allele. Some studies on folding and display have consequently been performed on both HLA-A2 and HLA-A3 scMHC molecules.

2.5.2 Optimisation of Display Level for the scMHC Construct

As stated in the introduction the display level is a parameter describing the fraction of phage displaying a gIIIp fusion protein. In the phagemid type 3+3 system, which is the method of choice in the presented work, the display level is highly influenced by the size and toxicity of the gIIIp fusion protein. Normally the display level varies between 0.1%-10% for scFv. Measurements showed display levels less than 0.1% for the scMHC fusion protein, and effort was therefore put into increasing the display level.

The general parameters that have been varied in other studies on display level and phage production are temperature, IPTG induction, leader sequence and incubation time. Observations showed that low temperature (below 30°C) and optimised pelB leader mutants could increase the display. Furthermore, the lower the growth temperature the better it was to induce phage production with IPTG (Jestin et al., 2001). Two studies on phage display of scMHC also describe the growth condition used – interestingly the conditions were not identical. In the first study (peptide-H-$2K^d$-β2MG) phage were produced at 30°C, mentioning that a 5 time decrease in display level was observed, when phage were produced at 37°C (Le Doussal et al., 2000). The later study (peptide-β2MG-H-$2K^k$) reports that phage produced at 30-37°C gives no signal in ELISA, whereas phage produced at up to 24°C reaching maximum at 18°C gives signals in ELISA. Consequently the authors suggest overnight phage production at low temperatures (Vest Hansen et al., 2001).

The optimisation study performed in this report was done on a scMHC clone encoding the HLA-A3 allele. The study showed that the display level increased with decreasing growth temperature (table 7), thereby supporting the observations made by Vest-Hansen NJ. We further performed a time-laps study on the phage production measuring phage titer and display level after varying periods of time

at 18°C. The data indicates that a long production period (at least over night) is beneficial for the yield of displaying phage (figure 24). Although this suggests that prolonged incubation is somewhat beneficial for the total phage titer, we performed the following study with incubation times ranging from 18-24 hours for practical reasons.

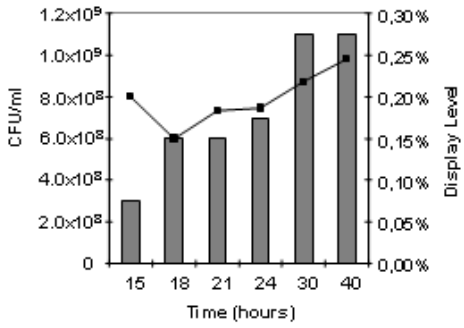


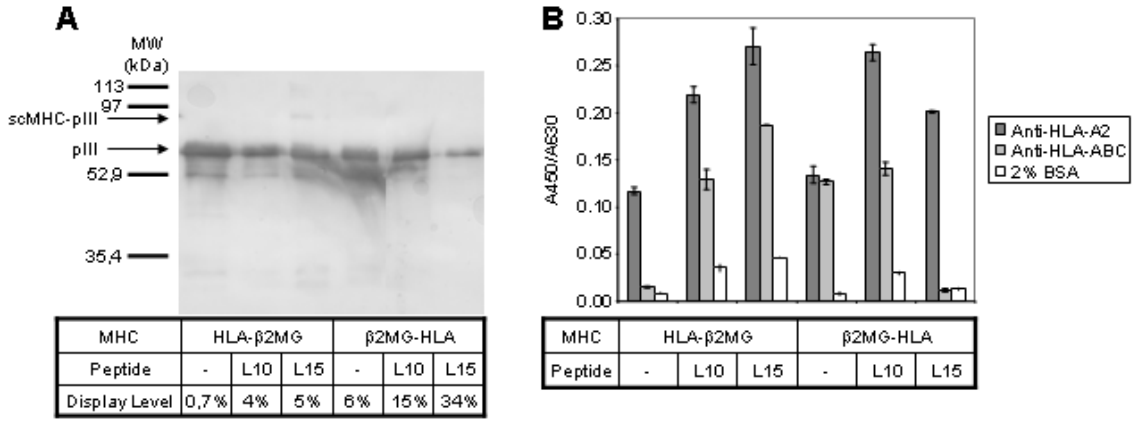
Figure 24 – Time-laps study of display level and phage production of scMHC-gIIIp fusion phages. The HLA-A3-β2MG pHEN2 clone not including any genetically fused peptide was arbitrarily chosen as model clone. Phage production was performed at 18°C in a 250ml culture flask including 50ml culture. Samples were collected after varying time intervals as indicated and total phage titer as well as display level was measured. The β2MG-HLA-A3 L10 pHEN2 clone including the MART-1 peptide covalently linked through a 10mer glycine rich linker was tested as well with the same take-home message, but with display levels that could first be detected after 24h (Data not shown).

Temperature	- trypsin	+ trypsin	Display level
18 °C	$2{,}0\times10^{11}$ cfu/mL	$1{,}0\times10^{9}$ cfu/mL	0,500%
25 °C	$4{,}0\times10^{11}$ cfu/mL	$7{,}0\times10^{8}$ cfu/mL	0,175%
32 °C	$3{,}0\times10^{12}$ cfu/mL	$1{,}0\times10^{8}$ cfu/mL	0,003%

Table 7 - Temperature optimisation of display level. Analysis of the HLA-A3-β2MG scMHC construct shows that low growth temperature greatly increases the display level although decreases the phage titer slightly. The number of colony forming units (cfu) resembles the amount of infective phage.

The study was extended to the scMHC constructs encoding the HLA-A2 gene with and without genetically fused peptide. The phage were produced by over night incubation at 18°C and were subsequently precipitated. The phage stock was then analysed for display level by western blotting detecting protein III as fusion or wild-type protein and by proteolytically treatment with trypsin (figure 25.A). Our proteolytically determined display levels suggest that fused peptide scMHC constructs give higher display levels than unfused peptide scMHC constructs. This observation could be explained by the fact that empty MHC class I molecules are less stable than peptide-loaded ones (Abdel-Motal et al., 1996). The HLA-A2-β2MG L15 phage showed a very weak band by western blotting, although no fusion protein III band was observed for the remaining scMHC phage (figure 25.A). The discrepancy of the proteolytically determined display level and the western blot data is most probably due to proteolytic degradation of the scMHC protein during propagation, but also due to proteases still present after PEG precipitation. Cellular expressed MHC molecules are extensively glycosylated, which is though to protect against proteases (Rudd et al., 2001). The problem of proteolytic degradation of phage displayed scMHC molecules was similarly observed by Gorochov and co-workers, who despite using a phage vector system did not observe display levels

above 1% (Le Doussal et al., 2000). Further investigation into parameters important for decreasing proteolytic degradation during phage production might be able to increase the number of full length fusion proteins displayed on phage. A phage production setup that would be interesting to investigate includes decreased propagation time and immediate and repetitive PEG precipitation.



MHC	HLA-β2MG			β2MG-HLA		
Peptide	-	L10	L15	-	L10	L15
Display Level	0,7%	4%	5%	6%	15%	34%

MHC	HLA-β2MG			β2MG-HLA		
Peptide	-	L10	L15	-	L10	L15

Figure 25 – Analysing the display of the scMHC molecules. (A) The phage protein III (theoretically 44 kDa) as well as protein III fusion molecules (theoretically 92 kDa) are detected by western blotting of each scMHC phage variant. The actual migration length of protein III and scMHC protein III fusion molecules are indicated. Display levels determined by trypsin treatment are also specified. (B) Correct folding of the displayed scMHC protein was determined in an ELISA showing phage binding to coated HLA-A2 specific antibody and coated HLA-ABC (W6/32) antibody. Non-coated wells are negative control.

2.5.3 Verification of correct folding of displayed single-chain MHC molecules.

Next step was to verify that the displayed fusion proteins were actively presented. The ultimate test for activity is to show that the constructs can initiate T cell activation. This will be further explored in chapter 2.5.7. An indirect technique for measuring correct protein folding is to test whether it is recognized by monoclonal antibodies against a conformational epitope on the native protein. The antibody used for this purpose is the W6/32 hybridoma antibody, named anti-HLA-ABC antibody, which recognises the human lymphocyte antigen (HLA) for the three families of alleles A, B and C. As positive control a non-conformational specific antibody against HLA-A2 was applied. The data show that the HLA-A2-β2MG displaying phage were not recognised. This could be due to the lacking peptide, although the β2MG-HLA-A2 construct did give signals in ELISA. Unexpectedly the peptide-β2MG-HLA-A2 displaying phage with a 15 a.a. glycine-rich peptide linker were also not recognised by the conformational antibody (figure 25.B). The reason for this phenomenon could be that L1 (the peptide linker) interferes or covers the epitope on the HLA molecule. This is supported by other studies, where the N-terminal elongation of β2MG has been observed to interfere with binding of W6/32 (Barouch et al., 1995; Shields and Ribaudo, 1998).

2.5.4 Phenotypic characterisation of the $CD3^+/CD8^+$ Jurkat T cell line.

In the introduction to T cell activation it was described how the mechanisms responsible for T cell activation are due to a diverse set of cell-cell interactions. These interactions are reviewed by Geppert (Geppert et al., 1990), and it is indicated that activation of T cells requires colocalisation of multiple TCRs on the T cell surface. Furthermore the requirement for cell-cell interactions can partly be supplied *in trans* by an accessory cell or agonistic antibodies against cell surface molecules on the T cells. The T cell clone employed in this study is the $CD3^+/CD8^+$ Jurkat T cell line transfected with a MART-1 specific T cell receptor (Unpublished results, Maeurer MJ, Mainz, Germany). The positive control for the T cell activation assay was the T cell activation with a tap deficient T2 cell line (Salter et al., 1985) loaded with peptide as reported by Nishimura and co-workers (Cole et al., 1995). Due to the tap deficiency no endogenous peptides are presented by the MHC Class I molecules. Instead the T2 cells can be loaded *in vitro* with HLA-A2 specific peptides. Due to their ability to activate T cell clones when loaded with a T cell specific peptide, we concluded that unloaded T2 cells contains the required costimulatory molecules, but not the primary peptide presenting MHC class I molecule. The cell line is therefore suitable as an accessory cell line.

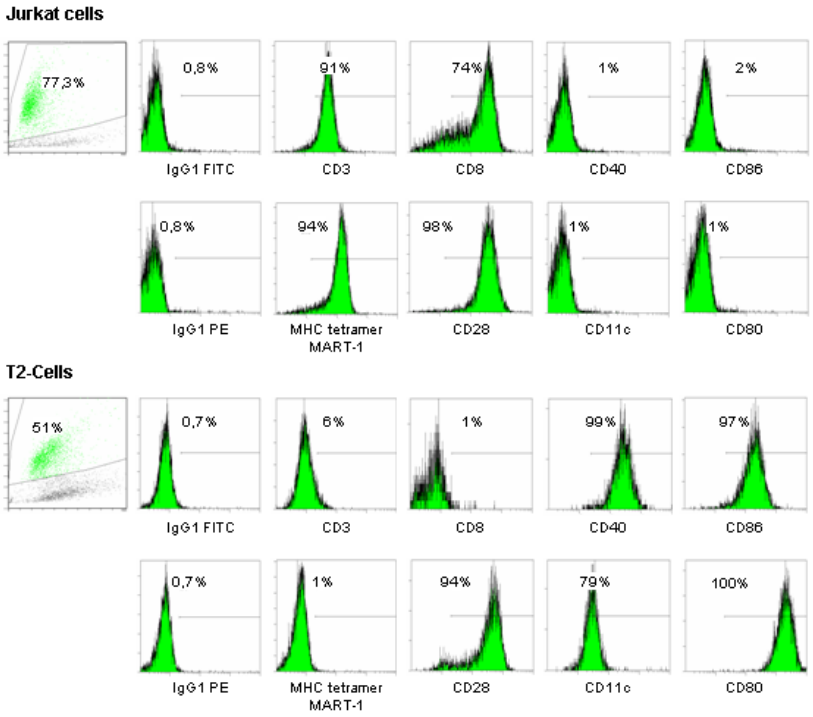


Figure 26 – Flow cytometry analysis of cell surface proteins on $CD3^+/CD8^+$ Jurkat cells and T2 cells. $CD3^+/CD8^+$ Jurkat cells or T2 cells were labelled with fluorescent labelled IgG1 subclass antibodies against CD3 (FITC), CD8 (FITC), CD11c (PE), CD28 (PE), CD40 (FITC), CD80 (PE) and CD86 (FITC). Also MART-1 specific TCR was detected utilising a MART-1 loaded scMHC tetramer (PE). Subsequently positive cells out of totally 10.000 cells gated for vitality were detected in flow cytometry. A fluorescently labelled non-specific IgG1 antibody was used as negative control. The MART-1 loaded scMHC tetramer had no cell specific negative control, only was it positive for the $CD3^+/CD8^+$ cell line and negative for the T2 cell line.

Flow cytometry on the Jurkat T cell line as well as the antigen presenting T2 cell line was performed to verify the presence or absence of costimulatory molecules as well as the surface

antigens recognised by the agonistic antibodies mediating T cell activation (cf. chapter 2.5.7.4). We observed that CD3 and CD8 were only present on the Jurkat cells and not on the T2 cells. The co stimulatory molecules CD40, CD80 and CD86 were on the other hand only present on T2 cells, whereas CD11c and CD28 were present on both cell types. We further analysed the binding of a soluble MHC class I molecule loaded with the MART-$1_{(27-35)}$ peptide and cross linked with streptavidin into a tetramer. The tetramer bound to the Jurkat T cells but not to the T2 cells (figure 26).

2.5.5 The importance of TCR clustering

The need for TCR clustering (Sedwick et al., 1999) suggests that the MHC Class I molecules need to be clustered. The phage displayed MHC class I molecules are primarily expressed monovalently, and may therefore not be able to induce the necessary cross-linking of receptors on the T cell surface. The studies using purified scMHC protein to activate T cells have been successful. In one of the first studies it was determined that monomeric mouse derived scMHC loaded with specific peptide could bind to a specific T cell hybridoma, although activation depended on dimerisation and was further increased by higher order multimers (Motta et al., 1998). In most studies scMHCs were biotinylated and bound to streptavidin in a tetrameric form, thereby increasing the avidity and possibility for receptor cross-linking (Denkberg et al., 2000; Lone et al., 1998). Other soluble constructs of the MHC class I molecule have likewise been tested positive for T cell activation (Cullen et al., 1999; McCluskey et al., 1988). A later study by Busch and co workers (Knabel et al., 2002) indicated that TCR bound tetrameric MHC class I molecules could be released from the TCR by elution with biotin, thereby removing the tetramerisation agent, streptavidin. It therefore seems that it will only be possible to establish binding of monomeric MHC class I molecules for very high affinity TCR. The ability to bind monomeric MHC Class I molecules have not been determined for the $CD3^+/CD8^+$ Jurkat T cell line employed in our studies, but observations by Nishimura and co-workers (Cole et al., 1995) suggest that multivalent display of MHC class I molecules loaded with the MART-1 peptide is needed for activation.

2.5.6 T cell activation with peptide loaded T2 cells

T2 cell activation of primed $CD8^+$ T cells have been standardised in our laboratory to a 1:10 ratio of T2 cells to $CD8^+$ T cells for a number of frequently used peptides (cf. chapter 4.5). For the T2 activation of Jurkat T cells we therefore analysed a range of T2:T cell ratios with 1:10 as reference.

Of the ratios tested a 1:5 ratio (40.000 T2 cells per 200.000 T cells) gives the highest activation although it seems that a higher amount of T2 cells could increase the activation further. The activation was determined as the rate of IL-2 secretion (figure 27.A). Addition of higher amounts of T2 cells was not pursued due to the signal saturation in ELISPOT assays obtained with the 1:5 ratio when 200.000 T cells are employed (figure 27.B). Therefore the 1:5 ratio was applied in the remaining studies. We further tested the specificity of the response measuring the activity as the number of IFN-γ secreting Jurkat cells. The specificity was determined by activation with unloaded T2 cells as well as T2 cells loaded with three unspecific peptides and the specific MART-1 peptide. As can be observed the response seems not to be very specific since responses towards MART-1 and HIV-GAG peptides seems equally strong and only slightly stronger than responses towards the MUC-1 and HIV-ENV peptides. Unloaded T2 cells do not activate the T cells to a level comparable to peptide loaded T2 cells (figure 27.B). The Jurkat T cell line was earlier reported to be specific, although this analysis was only on two unspecific peptides (MART-$1_{(22-30)}$ and MART-$1_{(32-40)}$) derived from the transmembrane domain of MART-1 compared to the specific peptide (MART-$1_{(27-35)}$) (Cole et al., 1995).

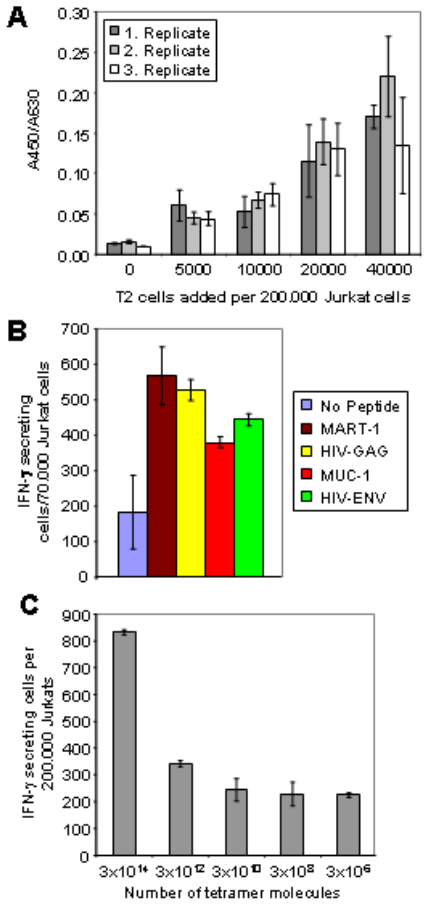


Figure 27 – Activation of the $CD3^+/CD8^+$ Jurkat cell line with peptide loaded T2 cells as well as MART-1 loaded tetramer scMHC molecules. (A) Activation of 200.000 MART-1 specific $CD3^+/CD8^+$ Jurkat cells with varying amount of MART-1 loaded T2 cells. 40.000 MART-1 loaded T2 cells were incubated without $CD3^+/CD8^+$ Jurkat cells as a negative control (data not shown). (B) The peptide specificity of the $CD3^+/CD8^+$ Jurkat cell line was assayed by activation of 70.000 Jurkat cells with 40.000 unloaded T2 cells as well as 40.000 T2 cells loaded with either one of four peptides. (C) 200.000 MART-1 specific $CD3^+/CD8^+$ Jurkat cells were incubated with varying numbers of MART-1 loaded tetramer scMHC molecules. After over night incubation activation of $CD3^+/CD8^+$ Jurkat cells was detected either as secreted IL-2 in the supernatant detected in a sandwich ELISA (A), or as the number of IFN-γ secreting cells determined in an ELISPOT assay (B and C).

2.5.7 The ability of the single-chain MHC as an activator of T Cells

2.5.7.1 Peptide specific enrichment by binding to CD3$^+$/CD8$^+$ Jurkat T cells

To activate T cells the interaction between peptide loaded MHC molecules and the T cell receptor is crucial. The interaction between the MART-1 specific TCR and phage displayed single-chain MHC Class I molecules was therefore explored in an enrichment study. First, irrelevant scFv phage as well as scMHC phage with or without covalently linked peptide were mixed equally (10^8 displaying phage/$5x10^5$ CD3$^+$/CD8$^+$ Jurkat cells). Secondarily, the mixture was enriched for binding to either $5x10^5$ CD3$^+$/CD8$^+$ Jurkat cells or as negative control $5x10^5$ T2 cells. And finally, the identity of a number of randomly picked clones before and after enrichment was identified by PCR (table 8). The experiment showed that HLA-A2-β2MG L10 phage could be enriched over the HLA-A2-β2MG phage lacking peptide, and even more significantly enriched over the irrelevant scFv phage. However, a similar enrichment of HLA-A2-β2MG L10 phage over the irrelevant scFv phage was observed when selected on T2 cells, thus suggesting that the HLA-A2-β2MG L10 phage binds CD3$^+$/CD8$^+$ Jurkat cells and T2 cells in a non-TCR dependent fashion. Whether the enrichment of HLA-A2-β2MG L10 phage over HLA-A2-β2MG phage is specific for CD3$^+$/CD8$^+$ Jurkat cells, or if a similar enrichment can be accomplished employing T2 cells, remains to be determined. For the reversed construct, β2MG-HLA-A2, our data shows that the non-peptide containing phage is enriched over the peptide containing phage both when selected for binding to CD3$^+$/CD8$^+$ Jurkat cells and to T2 cells. It was however possible to enrich β2MG-HLA-A2 L10 phage over the irrelevant scFv phage, although to a smaller degree than for the HLA-A2-β2MG L10 phage. The enrichment of β2MG-HLA-A2 L10 over an irrelevant scFv was furthermore temperature dependent, since enrichment was only observed when selected at 37°C and not at 20°C. Interestingly, β2MG-HLA-A2 seems to bind cells better than HLA-A2-β2MG, which might correlate with the observed study on correct folding with the conformational HLA-ABC antibody, W6/32 (cf. chapter 2.5.3 and figure 25). Previous studies by Gorochov and co-workers showed that peptide specific enrichment of scMHC phage on T cell clones was only possible for immunopurified scMHC phage, and only when bound to the cell surface at 37°C. They hypothesise that the temperature dependence is due to the need for internalisation of binding phage, since subsequent washing steps might be too harsh on the low affinity interaction between scMHC and TCR (Le Doussal et al., 2000). In our study we did not perform immunopurification, since the analysis was performed with the novel helper phage selection system (Kristensen and Winter,

1998), which has been reported to reduce background binding in cell surface selections (Jensen et al., 2003). Despite the improved selection method, complex selections are hampered by a high amount of sticky unspecific phage. With such low affinities as expected for the scMHC TCR interaction such background is expected to be high. The enrichment study did not offer a clear conclusion, but since the assay was sensitive towards a range of parameters, such as washing, temperature etc., we chose to proceed with an investigation of T cell activation due to interacting scMHC phage and TCRs. A T cell activation study would benefit from the fact that the scMHC TCR interaction is not challenged by harsh washing, although it is expected that a longer lasting scMHC TCR interaction is required as compared with enrichment studies. In future binding studies the mixture of specific and unspecific phage should be biased towards the unspecific phage, since a potential low enrichment of a specific phage is statistically better determined if the specific phage is underrepresented in the phage mixture.

Phage mixture	**Temp (°C)**	**Cell type**	**Pre enrichment**			**Post enrichment**		
			+L10	**-L10**	**scFv**	**+L10**	**-L10**	**scFv**
HLA-A2-β2MG HLA-A2-β2MG L10	37	Jurkat	7 (44%)	9 (56%)		8 (57%)	6 (43%)	
scFv HLA-A2-β2MG L10	37	Jurkat	5 (20%)		20 (80%)	18 (72%)		7 (28%)
scFv HLA-A2-β2MG L10	37	T2	5 (20%)		20 (80%)	6 (75%)		2 (25%)
β2MG-HLA-A2 β2MG-HLA-A2 L10	20	Jurkat	13 (81%)	3 (19%)		11 (69%)	5 (31%)	
scFv β2MG-HLA-A2 L10	20	Jurkat	6 (35%)		11 (65%)	6 (33%)		12 (67%)
β2MG-HLA-A2 β2MG-HLA-A2 L10	37	Jurkat	13 (57%)	10 (43%)		2 (13%)	14 (87%)	
scFv [a] β2MG-HLA-A2	37	Jurkat		3 (21%)	11 (79%)		11 (69%)	5 (31%)
scFv [a] β2MG-HLA-A2 L10	37	Jurkat	2 (15%)		11 (85%)	4 (44%)		5 (66%)
β2MG-HLA-A2 β2MG-HLA-A2 L10	37	T2	13 (57%)	10 (43%)		3 (38%)	5 (62%)	

Table 8 – Enrichment study analysing the peptide specificity of scMHC TCR interaction.
[a] The experiment is perfomed with a mix of three phage variants, β2MG-HLA-A2 L10, β2MG-HLA-A2 and scFv. In the table the result is divided into two rows, describing the enrichment of the two scMHC variants over the irrelevant scFv, respectively.

2.5.7.2 Multivalent display of scMHC molecules

Activation of $CD3^+/CD8^+$ Jurkat T cells with a scMHC construct is reported to be dependent on multimerisation as discussed in the previous paragraphs. It is therefore valuable to estimate the valency of phage presented scMHC molecules. To make such estimate it is hypothesised that phage

assembly does not discriminate between protein III wild-type and fusion molecule, and that the five-fold symmetry of the phage particle imply that it contains five protein III molecules (cf. chapter 1.1). The display level of a phage pool refers to the percentage of phage presenting one or more fusion protein III molecules; in statistical terms this is designated cumulated probability. If the number of protein III molecules (fused or wild-type) is theoretically unlimited the statistical approach applied should be the binomial distribution approach. The phage assembly process described statistically would thus be the random extraction of five protein III molecules from an unlimited pool of protein III with a fixed probability of getting a fusion protein III. The probability can for small display levels be estimated by neglecting the presence of phage with 2 or more fusion protein III molecules. For a 1% display level the situation would thus be that 1 out of 100 phage displays a fusion molecule or in other words that 1 out of 500 protein III molecules are fusion molecules. In table 9 the calculation was made for phage with 1%, 5% and 10% display level.

pIII fusion/pIII wt	1/498	1/98	1/48
Display level	1,0%	5,0%	10,0%
Multivalent Display	0,004%	0,102%	0,416%

Table 9 – Statistical analysis of the correlation between display level and multivalent display.

From table 9 it is obvious that for natural multivalent display the difference between 1% and 10% display is crucial. We observed that approximately 10^{12} soluble tetramer scMHC molecules loaded with MART-1 were required for detectable activation of IFN-γ secretion by 200.000 $CD3^+/CD8^+$ Jurkat T cells (figure 27.C). These data are supported by previous studies with a soluble scMHC molecule, where around 10^{12} molecules were added to 1000 T cells to induce activation (Abastado et al., 1995). Such high numbers of scMHC molecules are incompatible with the phage system due to the limited phage titer. With a phage stock of 10^{12} cfu/ml a maximum of $2x10^{11}$ phage can be added to 200.000 Jurkat T cells in the employed setup. With a 5% display level we would thus add $2x10^8$ multivalent phage to 200.000 Jurkat T cells. In our experiments however we added around 10^9 phage per 200.000 Jurkat T cells of practical reasons, thereby reducing the multivalent fraction at a 5% display level to 10^6 phage. Although the numbers of phage displayed scMHC molecules will never reach the numbers required for the soluble tetramer scMHC molecules, it is unknown if the two systems can be compared. One argument is that the stickiness of the phage might induce a high local concentration of phage on the cell surface and thus in proximity of the TCRs. The questions regarding what is required for T cell activation with phage displayed scMHC molecules

therefore remain. The importance of monovalent display, multivalent display and co stimulatory molecules need to be investigated. Similarly the required number of scMHC molecules per T cell has to be determined. The activation is further hampered by the number of proteolytically degraded and incorrectly folded (cf. chapter 2.5.3) single-chain MHC molecules.

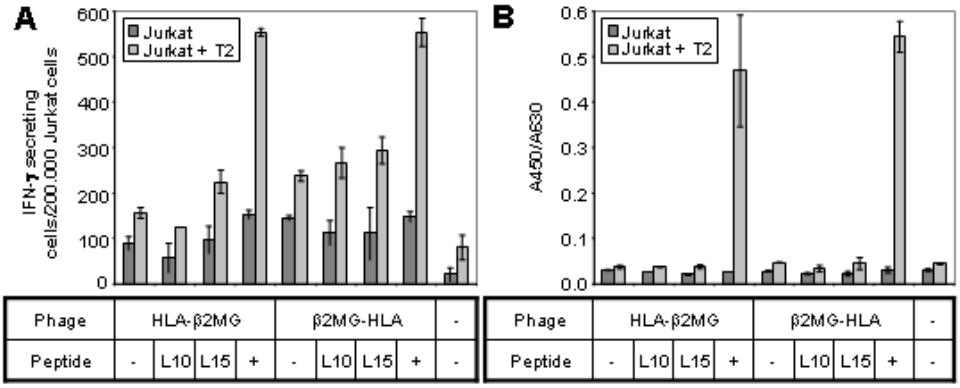


Figure 28 – ScMHC phage activation of CD3$^+$/CD8$^+$ Jurkat cells. ScMHC phages without peptide (-) or loaded with non-covalently bound peptide (+) as well as covalently bound peptide through either a 10mer (L10) or 15mer linker (L15) was assayed for their ability to activate 200.000 CD3$^+$/CD8$^+$ Jurkat cells in the presence or absence of 40.000 non-loaded T2 cells contributing with costimulatory molecules. Activation without phages was applied as negative control. (A) Activation measured by IFN-γ secretion detected in an ELISPOT assay. (B) Activation measured by IL-2 secretion detected in a sandwich ELISA.

2.5.7.3 Activation of CD3$^+$/CD8$^+$ Jurkat T cells by scMHC phage

We first investigated the T cell activating ability of scMHC phage either with no peptide or with peptide covalently linked through a 10mer or 15mer glycine-rich linker. The constructs without peptide covalently bound were further tested after *in vitro* loading with soluble peptide. All the constellations were analysed with and without 40.000 unloaded T2 cells as accessory cells. The activation was measured both as the IFN-γ secretion (ELISPOT) (figure 28.A) as well as the IL-2 secretion (Sandwich ELISA) (figure 28.B). The ELISPOT assay was clearly the more sensitive technique. It seems as if the addition of phage with or without T2 cells generally has an effect on the IFN-γ secretion, but a peptide specific induction of IFN-γ secretion was only very weakly observed for the HLA-β2MG and β2MG-HLA constructs with peptide covalently bound with a 15mer glycine-rich linker supplemented with unloaded T2 cells. The scMHC phage *in vitro* loaded with soluble peptide and supplemented with unloaded T2 cells strongly induced the IFN-γ as well as IL-2 secretion This induction is caused by the trans loading of T2 cells with free peptide and gives as high activation as our positive control - T2 cells pre incubated with soluble peptide (data not shown). In the succeeding experiments we therefore did not pre incubate the T2 cells with

peptide, but simply added peptide to the mixture of T2 cells and T cells representing our positive control. The trans loading effect draws attention to the possible trans loading of T2 cells with peptide covalently attached to the phage. Such trans loading can not be excluded, but sterical hindrance theoretically suggest that such effect would be highly unlikely.

Since our data were not very convincing we investigated whether multimerisation of the scMHC displaying phage could benefit activation. Multimerisation was accomplished by cross linking with an anti-cMYC antibody (9E10), which is specific for displaying phage. Single-chain MHC displaying phage with and without cross-linking were mixed with T cells and T2 cells if indicated and IFN-γ secretion was measured (figure 29). Preliminary results show that the HLA-β2MG and the β2MG-HLA constructs induce a peptide specific activation of T cells. The experiment further shows that cross-linking is critical.

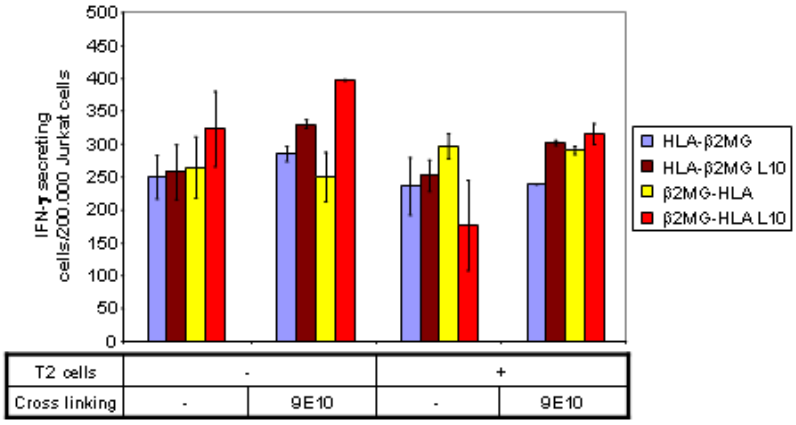


Figure 29 – Rendering scMHC phages multivalent to improve activation of $CD3^+/CD8^+$ Jurkat cells. 10^9 scMHC phages without peptide or with covalently bound peptide through a 10mer (L10) linker were cross-linked with 1µg anti-cMYC antibody as indicated and added to a mixture of 200.000 $CD3^+/CD8^+$ Jurkat cells and when indicated 40.000 non-loaded T2 cells. T2 cells either unloaded or loaded with MART-1 Peptide were used as negative and positive controls, respectively (data not shown). Activation of $CD3^+/CD8^+$ Jurkat cells was determined by the number of IFN-γ secreting cells in an ELISPOT assay. The display level of the scMHC phages was 10% except for HLA-β2MG without peptide, for which it was only 1%.

The theoretic beneficial effects of multivalency drew our attention to the established FuncFAb system (chapter 2.1) that could induce such multivalency. We therefore cloned the HLA-β2MG construct with the MART-1 peptide linked through a 10mer glycine-rich linker into the FuncFAb plasmid fusing the construct to DI of protein III from the filamentous bacteriophage. The fusion protein was purified and analysed for folding in ELISA as well as its ability to induce secretion of IL-2 from $CD3^+/CD8^+$ Jurkat T cells. Preliminary results showed that purified protein did not show folding specific binding in ELISA nor activation of T cells. Further experiments should elucidate if the DI fused scMHC could be actively expressed and purified.

2.5.7.4 Cell surface specific antibodies as costimulatory molecules

The observed activation by scMHC displaying phage encouraged us to perform further optimisation studies of the T cell activation. Antibodies against proteins on the T cell surface have been reported to have agonistic effects on T cell activation. One example is antibodies against CD3, which were reported to function as primary signal in T cell activation. The primary effect of adding anti-CD3 antibodies is proliferation and induction of IL-2, IL-3 and IFN-γ, although less prominent than APCs loaded with antigen (Jenkins et al., 1990). If anti-CD3 antibody is employed in the secondary boost of T cells without addition of costimulatory molecules TCRs are down regulated, thereby inducing immunosuppression. CD3 antibodies have therefore been utilized to avoid rejection of transplantations (Hirsch et al., 1990). In a more recent study it was showed that the epitope recognised by the anti-CD3 antibodies is of great importance for the agonistic effect, thus suggesting differences in the cytokine induction in Jurkat cells compared to primary T cells (Lafont et al., 1999). Likewise antibodies against CD28 seem to confer antagonistic activity and induce the secondary activation signal for T cells (Favero and Lafont, 1998; Rivas et al., 2001; Tacke et al., 1997). We analysed the agonistic effect of antibodies directed against CD3 and CD28 on $CD3^+/CD8^+$ Jurkat T cells. Our intention was to use such agonistic antibodies to augment the activating effect of scMHC displaying phage.

We analysed the effect of combinations of the two antibodies with or without unloaded T2 cells on the secretion of IFN-γ (figure 30.A and C) and IL-2 (figure 30.B and D). As shown in figure 26 only the $CD3^+/CD8^+$ Jurkat T cells were positive for CD3, whereas CD28 was present on both cell types. It has previously been reported that the effect of such antibodies are greater when they are statically coated on a plastic surface (figure 30.C and D) compared to soluble antibodies (figure 30.A and B). Such difference is most probably due to the high local antibody concentration obtained by coating, which resemble multivalency. Our observations show that soluble and coated antibodies give similar effect for IFN-γ production, whereas IL-2 secretion increase when antibodies are coated, although only slightly for the anti-CD28 antibody. Our data confirm earlier studies reporting that anti-CD3 antibodies affect IL-2 secretion but not IFN-γ secretion (Jenkins et al., 1990). The presence of unloaded T2 cells seems to increase the secretion of IFN-γ and IL-2. Anti-CD28 antibodies have only a vague activating effect on the $CD3^+/CD8^+$ Jurkat T cells, but in combination with the anti-CD3 antibody (both in soluble form and without unloaded T2 cells) our observations interestingly show an increased effect compared to anti-CD3 alone. The antibody

mixture supplemented with unloaded T2 cells on the other hand decreases the effect compared to anti-CD3 antibody alone. The observation is difficult to explain, but one speculative argument could be that the polarisation of T cells is inhibited due to the cross-linking of CD28 molecules on both T2 and T cells, thereby attaching T2 cells to several sites on the T cell surface.

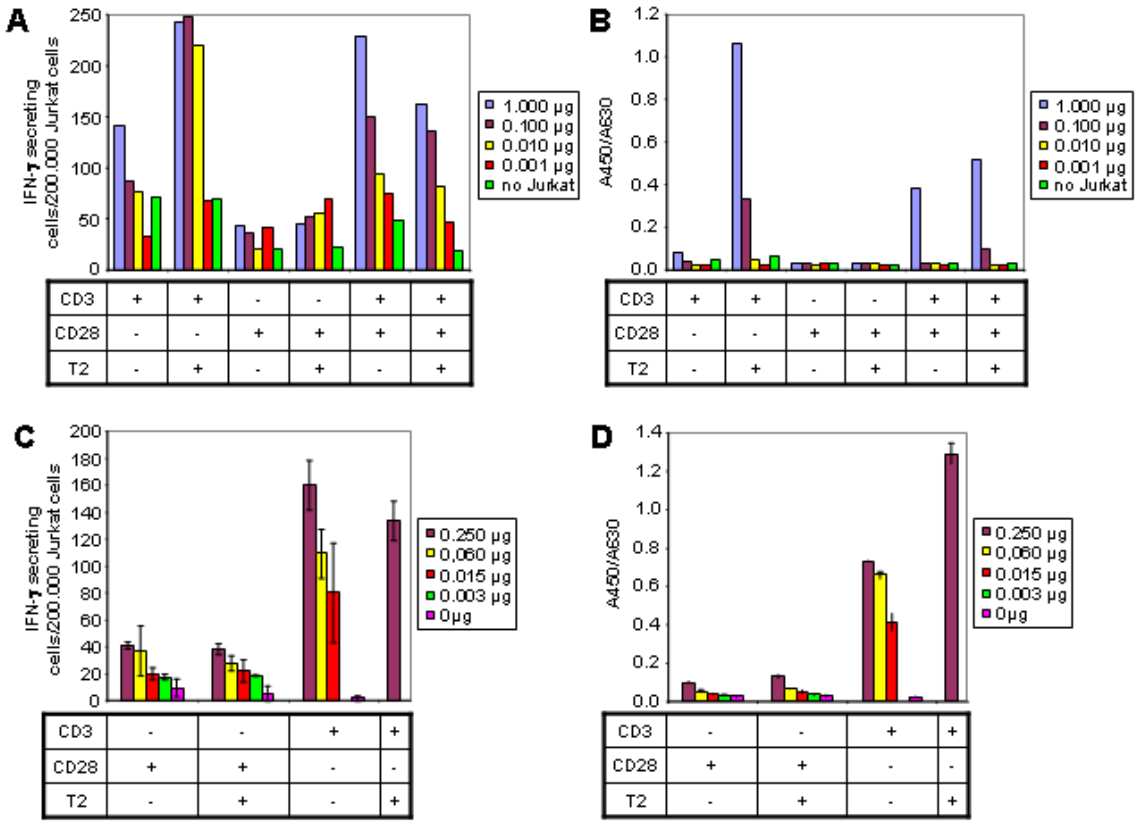


Figure 30 – Anti-CD3 and anti-CD28 antibodies as artificial costimulatory molecules. Varying amounts of antibodies against CD3 and CD28 were incubated with 200.000 $CD3^+/CD8^+$ Jurkat cells either in a soluble form (A and B) or coated to the membrane of the ELISPOT plate along side with the anti-human IFN-γ antibody (C and D). 40.000 non-loaded T2 cells were added as indicated. Activation of the $CD3^+/CD8^+$ Jurkat cells was determined as the number of IFN-γ secreting cells detected in an ELISPOT assay (A and C) and as the total amount of IL-2 secreted detected in a sandwich ELISA (B and D). Negative controls with no $CD3^+/CD8^+$ Jurkat cells but maximum amount of anti-CD3 and anti-CD28 antibodies (1µg each) as indicated are shown with green bars.

The optimisation study established an agonistic effect of coated anti-CD3 and coated anti-CD28 antibodies on $CD3^+/CD8^+$ Jurkat T cells. These antibodies were therefore subsequently employed in a T cell activation study using phage displaying β2MG-HLA with and without MART-1 peptide covalently attached through a 10mer glycine-rich linker (figure 31.A). The effect of multivalency of the scMHC molecules combined with the agonistic antibodies was investigated with anti-cMYC antibody (figure 31.B) as well as polyclonal anti-β2MG antibody (figure 31.C). Our data suggest that anti-cMYC antibody induce a peptide specific T cell activation, whereas the addition of anti-

CD3 and anti-CD28 antibodies seems to increase the peptide unspecific activation, without a proportional increase in the peptide specific activation. The addition of T2 cells has a similar effect on the peptide unspecific activation. Interestingly, scMHC phage cross-linked with the polyclonal anti-β2MG antibody did not induce a peptide specific T cell activation. This observation could be explained by the potential cross linking of MHC Class I molecules presented on T2 cells and T cells. Cross linking of T2 cells and T cells could eventually lead to T cell activation independent of the scMHC phage.

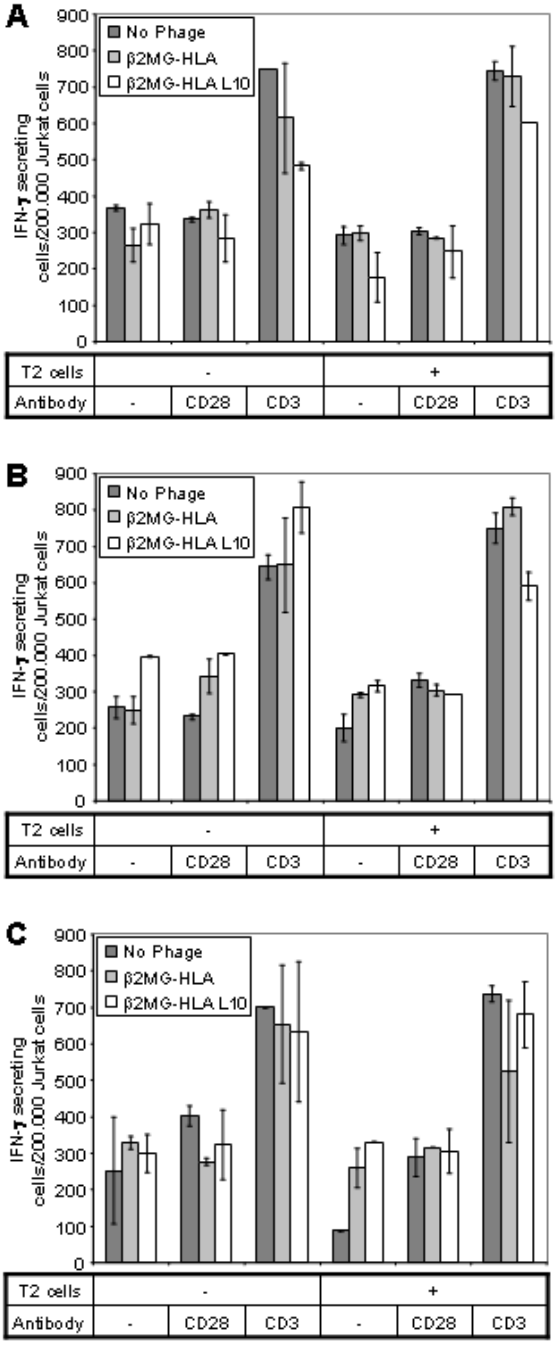


Figure 31 – Activation of $CD3^+/CD8^+$ Jurkat cells with cross-linked scMHC phages and costimulatory anti-CD3 and anti-CD28 antibody. 10^9 scMHC phages (10% display level) without peptide or with covalently bound peptide through a 10mer (L10) linker were added to a mixture of 200.000 $CD3^+/CD8^+$ Jurkat cells and when indicated 40.000 non-loaded T2 cells (A). In addition phages were cross-linked with either 1µg anti-cMYC antibody (B) or 1µg polyclonal anti-β2MG antibody (C). Anti-CD28 antibody (1µg/well) and coated anti-CD3 antibody (0.6µg/well) was coated on the ELISPOT wells as indicated. T2 cells either unloaded or loaded with MART-1 Peptide were used as negative and positive controls, respectively (data not shown). The Jurkat activation induced by the supplemented antibodies without the presence of phages was additionally analysed. Activation of $CD3^+/CD8^+$ Jurkat cells was determined by the number of IFN-γ secreting cells in an ELISPOT assay.

2.6 Concluding remarks on the scMHC system

We established a range of single-chain MHC class I constructs that were shown to be expressed and displayed as a fusion to protein III in a filamentous bacteriophage context. The native folding of the fusion protein was verified in ELISA and a cellular system to test the T cell binding and activating ability of the scMHC phage was established.

The primary reasons for the limited T cell binding and activating ability of scMHC displaying phage is most likely the low number of correctly folded molecules per T cell as well as the monovalent nature of the phagemid system. We showed that multimerisation of scMHC phage employing a cross-linking antibody enable T cell activation. Such requirement would though decrease the usefulness of future peptide phage libraries on a single-chain MHC scaffold, since the multivalent phage complexes generated with cross-linking antibodies would be heterogeneous with regard to the loaded peptide. One solution to this problem is the phage vector system, which enables homogeneous multivalent display. The scMHC construct has been investigated in phage vector systems by Gorochov and co-workers. They however observed similar display levels as in our study due to proteolytically degradation. Despite the low display level they were able to show enrichment of two phage constructs displaying each their peptide. The enrichment study was performed on two different T cell hybridomas specific for the two peptides, respectively. Interestingly, the enrichment was only observed at 37°C and not 4°C indicating that internalisation of the TCR-scMHC phage complex is important, and that subsequent washing steps are to harsh for the low affinity interaction (Le Doussal et al., 2000). Our enrichment experiments at 20°C and 37°C showed no peptide specific enrichment, but future experiments will show whether the successful cross-linking strategy employed in the T cell activation experiments could benefit the enrichment studies in a similar manner.

Since the late 1980s several soluble recombinant T cell receptor molecules have been reported, such as the HIV-GAG specific T cell receptor reported by Sykulev and co-workers (Anikeeva et al., 2003). The area is reviewed by Kozono and co-workers (Fremont et al., 1996). The use of such T cell receptors in an ELISA setup could confirm the native folding of the scMHC molecule on the phage, thus bypassing the background problems with complex selections and T cell activation as reported in chapter 2.5.7.1. A similar approach is the use of recombinant antibodies with MHC-restricted, peptide-specific, T cell receptor-like specificity (Cohen et al., 2003a).

As for most optimisation studies a range of parameters were not tested. The stability of MHC class I molecules have been reported to be dependent on physiological salt concentrations (Batalia et al., 2000) and its peptide binding ability is pH dependent (Ostergaard Pedersen et al., 2001). The most common assays employed to determine T cell activation utilize cytokine production as indirect indicators of activation. IFN-γ and IL-2 are such reporter molecules and can be analysed either as secreted (ELISPOT and sandwich ELISA) or intracellular (FACS) (Prussin and Metcalfe, 1995) molecules. Although cytokine related assays are the most frequently employed indirect markers for T cell activation, alternative indirect assays for T cell activation exist, such as analysing calcium mobilisation and activation of kinases (Judd and Koretzky, 2000; Lewis, 2001) as well as the up-regulation of early activation marker CD69 (Brophy et al., 2003; Cullen et al., 1999). Finally, T cell activations determined based on indirect reporter molecules should be tested for inducing CTL response in a standard ^{51}Cr (reviewed by Cerottini and Brunner (Cerottini and Brunner, 1974)) or europium (Blomberg et al., 1986a; Blomberg et al., 1986b) release assay. Alternatively the high-throughput T cell activation assay developed by Reiter and co-workers (Cohen et al., 2003b) based on the detection of changes in the cytoplasmic matrix could be useful. In their study they further use an alternative MART-$1_{(27-35)}$ specific human CTL cell line 5/195 (Kirkin et al., 1999), which might have other beneficial properties than the MART-$1_{(27-35)}$ specific $CD3^+/CD8^+$ Jurkat T cell line employed in our study.

The setup could have been considerable improved if the fused scMHC molecule was derived from earlier reports on soluble MHC molecule used for T cell activation and staining. The soluble protein could in such case have been used as positive control. Something we lacked in the presented study.

2.7 Gene transfected dendritic cells stimulate antigen specific CD8$^+$ T cells

The peptide specific interaction between an MHC class I molecule and the TCR of a CD8$^+$ T cell is the primary signal for activation of T cells as described in chapter 1.2.7. The primary signal should theoretically be enough to activate primed T cells, whereas naive T cells need a secondary signal through costimulatory molecules such as CD28 (Bromley et al., 2001; June et al., 1990) and a range of cytokines (Alves et al., 2003; Ruckert et al., 2003; Wesa and Galy, 2002). Naive T cells can therefore only be activated by accessory cells or highly specialised cross-priming professional antigen presenting cells (APCs) like dendritic cells (reviewed by Thery and Amigorena (Thery and Amigorena, 2001)).

2.7.1 Non-viral transfer of genetic material to dendritic cells

Mature dendritic cells derived from the NemodDC precursor cells (Nemod-mDCs) have been tested positive for their ability to stimulate naïve CD4$^+$ and CD8$^+$ T cells when loaded with either peptide, protein or tumour cell lysate (unpublished results, Baumeister H). In the presented study we analyse the ability of loading NemodDCs through transfection with antigen encoding DNA and RNA. A range of alternative nucleic acid transfer strategies for eukaryotic cells are established, like viral transfer, phosphate precipitation of DNA, lipid based transfer, microinjection and electroporation (reviewed by Gruenert and co-workers (Colosimo et al., 2000)), as well as an ever increasing number of novel methods, such as the newly reported histone based nucleic acid delivery system (Puebla et al., 2003). Non-viral transfection of primary dendritic cells has been reported by others utilising lipid-based transfection systems (Rughetti et al., 2000; Van Tendeloo et al., 2001), DNA complexes induced by the cationic peptide CL22 (Irvine et al., 2000), electroporation of RNA with the Easyject Plus system (Muller et al., 2003; Van Tendeloo et al., 2001), the Electro Square Porator ECM 830 (Ueno et al., 2004) and the Gene Pulser from Biorad (Racanelli et al., 2004) as well as electroporation of both RNA and DNA with the AMAXA Nucleofector (Lenz et al., 2003). An excellent review by Lyerly and co-workers summarise the studies on DNA and RNA delivery to dendritic cells, and conclude that non-viral delivery of DNA and RNA to dendritic cells is inefficient, thus leaving room for optimisation (Morse and Lyerly, 2002). Interestingly, it was shown that even with inefficient gene transfer the encoded antigen is presented by the dendritic cells. The more recent studies employing the novel AMAXA nucleofector technology seems to

fulfil the demands for efficient DNA and RNA transfer into dendritic cells. In the present study we thus seek to transfect NemodDC and its differentiated derivatives employing the AMAXA nucleofection system. The system has been observed to induce translation of reporter genes within 2-4 hours after nucleofection, and it is therefore suggested that the nucleic acids are delivered directly to the nuclear compartment, thereby potentially short-cutting the initiation of transcription (Trompeter et al., 2003). This result has been further confirmed in a subsequent study by Kockx and co-workers, showing that whereas both electroporation and nucleofection enable efficient expression of enhanced green fluorescent protein (EGFP) in human monocyte cell lines transfected with EGFP encoding mRNA, EGFP encoding DNA plasmids only induces appreciable EGFP expression using the nucleofection system (Martinet et al., 2003).

In a time-laps study the rate of transfection as well as cell vitality was assayed for precursor NemodDCs (figure 32), immature dendritic cells derived from NemodDC (Nemod-iDC) (figure 33) and Nemod-mDCs (figure 34) using an EGFP encoding reporter plasmid. The transfection rate corresponds to the percentage of EGFP expressing cells that were determined by flow cytometry. The transfection of precursor NemodDCs was performed by nucleofection as well as electroporation and lipofection. Whereas the nucleofector transfected 90% of the cells, the Eppendorf Multiporator system only reached an avarage transfection rate of 10% in four experiments (figure 32.B and data not shown). Preliminary results employing the Lipofectamine transfection system suggest that this system is unable to transfect NemodDCs (data not shown). The lacking ability to transfer DNA to dendritic cells via lipofection has been confirmed by others (Van Tendeloo et al., 2001; Van Tendeloo et al., 1998). The viability is higher for electroporated cells compared to nucleofected cells, which most likely is due to the stress effect of efficient DNA uptake and the toxic effect of stressing the cellular machinery with EGFP expression (figure 32.A). The time-laps study further shows that cell viability decreases up to 20% over a three day period. In fact recent studies suggest that the nucleofection technology only allows for short-term experiments with transiently nucleofected cells (Lenz et al., 2003). This limitation constitutes one of the major drawbacks of the nucleofection technology. However, nucleofection under optimised conditions enable high transfection rates and reasonable cell viabilities of 82% and 58%, 6 and 72 hours after nucleofection, respectively. Nucleofection therefore is a very strong technique for short term analysis of foreign gene expression in dendritic cells.

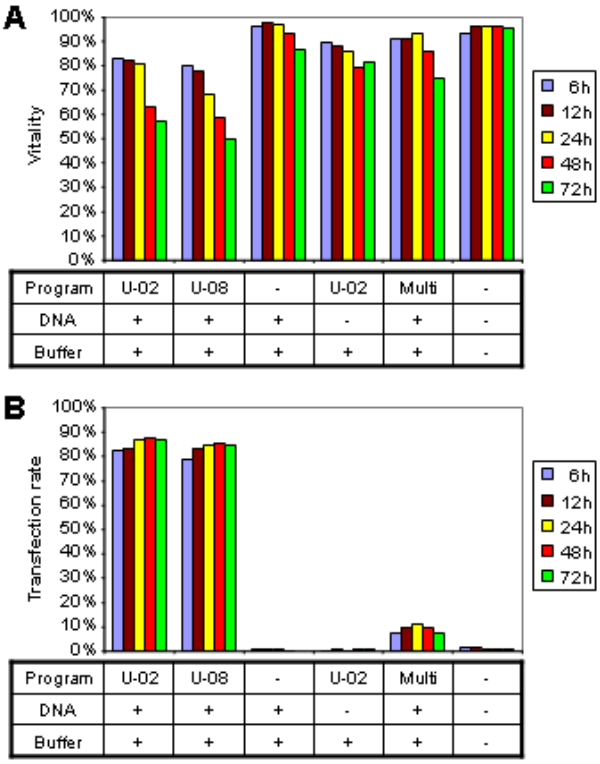


Figure 32 – Gene transfer into precursor NemodDCs. The pEGFP N1 plasmid is introduced into precursor NemodDCs either by nucleofection with the AMAXA programs U-02 (optimal for precursor NemodDCs) and U-08 (recommended by AMAXA GmbH for primary dendritic cells) or by transfection using Eppendorfs Multiporator. Negative controls are precursor NemodDCs mixed with DNA but no electroporation (- Program) and NemodDCs without DNA nucleofected with program U-02. The buffer used for nucleofection is Nucleofection Buffer V and for transfection with Eppendorfs Multiporator a 40:60 Hypo:Iso osmolar buffer is used. As negative control cultivated precursor NemodDCs neither dissolved in buffer nor mixed with DNA were analysed. The cell vitality (A) and EGFP expression (B) is measured by flow cytometry after varying periods of cultivation in non-differentiation medium.

A similar study performed on Nemod-iDCs either from thawed cells (figure 33.A and B) or freshly differentiated from precursor NemodDCs (figure 33.C and D) showed maximal transfection efficiencies 22 hours after nucleofection in the range of 60% and 70%, respectively. Once again the viability of the cells decreases upon electric shock, when comparing with cells similarly diluted in buffer including reporter plasmid but without nucleofection. The initial viability of the thawn cells was 83% compared with 90% for freshly grown Nemod-iDCs, and decreased further approximately 15% after over night cultivation before nucleofection (data not shown). The viability seemed to decrease an additional 15% due to the dilution in DNA containing nucleofection buffer both for thawed and freshly cultivated Nemod-iDCs. After nucleofection the viability further decreases for both thawed and freshly prepared Nemod-iDCs, whereas cells not exposed to the electric shock stabilises their viability. An important difference to the study on nucleofection of precursor NemodDC is that the nucleofected Nemod-iDCs are cultivated under maturation conditions (medium supplemented with TNF-α), whereas the precursor cells were cultivated with standard medium containing no additional cytokines. Therefore the Nemod-iDCs are committed to cell maturation and finally cell death.

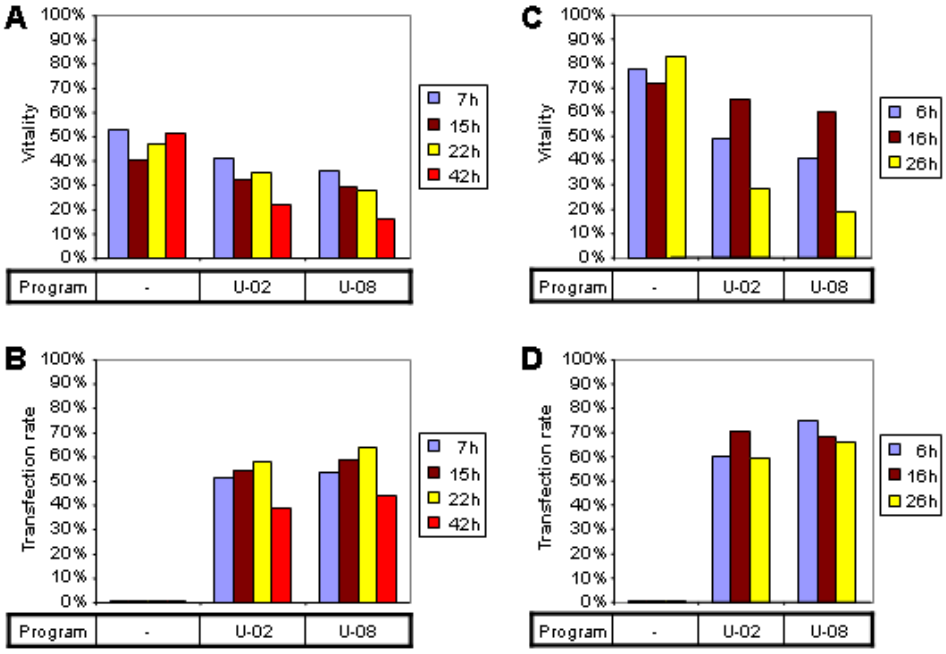


Figure 33 – Nucleofection of Nemod-iDCs. The pEGFP N1 plasmid is nucleofected into Nemod-iDCs thawn from a frozen batch and cultivated under differentiation conditions over night to recover from the harsh environmental changes (A and B) or into Nemod-iDCs freshly differentiated (C and D). Gene transfer was performed by nucleofection with the AMAXA programs U-02 (optimal for precursor NemodDCs), U-08 (recommended by AMAXA GmbH for primary dendritic cells) or no electroporation as negative control. The cell vitality (A and C) and EGFP expression (B and D) is measured by flow cytometry after varying periods of cultivation under maturation conditions.

Finally, we performed nucleofection of Nemod-mDCs matured from either thawed or freshly differentiated Nemod-iDCs (figure 34). The viability of nucleofected Nemod-mDCs origining from thawed iDCs was close to 0% and therefore not further pursued (data not shown). Approximately 50% of the Nemod-mDCs matured from freshly prepared Nemod-iDCs survived the nucleofection, whereas 80% of the non-nucleofected Nemod-mDCs survived incubation in buffer supplemented with reporter plasmid (figure 34.A). The transfection rate seems to peek after 24 hours at around 70%, although it drops to 30-40% after another day, which is not surprising because the cells are already committed to the mature cell stage that relentlessly leads to cell death (figure 34.B).

The nucleofection study was performed with two different programs, U-02 and U-08 described as being optimal for human dendritic cells and human $CD34^+$ cells respectively (Unpublished results, AMAXA GmbH). The term program reflects the characteristics of the electric pulse, whereas the number of repeated pulses could be manually determined. Also a range of different nucleofection buffers are available. A standard AMAXA 2 step iteration optimisation study determined the

optimal nucleofection conditions to be program U-02 with $2x10^6$ cells per 100µl Nucleofector Buffer V supplemented with 5µg plasmid (Data not shown). The program U-08 was included in the nucleofection study due to the fact that it was reported to be optimal for human $CD34^+$ cells, a marker that was also observed on precursor NemodDCs. No major difference was observed for the two programs although U-02 seemed to be slightly better for cell viability. U-02 was employed in all subsequent studies.

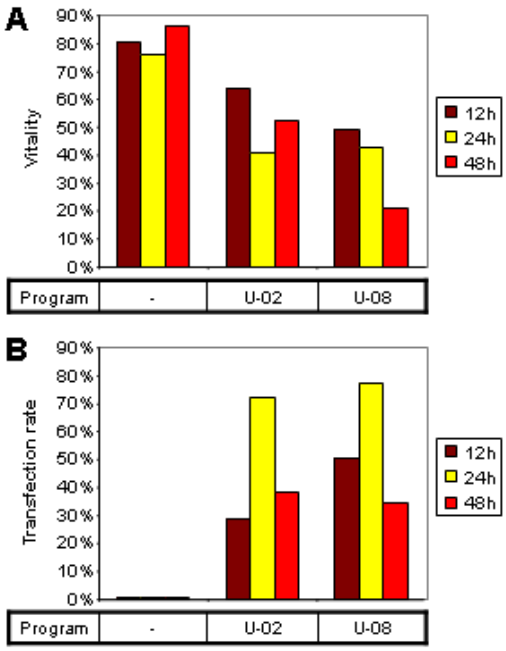


Figure 34 – Nucleofection of Nemod-mDCs. The pEGFP N1 plasmid is introduced into Nemod-mDCs differentiated and matured from precursor NemodDCs without intermediate freezing. Gene transfer was performed by nucleofection with the AMAXA programs U-02 (optimal for precursor NemodDCs), U-08 (recommended by AMAXA GmbH for primary dendritic cells) or no electroporation as negative control. The cell vitality (A) and EGFP expression (B) is measured by flow cytometry after varying periods of cultivation in non-differentiation medium.

2.7.2 Delivering genes encoding single-chain hIL-12, HLA-A1 and GPA into DCs

Before turning our interest towards loading of dendritic cells via nucleofection with DNA and RNA, we studied if nucleofection caused changes of the dendritic cell phenotype (cf. chapter 2.7.6.2). In addition we investigated the ability of altering the phenotype by induction of foreign genes employing the nucleofection technology. The nucleofection study described in chapter 2.7 employed a reporter plasmid, which express EGFP as soluble protein located in the cytoplasm. In the following study we investigated the feasibility of expressing membrane bound proteins like wild-type HLA-A1 and wild-type Glycophorin A (GPA) as well as secreted biologically active IL-12 single chain construct of the two human IL-12 subunits P40 (N terminal) and P35 (C terminal), covalently fused with a 10 a.a. linker derived from bovine elastin (Anderson et al., 1997). The constructs were delivered into precursor NemodDCs using the optimised conditions described above, and gene transcription was analysed by RT-PCR with gene specific primers. Gene

translation was further assayed in cell staining studies with monoclonal antibodies visualised by fluorescence microscopy and flow cytometry. Secreted IL-12 was detected by ELISA. The main reasons for introducing IL-12 into the dendritic cells are elucidated in a comprehensive phenotypic study described in the next paragraphs, showing that several immunological important interleukins are not secreted by Nemod-mDC when matured under various conditions.

2.7.3 Phenotype of Nemod-mDCs generated under various maturation conditions.

IL-12 secretion by DCs is considered an important event during cellular immune responses induced by viral infections or immune responses against tumour cells induced by immunotherapy (Valenzuela et al., 2002). Therefore we tested various maturation cocktails known to induce DC maturation and cytokine release for their potential to induce Nemod-iDC maturation, IL-12 secretion and additionally IL-4, IL-6 and IL-10 secretion. IL-4 is known to induce class switching in B cells (reviewed by Snapper and Mond (Snapper and Mond, 1993)), IL-10 is known to be important for immunological tolerance (Groux et al., 1997) and IL-6 is important for B cell differentiation (Jego et al., 2003), induction of $CD4^+$ Th2 cells (Dodge et al., 2003) and $CD8^+$ T cell activation (Renauld et al., 1989). The study revealed that the varying maturation protocols were not able to induce secretion of the human cytokines IL-4, IL-10 and IL-12 to an extend higher than the detection limit of around 80 pg/ml (data not shown), whereas IL-6 was highly expressed (971 pg/ml), when cultivated in the standard maturation medium containing TNF-α supplemented with a CD40 ligand expressing cell line ($CD40L^+$ J558). IL-6 has been reported to downregulate IL-12 production, which explains the lacking IL-12 in the experiment (Dodge et al., 2003). Supplementing the maturation medium with $CD40L^+$ J558 and either IFN-γ or Bacillus Calmette-Guerin mycobacteria (BCG) augmented the IL-6 secretion. In presence of IFN-γ or BCG secreted IL-6 was detectable even in the absence of $CD40L^+$ J558. Dexamethasone did not effect IL-6 secretion (figure 35). $CD40L^+$ J558 resemble the biological interaction between CD40L on T cells and CD40 on dendritic cells. Induction of IL-10 and IL-12 by dexamethasone and IFN-γ respectively is reported for the MUTZ-3 cell line, although IL-10 secretion (35-45 pg/ml) was below our detection limit (Masterson et al., 2002). Interestingly IL-6 secretion is undetectable for all serum free conditions except when supplemented with $CD40L^+$ J558 in combination with either IFN-γ or BCG (figure 35). Our maturation optimisation data for the serum free NemodDC and the serum

containing NemodDC thus suggest that an IL-6 inducing factor other than the compounds used for supplementation is present in the serum or the conditioned medium.

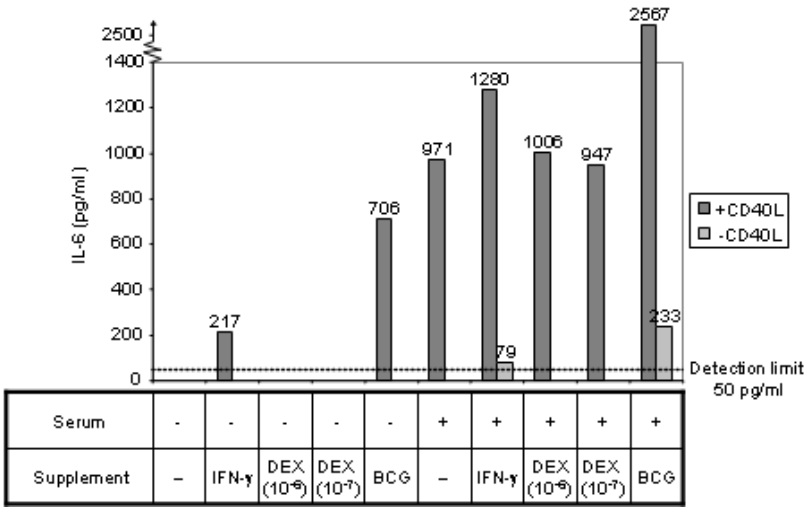


Figure 35 – Optimisation of maturation conditions for IL-6 secretion. Bar diagram showing the induction of IL-6 secretion due to varying maturation conditions. Nemod-iDCs are matured into Nemod-mDCs in 48 hours either with the standard supplement of TNF-α (75 ng/ml) alone or with TNF-α (75 ng/ml) supplemented with alternative maturation components like IFN-γ (1000 U/ml), Dexamethasone (DEX) (10^{-6}M or 10^{-7}M) or Bacillus Calmette-Guerin mycobacteria (BCG) (10^6 CFU/ml). The CD40L expressing cell line J558 was cocultivated in ratio 1:1 with the dendritic cells as indicated. The IL-6 secreted into the medium during the 48 hours was measured in a sandwich ELISA with a detection limit of 50 pg/ml.

The supplements to the standard maturation cocktail did in most cases not change the level of CD86, CD116, CD83 and CD1a cell surface markers compared to the standard differentiation and maturation pathway (figure 36). Our observations for the standard maturation pathway show that markers of the monocytic precursors, like CD14 (a receptor for lipopolysaccharides, which also mediates phagocytosis of apoptotic cells (Devitt et al., 1998)) and CD34 (cell adhesion through binding of L-selectin) as well as TNF receptor related CD30 (involved in TCR mediated T cell death) decreases during differentiation and maturation, whereas the costimulatory molecules CD80, CD86 and CD40 as well as maturation marker CD83 and Langerhans like cell marker CD1a are up-regulated (Banchereau et al., 2000; Robinson et al., 1998). The primary stimulatory molecules MHC class I and MHC class II are expressed on approximately 90% of the cells at all stages of cell differentiation. However, according to Sallusto and Lanzavecchia the presence of MHC class II on the cell surface of *in vitro* cultivated DCs should increase during maturation (Sallusto and Lanzavecchia, 1994). The DC differentiation markers DC SIGN (CD209), a C-type surface lectin mediating DC binding to ICAM-3 on resting T cells (Geijtenbeek et al., 2000), and the complement-mediated antigen capturing agent CD11c are induced by differentiation and remain unchanged during maturation (Corbi and Lopez-Rodriguez, 1997). The GM-CSF receptor CD116 is down-regulated during differentiation most likely due to a feed-back mechanism activated by the presence of GM-CSF in the differentiation medium. The CD116 level is restored after removal of

GM-CSF in the maturation phase as observed by others (Rosenzwajg et al., 2002). As mentioned most maturation conditions tested were similar to the standard maturation pathway in regard to the level of CD86, CD116, CD83 and CD1a. Three exceptions were observed. Firstly, 10^{-6}M Dexamethasone combined with CD40L^{+} J558, leads to very low cell viability, which makes the detection of cell surface markers difficult. This is also observed by Claesson and co-workers (Pedersen et al., 2004). Secondly and thirdly the supplements BCG and IFN-γ + CD40L J558, induce increased and reduced expression of the maturation marker CD83, respectively.

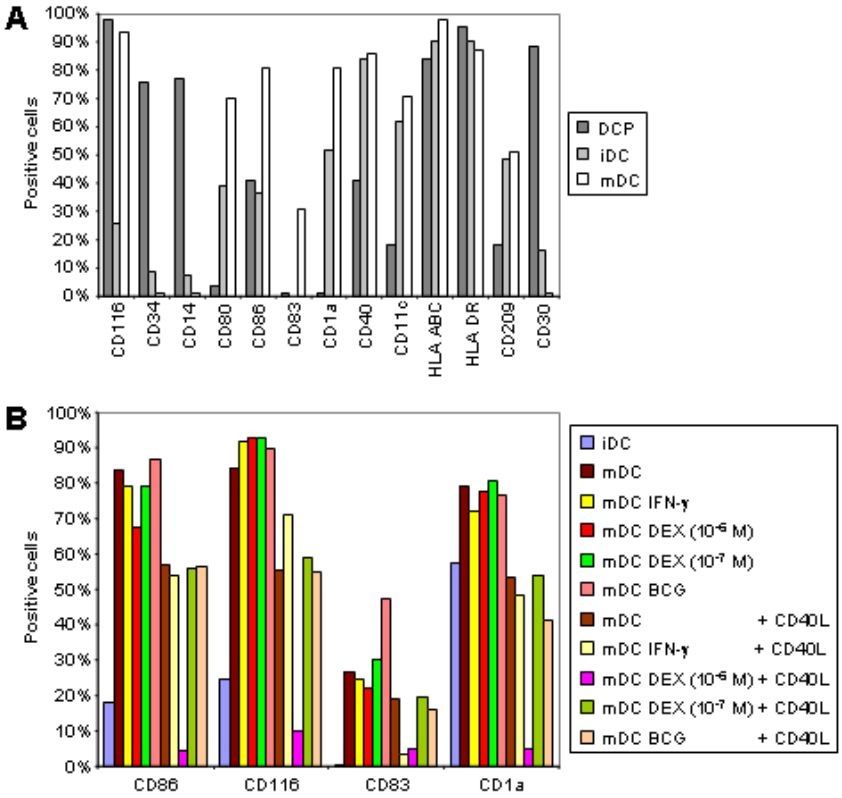


Figure 36 – Clusters of differentiation markers detected on precursor Nemod-DCs, Nemod-iDCs and Nemod-mDCs matured under various conditions. Bar diagrams showing the changes in cell surface expression of various differentiation markers on precursor Nemod-DCs (DCP), Nemod-iDCs (iDC) and Nemod-mDCs (mDC). The dendritic cells were differentiated by the standard protocol and either matured by the standard protocol (A) or matured under standard conditions with various supplements (B). The markers were labelled with specific antibodies and detected by flow cytometry. See text for further explanations. Abbreviations: Interferon gamma (IFN-γ), Dexamethasone (DEX), Bacillus Calmette-Guerin mycobacteria (BCG) and Accessory CD40L^{+} cell line (CD40L).

Our successful nucleofection studies encouraged us to investigate if the technique could be used to change the phenotype of the dendritic cells. Such changes could either be performed by recombinant expression of molecules that are lacking or by silencing of highly expressed genes with RNA-mediated interference (RNAi) (Fire, 1999). We turned our attention to the former method.

2.7.4 Induction of IL-12 secretion by nucleofection

Since a Th1 response is characterised by the three cytokines IFN-γ, IL-2 and IL-12 (Czerniecki et al., 2001), the lack of IL-12 secretion led us to assay the ability of inducing artificial IL-12 expression. A Th1 response is particularly important for immunotherapy against viruses and tumours, since it induces activation of cytotoxic CD8^{+} T cells (Dredge et al., 2002). This was

exemplified in a recent study showing that DCs engineered to express both IL-12 and IL-18 increase the Tc1 response against tumours when injected into the tumour (Tatsumi et al., 2003). The general induction of secreted cytokine expression would further suggest that we are able to skew the immune response in any desired direction. Precursor NemodDCs nucleofected with an IL-12 encoding plasmid were analysed for IL-12 secretion by a classic sandwich ELISA assay. Three independent experiments showed IL-12 secretions of 832 pg/ml, 966 pg/ml and 1500 pg/ml respectively (figure 37.B and data not shown). One experiment was performed as a time-laps study and shows that the amount of secreted IL-12 increases over a three day period. In the same period of time the cell viability decreases from 65% to 25% for nucleofected cells, whereas non-nucleofected cells remain 85% vital during the entire experiment (figure 37.A). The increase in secreted IL-12 over time can partly be due to release of intracellular IL-12 from bursting cells as a result of massive cell death. This would particularly explain the major jump in secreted IL-12 from 48 hours to 72 hours, as cell viability is strongly reduced in this timeframe. The declining viability is a serious obstacle in the pursuit of a stable IL-12 expressing precursor NemodDC clone, and seems to be an intrinsic feature of the nucleofection system as also reported by Feldman and co-workers (Lenz et al., 2003). So far cloning of nucleofected NemodDCs by limited dilution has been unsuccessful (data not shown), although non-nucleofected NemodDCs can be cloned by limited dilution (unpublished results, Baumeister H).

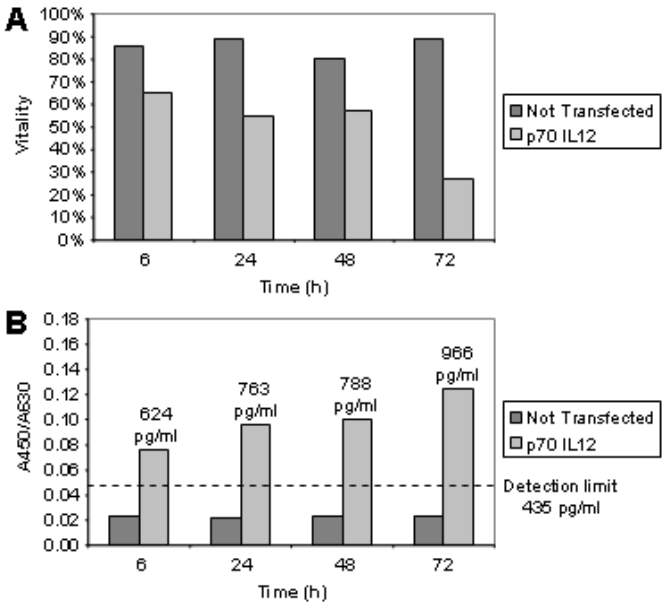


Figure 37 – IL-12 secretion from nucleofected precursor NemodDCs. Precursor NemodDCs were nucleofected with a p70 IL-12 encoding plasmid. Nucleofected and non-nucleofected cells were analysed for vitality (A) measured by trypan blue staining and for IL-12 secretion (B) detected after varying time intervals employing a sandwich ELISA setup. The 24 hour value is representative of 3 independent experiments. The detection limit of this experiment was 435 pg/ml referring to the lowest concentration included in the standard curve.

Since we were unable to obtain an IL-12 secreting NemodDC clone, and since the transient expression of IL-12 from NemodDC would not last the 10 days needed for differentiation and

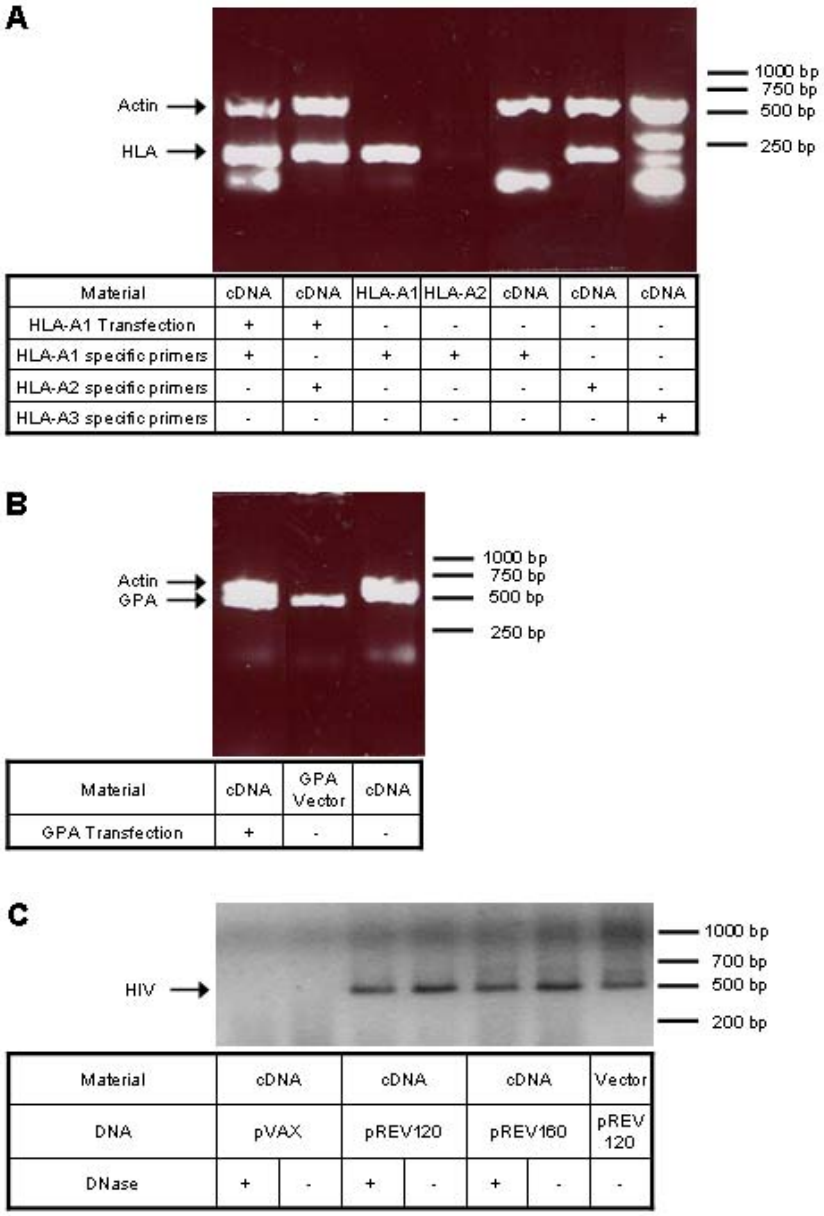
maturation, it became increasingly interesting to induce IL-12 in Nemod-mDCs. This is investigated in chapter 2.7.6.4 in a cotransfection study of an HIV DNA vaccine and the IL-12 encoding plasmid.

2.7.5 Induction of HLA-A1 and GPA in NemodDC by nucleofection

For dendritic cells to stimulate T cells they have to match the HLA type of the T cells. The HLA-A genotype of the cell line NemodDC has been determined to be HLA-$A2^+$ and HLA-$A3^+$ (unpublished results, Trinder P). We were therefore interested in broadening the range of T cells applicable for DC stimulation. Experimentally we investigated if HLA-A1 could be induced in NemodDCs by nucleofection. RT-PCR on precursor NemodDCs nucleofected with pHLA-A1 (cf. Appendix F) confirmed the presence of HLA-A1 mRNA, whereas non-transfected cells were negative (figure 38.A). At the same time both nucleofected and non-nucleofected cells were positive for the presence of HLA-A2 mRNA, and the non-nucleofected cells were further tested to be weakly positive for HLA-A3 although other non-specific bands were also amplified in this PCR. The specificity of the HLA-A1 gene specific primer set was further confirmed by analysis of cross reaction with an HLA-A2 encoding control plasmid (figure 38.A). Primers specific for the house keeping gene actin was included to verify that the PCR worked. Following, unsuccessful attempts were made to detect HLA-A1 on the cell surface. The functionality of the HLA-A1 antibody was confirmed by staining of the HLA-$A1^+$ C1R.A1 cell line. HLA-A2 specific antibodies showed cell staining of both nucleofected and non-nucleofected cells (Data not shown). Although both HLA-A1 and HLA-A2 seems to be transcribed to approximately the same extend the HLA-A1 gene is either not translated or delivered to the cell surface. It was so far not possible to detect HLA-A3 on the cell surface of precursor NemodDCs (personal communication, Baumeister H) probably due to the limited mRNA level, but maybe also for other unknown reasons that might similarly influence the HLA-A1 gene.

The transcription and translation of glycophorin A was analysed in a similar manner. The RT-PCR analysis successfully revealed the presence of GPA mRNA in nucleofected cells and not in non-nucleofected cells. The size of the GPA specific DNA band was nearly similar to the actin control band, but it is possible to detect the presence of two bands in the first lane containing cDNA from nucleofected cells that can be split in a GPA specific band (lane 2) and an actin specific band (lane 3) (figure 38.B). The presence of membrane bound GPA on the cell surface was subsequently

analysed by flow cytometry utilising the two monoclonal antibodies A63-C/A9 specific for asialoglycophorin A and A83-C/B12 binding both asialoglycophorin A and glycophorin A (unpublished results, Karsten U). We were unable to detect GPA expressing cells, although the GPA^+ and $aGPA^+$ NM-F9 cell line showed intensive staining with the two antibodies (data not shown).



Material	cDNA	cDNA	HLA-A1	HLA-A2	cDNA	cDNA	cDNA
HLA-A1 Transfection	+	+	-	-	-	-	-
HLA-A1 specific primers	+	-	+	+	+	-	-
HLA-A2 specific primers	-	+	-	-	-	+	-
HLA-A3 specific primers	-	-	-	-	-	-	+

Material	cDNA	GPA Vector	cDNA
GPA Transfection	+	-	-

Material	cDNA		cDNA		cDNA		Vector
DNA	pVAX		pREV120		pREV160		pREV 120
DNase	+	-	+	-	+	-	-

Figure 38 – Transcription analysis of nucleofected Nemod-DCs. HLA-A2 and HLA-A3 positive precursor NemodDCs were nucleofected with plasmids encoding (A) the HLA-A1 full length gene and (B) glycophorine A (GPA) as well as (C) the HIV vaccine plasmids pREV120, pREV160 and pVAX encoding gp120, gp160 or being empty respectively. After over night incubation total mRNA was isolated from both nucleofected and non-nucleofected cells and analysed for the presence of the transfected gene products by RT-PCR and gene specific primers. As a control for successful RNA purification and subsequent cDNA synthesis actin specific primers were added. (A) The presence of mRNA encoding HLA-A1, A2 and A3 was determined by specific primer pairs and the quality of the HLA-A1 specific primer pair was assayed with HLA-A1 and HLA-A2 encoding plasmids as template. (B) The GPA gene was detected with GPA specific primer. (C) The gp120 and gp160 gene products were detected by a specific primer pair recognising a gene fragment present in both gp120 and gp160 but not in the empty pVAX plasmid.To exclude the risk of DNA contamination the purified mRNA was treated with or without DNase.

The unsuccessful expression of membrane bound proteins might be due to features such as enhancers, signal peptide, codon usage and polyadenylation site utilised by the gene constructs, intrinsic characteristics of the cell line as well as disturbances in the cell membrane caused by the electric shock. The usefulness of an $HLA\text{-}A1^+$, $HLA\text{-}A2^+$ and $HLA\text{-}A3^+$ human dendritic cell line

are immense due to the compatibility with a major part of the human population. The surplus HLA alleles both within the A, B and C sub-families would induce some alloreactivity, and they therefore confer a technical challenge. It remains to be determined if such alloreactivity would paramount the specific response *in vivo*, but several *in vitro* studies show a specific T cell response although background is observed probably due to alloreactivity (unpublished results, Baumeister H).

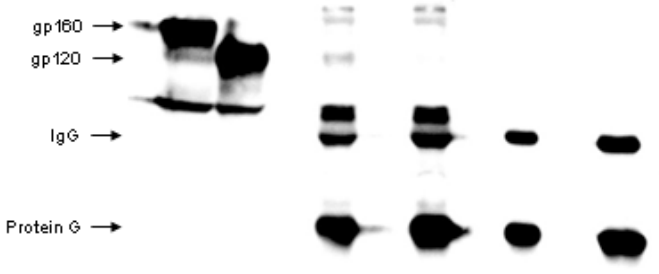
2.7.6 DNA vaccine comprising the HIV envelope gene

The successful expression of both soluble and secreted proteins in precursor NemodDCs encouraged the initiation of an experimental verification of a potential novel genetic HIV vaccine based on the HIV envelope gene gp160 and the related gp120. The wild-type envelope gene is overlapping with the rev gene, which is responsible for the transport of full length envelope encoding mRNA. A range of studies have confirmed the need for the rev protein to generate immunogenic amounts of gp160. The studies have dealt with the problem either by optimising the codon usage for mammalian expression (Fomsgaard, 1999) or by including the rev gene in the vaccine constructs. The latter strategy was analysed in a recent study by Xin and co-workers, showing that rev-env in its native gene structure was indeed important for the potency of HIV vaccines, when compared with vaccines without rev or even adding rev as an independent gene driven by an IRES (Jounai et al., 2003). In our study we employed a wild-type rev-env gene construct, which was observed to express gp160 more efficiently than the construct without the rev gene (Unpublished results, Schilling R, Strathmann AG, Hamburg). A construct, which solely express the gp120 protein, was generated by insertion of a stop-codon terminating the translation of gp160 at the glycin in position 512.

The first studies of the HIV gp160 vaccine by Ralf Schilling was performed in HEK293 cells, showing both transcription and translation of gp160 and gp120 from the respective vaccine plasmids (Unpublished results, Schilling R, Strathmann AG, Hamburg). The functionality of the vaccine plasmids in NemodDC was confirmed by nucleofecting precursor NemodDCs with the vaccine plasmids encoding gp160 and gp120, respectively as well as empty plasmid. The nucleofected cells were cultivated for 24 hours before isolation of total RNA. The analysis of transcription of gp160 and gp120 was subsequently performed by RT-PCR using gp160 specific primers. Since the vaccine plasmids encoding gp160 and gp120, respectively are identical except for a point mutation the PCR does not differentiate between the transcription product of the two

plasmids, and the fragments observed are correspondingly of the same size (figure 38.C). The total RNA was treated with DNase to exclude that the positive DNA bands could be artefacts caused by plasmid or genomic DNA contaminations. Our observations show that gp160 and gp120 are indeed transcribed by precursor NemodDCs upon nucleofection with the vaccine plasmids, whereas empty plasmid induces no transcription of gp160 or gp120.

We further analysed the protein expression in nucleofected cells after 72 hours. Immunoprecipitated gp120 from cytoplasmic cell extract as well as from medium obtained from 3x10^6 cells was subsequently detected by western blotting using anti-gp120 antibody, which was also employed for immunoprecipitation. HEK293 cells transfected with the vaccine vectors were included as positive controls. We were able to detect very small amounts of gp120 in the supernatant although no gp120 could be detected in the cell extract (figure 39). The first protein detection experiments analysed gp120 and gp160 in 10^6 nucleofected cells both after 24 hours and 72 hours. In these experiments neither gp120 nor gp160 were detected (data not shown). In light of the successful experiment employing 3x10^6 cells these results are likely due to the use of an inadequate amount of cells. Whether gp160 is also detectable when 3x10^6 cells are employed remains to be elucidated. An explanation for the much reduced protein expression is presented by Hayakawa and co-workers, who shows that DNA plasmids for protein expression in mammalian cells and tissue are tightly dependent on parameters such as enhancers, promoters, introns and poly A signal. They further show that these parameters need to be optimised for each cell and tissue type (Xu et al., 2001). A recent mouse immunisation study confirms the importance of the promoter for gp160 encoding vaccine plasmids (Jounai et al., 2003). Optimisation studies are required to determine if expression of gp120 and gp160 can be augmented.



Cell type	HEK293		NemodDC		NemodDC	
Preparation	Cell pellet		IP supernatant		IP cell extract	
Plasmid	pREV160	pREV120	pREV120	pVAX	pREV120	pVAX

Figure 39 – Expression analysis of nucleofected Nemod-DCs. Western blot detection of anti-env polyclonal antibody immunoprecipitated HIV env gp120 obtained from 3x10^6 Nemod-DCs 72 hours after nucleofection with pREV120 or empty plasmid (pVAX). The nucleofection efficiency determined by flow cytometry of cells nucleofected with an EGFP reporter plasmid was 80% EGFP expressing cells and the vitality was 65%. The protein was prepared from supernatant and cytoplasmic cell extract. As positive controls human embryonal kidney cells (HEK293) transfected with pREV120 and pREV160 were included.

Since EGFP expression in the cells was observed to be very efficient, we compared the plasmids pEGFP N1 and pVAX1. Both include the CMV promoter, although the CMV promoter in pEGFP N1 is 27bp longer at the 5' end, whereas the 3' end is 21 bp shorter compared to pVAX1. The TATA box in pEGFP N1 is around 120 bp from translation start, compared to around 400 bp for the vaccine plasmids. Interestingly, the Kozak sequence is optimal for pEGFP N1, but sub-optimal in the vaccine plasmids. The polyadenylation signal differs for the two plasmids, being SV40 pA for pEGFP N1 and BGH pA for pVAX1. These observations suggest that the vaccine plasmid might be optimised by improving the Kozak sequence and specifically for NemodDC by changing the polyadenylation signal. Also the minor differences for the CMV promoter sequence and localisation of the TATA box might be of importance, although the gene transcription does not seem to be the problem as shown in figure 38.C.

Despite the very weak expression of envelope protein encoded by the HIV vaccine plasmids, we chose to proceed with an analysis of T cell activation induced by nucleofected NemodDCs. The arguments for this decision were primarily that even very small amounts of protein can lead to active presentation of antigen on MHC class I as discussed by Lyerly and co-workers (Morse and Lyerly, 2002). Indeed APCs need to display approximately 100 MHC complexes to establish recognition by T helper cells and as few as one complex for recognition by cytotoxic T cells. Despite the motility of the cells and the small number of MHC complexes it has interestingly been reported that as many as 20.000 TCRs can be triggered by as few as 100 peptide-MHC complexes (Lanzavecchia, 1997).

2.7.6.1 Identification of the optimal day of nucleofection for $CD8^+$ T cell priming experiments

As described in chapter 1.2.12 the $CD34^+$ precursor NemodDCs can be differentiated *in vitro*, thereby generating immature dendritic cells and subsequent mature dendritic cells. Immature dendritic cells are established after 7 days of culture in medium containing GM-CSF and IL-4. Replacing the cytokines with TNF-α on day 8 induce maturation of the Nemod-iDCs within two days. On day 10 the Nemod-mDCs either loaded with peptide, protein, lysate, DNA or RNA are ready for priming naïve T cells. In our study we have focused on stimulation of naïve $CD8^+$ T cells. As mentioned above nucleofection of cells did so far not result in cells stably expressing the protein of interest. Since DC differentiation and maturation takes 10 days it is not feasible to nucleofect the

precursor cells that would lose the recombinant gene during the 10 days of cultivation. Instead it is necessary to optimise the time of nucleofection to reach maximal immunostimulatory capacity on day 10 and forward. To identify the optimal day of nucleofection the decreasing expression rate of the recombinant gene as well as the cell viability after nucleofection have to be considered.

In the optimisation protocol dendritic cells on day 4, 5, 6 and 7 of differentiation were nucleofected with the EGFP reporter plasmid. Cells were re-cultivated after nucleofection under the same conditions as before nucleofection. On day 8, GM-CSF and IL-4 were replaced with TNF-α and the cells were cultivated for further 2 days. The percentage of EGFP expressing cells as well as cell viability was measured on all subsequent days after nucleofection until full maturation (figure 40). The optimisation study suggested that nucleofection should be performed on day 4 or 5. Interestingly the maximum percentage of EGFP expressing cells was detected on day 7 no matter the day of nucleofection. The cells nucleofected on day 7 were not measured on the same day, but they peaked on day 8 indicating that maximum protein expression capacity was already reached. These observations fit nicely with the model of dendritic cell differentiation being transcriptionally active in the differentiation phase, whereas the maturation process is acknowledged for augmenting post transcriptional and post translational modifications (Richards et al., 2002). Viability seemed to be optimal for nucleofection on day 6, however the expression rate is much higher when cells were nucleofected at day 4 or 5 during differentiation. Of course these data describe the optimal day of nucleofection for pEGFP N1. This may therefore be different for other expression plasmids. The study would have improved by using an EGFP encoding vaccine vector (pVAX1 encoding EGFP).

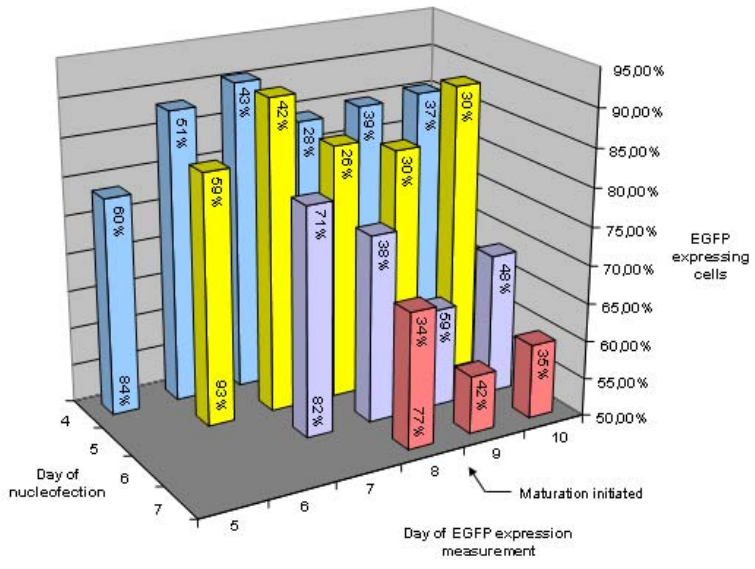


Figure 40 – Optimising the day of nucleofection for differentiating and maturating dendritic cells. To reach good expression and vitality on the day of full maturation, NemodDCs were nucleofected with an EGFP encoding plasmid at day 4 (blue bars), 5 (yellow bars), 6 (gray bars) and 7 (red bars) during the differentiation period. Day numbers stated in the figure all refers to day 1 as day of differentiation initiation. The vitality before nucleofection are stated in the bottom of the first bar representing each day of nucleofection tested. The cells were analysed for vitality (stated in the top of each bar) and the percentage of EGFP expressing cells on each day after nucleofection until full maturation on day 10. All measurements are obtained by flow cytometry and the data presented are representative of two identical studies.

2.7.6.2 Phenotypic changes due to the nucleofection protocol

Choosing day 5 as optimal day of nucleofection we turned our interest to possible phenotypic changes due to the electric shock as well as expression of the foreign gp160 or gp120. Dendritic cells were nucleofected with the vaccine plasmid encoding gp120 as well as empty plasmid. Non-nucleofected cells were included as control. The cells were subsequently cultivated for another 5 days to finish differentiation and maturation. The nucleofected cells were analysed for expression of various cell surface markers by flow cytometry on day 8 as Nemod-iDCs (figure 41.A) and day 10 as Nemod-mDCs (figure 41.B). Interestingly our data shows that precursor markers like CD34, CD14 and CD30 seems to reach their minimum already at day 8 for transfected cells, whereas the non-nucleofected culture reach minimum two days later at day 10. The costimulatory molecules CD80, CD86 and CD40 likewise reach their maximum at day 8 compared to day 10 for non-nucleofected cells. Basically our data indicate that the electric shock induces maturation of dendritic cells, although the maturation marker CD83 is not expressed on nucleofected cells. This could be due to the advanced differentiation state, and thereby simply indicating that day 8 is already post maturation. In chapter 2.7.6.4 we pursue the effect of reduced differentiation time, indicating that although phenotypic markers indeed indicate a more mature DC, their T cell stimulating capacity is reduced. Immuno escape mechanisms like down regulation of MHC molecules have been observed for HIV. Such down regulations have been pinpointed to the Vpu and Nef proteins, and since the Vpu gene is encoded within the rev-env gene, although in a seperate reading frame, it is unavoidable to include the Vpu gene in a native rev-env encoding vaccine construct (Frankel and Young, 1998). Luckily, we detected high expression of MHC class molecules on dendritic cells nucleofected with gp120 or gp160 encoding plasmids (figure 41 and data not shown).

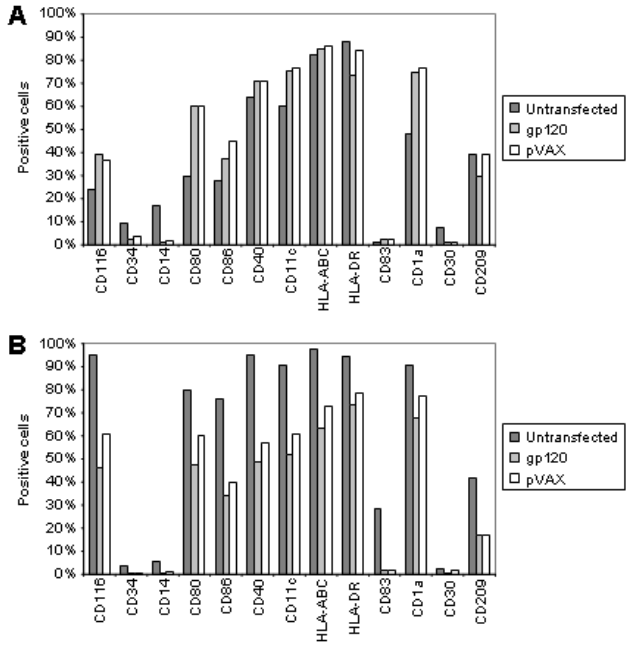


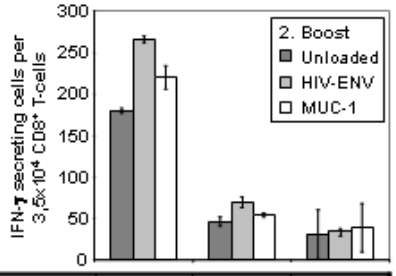
Figure 41 – Nemod-mDC phenotype changes due to nucleofection. Nemod-iDCs (A) and Nemod-mDCs (B) nucleofected on day 5 of differentiation with the HIV vaccine plasmid encoding gp120 and the empty plasmid as well as non-nucleofected cells are assayed for their expression of clusters of differentiation markers by flow cytometry. The percentage of cells expressing each marker is depicted.

2.7.6.3 $CD8^+$ T cell priming experiments employing the HIV DNA vaccines

$CD8^+$ T cells from an HLA-$A2^+$ donor were isolated by positive selection for the presence of the CD8 marker utilising magnetic cell sorting (MACS) technology with anti-CD8 antibody coupled microbeads. The donor phenotype with respect to MHC Class I is absolutely essential for the assay. T cells are only able to specifically recognise antigens presented by dendritic cells in the context of matching MHC class molecules. Indeed non-matching HLA alleles would theoretically induce an allogene immune response contributing to the intrinsic background of the system. The HLA genotype of the precursor NemodDC is HLA-A*02 (-A2), -A*03 (-A3), -B*44 (-B44), -B*56 (-B56), -Cw*04 (-Cw4), -Cw*07 (-Cw7), -DRB1*10 (-DR10), -DRB1*11 (-DR11), -DRB3*02 (-DR52), -DQB1*03 (-DQ5), -DQB1*05 (-DQ7), -DPB1*03 (-DPw3) and –DPB1*04 (-DPw4) including the serological names of the corresponding alleles in brackets. Some of the HLA alleles have been studied serologically to determine the HLA phenotype of the precursor NemodDC. The phenotype is HLA-A2, -B44, -DR10, -DR11, -DR52 and -DQ7, HLA-A3 is undetectable and the remaining alleles have not been analysed (unpublished results, Trinder P). The donor was screened for the presence of HLA-A2, but the phenotype was not further characterised, and mismatching between the phenotypes therefore could be responsible for the elevated background. On the other hand the assay describes the usefulness of a DC therapy against HIV in all HLA-$A2^+$ patients. The unsuccessful HLA-A1 nucleofection study was initiated with the goals of circumventing such allogene response and broaden the feasibility of a DC therapy to a larger range of patients (cf. chapter 2.7.2).

Purified naïve $CD8^+$ T cells were mixed with Nemod-mDCs, which had been nucleofected on the fifth day of differentiation with the gp160 and gp120 encoding vaccine plasmids as well as the empty vaccine plasmid (mDC:T cell ratio was 1:10). After a five day prime stimulation the surviving T cells were collected and T cell activation due to antigen presentation on T2 cells was measured as the number of IFN-γ secreting cells in a standard ELISPOT assay. We employed unloaded T2 cells as well as T2 cells loaded with either an irrelevant MUC-1 peptide or an HIV-ENV peptide derived from the V3 loop of gp120 and gp160. Both $CD4^+$ and $CD8^+$ T cells are reported to recognise V3 derived peptides (Moore and Fox, 1993; Moukrim et al., 1996; Ratto et al., 1996; Warren and Thomas, 1992). Therefore a qualified verification of T cell response can be obtained from restimulation studies with the HIV-ENV peptide, although it does not exclude T cell responses against other envelope derived peptides (Chitnis et al., 2003).

Our data suggest that dendritic cells nucleofected with the gp120 encoding vaccine plasmid induce a rather high number of IFN-γ secreting CD8+ T cells. Dendritic cells nucleofected with gp160 encoding or empty vaccine plasmid both are unable to induce specific T cell activation. The specificity of these reactions is shown by restimulation of the primed T cells with T2 cells presenting the gp160 V3 derived HIV-ENV peptide or the irrelevant MUC1 peptide or with unloaded T2 cells. Although a significant portion of the T cell response after priming with gp120 nucleofected dendritic cells is specifically directed against the gp120 protein, the number of stimulated unspecific T cells seems to be rather high. This may be caused by a strong specific prime reaction still active after restimulation with T2 cells loaded with irrelevant or no peptide. Another explanation would be the allogenic reaction due to mismatching HLA genotypes. Studying dendritic cell viability before T cell priming shows that cells nucleofected with empty plasmid are almost three times more viable than the ones nucleofected with gp120 or gp160 encoding plasmids. This could be due to a toxic effect of the encoded proteins, thereby offering an explanation for the low expression level of gp120 and gp160 in nucleofected precursor NemodDCs (cf. chapter 2.7.6). From the viability of the T cells as well as yield no noticeable difference is observed, which could indicate augmented proliferation of T cells activated by dendritic cells nucleofected with gp120 or gp160 encoding plasmids compared to dendritic cells nucleofected with empty plasmid (figure 42). The fact that we are unable to detect such differences in T cell viability most likely is caused by the background proliferation induced by allogenic T cell activation.



DNA Vaccine	pREV120	pREV160	pVAX
mDC vitality before primary boost	17,7%	24,1%	57,9%
CD8+ T-Cell vitality after primary boost	20,9%	22,2%	17,9%
CD8+ T-Cell yield after primary boost	7,5%	13,3%	6,3%

Figure 42 – CD8+ T cell activation by dendritic cells nucleofected with an HIV vaccine. CD8+ T cells purified from an HLA-A2 donor are stimulated for five days (primary boost) with Nemod-mDCs nucleofected on day 5 after differentiation initiation with HIV vaccine plasmids encoding HIV envelope genes gp120 and gp160 as well as the empty plasmid pVAX. Nucleofected Nemod-mDCs and naïve CD8+ T cells are mixed in a 1:10 ratio. The surviving CD8+ T cells are restimulated to test for specific recognition of the envelope related peptide (HIV-ENV) encoded by both the gp120 and the gp160 vaccine but not the empty pVAX plasmid. The peptide is *in vitro* loaded onto MHC class I molecules on HLA-A2 positive T2 cells. As negative controls CD8+ T cells are stimulated with T2 cells loaded with an irrelevant MUC-1 peptide as well as non-loaded T2 cells. 35.000 CD8+ T cells are restimulated with 7.000 T2 cells in an IFN-γ ELISPOT assay, and the number of IFN-γ secreting cells is detected. Vitality numbers are counted by trypan blue staining. The experiment is representative of two independent experiments.

The structural studies of gp41 and gp120 describe gp120 as non-covalently attached to the membrane spanning gp41 (Lawless et al., 1996). In the HIV DNA vaccine encoding only gp120 the

gp120 will thus be secreted as opposed to the membrane bound situation for the full length gp160 encoding vaccine. Aside from misfolded antigens, which are degraded by the proteasome and loaded onto MHC class I complexes, antigens will be collected by endocytosis of secreted soluble gp120 or membrane bound gp120. This difference might enable varying degrees of MHC class I presentation of ENV derived peptides and explain the varying ability to induce $CD8^+$ T cell activation. Indeed the pathway for receptor mediated endocytosis recycles receptors, but leaves the caught antigen behind for processing and presentation (Aderem and Underhill, 1999). To support the hypothesis the non-covalent interaction between gp41 and gp120 must be strong enough to withstand the rather harsh conditions in the endocytic compartments. Furthermore, the dendritic cells must be able to present endocytosed material on MHC class I molecules via cross-priming. Although the theory is speculative observations of the immune response in gene gun immunised mice, with gp120 and gp160 encoding DNA vaccines, respectively suggest that gp120 is inducing humoral immune response more effectively than gp160, although no difference in CTL response was observed (Fomsgaard, 1999; Vinner et al., 1999).

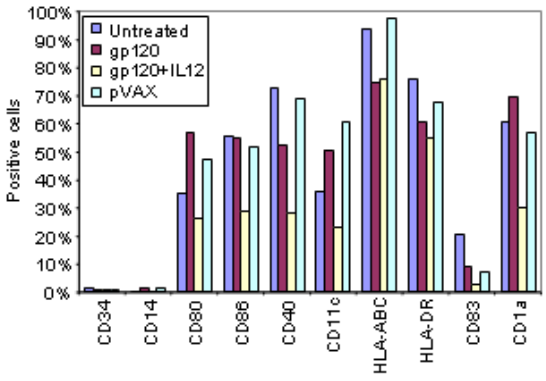


Figure 43 – Nemod-mDC phenotype changes due to nucleofection and reduced differentiation time. Nemod-mDCs nucleofected on day 5 of differentiation with the HIV vaccine plasmid encoding gp120 in combination with an IL-12 encoding plasmid as indicated are analysed for their expression of clusters of differentiation markers by flow cytometry. As controls mDCs nucleofected with empty vaccine plasmid (pVAX) as well as non-nucleofected cells are analysed. The percentage of cells expressing each marker is depicted.

2.7.6.4 Effect on T cell activation by reduced differentiation time and IL-12 induction

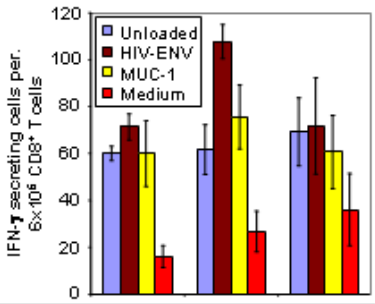
As described in chapter 2.7.6.2 we observed that nucleofected cells seemed to mature faster than untreated cells, when judged by the phenotypic surface markers. We therefore reduced the differentiation time to five days succeeded by two days of maturation. The cells were nucleofected on day five showing a stable transfection rate of approximately 70% during maturation and a final viability of approximately 20%. The phenotype of the obtained mDCs was analysed by flow cytometry (figure 43), showing that the nucleofected cells indeed seemed more mature than when differentiated for 7 days (figure 41). Especially, the presence of maturation marker, CD83 on nucleofected cells was striking. Interestingly, also the untreated cells seemed to mature with the

reduced time of differentiation, although CD80 was slightly reduced both compared to nucleofected cells and to the normal level obtained by the standard maturation protocol.

The lacking IL-12 secretion of the mature DCs was discussed in chapter 2.7.4. We also showed that it is possible to induce IL-12 secretion by nucleofection with an IL-12 encoding plasmid. We therefore set out to investigate, whether conucleofection of the HIV DNA vaccine and the IL-12 encoding plasmid would augment the HIV specific response compared to the HIV DNA vaccine alone. Using the conditions of the previous T cell activation study (chapter 2.7.6.3) the expected IL-12 secretion would be reduced to virtually zero after the 5 day period from nucleofection to T cell stimulation. In the new setup this period is therefore reduced to 3 days, and it was thus more likely that the nucleofected cells would still be secreting IL-12 when added to the T cells. The IL-12 secretion that we observed 24 hours after nucleofection, at which time the medium was changed to TNF-α containing medium, was approximately 250 pg/ml. Subsequently, IL-12 secreted during the two days of maturation resulted in an IL-12 concentration of approximately 50 pg/ml. Clearly, IL-12 secretion is at its maximum right after nucleofection, but IL-12 is also being secreted during maturation. Whether the IL-12 secretion from nucleofected mDCs is high enough to effect the T cell activation was investigated in a $CD8^+$ T cell activation study.

$CD8^+$ T cells from an HLA-$A2^+$ donor was mixed with dendritic cells nucleofected with empty vaccine vector or the gp120 encoding HIV DNA vaccine with or without conucleofection with the IL-12 encoding plasmid. After five days of primary stimulation the surviving T cells were restimulated with T2 cells loaded with the gp120 derived HIV-ENV peptide. As negative controls T cells were restimulated with unloaded T2 cells as well as T2 cells loaded with irrelevant MUC1 peptide. Non-restimulated T cells were similarly analysed, to determine the strength of the primary stimulation. Active T cells were detected by their ability to secrete IFN-γ in an ELISPOT assay (figure 44). Although our phenotypic analysis by flow cytometry indicated that the nucleofected cells were indeed mature, a HIV-ENV specific $CD8^+$ T cell response was absent for T cells stimulated with dendritic cells nucleofected with the HIV DNA vaccine. This observation is yet another clear indication that the interpretation of cell surface marker molecules is connected with considerable uncertainty, and that such interpretations should always be verified with cellular assays. Interestingly, the dendritic cells conucleofected with the HIV DNA vaccine and the IL-12 encoding plasmid were able to induce HIV-ENV specific T cell activation (figure 44). It therefore

seems that an even very small amount of secreted IL-12 from dendritic cells is sufficient to induce the primary T cell activation. Our observation is confirmed by a similar study by Storkus and co-workers, in which both antigen and immuno modulatory genes are transferred in parallel to dendritic cells (Wilson et al., 1999). Briefly, an HIV-nef vaccine combined with genetic constructs encoding Th1-biasing IL-12 or IFN-α improved the CTL response compared with HIV-nef vaccine alone. These data of course increase the interest of generating an IL-12 secreting variant of NemodDC. Such variant could be obtained through a yet unknown maturation protocol or by cloning of cells transfected with the IL-12 encoding plasmid. So far cloning of nucleofected cells has been unsuccessful, and therefore alternative delivery systems, such as viral delivery should be considered (Morse and Lyerly, 2002).



DNA Vaccine	pREV120	pREV120 + pGT60 hIL12	pVAX
mDC vitality before primary boost	24%	18%	20%
CD8⁺ T-Cell vitality after primary boost	47%	42%	38%
CD8⁺ T-Cell yield after primary boost	14%	12%	14%

Figure 44 – CD8+ T cell activation by dendritic cells nucleofected with the HIV vaccine in combination with the IL-12 encoding plasmid. CD8+ T cells purified from an HLA-A2 donor are stimulated for five days (primary boost) with Nemod-mDCs nucleofected on day 5 after differentiation initiation with HIV vaccine plasmids encoding HIV envelope genes gp120 in combination with an IL-12 encoding plasmid as indicated as well as empty vaccine plasmid (pVAX). Nucleofected Nemod-mDCs and naïve CD8+ T cells are mixed in a 1:10 ratio. The surviving CD8+ T cells are restimulated to test for specific recognition of the envelope related peptide (HIV-ENV) encoded by the gp120 vaccine, but not the empty pVAX plasmid. The peptide is *in vitro* loaded onto MHC class I molecules on HLA-A2 positive T2 cells. As negative controls CD8+ T cells are stimulated with T2 cells loaded with an irrelevant MUC-1 peptide as well as non-loaded T2 cells. Finally non-restimulated CD8+ T cells are analysed to determine the number of T cells still secreting IFN-γ due to the primary activation. 60.000 CD8+ T cells are restimulated with 6.000 T2 cells in an IFN-γ ELISPOT assay, and the number of IFN-γ secreting cells is detected. Vitality numbers are counted by trypan blue staining.

2.7.7 Comparative study on CD8+ T cell priming employing RNA, lysate and peptide

It is known that immune responses towards tumours are very weak. Tumour immune escape is partly caused by the lacking presentation of tumour antigens due to down-regulation of MHC class I molecules on the cell surface (Moingeon, 2001). The need for professional antigen presenting cells to take action is evident, and a range of studies did induce CTL response against various tumours using APCs loaded in various ways including RNA transfection, lysate treatment and peptide pulsing (reviewed by Steinman and co-workers (Schuler et al., 2003)). NemodDC has in a large number of *in vitro* experiments been shown to induce T cell activation when loaded with tumour

lysate, proteins, a ganglioside as well as by peptide pulsing (Paper in preparation, Baumeister H). The successful T cell activation induced by nucleofected dendritic cells (cf. chapter 2.7.6) therefore encouraged us to make a comparative study of the T cell activation efficiency of dendritic cells loaded via RNA nucleofection, tumour lysate and peptide pulsing. The mammary epithelium MCF-7 breast tumour cells (ATCC: HTB-22) was used as a model system. A recent study reports that dendritic cells transfected with MCF-7 RNA indeed induces a CTL response against MCF-7 cells (Muller et al., 2003). Earlier studies with NemodDC loaded with the MCF-7 related MUC-1 peptide as well as non-necrotic MCF-7 lysate also showed $CD8^+$ T cell activation (Unpublished results, Baumeister H).

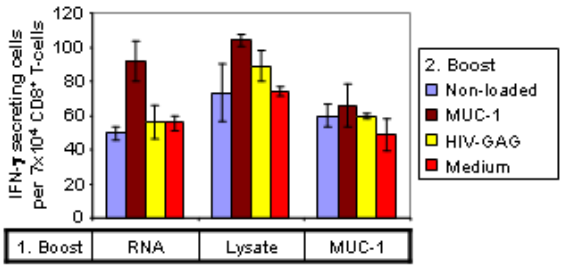


Figure 45 – Analysis of the $CD8^+$ T cell stimulatory ability of mRNA nucleofection, lysate treated and peptide loaded dendritic cells. $CD8^+$ T cells purified from an HLA-A2 donor are stimulated for five days (primary boost) with Nemod-mDCs loaded with antigens related to the MUC-1 positive MCF-7 cell line (A) or the MART-1 positive MEL624 cell line (B). The Nemod-mDCs were loaded either by nucleofection with total mRNA from the respective cell lines on day 5 after differentiation, treatment with cell lysate or incubated with peptide. The surviving $CD8^+$ T cells are restimulated to test for specific recognition of the oncogen related peptides MUC-1 and MART-1. The peptides are *in vitro* loaded onto MHC class I molecules on HLA-A2 positive T2 cells. As negative controls $CD8^+$ T cells are stimulated with T2 cells loaded with an irrelevant HIV-GAG peptide as well as non-loaded T2 cells. The activity level of the $CD8^+$ T cells origining from the primary bost is assayed by adding only medium to the cells. 70.000 $CD8^+$ T cells are restimulated with 7.000 T2 cells in an IFN-γ ELISPOT assay, and the number of IFN-γ secreting cells is detected.

In our study total RNA from MUC1 positive MCF-7 cells was nucleofected into dendritic cells on day 5 of the differentiation. We used the optimised conditions from the pEGFP N1 plasmid studies (cf. chapter 2.7.1 and 2.7.6.1), and it is therefore likely that more optimal conditions could be found in a new optimisation study with RNA from EGFP expressing cells. Most likely the response time for RNA would be shorter than for DNA, since transcription is not required. Dendritic cells from the same pool were analysed for their capacity to present antigens derived from MCF-7 lysate as well as their capacity to be pulsed with MUC-1 peptide. Lysate and peptide were loaded according to a standard protocol, which have worked in earlier studies with the same lysate and peptide (unpublished results, Baumeister H). Interestingly, the data show that RNA nucleofected dendritic cells induce a specific $CD8^+$ T cell response, which can be restimulated with T2 cells loaded with

MUC-1 peptide compared to T2 cells loaded with the irrelevant HIV-GAG peptide as well as unloaded T2 cells (figure 45). In the case of lysate a much higher background was observed although the MUC-1 peptide loaded T2 cells gave a slightly higher response than the negative controls. For some reason the MUC-1 peptide pulsed dendritic cells did not induce specific T cell activation. We also observed that the number of IFN-γ secreting T cells without restimulation and T cells restimulated with unloaded T2 cells were very much alike. Such background is most likely due to high prime stimulation as well as allogenic immune response caused by a non-compatible HLA phenotype.

We further analysed the CTL activity of the stimulated and IFN-γ secreting $CD8^+$ T cells in a standard Europium assay (Blomberg et al., 1986a; Blomberg et al., 1986b) targeting MCF-7 cells that were treated with IFN-γ to up-regulate the MHC class I expression (Brown et al., 1988; Giacomini et al., 1988). Preliminary results did not show any CTL response for T cells stimulated with RNA transfected dendritic cells as well as for T cells stimulated with MUC-1 loaded dendritic cells employed as positive control. The unsuccessful result was most likely due to a very low number of vital $CD8^+$ T cells (data not shown), and should be repeated in future experiments.

These preliminary data suggest that RNA nucleofection is a more effective loading method than lysate or peptide loading, although it might be possible to optimise the conditions for loading with lysate and peptide, for example by lowering the pH to augment endocytosis of antigens (Vermeulen et al., 2004). Furthermore, the scavenger CD36 receptor responsible for phagocytosis of apoptotic cells should in future studies be analysed by flow cytometry before lysate treatment, to render the internalisation of lysate probable. Preliminary results obtained with the Affymetrix DNA microarray technology confirmed that mRNA encoding CD36 was present in Nemod-iDC and subsequently down regulated upon maturation (data not shown), which has also been reported by Bhardwaj and co-workers (Albert et al., 1998). We observe a small specific response and a non-specific response for lysate and peptide, respectively. However, the dendritic cells might not be responsible for the non-responsiveness, which could also be due to the fitness of the $CD8^+$ T cells, which would back up the conclusion that RNA nucleofection is the most effective loading method. The T cell fitness has been one of the major obstacles of the assay, since this parameter is donor dependent and undetectable until the conclusion of the comprehensive assay. The fitness of the

$CD8^+$ T cells was much improved after implementation of one single donor delivering all the T cells, although even T cells from the same donor from various retrieval dates vary in fitness.

2.8 Concluding remarks on the nucleofection of NemodDC

The presented data confirm that it is indeed possible to transfer genetic material to the functional human dendritic cell line, NemodDC, at varying differentiation stages. The transfer of DNA was extensively improved employing the nucleofection technology compared to both lipofection and standard electroporation. Reaching transfection rates in the range of 90% the technique is almost as efficient as viral delivery systems. Unfortunately, the success is short-lived due to decreasing viability, which hampers the generation of stably transfected clones.

In $CD8^+$ T cell activation studies we show that the nucleofection of DNA and RNA into dendritic cells can induce appropriate antigen presentation and subsequently T cell activation. Preliminary results further suggest that the nucleofection technology is a more efficient DC loading technique than loading via lysate or peptide pulsing, at least for the antigen material employed in this particular study.

Our optimisation studies of nucleofection of NemodDC intended for $CD8^+$ T cell priming showed that nucleofection on day 5 of differentiation was optimal. A major technological difficulty was that the viability on day 10 was very low, thereby forcing us to use non-optimal T cell/mDC ratios in some T cell priming experiments. It would consequently be very beneficial if the T cell priming could be initiated sooner after DC nucleofection. Our phenotypic characterisation suggests that this is possible, since the nucleofection seems to induce faster differentiation and maturation. However, dendritic cells that were differentiated for 5 days instead of 7 did not stimulate $CD8^+$ T cells as efficiently as 7 days differentiated dendritic cells.

The phenotypic characterisation of the dendritic cell line showed that cytokines like Th2 biasing IL-4 and IL-10 as well as IL-12 inducing Th1 response are not secreted. Our studies of IL-12 gene transfer show that the nucleofection technique can induce immuno modulatory genes, which improve the adjuvant effect of the dendritic cells. We further showed that dendritic cells nucleofected with a mixture of the gp120 encoding HIV DNA vaccine and the IL-12 encoding plasmid are stimulating T cells more efficiently than dendritic cells only nucleofected with the vaccine vector.

To our knowledge this is the first report on the generation of a highly efficient gene transfer technique aimed at a human functional dendritic cell line. The easy production of large numbers of functional human dendritic cells combined with a highly effective gene transfer has great perspectives, and could in the near future be of utmost importance for the clinical utilisation of DC therapy.

3 General conclusions and future perspectives

3.1 General conclusions and future perspectives

In chapter 2.1 we describe the generation of a novel fusion system, designated the FuncFAb system, which has beneficial biochemical properties, such as multimerisation and the ability to rescue phage displayed scFvs as soluble active fusion proteins. Further described in chapters 2.2 and 2.3 this fusion system is also highly immunogenic, inducing strong adaptive humoral immune responses upon administration either as a protein or DNA vaccine. In either case adjuvanticity is retained within the vaccines, which therefore require no supplementary adjuvants, such as incomplete Freund's adjuvant and alum-derived adjuvants. The filamentous phage protein III fragment employed in the FuncFAb system is supposedly non-harmful, since animals and humans are constantly challenged with a plethora of phage strains in our colon, upper respiratory system and on our skin (Bergh et al., 1989). Indeed, non-immunised rabbit sera contain inactivating filamentous bacteriophage antibodies (Salivar et al., 1964). Further studies have shown intravenous whole-phage administration to be non-harmful, although the human experiments were performed with other phage strains (in mice (Willis et al., 1993) and in humans (Merril et al., 2003; Pyun et al., 1989)). Until recently, only relatively weak adjuvants, such as alum-derived adjuvants, have been approved for human vaccination, whereas strong adjuvants, such as complete or incomplete Freund's adjuvants, have not. The adjuvant strength of the FuncFAb system is comparable to incomplete Freund's adjuvant, which makes it an important candidate for future vaccine approaches. In the future experiments should therefore be performed to verify the adjuvant effect of the FuncFAb system by employing a larger number of antigens as well as larger pools of animals.

The most potent adjuvant discovered so far is the dendritic cell. It exists with the sole purpose of presenting antigens to $CD4^+$ T cells and via cross priming also $CD8^+$ T cells. An extensive number of studies have investigated the potential use of dendritic cell therapy, including the exploration of various antigen loading techniques onto dendritic cells *in vitro* (reviewed by Lyerly and co-workers (Morse and Lyerly, 2002) and Steinman and co-workers (Schuler et al., 2003)). In chapter 2.7 we explored the potential antigen loading of a human dendritic cell line with the novel nucleofection technique employing antigens encoded by DNA and RNA. We established $CD8^+$ T cell activation against a HIV envelope derived peptide (HIV-ENV) employing a gp120 encoding HIV DNA vaccine, and against a MUC-1 derived peptide employing total RNA from MUC-1 expressing MCF-7 cells. Future experiments should verify the generality of the technique by employing various other antigens. We further show that the nucleofection technology can modify the

phenotype of the dendritic cells, for instance by induction of cytokines, such as IL-12, that are able to modulate the type of immune response induced. In the future gene knock-in and knock-out modified dendritic cells may have great potential in modulating the type of immune response. Knock-in modified DCs can be generated by nucleofection with DNA as shown in chapter 2.7.2 and knock-out modifications may be generated by transfection of small interfering RNA molecules (siRNA) (Elbashir et al., 2001). These model cells are ideal for investigating dendritic cell biology, although low viability and inability to generate stable clones are technical issues that should be addressed in future experiments. The fact that IL-4, IL-10 and IL-12 are not produced under the conditions tested so far, shows that the model system is not a perfect model. Therefore, as for all model systems comparison with the situation *in vivo* should be performed with great prudence.

When the immune system raises a CTL response against a pathogen, it presents peptide fragments of pathogen derived proteins in the context of the MHC class I molecule to be recognised by $CD8^+$ T cells. Several pathogen derived peptides are normally presented simultaneously, although the applicability of peptides to be presented by MHC class I complexes is tightly linked to the HLA allele of the MHC complex. The dendritic cell vaccine loaded via nucleofection with antigen encoding DNA or RNA similarly presents a number of antigen derived peptides. When we estimate the $CD8^+$ T cell activation induced by the dendritic cell vaccine using an IFN-γ ELISPOT assay, our assay measure the activity towards one single antigen derived peptide, which is chosen due to earlier reports on immunisations with that specific antigen in the context of the same HLA phenotype. It is therefore obvious that the actual antigen specific T cell activation might be much higher, and that knowledge on which antigen derived peptides are inducing T cell response is of great importance. So far identification of such peptides is done in two steps, 1) identification of potential antigen derived peptides and 2) verification in T cell stimulation assays. The first step is normally approached either with bioinformatics on the basis of peptide databases (Brusic et al., 1998) or employing proteomics using a technique involving elution of peptides bound to cell surface MHC complexes followed by mass spectrometry (Crotzer et al., 2000; de Jong, 1998). The latter technique further enables detection of peptides generated by protein splicing that are not predictable from the protein sequence (Hanada et al., 2004)).

In chapter 2.5 we approached a technique theoretically capable of identifying any peptide, which in the context of an MHC class I complex, can bind to a sample of $CD8^+$ T cells. The technique

theoretically enable selection of peptides binding to a T cell receptor employing a phage displayed peptide library based on single-chain MHC class I molecules as scaffold. Our study shows that with the employed setup it is not possible to detect binding of a peptide specific T cell clone in a peptide specific manner. However, preliminary T cell activation data with cross-linked scMHC phage show weak peptide specific activation, which suggests that T cell binding, might be possible with cross-linked scMHC phage. The need for cross-linking however hampers the applicability of the system in regard to peptide identification. In the future correct folding and efficient display of the scMHC molecule should be further addressed, in particular investigation of various multivalent display systems should be of high priority.

4 Materials and methods

4.1 Antibodies and primers

Utilised antibodies

Primary Ab:

Rabbit-anti-human beta-2-Microglobulin with or without conjugation to HRP (DAKO, Denmark)
Mouse anti-human HLA-ABC antibody (W6/32) (DAKO, Denmark)
Mouse anti-human HLA-A2 antibody (OneLambda, Germany)
Mouse Anti-pIII antibody (MoBiTec, Germany)
Mouse anti-c-Myc antibody/9E10 (grown according to the instructions provided by European Collection of Animal Cell Cultures and purified protein A column according to manufacturer's protocol (Pharmacia)
Anti-Myc: 9E10, Mouse Ascites Fluid (Sigma, Saint Louis, Missouri, USA)
Anti-His/HRP: Tetra HisTM HRP conjugate (Qiagen GmbH, Hilden, Germany)
Goat anti-gp120 HIV IIIB polyclonal antibody BP1035 (DPC Biermann, Germany)

Secondary Ab:

Anti-Rabbit/HRP Pork Anti-Rabbit Ig (DAKO, Denmark)
Anti-Mouse/HRP Rabbit Anti-Mouse Ig (DAKO, Denmark)
Anit-Goat/HRP Rabbit Anti-Goat Ig (DAKO, Denmark)
Anti-M13/HRP: (Amersham Biosciences, Freiburg, Germany)
Anti-pIII/HRP: PSKAN3 (MoBiTec, Göttingen, Germany)
Anti-Mouse/HRP Goat Anti-Mouse IgG + IgM (H+L) (Jackson Immunoresearch Lab. Inc, PA, USA)
Anti-Mouse/AP Goat Anti-Mouse IgG + IgM (H+L) (Jackson Immunoresearch Lab. Inc, PA, USA)
Anti-Human/HRP Goat anti-Human Ig HRP (Jackson Immunoresearch Lab. Inc, PA, USA)
Anti-Mouse IgG/HRP: Goat anti-Mouse IgG (Fcγ) HRP (Jackson Immunoresearch Lab. Inc, PA, USA)
Anti-Mouse IgM/HRP: Goat anti-Mouse IgM (μ) HRP (Jackson Immunoresearch Lab. Inc, PA, USA)
Anti-Human IgG/HRP: Goat anti-Human IgG, F(ab')2 HRP (Jackson Imm. Lab. Inc, PA, USA)

Cell Staining Ab:

Mouse IgM anti-human HLA-A1, A11, A26+ (OneLambda, Germany)
Mouse IgM anti-human HLA-A1, A36 (OneLambda, Germany)
Anti-IgG Cy3: Goat anti-Mouse IgG (Fcγ) Cy3 (Jackson Immunoresearch Lab. Inc, PA, USA)
Anti-IgM Cy3: Goat anti-Mouse IgM (μ) Cy3 (Jackson Immunoresearch Lab. Inc, PA, USA)
Anti-Ig Cy3: Goat Anti-Mouse IgG + IgM (H+L) (Jackson Immunoresearch Lab. Inc, PA, USA)
Mouse IgM Anti-a-GPA A63-C/A9 specific for asialoglycophorin A (kind gift from Dr. Karsten U)
Mouse IgG1 A83-C/B12 recognising both asialoglycophorin A and glycophorin A (kind gift from Dr. Karsten U).
The Mouse IgG1 anti-human antibodies, IgG1-FITC, anti-CD3-FITC, anti-CD8-FITC, anti-CD11c-PE, anti-CD28-PE, anti-CD30-PE, anti-CD34-FITC, anti-CD40-FITC, anti-CD80-PE, anti-CD83-FITC and anti-CD209 (DC SIGN)-Cy2-FITC are all purchased from PharMingen, USA.
The Mouse IgG2a anti-human antibodies, IgG2a-FITC, IgG2a-PE and anti-CD14-PE are likewise purchased from PharMingen, USA.
The Mouse IgG2a anti-human antibodies, anti-HLA-ABC-FITC and anti-HLA-DP+DQ+DR-PE are purchased from Serotec GmbH, Düsseldorf, Germany.
The Mouse IgG1 anti-human antibodies, anti-CD54-PE and anti-CD86-FITC are purchased from Serotec GmbH, Düsseldorf, Germany.
The Mouse IgG1 anti-human antibodies, anti-CD1a-PE and anti-CD116-PE are purchased from Immunotech, Marseille, France.
Cy^{TM}2 conjugated F(ab')$_2$ Goat anti-Mouse IgG, Fc (Jackson Immunoresearch Lab. Inc, PA, USA)

All primary and secondary antibodies were used in concentrations according to manufacturer's instructions.

Utilised primers

Table 10 - List of primers used for DNA cloning of scMHC

Name	Sequence (5' – 3')
Beta2-Mikroglobulin forward + NcoI (β2MG-HLA)	CATGCATGCCATGGCGATCCAGCGTACTCCAAAGAT
Beta2-Mikroglobulin back + 5' linker (β2MG-HLA)	GCTACCGCCACCGCCGGATCCACCTCCGCCGGA TCCGATTCCACCCATGTCTCGATCCCACTTAAC
Beta2-Mikroglobulin forward + 5' linker	GGTGGAATCGGATCCGGCGGAGGTGGATCCGGC GGTGGCGGTAGCATCCAGCGTACTCCAAAGAT
Beta2-Mikroglobulin back + NotI	CCTTTTCCTTTTGCGGCCGCCATGTCTCGATCCCACTTAAC
HLA-A*02011 forward + 5' linker (β2MG-HLA)	GGTGGAATCGGATCCGGCGGAGGTGGATCCGG CGGTGGCGGTAGCGGCTCTCACTCCATGAGGTA
HLA-A*02011 back + NotI (β2MG-HLA)	CCTTTTCCTTTTGCGGCCGCGGTGAGGGGCTTGGGCA
HLA-A*02011 forward NcoI	CATGCATGCCATGGGCTCTCACTCCATGAGGTA
HLA-A*02011 back + linker	GCTACCGCCACCGCCGGATCCACCTCCGCCGG ATCCGATTCCACCGGTGAGGGGCTTGGGCA
MART-1 A2 peptide with linker 10 for	CATGGCTGCCGCGGGCATTGGCATCTTAACAG TAGGTGGTGGAGGCTCGAGCGGCGGTGGGAG
MART-1 A2 peptide with linker 10 back	CATGCTCCCACCGCCGCTCGAGCCTCCACCAC CTACTGTTAAGATGCCAATGCCCGCGGCAGC
MART-1 A2 peptide with linker 15 for	CATGGCTGCCGCGGGCATTGGCATCTTAACAGTAGGTGG TGGAGGCTCGAGCGGCGGTGGGAGCGGGGGCGGTGGTAG
MART-1 A2 peptide with linker 15 back	CATGCTACCACCGCCCCCGCTCCCACCGCCGCTCGAGCC TCCACCACCTACTGTTAAGATGCCAATGCCCGCGGCAGC

Table 11 - List of standard primers for standard plasmids

Name	Sequence (5' – 3')
Rev	AAACAGCTATGACCATG
M13-back	TGAATTTTCTGTATGAGGTTTTG
M13-20	GTAAAACGACGGCCAGT
T7	TAATACGACTCACTATAGGG
PVAC2for	TTCGTTGTCGACCCAACA

Table 12 - List of primers employed in NemodDC study

Name	Sequence (5' – 3')
HLA-A1 Kozak HindIII For	ATCCCAAGCTTACCATGGCCGTCATGGCGCCCCG
HLA-A1 full length XbaI Back	CTCTAGTCTAGATCACACTTTACAAGCTGTGA
ENV-STOP	GGGGCGCGCAGTGTGAATAGGAGCTCTGTTCCTTGGG
BCG reverse primer	TAGAAGGCACAGTCGAGG
HLA-A1/A3 for SSP	CGCTTCCTCCGCGGGTACCGG
HLA-A2 for SSP	CGCTTCCTCCGCGGGTACCAC
HLA-A2/A3 back SSP	GCGGAGCCACTCCACGCACGT
HLA-A1 back SSP	GCGGAGCCCGTCCACGCACCG
p1u GPA for	GCTAGAATTCGATGTATGGAAAAATAATCT
p3r GPA back	TATTGCGGCCGCATTGATCACTTGTCTCT
Gp160 for	ATCCAGAGGGGACCA
Gp160 back	TTGCAACAGATGCTG
β Actin for	GGCATCGTGATGGACTCCG
β Actin back	GCTGGAAGGTGGACAGCGA

All primers are purchased from DNA Technology A/S, Aarhus, Denmark.

4.2 FuncFAb – biochemical experiments

Thermostability of FuncFAbs

ScFv and derivatives thereof were incubated at room temperature, 30°C and 37°C for different intervals in a concentration of 0,3 mg/ml and were subsequently tested in ELISA as described in appendix A, in order to determine whether exposure to elevated temperatures affected the functionality of the antibodies.

SDS-PAGE analysis detecting soluble and insoluble protein.

Protein was expressed as described in Appendix A. Cells were subsequently spun down and resuspended in 50mM Na_xH_yPO4, pH 8.0, before lysis in French Press (American Instruments, Silver Spring, MD, USA). The suspension was subsequently cleared by centrifugation (26,000g) and the cell pellet was resuspended in 50mM Na_xH_yPO4, pH 8.0 supplemented with 8M Urea in a volume identical to the supernatant. Similar sample volumes were supplemented with 2xloading buffer (4% SDS, 20% glycerol, 10mM Tris, 0,2% bromphenol blue) containing 200 mM DTT, when not stated otherwise. The samples were applied to a 12% denaturating gel and separated by SDS-PAGE (Laemmli, 1970) and subsequently transferred onto PVDF membrane (Millipore GmbH, Gemany) by semi-dry electroblotting according to (Sambrook et al., 1989). Membranes were incubated over night in PBS supplemented with 2% Milk Powder (MPBS), and protein was detected by incubation with the c-Myc tag specific murine mAb 9E10 (provided by the European collection of animal cell cultures, ECACCs) and subsequently a secondary HRP conjugated rabbit anti-mouse antibody (DAKO, Denmark) both diluted 1:1000 in 2% MPBS. The blots were finally developed utilising chemoluminiscence (Supersignal, Pierce, USA).

Gel filtration analysis

Gel filtration analysis was performed to determine whether antibody fusions multimerised. Gel filtration was performed on a TSK-gel G4000 SW column with a precolumn (ToSoHaas) using HPLC (Biotek Instruments). For correlation of retention times with globular molecular mass, blue dextran and β-amylase were applied onto the column and protein detected with a Diode Array Detector 540+ (Biotek Instruments). At least 1mg scFv-fusion protein was applied to the column of each of the constructs.

Iso-electric focusing

Protein was analysed for multimer composition employing iso-electric focusing of native protein. Purified protein was supplemented with non-denaturing and non-reducing loading buffer and analysed on a 0.8% Agarose gel according to Kim and co-workers (Kim, 2000 #84). Briefly the protein was loaded in the middle of the agarose gel enabling the band migration towards both negative and positive electrode. The protein is detected by traditional staining with Coomassie Brilliant Blue.

Bacterial Strains and the Vector Used for Phage Display

The *E. coli* strain used as host for the phage production was the amber codon suppressing E.coli strain TG-1 (K12, supE, hsdD5, Δ(lac-proAB), thi, F'{traD36 pro A^+B^+ $lacI^q$ lacZΔM15}).
The utilised phage display systems were based on the pHEN2 phagemid vector, the pUC119 derived FuncFAb vectors and the cleavable KM13 helper phage (Kristensen and Winter, 1998). The pHEN2 vector and the TG-1 were kind gifts from Greg Winter, MRC Cambridge, UK.

Rescue with the novel helper phage KM13

Phagemid or FuncFAb plasmids are rescued by the novel protease sensitive helperphage, KM13, essentially as described by Kristensen (Kristensen and Winter, 1998; Ravn et al., 2000). Briefly the bacteria containing phagemid or FuncFAb plasmid were grown to log phase (OD_{600} ~ 0,6) at 37°C in 2×TY (Sambrook, 1989) supplemented with 100µg/ml ampicillin and 1% glucose and subsequently superinfected with a surplus (multiplicity of infection (MOI) ~ 20 times) of helper phage, KM13, for 1 hour a 37°C. Afters superinfection bacteria were pelleted by centrifugation (2700×g, 10 min), resuspended in 2×TY supplemented with 100µg/ml ampicillin and 25µg/ml kanamycin and grown over night at 18°C unless otherwise stated. For the rescued FuncFAb plasmid containing bacteria 1mM IPTG is added to the over night incubation. After ON phage production the cultures were cleared and the phage containing supernatant precipitated by addition of 1/5 v/v 20% w/v PEG 6000, 2,5M NaCl over night at 4°C. The phages were subsequently pelleted by centrifugation (2700×g, 30 min) and resuspended in 1/100 volume PBS compared to culture volume.

Determination of phage titer and display level

Display levels were determined as the fraction of colony forming units (CFU) after versus before trypsin treatment. Phages were trypsinised for 20 minutes at room temperature in 50 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$ supplemented with trypsin to a final concentration of 1µg/ml trypsin (Sigma-Aldrich). Following, a log phase TG-1 was infected with phages and CFUs determined on 1xTY agar plates supplemented with 100µg/ml ampicillin and 1% glucose.

Determination of display level by western blotting

For western blotting phages were separated by SDS-PAGE (Laemmli, 1970), and electroblotted onto PVDF membrane (Millipore GmbH, Gemany) (Sambrook et al., 1989). After over night incubation in PBS supplemented with 2% fat free milk powder (MPBS), the membrane was probed with a murine anti-pIII antibody (MoBiTech, Germany) for 1 hour. The primary antibody was subsequently detected with a HRP-conjugated rabbit anti-mouse antibody (DAKO), and blots developed using chemiluminescence (SuperSignal, Pierce, USA).

Phage ELISA

Phages displaying either scFv or single-chain MHC were screened for binding in ELISA as described by Marks (Marks et al., 1991a). ELISA plates (MAXI-sorp^{TM}, Nunc, Roskilde, Denmark) were coated over night at 4°C with 1-10 µg/mL of hybridoma antibody in 100 µL PBS. The plates were blocked for 2 hours in 2% BSA PBS. Subsequently around 10^9-10^{11} displaying phages were added pr. well (either compiled directly form the culture supernatant or from a PEG precipitated phage stock) dissolved in 2% BSA PBS and incubated for 2 hours. The plate was washed five times with PBS and incubated with 100µl HRP conjugated Anti-M13 antibody (Amersham Pharmacia, Uppsala, Sweden) against the major phage coat protein (pVIII) diluted 1:5000 in 2% BSA PBS for 1 hour. After washing with PBS the ELISA was developed with o-phenylenediamine (OPD) tablets (DAKO, Denmark) according to manufacturer's instructions and light absorbance at 490 nm and 655 nm (reference) were measured (Bio-Rad, USA).

FuncFAb rescue with three deletion phages

The deletion phage vectors were a kind gift from Dr. Philipp Holliger, MRC, Cambridge, UK (Riechmann, Cell, 1997). Three deletion phage vectors including deletions of protein III domains, DI (fd-DII-DIII), DII (fd-DI-DIII) or DI-DII (fd-DIII), of the filamentous bacteriophage were purified from bacteria cultures utilising the Qiagen Plasmid Midi kit according to manufacturer's protocol (Qiagen GmbH, Hilden, Germany) including the extra washing step with STE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) to avoid ssDNA also mentioned in manufacturer's instructions. The phage deletion vectors encoding tetracycline resistance were subsequently coelectroporated with the FuncFAb vectors encoding ampicillin resistance. Double resistant TG-1 bacteria were subsequently selected on 1xTY agar plates supplemented with 100µg/ml ampicillin, 50µg/ml tetracycline and 1% glucose. Phages were expressed from the double resistant bacteria by growing to log phase (OD_{600} ~ 0,6) at 37°C in 2×TY (Sambrook, 1989) supplemented with 100µg/ml ampicillin, 50µg/ml tetracycline and 1% glucose. Bacteria were then pelleted by centrifugation (2700×g, 10 min), resuspended in 2×TY supplemented with 100µg/ml ampicillin, 50µg/ml tetracycline and 1mM IPTG and grown over night at 18°C. After ON phage production the cultures were cleared but not precipitated to retain the potentially weak binding of FuncFAb proteins to the phage coat protein III.

Crystallisation of R5 scFv-DI protein

The R5 scFv fused to DI was IMAC purified and further purified on a preparative gel filtration column essentially as described in Appendix A. The void volume was concentrated to 3 mg/ml using centricon spin-tubes (Millipore GmbH, Gemany) and a crystallisation screen (Hampton crystal screen kit and Hampton crystal screen kit 2) was conducted, whereby 96 different crystallisation conditions were tested, leaving out conditions 25 and 27 of Hampton crystal screen kit that are reported not to generate crystals. The samples were observed after 5 hours, 1 day, 7 days, 14 days, 1 month and 3 months.

Cloning of G/A7 scFv into FuncFAb plasmids and subsequent protein analysis

The G/A7 scFv (18 a.a. linker) was a kind gift from Dr. Peter Ravn derived from the TF specific A78-G/A7 mAb (Karsten et al., 1995). It was subcloned into the FuncFAb plasmids employing restriction sites NcoI and NotI. Expressed protein was purified according to appendix A and analysed in ELISA. The ELISA detected the binding of G/A7 derivatives to TF conjugated to poly(acrylic acid) (TF-PAA), asialoglycophorin A (a-GP), glycophorine A (GP) and 2% BSA. All compounds were coated over night in PBS at a concentration of 10µg/ml. 5µg protein is added at a concentration of 50 µg/ml to each ELISA well. Protein detection in ELISA was performed according to appendix A.

4.3 FuncFAb – adjuvant

Specificity of anti-idiotypic response against PACA17 and R5

10 µg/ml carbohydrate PAA conjugate in PBS were coated in ELISA wells over night. Binding of sera diluted 1:30 was detected with HRP-conjugated goat-anti-mouse IgM antibody (Sigma) or HRP-conjugated goat-anti-mouse IgG antibody (Sigma-Aldrich Chemie GmbH, Munich, Germany). Besides that ELISA was performed according to appendix B.

Binding of sera to α2 I-domain from α2β1 integrin

α2 I-domain fused or non-fused to GST was coated in ELISA wells along side with GST alone. Binding of sera (diluted 1:25) to the α2 I-domain variants were detected with HRP-conjugated goat-anti-mouse IgG antibody (Sigma-Aldrich Chemie GmbH, Munich, Germany). Besides that ELISA was performed according to appendix B.

4.4 Single-chain MHC

Creation of the single-chain MHC constructs

HLA-β2MG scMHC: The cDNA template for β2MG, HLA-A2 and HLA-A3 was made by bleeding human donors, purifying mRNA using the Trizol method provided by Life Technologies (Manufactors protocol) or Qiagens RNeasy Kit (Qiagen GmbH, Hilden, Germany), and performing RT PCR with pT primers on magnetic beads following the protocol provided by (Dynal, Oslo, Norway). The genes encoding β2MG, HLA-A2 and HLA-A3 were amplified with PCR from the cDNA employing the primers "Beta2-Mikroglobulin forward + 5' linker" and "Beta2-Mikroglobulin back + NotI" for the β2MG gene and using the "HLA-A*02011 forward NcoI" and "HLA-A*02011 back + linker" for the two HLA genes. The genes for the HLA and the β2MG were fused in a Single-Strand Overlap Extension Reaction (SOE Reaction) as described in (Toshitani et al., 1996). The fusion reaction joined the HLA-A2 or HLA-A3 gene to the 5' terminus of the β2MG gene. The fusion DNA fragment were cloned into the pHEN2 vector utilising the restriction sites NcoI and NotI (New England BioLabs, MA) according to the manufacturer's instructions, and finally electroporated into TG-1. The clones were screened by PCR using the two vector primers Rev and M13-back. Additionally the clones were sequenced using the method provided by (Sanger and Coulson, 1975). Sequencing facilities were provided by the Core Facility at the department for Molecular Biology, University of Aarhus and MWG Biotech AG (www.mwg-biotech.com)

β2MG-HLA scMHC: The reverse construction, with the β2MG gene fused to the 5' terminus of the HLA gene, were made in a similar fashion. The template for both genes of the fusion was the pHEN2 vector with the HLA-A-β2MG fusion gene inserted. The primers used were "Beta2-Mikroglobulin forward + NcoI (β2MG-HLA)" and "Beta2-Mikroglobulin back + 5' linker (β2MG-HLA)" for the β2MG gene and "HLA-A*02011 forward + 5' linker (β2MG-HLA)" and "HLA-A*02011 back + NotI (β2MG-HLA)" for the HLA genes. The subsequent recombinant manipulations are similar to those described for HLA-β2MG scMHC.

Insertion of Peptide and Peptide Linker (Construction of scpMHC Vectors)

The insertion of short DNA fragments encoding peptides and a peptide linker into the scMHC constructs were performed with a cassette cloning strategy. The cassette primers "MART-1 A2 peptide with linker 10 for" and "MART-1 A2 peptide with linker 10 back" and likewise with the complementary cassette primers "MART-1 A2 peptide with linker 15 for" and "MART-1 A2 peptide with linker 15 back" were mixed in water to a concentration of 1μM of each primer. The mixture was heated to 95°C for 5 min. followed by slow cooling for 8 hr. The cassettes were constructed in such a way that they had NcoI overhangs in both ends, but removing the NcoI site in the 3' end after ligation. Since the cassette could be inserted in both directions, the screening of positive clones was performed as PCR with the forward primer of the cassette and the M13-back vector primer.

The MART-1-L10-HLA-A2-β2MG pHEN2 construct was subcloned into pKBJ3 FuncFAb vector, utilising the NcoI and NotI cloning sites.

Detection of folded MHC molecules via phage ELISA

The phage ELISA was performed as described in chapter 4.2 employing coated antibodies against HLA-A2 (OneLambda, Germany) and HLA-ABC (W6/32) (Dako, Denmark).

Flow cytometry detection of cell markers recognised by fluorescence labelled antibodies and PE conjugated MHC tetramer

Cells were stained with 0,25μg/sample fluorescent labelled antibodies against a range of cell surface markers. MART-1 specific TCRs were recognised with 1μg commercial PE conjugated recombinant MART-1 loaded biotinylated MHC class I molecules, which are tetramerised with streptavidin (ProImmune, Oxford, UK). After 2 hours of incubation at 4°C the cells were washed, and flow cytometric analysis was performed employing a Beckman Coulter Epics XL flow cytometer. Data was subsequently analysed with the EXPO^{TM}32 MultiCOMP Software (Beckamn Coulter, Krefeld, Germany). Vitality was determined by the gating of the flow cytometry data correlated to a standard culture with vitality measured by trypan blue staining.

T Cell Activation Assay

The activation assay was compiled in a total volume of 200µl added to each well in a 96 well ELISPOT plate. Each well contained 200.000 $CD3^+/CD8^+$ Jurkat cells mixed with either purified MART-1 peptide loaded MHC class I protein (50µg/ml MART-1-L10-HLA-A2-β2MG-DI or 1.25µg/ml MART-1 peptide loaded tetramer MHC class I (ProImmune, Oxford, UK)) or 10^7-10^8 displaying sterile filtered phages (0,4µm filter) diluted in AIM V with or without 1µg cross-linking antibody (polyclonal anti-β2MG antibody and anti-c-Myc antibody). In some experiments 40.000 unloaded T2 cells, 0.6µg anti-CD3 or 1µg anti-CD28 were added. When clearly stated the amounts can vary and the T2 cells can be loaded with MART-1. The plate was sealed and incubated at 37°C for 16-40 hours. Afterwards the cultures were transferred to new 96 well plates (TPP®, Trasadingen, Switzerland), cleared by centrifugation (1000×g, 15 min.) and supernatant was removed for analysis (IFN-γ and IL-2 detection) and freezing.

ELISPOT Analysis for detection of IFN-γ secreting cells

IFN-γ secreting cells were detected with a hIFN-γ ELISPOT kit (# 3420-2A-44; Mabtech AB, Germany) according to manufacturer's protocol. Briefly capture anti-hIFN-γ antibody was coated on a multiscreen 96 well plate with PVDF-membrane (#MAIPS 4510; Millipore GmbH, Gemany) over night at 4°C. After blocking with 3% BSA in sample medium the cell samples were added and incubated over night unless otherwise stated. Cells are removed by washing and secreted hIFN-γ is detected with a biotinylated detection anti-hIFN-γ antibody. The sandwich is detected with streptavidin coupled Alkaline Phosphatase (AP) and subsequently a precipitating AP substrate (5-bromo,4-chloro,3-indolylphosphate (BCIP) and colour enhancer nitroblue tetrazolium (NBT)). The result is analysed in an AID-reader (Autoimmun Diagnostika GmbH, Strassberg, Germany).

ELISA Detection of secreted interleukins

The sandwich ELISA for IL-2, IL-4, IL-6, IL-10 and IL-12 is performed according to Manufacturer's protocol (OptEIA™ Set, PharMingen, USA). Briefly Capture Antibody Anti-human IL-X is coated over night at 4°C in a 96 well format ELISA. The plate was then blocked with complete Jurkat media for 2 hours at RT and 100µL supernatant was added for further 2 hours. The bound IL-X was recognised by the detection antibody (Biotinylated anti-human IL-X monoclonal antibody) incubated for 1 hour at RT, and linkage to HRP was accomplished by adding the Enzyme reagent (Avidin-horseradish peroxidase conjugate) for 1 hour at RT. After each incubation the ELISA plate was washed five times with PBS. The ELISA was developed as described in chapter 4.2. The theoretical detection range according to the manufacturer is 7,8-500 pg/ml.

4.5 Gene transfer to NemodDC

Cell culture

The first Jurkat cell line (hybridoma) was established from the peripheral blood of a 14-year-old boy by Schneider et al., and was originally designated JM having genotype $CD3^+$, $CD4^+$ and $CD8^-$. The specific cell line used for this activation assay is $CD3^+$, $CD4^+$ and $CD8^+$. It has furthermore been supplemented with a specific TCR recognising the MART-1 sequence and the homologue superpeptide in the context of HLA-A2 as shown by Mauerer (Unpublished results, Maeurer, Mainz). The cell line was grown in RPMI medium from Biochrom KG (Berlin, Germany) periodically containing 1,5mg/ml G418 and 1,5mg/ml L-Histidinol (both Sigma tissue culture reagents) at 37°C with 8% CO_2 in the atmosphere.

The human breast adenocarcinoma cell line, MCF-7 (ATCC No. HTB-22) and the melanoma cell line MEL624 (Rivoltini et al., 1995) were cultivated in Dulbecco's Modified Eagle Media (DMEM) (Biochrom KG, Berlin, Germany) supplemented with 10% Fetal Calf Serum (FCS) (HyClone, PERBIO) and 1mM L-Glutamine (Biochrom KG, Berlin, Germany). The HLA-A1 positive C1R.A1 (JBC 1996; 271(21):12463-71) cell line was established by transfection of the parental human B-cell lymphoblastoid cell line (C1R, (PNAS 2361-64, 1989)) lacking surface HLA A and B antigens. C1R.A1 as well as the TAP-deficient T2 (ATCC No. CRL-1992) cell line was cultivated in RPMI 1640 (Biochrom KG, Berlin, Germany) supplemented with 10% FCS and 1mM L-Glutamine. The glyco-engineered asialoglycophorin A positive NM-F9 cell line, which is derived from the glycophorine A positive K562 cell line (ATCC No. CCL-243), was employed as positive control for the GPA and aGPA flow cytometry detection study (Ute Schöber in press).

The HLA-A2 positive dendritic cell line, Nemod-DC was cultivated, differentiated and maturated according to Scheper and co-workers (Masterson et al., 2002) and manufacturer's instructions (Nemod Biotherapeutics GmbH & Co. KG, Berlin, Germany.). Briefly, the Nemod-DC cell line was cultivated in Minimum Essential Media α modified (including 2mM L-Glutamine) (MEMα) (Gibco™, Invitrogen GmbH, Kalsruhe, Germany) supplemented with 20% FCS and 10% conditioned medium from 5637 cells (ATCC No. HTB-9)(Quentmeier et al., 1997) (Nemod-DC medium). Nemod-iDCs were obtained by cultivating Nemod-DC precursor cells for 7 days (or 5 days if indicated) in Nemod-DC medium supplemented with 1000 U/ml GM-CSF (Leukomax®, Schering-Plough, Basel, Switzerland), 100 U/ml IL-4 (PeproTech EC, London, UK) and 2,5 ng/ml TNF-α (tebu-bio GmbH, Offenbach, Germany). Cultivating for another 48h in Nemod-DC medium supplemented with 75 ng/ml TNF-α further maturates the differentiated cells into Nemod-mDCs. The mature dendritic cells were secondarily used for antigen presentation and activation of MACS purified $CD8^+$ T cells from buffy coats from HLA-$A2^+$ healthy donors (DRK, Berlin, Germany) using CD8 MicroBeads according to manufacturer's protocol (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

The *maturation optimisation study* was performed by supplementing the standard maturation conditions including 75 ng/ml TNF-α with 1000 U/ml IFN-γ (PeproTech EC, London, UK), 10^{-6}M or 10^{-7}M Dexamethasone (DEX) (Sigma-Aldrich Chemie GmbH, Munich, Germany) or 10^6 CFU/ml *Bacillus Calmette-Guerin mycobacteria* (BCG) (CALBIOCHEM®, CN Biosciences, Inc. La Jolla. Canada). The study was performed on 7 days differentiated Nemod-iDCs.

Genetic material, lysate and peptides

The transfection study of Nemod-DC employed six vectors: pEGFP N1 (Clontech, Palo Alto, USA), pGT60 hIL12 (Invivogen, San Diego, USA), pGPA2 (kind gift from Dr. Hans Baumeister, Nemod Biotherapeutics GmbH & Co. KG), pHLA-A1, pHIV gp160 rev (Strathmann AG, Hamburg, Germany), pHIV gp120 rev (Strathmann AG, Hamburg, Germany). The plasmid pEGFP-N1 (Clontech) encoding the Enhanced Green Fluorescent Protein (EGFP) behind a CMV promoter was used as reporter plasmid. The pGPA2 plasmid encodes the wild type human glycophorin A gene derived from K562 cells (ATCC No. CCL-243) directionally inserted into the pIRESpuro2 vector (Clontech, Palo Alto, USA) using the Nhe1 and EcoRI cloning sites (unpublished data, Hans Baumeister). The pHLA-A1 vector was constructed by directional insertion of the wild type HLA-A1 gene into the restriction sites HindIII and XbaI of the pcDNA3 vector. The HLA-A1 gene was isolated from the C1R.A1 cell line by pT primed RT-PCR with two gene specific primers containing the two restriction sites HindIII and XbaI respectively, HLA-A1 Kozak HindIII For and HLA-A1 full length XbaI Back (cf. table 12). The HLA-A1 gene is thereby under control of the constitutive CMV promoter as is the case for all six vectors employed in our study. The cloning was verified by sequencing (MWG Biotech AG, Ebersberg, Germany). Construction of the pHIV gp160 rev and pHIV gp120 rev vectors was performed by Dr. R. Schilling (Strathmann AG, Hamburg, Germany). Briefly, a 2867 bp fragment containing the HIV rev and HIV

envelope genes was cloned into the multiple cloning sites EcoRI and ApaI of pVAX1 (Invitrogen) generating the vector pHIV gp160 rev. Site directed mutagenesis changing the codon encoding G512 of the gp160 protein into an opal stop-codon further changed pHIV gp160 rev into pHIV gp120 rev encoding the somewhat smaller 1443 bp gene fragment – gp120. The mutagenesis was performed by PCR with mutation primer ENV-STOP and vector primer BCG reverse primer, and subsequent sub-cloning of the PCR fragment into pHIV gp160 rev employing the restriction sites BssHII and AvaI.

Total RNA was isolated from MCF-7 and MEL624 cells using the RNeasy Mini Kit according to manufacturer's instructions (Qiagen GmbH, Hilden, Germany). Lysate from the same two cell lines was prepared by freeze thawing the cells in AIM V (GIBCO^{TM}, Invitrogen GmbH, Karlsruhe, Germany) medium 4 times, each time frozen for 30 seconds in liquid nitrogen and thawn at room temperature.

The following peptides were used MART-1 (ELAGIGILTV), HIV-gag (SLYNTVATL), MUC-1 (LLLTVLTV), AGP (TNDTHKRDTY) and HIV-ENV (RGPGRAFVTI). All peptides were synthesised by Biotez AG (Berlin, Germany).

Transfection of Nemod-DC at various differentiation stages

The appropriate amount of cells ($2x10^6$/transfection) at different stages of differentiation or maturation were collected by centrifugation at 1200 g for 5 min and gently resuspended in an appropriate volume (100µl/transfection) of Cell Line Nucleofector Solution V (Amaxa GmbH, Cologne, FRG). 100µl of cells were transferred into a cuvette (4 mm gap) and 5µg of endotoxin-free, circular plasmid DNA (Qiagen GmbH, Hilden, Germany) or 5µg total RNA isolated from MCF-7 or MEL624 cells (see below) was immediately added. Transfection was performed with the Nucleofector device (Amaxa GmbH, Cologne, Germany) with electrical setting U-02. One pulse was administered (N=1). Immediately after nucleofection the cell suspension was transferred to a 12-well Costar® culture plate (Corning Inc, NY, USA) containing 2 ml of prewarmed Nemod-DC medium, which was supplemented with the appropriate cytokines but only when cells were cultivated for further differentiation or maturation. Succeedingly, the cells were incubated at 37 °C with 5% CO_2. Variations from the standard protocol described are clearly stated in the experimental description.

Cloning of precursor Nemod-DC by limited dilution

Precursor Nemod-DCs were diluted to an average of 10 cells per well in Nemod DC medium including appropriate antibiotics, neomycin (G418 Sulfate) (CALBIOCHEM®, CN Biosciences, Inc. La Jolla. Canada) or hygromycin B (Sigma-Aldrich Chemie GmbH, Munich, Germany). The cells were cultured for 7 days, and wells with growing cells were detected and cell culture was further expanded. The precursor Nemod-DCs are sensitive towards low cell concentration, and does therefore need accessory cells to retain vitality. The non-dividing accessory cells are radioactively irradiated (200 Gy for 200 seconds). The cell type used as accessory cell is not unveiled due to intellectual properties (IPs).

Gene transcription in dendritic cells detected by RT-PCR

Total RNA from 10^6 dendritic cells was purified utilising the Qiagens RNeasy Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's protocol. Subsequently cDNA was synthesised by reverse transcription with pT primers on magnetic beads following the protocol provided by (Dynal, Oslo, Norway). Sequence specific primers (SSP) (table 12) were employed in PCR to detect gene transcription of HLA-A1, HLA-A2, HLA-A3, Glycophorin A and gp160. DNA was separated on a 1% agarose gel by gel electrophoresis. As positive controls DNA templates containing the respective genes were used.

Employed primer combinations:

HLA-A1: HLA-A1/A3 for SSP + HLA-A1 back SSP
HLA-A2: HLA-A2 for SSP + HLA-A2/A3 back SSP
HLA-A3: HLA-A1/A3 for SSP + HLA-A2/A3 back SSP
Glycophorin A: GPA for + GPA back
Gp160: Gp160 for + Gp160 back

Detection of HLA-A1, gp120 and GPA by flow cytometry

Cells were analysed using the standard cell staining and flow cytometry analysis described above. Detection of HLA-A1 was accomplished with two mutually exclusive antibodies anti-HLA-A1,A11,A26 and anti-HLA-A1,A36 antibody (presence of HLA-A1 only when both antibodies bind). Detection of aGPA was accomplished with the antibodies A63-

C/A9. A83-C/B12 recognises GPA glycosylation independent. Detection of cell surface bound gp120 through interaction with gp41 membrane protein was accomplished with anti-gp120 antibody.

Detection of gp120 and gp160 protein by western blotting

Western blotting is performed according to chapter 4.2. Briefly, protein from supernatant and cytoplasmic cell extract fractions from $3x10^6$ cells are immunoprecipitated with 5µg goat anti-gp120 HIV IIIB polyclonal antibody, BP1035 (DPC Biermann, Germany) and captured with protein G Agarose beads (Sigma-Aldrich Chemie GmbH, Münich, Germany). Protein samples are then boiled in denaturing loading buffer, the samples are separated by SDS-PAGE and electroblotted onto PVDF membrane. Gp120 and Gp160 proteins were subsequently detected with 50µg/ml goat anti-gp120 HIV IIIB polyclonal antibody, BP1035 (DPC Biermann, Germany) and secondary HRP conjugated goat anti-mouse antibody. Blots were developed using chemiluminescence (SuperSignal, Pierce, USA).

Goat anti-gp120 HIV IIIB polyclonal antibody BP1035 (DPC Biermann, Germany) c = 4 – 5 µg/µl (>95% purity), 1 µl used for immune precipitation and for immune detection used as 1:100 diluted

Loading of antigens onto Nemod-DC using tumor cell lysate or peptides

The cell lysate loading of Nemod-DC is initiated on the last day of differentiation (day 7). Nemod-iDC (10^6 cells/sample) were collected by centrifugation at 1200 g for 5 min and gently resuspended in AIM V medium (106 cells per 500µl AIM V medium). Then the lysate from 10^6 cells (MCF-7 or MEL624) was added to the cells and incubated over night at 37°C with 5% CO2. The next day the cells are washed once in PBS and maturation is initiated.
For peptide loading the appropriate amount of Nemod-mDC were collected by centrifugation at 1200 g for 5 min and gently resuspended in AIM V medium (10^6 cells/ml) and 10µg/ml peptide was added. The mixture was incubated for 4h at 37°C with 5% CO2.

Peptide loading of T2 cells and a control assay for successful loading

Peptide loading was performed as described for Nemod-mDC. Correct loading was verified by measuring the amount of MHC class I molecules on the cell surface, because T2 cells are characterised by having increased numbers of MHC class I molecules on the cell surface in the presence of MHC bound peptides. MHC molecules were detected by incubation with the W6/32 antibody at 4°C and subsequent flow cytometry as described by Amlot and co-workers (Gricks et al., 2001).

IFN-γ ELISPOT T cell activation assay

Primary boost: Matured dendritic cells (Nemod-mDCs) and T cells were mixed in AIM V medium (DC:T cell ratio 1:10). Briefly 10^5 purified $CD8^+$ T cells were cultivated per well in 96 well plates (TPP®, Trasadingen, Switzerland) over night at 37°C with 5% CO_2 in a cell concentration of 10^6 cells/ml. After over night incubation 10^4 radioactively irradiated (30 Gy) antigen loaded Nemod-mDCs, loaded in any of the three methods mentioned above, are added to each well (10^5 cell/ml).
Secondary boost: After 6 days 100µl surviving T cells (approximately $7x10^5$ cells/ml) are mixed with 100µl radioactively irradiated (30 Gy) peptide loaded T2 cells ($7x10^4$ cells/ml) (T2:T cell ratio 1:10 in AIM V medium) in an ELISPOT well prepared according to chapter 4.4. The cell mixture is incubated at 37°C with 5% CO_2 over night and developed according to chapter 4.4. For the HIV-vaccine the vitality of the T cells necessitated the use of a non-optimal T2:T cell ratio of 1:5, thus mixing 100 µl surviving T cells ($3,5x10^5$ cells/ml) with 100 µl radioactively irradiated peptide loaded T2 cells ($7x10^4$ cells/ml).

Europium (Eu) CTL assay

The adherent target cells, MCF-7 and MEL624, were cultivated with 10 U/ml IFN-γ 48 hours before the assay start to increase expression of MHC molecules (reviewed by Meruelo and co-workers (Brown et al., 1988) and Natali and co-workers (Giacomini et al., 1988)). The cells were grown to 100% confluence on the day of assay initiation. Then the cells were treated with Trypsin/EDTA (GIBCO™), washed once with 10mL PBS and dissolved in culture medium for cell number measurement. $5x10^6$ cells were washed once with medium, resuspended in 800µl Europium buffer (50mM HEPES, 93mM NaCl, 5mM KCl, 2mM $MgCl_2$, 10mM DTPA, 2mM Europium-III-acetate pH 7.4) and incubated for 10 minutes on ice. The cells were then electroporated employing the Multiporater from Eppendorf (Hamborg, Germany) (MCF-7 and MEL624 specific parameters: 710V, N=1, 30µs in a 0.4mm cuvettes) and incubated for another 6-10 minutes on ice. After extensive washing 10.000 marked MCF-7 cells were mixed with theoretically 70.000 T cells taken directly from the ELISPOT plate after secondary boost. However, the number of T cells was not counted before mixing and a considerable loss is expected due to the transfer. The best estimate for the MCF-7 cell to T cell ratio would

therefore be in the range of 1:1 to 1:5. Optimally this ratio should be varied from 1:80 to 1:5 but such numbers of T cells were not available in our assay. The europium release was detected after varying amount of time (over a 36 hour period) employing an enhancer solution (#1244-105, PerkinElmer, Boston, USA) and subsequently read in a Victor^2 fluorometer (PerkinElmer, Boston, USA). As positive and negative control MCF-7 and MEL624 cells were incubated with Ethanol and Medium, respectively.

5 References

Abastado, J.P., Lone, Y.C., Casrouge, A., Boulot, G. and Kourilsky, P. (1995) Dimerization of soluble major histocompatibility complex-peptide complexes is sufficient for activation of T cell hybridoma and induction of unresponsiveness. *J Exp Med*, **182**, 439-447.

Abdel-Motal, U.M., Dahmen, J., Liu, T., Ljunggren, H.G. and Jondal, M. (1996) External glycopeptide binding to MHC class-I in relation to expression of TAP transporters, beta 2-microglobulin and to pH. *Immunol Lett*, **54**, 31-35.

Aderem, A. and Underhill, D.M. (1999) Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol*, **17**, 593-623.

Aderem, A.A., Wright, S.D., Silverstein, S.C. and Cohn, Z.A. (1985) Ligated complement receptors do not activate the arachidonic acid cascade in resident peritoneal macrophages. *J Exp Med*, **161**, 617-622.

Agadjanyan, M., Luo, P., Westerink, M.A., Carey, L.A., Hutchins, W., Steplewski, Z., Weiner, D.B. and Kieber-Emmons, T. (1997) Peptide mimicry of carbohydrate epitopes on human immunodeficiency virus. *Nat Biotechnol*, **15**, 547-551.

Albert, M.L., Pearce, S.F., Francisco, L.M., Sauter, B., Roy, P., Silverstein, R.L. and Bhardwaj, N. (1998) Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J Exp Med*, **188**, 1359-1368.

Alfonso, C. and Karlsson, L. (2000) Nonclassical MHC class II molecules. *Annu Rev Immunol*, **18**, 113-142.

Alfthan, K., Takkinen, K., Sizmann, D., Soderlund, H. and Teeri, T.T. (1995) Properties of a single-chain antibody containing different linker peptides. *Protein Eng*, **8**, 725-731.

Alpar, H.O. and Bramwell, V.W. (2002) Current status of DNA vaccines and their route of administration. *Crit Rev Ther Drug Carrier Syst*, **19**, 307-383.

Alves, N.L., Hooibrink, B., Arosa, F.A. and van Lier, R.A. (2003) IL-15 induces antigen-independent expansion and differentiation of human naive CD8+ T cells in vitro. *Blood*, **102**, 2541-2546.

Anderson, K.S. and Cresswell, P. (1994) A role for calnexin (IP90) in the assembly of class II MHC molecules. *Embo J*, **13**, 675-682.

Anderson, R., Macdonald, I., Corbett, T., Hacking, G., Lowdell, M.W. and Prentice, H.G. (1997) Construction and biological characterization of an interleukin-12 fusion protein (Flexi-12): delivery to acute myeloid leukemic blasts using adeno-associated virus. *Hum Gene Ther*, **8**, 1125-1135.

Anikeeva, N., Lebedeva, T., Sumaroka, M., Kalams, S.A. and Sykulev, Y. (2003) Soluble HIV-specific T cell receptor: expression, purification and analysis of the specificity. *J Immunol Methods*, **277**, 75-86.

Anthony, L.S., Wu, H., Sweet, H., Turnnir, C., Boux, L.J. and Mizzen, L.A. (1999) Priming of CD8+ CTL effector cells in mice by immunization with a stress protein-influenza virus nucleoprotein fusion molecule. *Vaccine*, **17**, 373-383.

Apostolopoulos, V., Sandrin, M.S. and McKenzie, I.F. (1999) Carbohydrate/peptide mimics: effect on MUC1 cancer immunotherapy. *J Mol Med*, **77**, 427-436.

Apostolopoulos, V., Sandrin, M.S. and McKenzie, I.F. (2000) Mimics and cross reactions of relevance to tumour immunotherapy. *Vaccine*, **18**, 268-275.

Armstrong, N., Adey, N.B., McConnell, S.J. and Kay, B.K. (1996) Vectors for Phage Display. In "Phage display of peptides and proteins - a laboratory manual". In Kay, B.K., Winter, J. and McCafferty, J. (eds.), *Phage Display of Peptides and Proteins - A Laboratory Manual*. Academic Press, San Diego, pp. 35-54.

Arndt, K.M., Muller, K.M. and Pluckthun, A. (1998) Factors influencing the dimer to monomer transition of an antibody single-chain Fv fragment. *Biochemistry*, **37**, 12918-12926.

Atwell, S. and Wells, J.A. (1999) Selection for improved subtiligases by phage display. *Proc Natl Acad Sci U S A*, **96**, 9497-9502.

Baek, H., Suk, K.H., Kim, Y.H. and Cha, S. (2002) An improved helper phage system for efficient isolation of specific antibody molecules in phage display. *Nucleic Acids Res*, **30**, e18.

Bambos, M., Charalambous, B.M. and Feavers, I.M. (2000) Peptide mimics elicit antibody responses against the outer-membrane lipooligosaccharide of group B neisseria meningitidis. *FEMS Microbiol Lett*, **191**, 45-50.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.J., Pulendran, B. and Palucka, K. (2000) Immunobiology of dendritic cells. *Annu Rev Immunol*, **18**, 767-811.

Barbas, C.F.d., Kang, A.S., Lerner, R.A. and Benkovic, S.J. (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc Natl Acad Sci U S A*, **88**, 7978-7982.

Barouch, D., Davenport, M., McMichael, A. and Reay, P. (1995) A mAb against HLA-A2 can be influenced both positively and negatively by the associated peptide. *Int Immunol*, **7**, 1599-1605.

Barr, T.A., McCormick, A.L., Carlring, J. and Heath, A.W. (2003) A potent adjuvant effect of CD40 antibody attached to antigen. *Immunology*, **109**, 87-92.

Bass, S., Greene, R. and Wells, J.A. (1990) Hormone phage: an enrichment method for variant proteins with altered binding properties. *Proteins*, **8**, 309-314.

Batalia, M.A., Kirksey, T.J., Sharma, A., Jiang, L., Abastado, J.P., Yan, S., Zhao, R. and Collins, E.J. (2000) Class I MHC is stabilized against thermal denaturation by physiological concentrations of NaCl. *Biochemistry*, **39**, 9030-9038.

Batova, A., Kamps, A., Gillies, S.D., Reisfeld, R.A. and Yu, A.L. (1999) The Ch14.18-GM-CSF fusion protein is effective at mediating antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity in vitro. *Clin Cancer Res*, **5**, 4259-4263.

Bauer, M. and Smith, G.P. (1988) Filamentous phage morphogenetic signal sequence and orientation of DNA in the virion and gene-V protein complex. *Virology*, **167**, 166-175.

Beck, E. and Zink, B. (1981) Nucleotide sequence and genome organisation of filamentous bacteriophages fl and fd. *Gene*, **16**, 35-58.

Beninati, C., Arseni, S., Mancuso, G., Magliani, W., Conti, S., Midiri, A., Biondo, C., Polonelli, L. and Teti, G. (2004) Protective immunization against Group B meningococci using anti-idiotypic mimics of the capsular polysaccharide. *J Immunol*, **172**, 2461-2468.

Beninga, J., Rock, K.L. and Goldberg, A.L. (1998) Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. *J Biol Chem*, **273**, 18734-18742.

Bergh, O., Borsheim, K.Y., Bratbak, G. and Heldal, M. (1989) High abundance of viruses found in aquatic environments. *Nature*, **340**, 467-468.

Betts, M.R., Yusim, K. and Koup, R.A. (2002) Optimal antigens for HIV vaccines based on CD8+ T response, protein length, and sequence variability. *DNA Cell Biol*, **21**, 665-670.

Bevan, M.J. (1976a) Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med*, **143**, 1283-1288.

Bevan, M.J. (1976b) Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during in vivo priming. *J Immunol*, **117**, 2233-2238.

Bhattacharya-Chatterjee, M., Chatterjee, S.K. and Foon, K.A. (2000) Anti-idiotype vaccine against cancer. *Immunol Lett*, **74**, 51-58.

Bhattacharya-Chatterjee, M., Mukerjee, S., Biddle, W., Foon, K.A. and Kohler, H. (1990) Murine monoclonal anti-idiotype antibody as a potential network antigen for human carcinoembryonic antigen. *J Immunol*, **145**, 2758-2765.

Binder, C.J., Horkko, S., Dewan, A., Chang, M.K., Kieu, E.P., Goodyear, C.S., Shaw, P.X., Palinski, W., Witztum, J.L. and Silverman, G.J. (2003) Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. *Nat Med*, **9**, 736-743.

Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee, S.M., Lee, T., Pope, S.H., Riordan, G.S. and Whitlow, M. (1988) Single-chain antigen-binding proteins. *Science*, **242**, 423-426.

Blomberg, K., Granberg, C., Hemmila, I. and Lovgren, T. (1986a) Europium-labelled target cells in an assay of natural killer cell activity. I. A novel non-radioactive method based on time-resolved fluorescence. *J Immunol Methods*, **86**, 225-229.

Blomberg, K., Granberg, C., Hemmila, I. and Lovgren, T. (1986b) Europium-labelled target cells in an assay of natural killer cell activity. II. A novel non-radioactive method based on time-resolved fluorescence. significance and specificity of the method. *J Immunol Methods*, **92**, 117-123.

Bothmann, H. and Pluckthun, A. (2000) The periplasmic Escherichia coli peptidylprolyl cis,trans-isomerase FkpA. I. Increased functional expression of antibody fragments with and without cis-prolines. *J Biol Chem*, **275**, 17100-17105.

Bousso, P. and Robey, E. (2003) Dynamics of CD8+ T cell priming by dendritic cells in intact lymph nodes. *Nat Immunol*, **4**, 579-585.

Bouvet, J.P., Decroix, N. and Pamonsinlapatham, P. (2002) Stimulation of local antibody production: parenteral or mucosal vaccination? *Trends Immunol*, **23**, 209-213.

Brasel, K., De Smedt, T., Smith, J.L. and Maliszewski, C.R. (2000) Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood*, **96**, 3029-3039.

Breitling, F., Dubel, S., Seehaus, T., Klewinghaus, I. and Little, M. (1991) A surface expression vector for antibody screening. *Gene*, **104**, 147-153.

Brinkmann, U., Chowdhury, P.S., Roscoe, D.M. and Pastan, I. (1995) Phage display of disulfide-stabilized Fv fragments. *J Immunol Methods*, **182**, 41-50.

Bromley, S.K., Iaboni, A., Davis, S.J., Whitty, A., Green, J.M., Shaw, A.S., Weiss, A. and Dustin, M.L. (2001) The immunological synapse and CD28-CD80 interactions. *Nat Immunol*, **2**, 1159-1166.

Brophy, S.E., Holler, P.D. and Kranz, D.M. (2003) A yeast display system for engineering functional peptide-MHC complexes. *J Immunol Methods*, **272**, 235-246.

Brown, G.D., Choi, Y., Pampeno, C. and Meruelo, D. (1988) Regulation of H-2 class I gene expression in virally transformed and infected cells. *Crit Rev Immunol*, **8**, 175-215.

Brusic, V., Rudy, G. and Harrison, L.C. (1998) MHCPEP, a database of MHC-binding peptides: update 1997. *Nucleic Acids Res*, **26**, 368-371.

Burton, D.R., Desrosiers, R.C., Doms, R.W., Koff, W.C., Kwong, P.D., Moore, J.P., Nabel, G.J., Sodroski, J., Wilson, I.A. and Wyatt, R.T. (2004) HIV vaccine design and the neutralizing antibody problem. *Nat Immunol*, **5**, 233-236.

Cao, Y., Schlag, P.M. and Karsten, U. (1997) Immunodetection of epithelial mucin (MUC1, MUC3) and mucin-associated glycotopes (TF, Tn, and sialosyl-Tn) in benign and malignant lesions of colonic epithelium: apolar localization corresponds to malignant transformation. *Virchows Arch*, **431**, 159-166.

Caramelo, J.J., Castro, O.A., Alonso, L.G., De Prat-Gay, G. and Parodi, A.J. (2003) UDP-Glc:glycoprotein glucosyltransferase recognizes structured and solvent accessible hydrophobic patches in molten globule-like folding intermediates. *Proc Natl Acad Sci U S A*, **100**, 86-91.

Carlton, R.M. (1999) Phage therapy: past history and future prospects. *Arch Immunol Ther Exp (Warsz)*, **47**, 267-274.

Carroll, M.C. (1998) The role of complement and complement receptors in induction and regulation of immunity. *Annu Rev Immunol*, **16**, 545-568.

Cascales, E., Gavioli, M., Sturgis, J.N. and Lloubes, R. (2000) Proton motive force drives the interaction of the inner membrane TolA and outer membrane pal proteins in Escherichia coli. *Mol Microbiol*, **38**, 904-915.

Cerottini, J.C. and Brunner, K.T. (1974) Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. *Adv Immunol*, **18**, 67-132.

Chakravarty, S., Mitra, N., Queitsch, I., Surolia, A., Varadarajan, R. and Dubel, S. (2000) Protein stabilization through phage display. *FEBS Lett*, **476**, 296-300.

Chang, H.J., Sheu, S.Y. and Lo, S.J. (1999) Expression of foreign antigens on the surface of Escherichia coli by fusion to the outer membrane protein traT. *J Biomed Sci*, **6**, 64-70.

Chefalo, P.J. and Harding, C.V. (2001) Processing of exogenous antigens for presentation by class I MHC molecules involves post-Golgi peptide exchange influenced by peptide-MHC complex stability and acidic pH. *J Immunol*, **167**, 1274-1282.

Chitnis, V., Pahwa, R. and Pahwa, S. (2003) Determinants of HIV-specific CD8 T-cell responses in HIV-infected pediatric patients and enhancement of HIV-gag-specific responses with exogenous IL-15. *Clin Immunol*, **107**, 36-45.

Choi, E.M., Chen, J.L., Wooldridge, L., Salio, M., Lissina, A., Lissin, N., Hermans, I.F., Silk, J.D., Mirza, F., Palmowski, M.J., Dunbar, P.R., Jakobsen, B.K., Sewell, A.K. and Cerundolo, V. (2003) High avidity antigen-specific CTL identified by CD8-independent tetramer staining. *J Immunol*, **171**, 5116-5123.

Christmann, A., Wentzel, A., Meyer, C., Meyers, G. and Kolmar, H. (2001) Epitope mapping and affinity purification of monospecific antibodies by Escherichia coli cell surface display of gene-derived random peptide libraries. *J Immunol Methods*, **257**, 163-173.

Clackson, T., Hoogenboom, H.R., Griffiths, A.D. and Winter, G. (1991) Making antibody fragments using phage display libraries. *Nature*, **352**, 624-628 issn: 0028-0836.

Click, E.M. and Webster, R.E. (1997) Filamentous phage infection: required interactions with the TolA protein. *J Bacteriol*, **179**, 6464-6471.

Click, E.M. and Webster, R.E. (1998) The TolQRA proteins are required for membrane insertion of the major capsid protein of the filamentous phage f1 during infection. *J Bacteriol*, **180**, 1723-1728.

Cochran, A.G., Tong, R.T., Starovasnik, M.A., Park, E.J., McDowell, R.S., Theaker, J.E. and Skelton, N.J. (2001) A minimal peptide scaffold for beta-turn display: optimizing a strand position in disulfide-cyclized beta-hairpins. *J Am Chem Soc*, **123**, 625-632.

Cohen, C.J., Denkberg, G., Lev, A., Epel, M. and Reiter, Y. (2003a) Recombinant antibodies with MHC-restricted, peptide-specific, T-cell receptor-like specificity: new tools to study antigen presentation and TCR-peptide-MHC interactions. *J Mol Recognit*, **16**, 324-332.

Cohen, C.J., Denkberg, G., Schiffenbauer, Y.S., Segal, D., Trubniykov, E., Berke, G. and Reiter, Y. (2003b) Simultaneous monitoring of binding to and activation of tumor-specific T lymphocytes by peptide-MHC. *J Immunol Methods*, **277**, 39-52.

Cohen, S., Haimovich, J. and Hollander, N. (2003c) Anti-idiotype x anti-LFA-1 bispecific antibodies inhibit metastasis of B cell lymphoma. *J Immunol*, **170**, 2695-2701.

Cole, D.J., Weil, D.P., Shilyansky, J., Custer, M., Kawakami, Y., Rosenberg, S.A. and Nishimura, M.I. (1995) Characterization of the functional specificity of a cloned T-cell receptor heterodimer recognizing the MART-1 melanoma antigen. *Cancer Res*, **55**, 748-752.

Colosimo, A., Goncz, K.K., Holmes, A.R., Kunzelmann, K., Novelli, G., Malone, R.W., Bennett, M.J. and Gruenert, D.C. (2000) Transfer and expression of foreign genes in mammalian cells. *Biotechniques*, **29**, 314-318, 320-312, 324 passim.

Cook, G.P. and Tomlinson, I.M. (1995) The human immunoglobulin VH repertoire. *Immunol Today*, **16**, 237-242.

Cook, G.P., Tomlinson, I.M., Walter, G., Riethman, H., Carter, N.P., Buluwela, L., Winter, G. and Rabbitts, T.H. (1994) A map of the human immunoglobulin VH locus completed by analysis of the telomeric region of chromosome 14q. *Nat Genet*, **7**, 162-168.

Corbett, S.J., Tomlinson, I.M., Sonnhammer, E.L., Buck, D. and Winter, G. (1997) Sequence of the human immunoglobulin diversity (D) segment locus: a systematic analysis provides no evidence for the use of DIR segments, inverted D segments, "minor" D segments or D-D recombination. *J Mol Biol*, **270**, 587-597.

Corbi, A.L. and Lopez-Rodriguez, C. (1997) CD11c integrin gene promoter activity during myeloid differentiation. *Leuk Lymphoma*, **25**, 415-425.

Cortese, R., Monaci, P., Luzzago, A., Santini, C., Bartoli, F., Cortese, I., Fortugno, P., Galfre, G., Nicosia, A. and Felici, F. (1996) Selection of biologically active peptides by phage display of random peptide libraries. *Curr Opin Biotechnol*, **7**, 616-621.

Cox, J.P., Tomlinson, I.M. and Winter, G. (1994) A directory of human germ-line V kappa segments reveals a strong bias in their usage. *Eur J Immunol*, **24**, 827-836.

Crameri, R., Jaussi, R., Menz, G. and Blaser, K. (1994) Display of expression products of cDNA libraries on phage surfaces. A versatile screening system for selective isolation of genes by specific gene-product/ligand interaction. *Eur-J-Biochem*, **226**, 53-58 issn: 0014-2956.

Crameri, R. and Suter, M. (1993) Display of biologically active proteins on the surface of filamentous phages: a cDNA cloning system for selection of functional gene products linked to the genetic information responsible for their production. *Gene*, **137**, 69-75.

Creighton, T.E. (1993) *Proteins - Structures and Molecular Properties*. W.H. Freeman and Company, New York.

Crissman, J.W. and Smith, G.P. (1984) Gene-III protein of filamentous phages: evidence for a carboxyl-terminal domain with a role in morphogenesis. *Virology*, **132**, 445-455.

Crotzer, V.L., Christian, R.E., Brooks, J.M., Shabanowitz, J., Settlage, R.E., Marto, J.A., White, F.M., Rickinson, A.B., Hunt, D.F. and Engelhard, V.H. (2000) Immunodominance among EBV-derived epitopes restricted by HLA-B27 does not correlate with epitope abundance in EBV-transformed B-lymphoblastoid cell lines. *J Immunol*, **164**, 6120-6129.

Cull, M.G., Miller, J.F. and Schatz, P.J. (1992) Screening for receptor ligands using large libraries of peptides linked to the C terminus of the lac repressor. *Proc Natl Acad Sci U S A*, **89**, 1865-1869.

Cullen, C.M., Jameson, S.C., DeLay, M., Cottrell, C., Becken, E.T., Choi, E. and Hirsch, R. (1999) A divalent major histocompatibility complex/IgG1 fusion protein induces antigen-specific T cell activation in vitro and in vivo. *Cell Immunol*, **192**, 54-62.

Curtsinger, J.M., Schmidt, C.S., Mondino, A., Lins, D.C., Kedl, R.M., Jenkins, M.K. and Mescher, M.F. (1999) Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J Immunol*, **162**, 3256-3262.

Cwirla, S.E., Peters, E.A., Barrett, R.W. and Dower, W.J. (1990) Peptides on phage: a vast library of peptides for identifying ligands. *Proc Natl Acad Sci U S A*, **87**, 6378-6382.

Czerniecki, B.J., Cohen, P.A., Faries, M., Xu, S., Roros, J.G. and Bedrosian, I. (2001) Diverse functional activity of CD83+ monocyte-derived dendritic cells and the implications for cancer vaccines. *Crit Rev Immunol*, **21**, 157-178.

Danielsen, S., Eklund, M., Deussen, H.J., Graslund, T., Nygren, P.A. and Borchert, T.V. (2001) In vitro selection of enzymatically active lipase variants from phage libraries using a mechanism-based inhibitor. *Gene*, **272**, 267-274.

Davis, H.L., Demeneix, B.A., Quantin, B., Coulombe, J. and Whalen, R.G. (1993) Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. *Hum Gene Ther*, **4**, 733-740.

Day, P.M., Yewdell, J.W., Porgador, A., Germain, R.N. and Bennink, J.R. (1997) Direct delivery of exogenous MHC class I molecule-binding oligopeptides to the endoplasmic reticulum of viable cells. *Proc Natl Acad Sci U S A*, **94**, 8064-8069.

De Berardinis, P., Sartorius, R., Fanutti, C., Perham, R.N., Del Pozzo, G. and Guardiola, J. (2000) Phage display of peptide epitopes from HIV-1 elicits strong cytolytic responses. *Nat Biotechnol*, **18**, 873-876.

De Bolle, X., Laurent, T., Tibor, A., Godfroid, F., Weynants, V., Letesson, J.J. and Mertens, P. (1999) Antigenic properties of peptidic mimics for epitopes of the lipopolysaccharide from Brucella. *J Mol Biol*, **294**, 181-191.

de Ines, C., Cochlovius, B., Schmidt, S. and Little, M. (2000) A cell surface-displayed anti-c-myc single-chain antibody: new perspectives for the genetic improvement of cellular tumor vaccines. *Cancer Gene Ther*, **7**, 1257-1262.

de Jong, A. (1998) Contribution of mass spectrometry to contemporary immunology. *Mass Spectrom Rev*, **17**, 311-335.

de Keyzer, J., van der Does, C. and Driessen, A.J. (2003) The bacterial translocase: a dynamic protein channel complex. *Cell Mol Life Sci*, **60**, 2034-2052.

de la Cruz, V.F., Lal, A.A. and McCutchan, T.F. (1988) Immunogenicity and epitope mapping of foreign sequences via genetically engineered filamentous phage. *J Biol Chem*, **263**, 4318-4322.
Deng, L.W., Malik, P. and Perham, R.N. (1999) Interaction of the globular domains of pIII protein of filamentous bacteriophage fd with the F-pilus of Escherichia coli. *Virology*, **253**, 271-277.
Deng, L.W. and Perham, R.N. (2002) Delineating the site of interaction on the pIII protein of filamentous bacteriophage fd with the F-pilus of Escherichia coli. *J Mol Biol*, **319**, 603-614.
Denisova, G.F., Zerwanitzer, M., Denisov, D.A., Spectorman, E., Mondor, I., Sattentau, Q. and Gershoni, J.M. (2000) Expansion of epitope cross-reactivity by anti-idiotype modulation of the primary humoral response. *Mol Immunol*, **37**, 53-58.
Denkberg, G., Cohen, C.J., Segal, D., Kirkin, A.F. and Reiter, Y. (2000) Recombinant human single-chain MHC-peptide complexes made from E. coli By in vitro refolding: functional single-chain MHC-peptide complexes and tetramers with tumor associated antigens. *Eur J Immunol*, **30**, 3522-3532.
Desplancq, D., King, D.J., Lawson, A.D. and Mountain, A. (1994) Multimerization behaviour of single chain Fv variants for the tumour-binding antibody B72.3. *Protein Eng*, **7**, 1027-1033.
Devitt, A., Moffatt, O.D., Raykundalia, C., Capra, J.D., Simmons, D.L. and Gregory, C.D. (1998) Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature*, **392**, 505-509.
Devlin, J.J., Panganiban, L.C. and Devlin, P.E. (1990) Random peptide libraries: a source of specific protein binding molecules. *Science*, **249**, 404-406.
Ditzel, H.J., Binley, J.M., Moore, J.P., Sodroski, J., Sullivan, N., Sawyer, L.S., Hendry, R.M., Yang, W.P., Barbas, C.F.r. and Burton, D.R. (1995) Neutralizing recombinant human antibodies to a conformational V2- and CD4-binding site-sensitive epitope of HIV-1 gp120 isolated by using an epitope-masking procedure. *J Immunol*, **154**, 893-906.
Dodge, I.L., Carr, M.W., Cernadas, M. and Brenner, M.B. (2003) IL-6 production by pulmonary dendritic cells impedes Th1 immune responses. *J Immunol*, **170**, 4457-4464.
Dong, C., Juedes, A.E., Temann, U.A., Shresta, S., Allison, J.P., Ruddle, N.H. and Flavell, R.A. (2001) ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature*, **409**, 97-101.
Dowty, M.E., Williams, P., Zhang, G., Hagstrom, J.E. and Wolff, J.A. (1995) Plasmid DNA entry into postmitotic nuclei of primary rat myotubes. *Proc Natl Acad Sci U S A*, **92**, 4572-4576.
Dredge, K., Marriott, J.B., Todryk, S.M. and Dalgleish, A.G. (2002) Adjuvants and the promotion of Th1-type cytokines in tumour immunotherapy. *Cancer Immunol Immunother*, **51**, 521-531.
Duenas, M. and Borrebaeck, C.A. (1994) Clonal selection and amplification of phage displayed antibodies by linking antigen recognition and phage replication. *Biotechnology (N Y)*, **12**, 999-1002.
Duenas, M. and Borrebaeck, C.A. (1995) Novel helper phage design: intergenic region affects the assembly of bacteriophages and the size of antibody libraries. *FEMS Microbiol Lett*, **125**, 317-321.
Durrant, L.G., Maxwell-Armstrong, C., Buckley, D., Amin, S., Robins, R.A., Carmichael, J. and Scholefield, J.H. (2000) A neoadjuvant clinical trial in colorectal cancer patients of the human anti-idiotypic antibody 105AD7, which mimics CD55. *Clin Cancer Res*, **6**, 422-430.
Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, **411**, 494-498.
Endemann, H. and Model, P. (1995) Location of filamentous phage minor coat proteins in phage and in infected cells. *J Mol Biol*, **250**, 496-506.
Enshell-Seijffers, D., Smelyanski, L. and Gershoni, J.M. (2001) The rational design of a 'type 88' genetically stable peptide display vector in the filamentous bacteriophage fd. *Nucleic Acids Res*, **29**, E50-50.
Ertl, H.C. and Xiang, Z. (1996) Novel vaccine approaches. *J Immunol*, **156**, 3579-3582.
Evan, G.I., Lewis, G.K., Ramsay, G. and Bishop, J.M. (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Biol*, **5**, 3610-3616.
Favero, J. and Lafont, V. (1998) Effector pathways regulating T cell activation. *Biochem Pharmacol*, **56**, 1539-1547.
Felici, F., Castagnoli, L., Musacchio, A., Jappelli, R. and Cesareni, G. (1991) Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. *J Mol Biol*, **222**, 301-310.
Felici, F., Luzzago, A., Folgori, A. and Cortese, R. (1993) Mimicking of discontinuous epitopes by phage-displayed peptides, II. Selection of clones recognized by a protective monoclonal antibody against the Bordetella pertussis toxin from phage peptide libraries. *Gene*, **128**, 21-27.
Fenouillet, E. and Gluckman, J.C. (1992) Immunological analysis of human immunodeficiency virus type 1 envelope glycoprotein proteolytic cleavage. *Virology*, **187**, 825-828.
Fields, B.A., Goldbaum, F.A., Ysern, X., Poljak, R.J. and Mariuzza, R.A. (1995) Molecular basis of antigen mimicry by an anti-idiotope. *Nature*, **374**, 739-742.
Fire, A. (1999) RNA-triggered gene silencing. *Trends Genet*, **15**, 358-363.
Foged, C., Sundblad, A. and Hovgaard, L. (2002) Targeting vaccines to dendritic cells. *Pharm Res*, **19**, 229-238.

Fomsgaard, A. (1999) HIV-1 DNA vaccines. *Immunol Lett*, **65**, 127-131.
Fourneau, J.M., Cohen, H. and Van Endert, P.M. (2004) A chaperone-assisted high yield system for the production of HLA-DR4 tetramers in insect cells. *J Immunol Methods*, **285**, 253-264.
Frankel, A.D. and Young, J.A. (1998) HIV-1: fifteen proteins and an RNA. *Annu Rev Biochem*, **67**, 1-25.
Fremont, D.H., Rees, W.A. and Kozono, H. (1996) Biophysical studies of T-cell receptors and their ligands. *Curr Opin Immunol*, **8**, 93-100.
Frippiat, J.P., Williams, S.C., Tomlinson, I.M., Cook, G.P., Cherif, D., Le Paslier, D., Collins, J.E., Dunham, I., Winter, G. and Lefranc, M.P. (1995) Organization of the human immunoglobulin lambda light-chain locus on chromosome 22q11.2. *Hum Mol Genet*, **4**, 983-991.
Frisch, C., Brocks, B., Ostendorp, R., Hoess, A., von Ruden, T. and Kretzschmar, T. (2003) From EST to IHC: human antibody pipeline for target research. *J Immunol Methods*, **275**, 203-212.
Fu, T.M., Ulmer, J.B., Caulfield, M.J., Deck, R.R., Friedman, A., Wang, S., Liu, X., Donnelly, J.J. and Liu, M.A. (1997) Priming of cytotoxic T lymphocytes by DNA vaccines: requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. *Mol Med*, **3**, 362-371.
Fuh, G., Pisabarro, M.T., Li, Y., Quan, C., Lasky, L.A. and Sidhu, S.S. (2000) Analysis of PDZ domain-ligand interactions using carboxyl-terminal phage display [In Process Citation]. *J Biol Chem*, **275**, 21486-21491.
Fukuda, M.N., Ohyama, C., Lowitz, K., Matsuo, O., Pasqualini, R., Ruoslahti, E. and Fukuda, M. (2000) A peptide mimic of E-selectin ligand inhibits sialyl Lewis X-dependent lung colonization of tumor cells. *Cancer Res*, **60**, 450-456.
Fulford, W. and Model, P. (1984) Gene X of bacteriophage f1 is required for phage DNA synthesis. Mutagenesis of in-frame overlapping genes. *J Mol Biol*, **178**, 137-153.
Fulford, W. and Model, P. (1988a) Bacteriophage f1 DNA replication genes. II. The roles of gene V protein and gene II protein in complementary strand synthesis. *J Mol Biol*, **203**, 39-48.
Fulford, W. and Model, P. (1988b) Regulation of bacteriophage f1 DNA replication. I. New functions for genes II and X. *J Mol Biol*, **203**, 49-62.
Gailus, V., Ramsperger, U., Johner, C., Kramer, H. and Rasched, I. (1994) The role of the adsorption complex in the termination of filamentous phage assembly. *Res Microbiol*, **145**, 699-709.
Galfre, G., Monaci, P., Nicosia, A., Luzzago, A., Felici, F. and Cortese, R. (1996) Immunization with phage-displayed mimotopes. *Methods Enzymol*, **267**, 109-115.
Gao, C., Mao, S., Ditzel, H.J., Farnaes, L., Wirsching, P., Lerner, R.A. and Janda, K.D. (2002) A cell-penetrating peptide from a novel pVII-pIX phage-displayed random peptide library. *Bioorg Med Chem*, **10**, 4057-4065.
Gao, C., Mao, S., Lo, C.H., Wirsching, P., Lerner, R.A. and Janda, K.D. (1999) Making artificial antibodies: a format for phage display of combinatorial heterodimeric arrays. *Proc Natl Acad Sci U S A*, **96**, 6025-6030.
Garcia, K.C., Scott, C.A., Brunmark, A., Carbone, F.R., Peterson, P.A., Wilson, I.A. and Teyton, L. (1996) CD8 enhances formation of stable T-cell receptor/MHC class I molecule complexes. *Nature*, **384**, 577-581.
Gates, C.M., Stemmer, W.P., Kaptein, R. and Schatz, P.J. (1996) Affinity selective isolation of ligands from peptide libraries through display on a lac repressor "headpiece dimer". *J Mol Biol*, **255**, 373-386.
Geider, K., Hohmeyer, C., Haas, R. and Meyer, T.F. (1985) A plasmid cloning system utilizing replication and packaging functions of the filamentous bacteriophage fd. *Gene*, **33**, 341-349.
Geijtenbeek, T.B., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Adema, G.J., van Kooyk, Y. and Figdor, C.G. (2000) Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell*, **100**, 575-585.
Geppert, T.D., Davis, L.S., Gur, H., Wacholtz, M.C. and Lipsky, P.E. (1990) Accessory cell signals involved in T-cell activation. *Immunol Rev*, **117**, 5-66.
Ghiran, I., Barbashov, S.F., Klickstein, L.B., Tas, S.W., Jensenius, J.C. and Nicholson-Weller, A. (2000) Complement receptor 1/CD35 is a receptor for mannan-binding lectin. *J Exp Med*, **192**, 1797-1808.
Giacomini, P., Fisher, P.B., Duigou, G.J., Gambari, R. and Natali, P.G. (1988) Regulation of class II MHC gene expression by interferons: insights into the mechanism of action of interferon (review). *Anticancer Res*, **8**, 1153-1161.
Giebel, L.B., Cass, R.T., Milligan, D.L., Young, D.C., Arze, R. and Johnson, C.R. (1995) Screening of cyclic peptide phage libraries identifies ligands that bind streptavidin with high affinities. *Biochemistry*, **34**, 15430-15435.
Gimmi, C.D., Freeman, G.J., Gribben, J.G., Gray, G. and Nadler, L.M. (1993) Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation. *Proc Natl Acad Sci U S A*, **90**, 6586-6590.
Glockshuber, R., Malia, M., Pfitzinger, I. and Pluckthun, A. (1990) A comparison of strategies to stabilize immunoglobulin Fv-fragments. *Biochemistry*, **29**, 1362-1367.
Godeau, F., Luescher, I.F., Ojcius, D.M., Saucier, C., Mottez, E., Cabanie, L. and Kourilsky, P. (1992) Purification and ligand binding of a soluble class I major histocompatibility complex molecule consisting of the first three

domains of H-2Kd fused to beta 2-microglobulin expressed in the baculovirus-insect cell system. *J Biol Chem*, **267**, 24223-24229.
Goldstein, S.A. and Mescher, M.F. (1985) Carbohydrate moieties of major histocompatibility complex class I alloantigens are not required for their recognition by T lymphocytes. *J Exp Med*, **162**, 1381-1386.
Goletz, S., Christensen, P.A., Kristensen, P., Blohm, D., Tomlinson, I., Winter, G. and Karsten, U. (2002) Selection of large diversities of antiidiotypic antibody fragments by phage display. *J Mol Biol*, **315**, 1087-1097.
Goodson, R.J., Doyle, M.V., Kaufman, S.E. and Rosenberg, S. (1994) High-affinity urokinase receptor antagonists identified with bacteriophage peptide display. *Proc Natl Acad Sci U S A*, **91**, 7129-7133.
Gramatikoff, K., Georgiev, O. and Schaffner, W. (1994) Direct interaction rescue, a novel filamentous phage technique to study protein-protein interactions. *Nucleic Acids Res*, **22**, 5761-5762.
Grant, H.W., Hadley, G.P., Adhikari, M. and Fernandes-Costa, F. (1998) T-cryptantigen (TCA) activation in surgical neonates: a hidden problem. *Pediatr Surg Int*, **14**, 204-207.
Greenwood, J., Willis, A.E. and Perham, R.N. (1991) Multiple display of foreign peptides on a filamentous bacteriophage. Peptides from Plasmodium falciparum circumsporozoite protein as antigens. *J Mol Biol*, **220**, 821-827.
Gricks, C.S., Rawlings, E., Foroni, L., Madrigal, J.A. and Amlot, P.L. (2001) Somatically mutated regions of immunoglobulin on human B-cell lymphomas code for peptides that bind to autologous major histocompatibility complex class I, providing a potential target for cytotoxic T cells. *Cancer Res*, **61**, 5145-5152.
Grothaus, M.C., Srivastava, N., Smithson, S.L., Kieber-Emmons, T., Williams, D.B., Carlone, G.M. and Westerink, M.A. (2000) Selection of an immunogenic peptide mimic of the capsular polysaccharide of Neisseria meningitidis serogroup A using a peptide display library. *Vaccine*, **18**, 1253-1263.
Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J.E. and Roncarolo, M.G. (1997) A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*, **389**, 737-742.
Hakim, I., Levy, S. and Levy, R. (1996) A nine-amino acid peptide from IL-1beta augments antitumor immune responses induced by protein and DNA vaccines. *J Immunol*, **157**, 5503-5511.
Hammer, J. (1995) New methods to predict MHC-binding sequences within protein antigens. *Curr Opin Immunol*, **7**, 263-269.
Hammer, J., Takacs, B. and Sinigaglia, F. (1992) Identification of a motif for HLA-DR1 binding peptides using M13 display libraries. *J Exp Med*, **176**, 1007-1013.
Hanada, K., Yewdell, J.W. and Yang, J.C. (2004) Immune recognition of a human renal cancer antigen through post-translational protein splicing. *Nature*, **427**, 252-256.
Hanes, J. and Pluckthun, A. (1997) In vitro selection and evolution of functional proteins by using ribosome display. *Proc Natl Acad Sci U S A*, **94**, 4937-4942.
Hanes, J., Schaffitzel, C., Knappik, A. and Pluckthun, A. (2000) Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display. *Nat Biotechnol*, **18**, 1287-1292.
Hanisch, F.G., Schwientek, T., Von Bergwelt-Baildon, M.S., Schultze, J.L. and Finn, O. (2003) O-Linked glycans control glycoprotein processing by antigen-presenting cells: a biochemical approach to the molecular aspects of MUC1 processing by dendritic cells. *Eur J Immunol*, **33**, 3242-3254.
Harris, L.J., Larson, S.B., Hasel, K.W. and McPherson, A. (1997) Refined structure of an intact IgG2a monoclonal antibody. *Biochemistry*, **36**, 1581-1597.
Hawkins, R.E., Russell, S.J. and Winter, G. (1992) Selection of phage antibodies by binding affinity. Mimicking affinity maturation. *J Mol Biol*, **226**, 889-896.
Hennecke, F., Krebber, C. and Pluckthun, A. (1998) Non-repetitive single-chain Fv linkers selected by selectively infective phage (SIP) technology. *Protein Eng*, **11**, 405-410.
Heyman, B. (2003) Feedback regulation by IgG antibodies. *Immunol Lett*, **88**, 157-161.
Hiltbold, E.M., Vlad, A.M., Ciborowski, P., Watkins, S.C. and Finn, O.J. (2000) The mechanism of unresponsiveness to circulating tumor antigen MUC1 is a block in intracellular sorting and processing by dendritic cells. *J Immunol*, **165**, 3730-3741.
Hirsch, R., Bluestone, J.A., DeNenno, L. and Gress, R.E. (1990) Anti-CD3 F(ab')2 fragments are immunosuppressive in vivo without evoking either the strong humoral response or morbidity associated with whole mAb. *Transplantation*, **49**, 1117-1123.
Hisamatsu, H., Shimbara, N., Saito, Y., Kristensen, P., Hendil, K.B., Fujiwara, T., Takahashi, E., Tanahashi, N., Tamura, T., Ichihara, A. and Tanaka, K. (1996) Newly identified pair of proteasomal subunits regulated reciprocally by interferon gamma. *J Exp Med*, **183**, 1807-1816.
Holliger, P. and Riechmann, L. (1997) A conserved infection pathway for filamentous bacteriophages is suggested by the structure of the membrane penetration domain of the minor coat protein g3p from phage fd. *Structure*, **5**, 265-275.

Holliger, P., Riechmann, L. and Williams, R.L. (1999) Crystal structure of the two N-terminal domains of g3p from filamentous phage fd at 1.9 A: evidence for conformational lability. *J Mol Biol*, **288**, 649-657.

Hoogenboom, H.R. (1997) Designing and optimizing library selection strategies for generating high-affinity antibodies. *Trends Biotechnol*, **15**, 62-70.

Hoogenboom, H.R., Griffiths, A.D., Johnson, K.S., Chiswell, D.J., Hudson, P. and Winter, G. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic-Acids-Res*, **19**, 4133-4137 issn: 0305-1048.

Hu, Z.B., Ma, W., Zaborski, M., MacLeod, R., Quentmeier, H. and Drexler, H.G. (1996) Establishment and characterization of two novel cytokine-responsive acute myeloid and monocytic leukemia cell lines, MUTZ-2 and MUTZ-3. *Leukemia*, **10**, 1025-1040.

Huang, J.F., Yang, Y., Sepulveda, H., Shi, W., Hwang, I., Peterson, P.A., Jackson, M.R., Sprent, J. and Cai, Z. (1999) TCR-Mediated internalization of peptide-MHC complexes acquired by T cells. *Science*, **286**, 952-954.

Huang, W., Petrosino, J. and Palzkill, T. (1998) Display of functional beta-lactamase inhibitory protein on the surface of M13 bacteriophage. *Antimicrob Agents Chemother*, **42**, 2893-2897.

Hufton, S.E., Moerkerk, P.T., Meulemans, E.V., de Bruine, A., Arends, J.W. and Hoogenboom, H.R. (1999) Phage display of cDNA repertoires: the pVI display system and its applications for the selection of immunogenic ligands. *J Immunol Methods*, **231**, 39-51.

Hultgren, S.J., Normark, S. and Abraham, S.N. (1991) Chaperone-assisted assembly and molecular architecture of adhesive pili. *Annu Rev Microbiol*, **45**, 383-415.

Huston, J.S., Levinson, D., Mudgett Hunter, M., Tai, M.S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R. and al., e. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. *Proc Natl Acad Sci U S A*, **85**, 5879-5883.

Iannolo, G., Minenkova, O., Petruzzelli, R. and Cesareni, G. (1995) Modifying filamentous phage capsid: limits in the size of the major capsid protein. *J Mol Biol*, **248**, 835-844.

Irvine, A.S., Trinder, P.K., Laughton, D.L., Ketteringham, H., McDermott, R.H., Reid, S.C., Haines, A.M., Amir, A., Husain, R., Doshi, R., Young, L.S. and Mountain, A. (2000) Efficient nonviral transfection of dendritic cells and their use for in vivo immunization. *Nat Biotechnol*, **18**, 1273-1278.

Jamieson, A.C., Kim, S.H. and Wells, J.A. (1994) In vitro selection of zinc fingers with altered DNA-binding specificity. *Biochemistry*, **33**, 5689-5695.

Jancarik, J., Scott, W.G., Milligan, D.L., Koshland, D.E., Jr. and Kim, S.H. (1991) Crystallization and preliminary X-ray diffraction study of the ligand-binding domain of the bacterial chemotaxis-mediating aspartate receptor of Salmonella typhimurium. *J Mol Biol*, **221**, 31-34.

Jego, G., Palucka, A.K., Blanck, J.P., Chalouni, C., Pascual, V. and Banchereau, J. (2003) Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. *Immunity*, **19**, 225-234.

Jenkins, M.K., Chen, C.A., Jung, G., Mueller, D.L. and Schwartz, R.H. (1990) Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *J Immunol*, **144**, 16-22.

Jensen, K.B., Jensen, O.N., Ravn, P., Clark, B.F. and Kristensen, P. (2003) Identification of Keratinocyte-specific Markers Using Phage Display and Mass Spectrometry. *Mol Cell Proteomics*, **2**, 61-69.

Jensen, K.B., Larsen, M., Pedersen, J.S., Christensen, P.A., Alvarez-Vallina, L., Goletz, S., Clark, B.F. and Kristensen, P. (2002) Functional improvement of antibody fragments using a novel phage coat protein III fusion system. *Biochem Biophys Res Commun*, **298**, 566-573.

Jerne, N.K. (1974) Towards a network theory of the immune system. *Ann Immunol (Paris)*, **125C**, 373-389.

Jespers, L.S., Messens, J.H., A, K.d. and al., e. (1995a) Surface expression and ligand-based selection of cDNAs fused to filamentous phage gene VI. *Biotechnology N Y*, **13**, 378-382.

Jespers, L.S., Messens, J.H., De Keyser, A., Eeckhout, D., Van den Brande, I., Gansemans, Y.G., Lauwereys, M.J., Vlasuk, G.P. and Stanssens, P.E. (1995b) Surface expression and ligand-based selection of cDNAs fused to filamentous phage gene VI. *Biotechnology (N Y)*, **13**, 378-382.

Jestin, J.-L., Kristensen, P. and Winter, G. (1999) A Method for the Selection of Catalytic Activity Using Phage Display and Proximity Coupling. *Angewandte Chemie International Edition*, **38**, 1124-1127.

Jestin, J.L., Volioti, G. and Winter, G. (2001) Improving the display of proteins on filamentous phage. *Res Microbiol*, **152**, 187-191.

Jiang, J., Abu-Shilbayeh, L. and Rao, V.B. (1997) Display of a PorA peptide from Neisseria meningitidis on the bacteriophage T4 capsid surface. *Infect Immun*, **65**, 4770-4777.

Jounai, N., Okuda, K., Kojima, Y., Toda, Y., Hamajima, K., Ohba, K., Klinman, D. and Xin, K.Q. (2003) Contribution of the rev gene to the immunogenicity of DNA vaccines targeting the envelope glycoprotein of HIV. *J Gene Med*, **5**, 609-617.

Judd, B.A. and Koretzky, G.A. (2000) Antigen specific T lymphocyte activation. *Rev Immunogenet*, **2**, 164-174.
June, C.H., Jackson, K.M., Ledbetter, J.A., Leiden, J.M., Lindsten, T. and Thompson, C.B. (1989) Two distinct mechanisms of interleukin-2 gene expression in human T lymphocytes. *J Autoimmun*, **2 Suppl**, 55-65.
June, C.H., Ledbetter, J.A., Linsley, P.S. and Thompson, C.B. (1990) Role of the CD28 receptor in T-cell activation. *Immunol Today*, **11**, 211-216.
Kang, A.S., Barbas, C.F., Janda, K.D., Benkovic, S.J. and Lerner, R.A. (1991) Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. *Proc Natl Acad Sci U S A*, **88**, 4363-4366.
Karsten, U., Butschak, G., Cao, Y., Goletz, S. and Hanisch, F.G. (1995) A new monoclonal antibody (A78-G/A7) to the Thomsen-Friedenreich pan-tumor antigen. *Hybridoma*, **14**, 37-44.
Karsten, U., Diotel, C., Klich, G., Paulsen, H., Goletz, S., Muller, S. and Hanisch, F.G. (1998) Enhanced binding of antibodies to the DTR motif of MUC1 tandem repeat peptide is mediated by site-specific glycosylation. *Cancer Res*, **58**, 2541-2549.
Karsten, U., Pilgrim, G., Hanisch, F.G., Uhlenbruck, G., Kasper, M., Stosiek, P., Papsdorf, G. and Pasternak, G. (1988) A new monoclonal antibody (A46-B/B10) highly specific for the blood group H type 2 epitope: generation, epitope analysis, serological and histological evaluation. *Br J Cancer*, **58**, 176-181.
Kato, I., Takai, T. and Kudo, A. (2002) The pre-B cell receptor signaling for apoptosis is negatively regulated by Fc gamma RIIB. *J Immunol*, **168**, 629-634.
Kay, B.K., Winter, J. and McCafferty, J. (eds.). (1996) *Phage display of Peptides and Proteins - A Laboratory Manual*. Academic Press, San Diego.
Kidd, P. (2003) Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Altern Med Rev*, **8**, 223-246.
Kieber-Emmons, T. (1998) Peptide mimotopes of carbohydrate antigens. *Immunol Res*, **17**, 95-108.
Kieber-Emmons, T., Luo, P., Qiu, J., Chang, T.Y., O, I., Blaszczyk-Thurin, M. and Steplewski, Z. (1999) Vaccination with carbohydrate peptide mimotopes promotes anti-tumor responses. *Nat Biotechnol*, **17**, 660-665.
Kieber-Emmons, T., Monzavi-Karbassi, B., Wang, B., Luo, P. and Weiner, D.B. (2000) Cutting edge: DNA immunization with minigenes of carbohydrate mimotopes induce functional anti-carbohydrate antibody response. *J Immunol*, **165**, 623-627.
Kieber-Emmons, T., Murali, R. and Greene, M.I. (1997) Therapeutic peptides and peptidomimetics. *Curr Opin Biotechnol*, **8**, 435-441.
Kim, R., Yokota, H. and Kim, S.H. (2000a) Electrophoresis of proteins and protein-protein complexes in a native agarose gel. *Anal Biochem*, **282**, 147-149.
Kim, Y.S., Jung, H.C. and Pan, J.G. (2000b) Bacterial cell surface display of an enzyme library for selective screening of improved cellulase variants. *Appl Environ Microbiol*, **66**, 788-793.
Kirkham, P.M., Neri, D. and Winter, G. (1999) Towards the design of an antibody that recognises a given protein epitope. *J Mol Biol*, **285**, 909-915.
Kirkin, A.F., thor Straten, P., Hansen, M.R., Barfoed, A., Dzhandzhugazyan, K.N. and Zeuthen, J. (1999) Establishment of gp100 and MART-1/Melan-A-specific cytotoxic T lymphocyte clones using in vitro immunization against preselected highly immunogenic melanoma cell clones. *Cancer Immunol Immunother*, **48**, 239-246.
Knabel, M., Franz, T.J., Schiemann, M., Wulf, A., Villmow, B., Schmidt, B., Bernhard, H., Wagner, H. and Busch, D.H. (2002) Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. *Nat Med*, **8**, 631-637.
Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Hoess, A., Wolle, J., Pluckthun, A. and Virnekas, B. (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J Mol Biol*, **296**, 57-86.
Kohler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, **256**, 495-497.
Krebber, C., Spada, S., Desplancq, D. and Pluckthun, A. (1995) Co-selection of cognate antibody-antigen pairs by selectively-infective phages. *FEBS Lett*, **377**, 227-231.
Krebs, B., Ackermann, B. and Rose-John, S. (1998) Specific targeting of cytokine-secreting cells: a bispecific diabody recognizing human interleukin-6 and CD3 induces T cell-mediated killing. *J Interferon Cytokine Res*, **18**, 783-791.
Kristensen, P. and Winter, G. (1998) Proteolytic selection for protein folding using filamentous bacteriophages. *Fold Des*, **3**, 321-328.
Kuhn, J.R. and Poenie, M. (2002) Dynamic polarization of the microtubule cytoskeleton during CTL-mediated killing. *Immunity*, **16**, 111-121.
Kurokawa, M.S., Ohoka, S., Matsui, T., Sekine, T., Yamamoto, K., Nishioka, K. and Kato, T. (2002) Expression of MHC class I molecules together with antigenic peptides on filamentous phages. *Immunol Lett*, **80**, 163-168.

Lafont, V., Ottones, F., Liautard, J. and Favero, J. (1999) Evidence for a p21(ras)/Raf-1/MEK-1/ERK-2-independent pathway in stimulation of IL-2 gene transcription in human primary T lymphocytes. *J Biol Chem*, **274**, 25743-25748.

Lalanne, J.L., Delarbre, C., Gachelin, G. and Kourilsky, P. (1983) A cDNA clone containing the entire coding sequence of a mouse H-2Kd histocompatibility antigen. *Nucleic Acids Res*, **11**, 1567-1577.

Lanzavecchia, A. (1997) Understanding the mechanisms of sustained signaling and T cell activation. *J Exp Med*, **185**, 1717-1719.

Larocca, D., Jensen-Pergakes, K., Burg, M.A. and Baird, A. (2001) Receptor-targeted gene delivery using multivalent phagemid particles. *Mol Ther*, **3**, 476-484.

Lattemann, C.T., Maurer, J., Gerland, E. and Meyer, T.F. (2000) Autodisplay: functional display of active beta-lactamase on the surface of Escherichia coli by the AIDA-I autotransporter. *J Bacteriol*, **182**, 3726-3733.

Lawless, M.K., Barney, S., Guthrie, K.I., Bucy, T.B., Petteway, S.R., Jr. and Merutka, G. (1996) HIV-1 membrane fusion mechanism: structural studies of the interactions between biologically-active peptides from gp41. *Biochemistry*, **35**, 13697-13708.

Le Doussal, J., Piqueras, B., Dogan, I., Debre, P. and Gorochov, G. (2000) Phage display of peptide/major histocompatibility complex. *J Immunol Methods*, **241**, 147-158.

Lebens, M., Sun, J.B., Sadeghi, H., Backstrom, M., Olsson, I., Mielcarek, N., Li, B.L., Capron, A., Czerkinsky, C. and Holmgren, J. (2003) A mucosally administered recombinant fusion protein vaccine against schistosomiasis protecting against immunopathology and infection. *Vaccine*, **21**, 514-520.

Lee, L., Loftus, D., Appella, E., Margulies, D.H. and Mage, M. (1996) A recombinant single-chain HLA-A2.1 molecule, with a cis active beta-2-microglobulin domain, is biologically active in peptide binding and antigen presentation. *Hum Immunol*, **49**, 28-37.

Lei, S.P., Lin, H.C., Wang, S.S., Callaway, J. and Wilcox, G. (1987) Characterization of the Erwinia carotovora pelB gene and its product pectate lyase. *J Bacteriol*, **169**, 4379-4383.

Lenz, P., Bacot, S.M., Frazier-Jessen, M.R. and Feldman, G.M. (2003) Nucleoporation of dendritic cells: efficient gene transfer by electroporation into human monocyte-derived dendritic cells. *FEBS Lett*, **538**, 149-154.

Lesinski, G.B., Smithson, S.L., Srivastava, N., Chen, D., Widera, G. and Westerink, M.A. (2001) A DNA vaccine encoding a peptide mimic of Streptococcus pneumoniae serotype 4 capsular polysaccharide induces specific anti-carbohydrate antibodies in Balb/c mice. *Vaccine*, **19**, 1717-1726.

Lesinski, G.B. and Westerink, M.A. (2001) Novel vaccine strategies to T-independent antigens. *J Microbiol Methods*, **47**, 135-149.

Lewis, R.S. (2001) Calcium signaling mechanisms in T lymphocytes. *Annu Rev Immunol*, **19**, 497-521.

Liljeqvist, S., Stahl, S., Andreoni, C., Binz, H., Uhlen, M. and Murby, M. (1997) Fusions to the cholera toxin B subunit: influence on pentamerization and GM1 binding. *J Immunol Methods*, **210**, 125-135.

Linderoth, N.A., Simon, M.N. and Russel, M. (1997) The filamentous phage pIV multimer visualized by scanning transmission electron microscopy. *Science*, **278**, 1635-1638.

Lipton, A. and Weissbach, A. (1969) The bacteriophages. *Am J Med*, **46**, 264-274.

Lone, Y.C., Motta, I., Mottez, E., Guilloux, Y., Lim, A., Demay, F., Levraud, J.P., Kourilsky, P. and Abastado, J.P. (1998) In vitro induction of specific cytotoxic T lymphocytes using recombinant single-chain MHC class I/peptide complexes. *J Immunother*, **21**, 283-294.

Lopez, J. and Webster, R.E. (1983) Morphogenesis of filamentous bacteriophage f1: orientation of extrusion and production of polyphage. *Virology*, **127**, 177-193.

Low, N.M., Holliger, P.H. and Winter, G. (1996) Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. *J Mol Biol*, **260**, 359-368.

Lubkowski, J., Hennecke, F., Pluckthun, A. and Wlodawer, A. (1998) The structural basis of phage display elucidated by the crystal structure of the N-terminal domains of g3p. *Nat Struct Biol*, **5**, 140-147.

Lubkowski, J., Hennecke, F., Pluckthun, A. and Wlodawer, A. (1999) Filamentous phage infection: crystal structure of g3p in complex with its coreceptor, the C-terminal domain of TolA. *Structure Fold Des*, **7**, 711-722.

Luo, P., Agadjanyan, M., Qiu, J., Westerink, M.A., Steplewski, Z. and Kieber-Emmons, T. (1998) Antigenic and immunological mimicry of peptide mimotopes of Lewis carbohydrate antigens. *Mol Immunol*, **35**, 865-879.

Luzzago, A., Felici, F., Tramontano, A., Pessi, A. and Cortese, R. (1993) Mimicking of discontinuous epitopes by phage-displayed peptides, I. Epitope mapping of human H ferritin using a phage library of constrained peptides. *Gene*, **128**, 51-57.

Madden, D.R., Garboczi, D.N. and Wiley, D.C. (1993) The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2. *Cell*, **75**, 693-708.

Mage, M.G., Lee, L., Ribaudo, R.K., Corr, M., Kozlowski, S., McHugh, L. and Margulies, D.H. (1992) A recombinant, soluble, single-chain class I major histocompatibility complex molecule with biological activity. *Proc Natl Acad Sci U S A*, **89**, 10658-10662.

Maneewannakul, S., Maneewannakul, K. and Ippen-Ihler, K. (1992) Characterization, localization, and sequence of F transfer region products: the pilus assembly gene product TraW and a new product, TrbI. *J Bacteriol*, **174**, 5567-5574.

Marks, J.D., Griffiths, A.D., Malmqvist, M., Clackson, T.P., Bye, J.M. and Winter, G. (1992) By-passing immunization: building high affinity human antibodies by chain shuffling. *Biotechnology (N Y)*, **10**, 779-783.

Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D. and Winter, G. (1991a) By-passing immunization. Human antibodies from V-gene libraries. *J Mol Biol*, **222**, 581-597.

Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D. and Winter, G. (1991b) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J Mol Biol*, **222**, 581-597.

Martin, A. and Schmid, F.X. (2003a) The folding mechanism of a two-domain protein: folding kinetics and domain docking of the gene-3 protein of phage fd. *J Mol Biol*, **329**, 599-610.

Martin, A. and Schmid, F.X. (2003b) A proline switch controls folding and domain interactions in the gene-3-protein of the filamentous phage fd. *J Mol Biol*, **331**, 1131-1140.

Martinet, W., Schrijvers, D.M. and Kockx, M.M. (2003) Nucleofection as an efficient nonviral transfection method for human monocytic cells. *Biotechnol Lett*, **25**, 1025-1029.

Marvin, D.A. (1998) Filamentous phage structure, infection and assembly. *Curr Opin Struct Biol*, **8**, 150-158.

Marvin, D.A. and Hohn, B. (1969) Filamentous bacterial viruses. *Bacteriol Rev*, **33**, 172-209.

Masterson, A.J., Sombroek, C.C., De Gruijl, T.D., Graus, Y.M., van der Vliet, H.J., Lougheed, S.M., van den Eertwegh, A.J., Pinedo, H.M. and Scheper, R.J. (2002) MUTZ-3, a human cell line model for the cytokine-induced differentiation of dendritic cells from CD34+ precursors. *Blood*, **100**, 701-703.

Matthews, D.J. and Wells, J.A. (1993) Substrate phage: selection of protease substrates by monovalent phage display. *Science*, **260**, 1113-1117.

McAdam, A.J., Greenwald, R.J., Levin, M.A., Chernova, T., Malenkovich, N., Ling, V., Freeman, G.J. and Sharpe, A.H. (2001) ICOS is critical for CD40-mediated antibody class switching. *Nature*, **409**, 102-105.

McCafferty, J., Griffiths, A.D., Winter, G. and Chiswell, D.J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature*, **348**, 552-554.

McCluskey, J., Boyd, L.F., Highet, P.F., Inman, J. and Margulies, D.H. (1988) T cell activation by purified, soluble, class I MHC molecules. Requirement for polyvalency. *J Immunol*, **141**, 1451-1455.

McColl, S.R. (2002) Chemokines and dendritic cells: a crucial alliance. *Immunol Cell Biol*, **80**, 489-496.

McConnell, S.J., Kendall, M.L., Reilly, T.M. and Hoess, R.H. (1994) Constrained peptide libraries as a tool for finding mimotopes. *Gene*, **151**, 115-118.

McCormick, A.L., Thomas, M.S. and Heath, A.W. (2001) Immunization with an interferon-gamma-gp120 fusion protein induces enhanced immune responses to human immunodeficiency virus gp120. *J Infect Dis*, **184**, 1423-1430.

McInerney, T.L., Brennan, F.R., Jones, T.D. and Dimmock, N.J. (1999) Analysis of the ability of five adjuvants to enhance immune responses to a chimeric plant virus displaying an HIV-1 peptide. *Vaccine*, **17**, 1359-1368.

McLafferty, M.A., Kent, R.B., Ladner, R.C. and Markland, W. (1993) M13 bacteriophage displaying disulfide-constrained microproteins. *Gene*, **128**, 29-36.

Mead, D.A. and Kemper, B. (1988) Chimeric single-stranded DNA phage-plasmid cloning vectors. *Biotechnology*, **10**, 85-102.

Melief, C.J. (2003) Mini-review: Regulation of cytotoxic T lymphocyte responses by dendritic cells: peaceful coexistence of cross-priming and direct priming? *Eur J Immunol*, **33**, 2645-2654.

Mempel, T.R., Henrickson, S.E. and Von Andrian, U.H. (2004) T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature*, **427**, 154-159.

Merril, C.R., Scholl, D. and Adhya, S.L. (2003) The prospect for bacteriophage therapy in Western medicine. *Nat Rev Drug Discov*, **2**, 489-497.

Minenkova, O.O., Ilyichev, A.A., Kishchenko, G.P. and Petrenko, V.A. (1993) Design of specific immunogens using filamentous phage as the carrier. *Gene*, **128**, 85-88.

Mitra-Kaushik, S., Shaila, M.S., Karande, A. and Nayak, R. (2001a) Idiotypic-anti-idiotypic B cell interactions generated against a protective antigen of a morbillivirus in mice. *Cell Immunol*, **209**, 10-18.

Mitra-Kaushik, S., Shaila, M.S., Karande, A.K. and Nayak, R. (2001b) Idiotype and antigen-specific T cell responses in mice on immunization with antigen, antibody, and anti-idiotypic antibody. *Cell Immunol*, **209**, 109-119.

Moe, G.R., Tan, S. and Granoff, D.M. (1999) Molecular mimetics of polysaccharide epitopes as vaccine candidates for prevention of Neisseria meningitidis serogroup B disease. *FEMS Immunol Med Microbiol*, **26**, 209-226.

Moingeon, P. (2001) Cancer vaccines. *Vaccine*, **19**, 1305-1326.

Monaco, J.J. and McDevitt, H.O. (1986) The LMP antigens: a stable MHC-controlled multisubunit protein complex. *Hum Immunol*, **15**, 416-426.

Moore, R.L. and Fox, B.S. (1993) Immunization of mice with human immunodeficiency virus glycoprotein gp160 peptide 315-329 induces both class I- and class II-restricted T cells: not all T cells can respond to whole molecule stimulation. *AIDS Res Hum Retroviruses*, **9**, 51-59.

Morse, M.A. and Lyerly, H.K. (2002) DNA and RNA modified dendritic cell vaccines. *World J Surg*, **26**, 819-825.

Mosca, P.J., Hobeika, A.C., Colling, K., Clay, T.M., Thomas, E.K., Caron, D., Lyerly, H.K. and Morse, M.A. (2002) Multiple signals are required for maturation of human dendritic cells mobilized in vivo with Flt3 ligand. *J Leukoc Biol*, **72**, 546-553.

Moser, M. and Murphy, K.M. (2000) Dendritic cell regulation of TH1-TH2 development. *Nat Immunol*, **1**, 199-205.

Motta, I., Lone, Y.C. and Kourilsky, P. (1998) In vitro induction of naive cytotoxic T lymphocytes with complexes of peptide and recombinant MHC class I molecules coated onto beads: role of TCR/ligand density. *Eur J Immunol*, **28**, 3685-3695.

Mottershead, D.G., Alfthan, K., Ojala, K., Takkinen, K. and Oker-Blom, C. (2000) Baculoviral display of functional scFv and synthetic IgG-binding domains. *Biochem Biophys Res Commun*, **275**, 84-90.

Mottez, E., Jaulin, C., Godeau, F., Choppin, J., Levy, J.P. and Kourilsky, P. (1991) A single-chain murine class I major transplantation antigen. *Eur J Immunol*, **21**, 467-471.

Moukrim, Z., Cho, Y.Y., Mbika, J.P. and Achour, A. (1996) Lymphoproliferative response to synthetic V3 loop P18 peptide and HIV-1 envelope glycoprotein among individuals immunized with gp160 candidate vaccines. *Biomed Pharmacother*, **50**, 494-499.

Muller, M.R., Grunebach, F., Nencioni, A. and Brossart, P. (2003) Transfection of dendritic cells with RNA induces CD4- and CD8-mediated T cell immunity against breast carcinomas and reveals the immunodominance of presented T cell epitopes. *J Immunol*, **170**, 5892-5896.

Nakagawa, Y., Takeshita, T., Berzofsky, J.A. and Takahashi, H. (2000) Analysis of the mechanism for extracellular processing in the presentation of human immunodeficiency virus-1 envelope protein-derived peptide to epitope-specific cytotoxic T lymphocytes. *Immunology*, **101**, 76-82.

Nishimura, T., Iwakabe, K., Sekimoto, M., Ohmi, Y., Yahata, T., Nakui, M., Sato, T., Habu, S., Tashiro, H., Sato, M. and Ohta, A. (1999) Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *J Exp Med*, **190**, 617-627.

Norbury, C.C., Chambers, B.J., Prescott, A.R., Ljunggren, H.G. and Watts, C. (1997) Constitutive macropinocytosis allows TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells. *Eur J Immunol*, **27**, 280-288.

Nord, K., Nilsson, J., Nilsson, B., Uhlen, M. and Nygren, P.A. (1995) A combinatorial library of an alpha-helical bacterial receptor domain. *Protein Eng*, **8**, 601-608.

O'Hagan, D.T., MacKichan, M.L. and Singh, M. (2001) Recent developments in adjuvants for vaccines against infectious diseases. *Biomol Eng*, **18**, 69-85.

Oldenburg, K.R., Loganathan, D., Goldstein, I.J., Schultz, P.G. and Gallop, M.A. (1992) Peptide ligands for a sugar-binding protein isolated from a random peptide library. *Proc Natl Acad Sci U S A*, **89**, 5393-5397.

Onaitis, M., Kalady, M.F., Pruitt, S. and Tyler, D.S. (2002) Dendritic cell gene therapy. *Surg Oncol Clin N Am*, **11**, 645-660.

Ostergaard Pedersen, L., Nissen, M.H., Hansen, N.J., Nielsen, L.L., Lauenmoller, S.L., Blicher, T., Nansen, A., Sylvester-Hvid, C., Thromsen, A.R. and Buus, S. (2001) Efficient assembly of recombinant major histocompatibility complex class I molecules with preformed disulfide bonds. *Eur J Immunol*, **31**, 2986-2996.

Pannekoek, H., van Meijer, M., Schleef, R.R., Loskutoff, D.J. and Barbas, C.F.d. (1993) Functional display of human plasminogen-activator inhibitor 1 (PAI-1) on phages: novel perspectives for structure-function analysis by error-prone DNA synthesis. *Gene*, **128**, 135-140.

Parmley, S.F. and Smith, G.P. (1989) Filamentous fusion phage cloning vectors for the study of epitopes and design of vaccines. *Adv Exp Med Biol*, **251**, 215-218.

Paul, S., Snary, D., Hoebeke, J., Allen, D., Balloul, J.M., Bizouarne, N., Dott, K., Geist, M., Hilgers, J., Kieny, M.P., Burchell, J., Taylor-Papadimitriou, J. and Acres, R.B. (2000) Targeted macrophage cytotoxicity using a nonreplicative live vector expressing a tumor-specific single-chain variable region fragment. *Hum Gene Ther*, **11**, 1417-1428.

Paulsson, K. and Wang, P. (2003) Chaperones and folding of MHC class I molecules in the endoplasmic reticulum. *Biochim Biophys Acta*, **1641**, 1-12.

Paulsson, K.M., Kleijmeer, M.J., Griffith, J., Jevon, M., Chen, S., Anderson, P.O., Sjogren, H.O., Li, S. and Wang, P. (2002) Association of tapasin and COPI provides a mechanism for the retrograde transport of major histocompatibility complex (MHC) class I molecules from the Golgi complex to the endoplasmic reticulum. *J Biol Chem*, **277**, 18266-18271.

Pedersen, A.E., Gad, M., Walter, M.R. and Claesson, M.H. (2004) Induction of regulatory dendritic cells by dexamethasone and 1alpha,25-Dihydroxyvitamin D(3). *Immunol Lett*, **91**, 63-69.

Pedersen, H., Holder, S., Sutherlin, D.P., Schwitter, U., King, D.S. and Schultz, P.G. (1998) A method for directed evolution and functional cloning of enzymes. *Proc Natl Acad Sci U S A*, **95**, 10523-10528.

Pedersen, J.S. (2001) Directed Evolution of Thermostable Enzymes Using Phage Display. *IMSB*. University of Aarhus, Aarhus.

Penichet, M.L. and Morrison, S.L. (2001) Antibody-cytokine fusion proteins for the therapy of cancer. *J Immunol Methods*, **248**, 91-101.

Phalipon, A., Folgori, A., Arondel, J., Sgaramella, G., Fortugno, P., Cortese, R., Sansonetti, P.J. and Felici, F. (1997) Induction of anti-carbohydrate antibodies by phage library-selected peptide mimics. *Eur J Immunol*, **27**, 2620-2625.

Pichler, W.J. and Wyss-Coray, T. (1994) T cells as antigen-presenting cells. *Immunol Today*, **15**, 312-315.

Pierce, H.H., Adey, N. and Kay, B.K. (1996) Identification of cyclized calmodulin antagonists from a phage display random peptide library. *Mol Divers*, **1**, 259-265.

Pini, A., Viti, F., Santucci, A., Carnemolla, B., Zardi, L., Neri, P. and Neri, D. (1998) Design and use of a phage display library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. *J Biol Chem*, **273**, 21769-21776.

Polakova, K. and Russ, G. (2001) Analysis of the extracellular processing of HIV-1 gp160-derived peptides using monoclonal antibodies specific to H-2Dd molecule complexed with p18-I10 peptide. *Acta Virol*, **45**, 227-234.

Poljak, R.J., Amzel, L.M., Avey, H.P., Chen, B.L., Phizackerley, R.P. and Saul, F. (1973) Three-dimensional structure of the Fab' fragment of a human immunoglobulin at 2,8-A resolution. *Proc Natl Acad Sci U S A*, **70**, 3305-3310.

Pomerantz, R.J. (2003) The HIV-1 Vif protein: a paradigm for viral:cell interactions. *Cell Mol Life Sci*, **60**, 2017-2019.

Price, M.R., Rye, P.D., Petrakou, E., Murray, A., Brady, K., Imai, S., Haga, S., Kiyozuka, Y., Schol, D., Meulenbroek, M.F., Snijdewint, F.G., von Mensdorff-Pouilly, S., Verstraeten, R.A., Kenemans, P., Blockzjil, A., Nilsson, K., Nilsson, O., Reddish, M., Suresh, M.R., Koganty, R.R., Fortier, S., Baronic, L., Berg, A., Longenecker, M.B., Hilgers, J. and et al. (1998) Summary report on the ISOBM TD-4 Workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin. San Diego, Calif., November 17-23, 1996. *Tumour Biol*, **19 Suppl 1**, 1-20.

Prussin, C. and Metcalfe, D.D. (1995) Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J Immunol Methods*, **188**, 117-128.

Puebla, I., Esseghir, S., Mortlock, A., Brown, A., Crisanti, A. and Low, W. (2003) A recombinant H1 histone-based system for efficient delivery of nucleic acids. *J Biotechnol*, **105**, 215-226.

Pyun, K.H., Ochs, H.D., Wedgwood, R.J., Yang, X.Q., Heller, S.R. and Reimer, C.B. (1989) Human antibody responses to bacteriophage phi X 174: sequential induction of IgM and IgG subclass antibody. *Clin Immunol Immunopathol*, **51**, 252-263.

Quentmeier, H., Zaborski, M. and Drexler, H.G. (1997) The human bladder carcinoma cell line 5637 constitutively secretes functional cytokines. *Leuk Res*, **21**, 343-350.

Racanelli, V., Behrens, S.E., Aliberti, J. and Rehermann, B. (2004) Dendritic cells transfected with cytopathic self-replicating RNA induce crosspriming of CD8+ T cells and antiviral immunity. *Immunity*, **20**, 47-58.

Rada, C. and Milstein, C. (2001) The intrinsic hypermutability of antibody heavy and light chain genes decays exponentially. *Embo J*, **20**, 4570-4576.

Rakonjac, J., Feng, J. and Model, P. (1999) Filamentous phage are released from the bacterial membrane by a two-step mechanism involving a short C-terminal fragment of pIII. *J Mol Biol*, **289**, 1253-1265.

Rakonjac, J., Jovanovic, G. and Model, P. (1997) Filamentous phage infection-mediated gene expression: construction and propagation of the gIII deletion mutant helper phage R408d3. *Gene*, **198**, 99-103.

Ramm, K., Gehrig, P. and Pluckthun, A. (1999) Removal of the conserved disulfide bridges from the scFv fragment of an antibody: effects on folding kinetics and aggregation. *J Mol Biol*, **290**, 535-546.

Rapoza, M.P. and Webster, R.E. (1995) The products of gene I and the overlapping in-frame gene XI are required for filamentous phage assembly. *J Mol Biol*, **248**, 627-638.

Ratto, S., Sitz, K.V., Scherer, A.M., Loomis, L.D., Cox, J.H., Redfield, R.R. and Birx, D.L. (1996) CD4+ T-lymphocyte lines developed from HIV-1-seropositive patients recognize different epitopes within the V3 loop. *J Acquir Immune Defic Syndr Hum Retrovirol*, **11**, 128-136.

Ravera, M.W., Carcamo, J., Brissette, R., Alam-Moghe, A., Dedova, O., Cheng, W., Hsiao, K.C., Klebanov, D., Shen, H., Tang, P., Blume, A. and Mandecki, W. (1998) Identification of an allosteric binding site on the transcription factor p53 using a phage-displayed peptide library. *Oncogene*, **16**, 1993-1999.

Ravn, P., Danielczyk, A., Jensen, K.B., Larsen, M., Kristensen, P., Karsten, U. and Goletz, S. Multivalent antibody fragments toward the tumor-associated core-1 disaccharide generated by phage display of multimeric scFv repertoires. *Ready for submission*.

Ravn, P., Kjaer, S., Jensen, K.H., Wind, T., Jensen, K.B., Kristensen, P., Brosh, R.M., Orren, D.K., Bohr, V.A. and Clark, B.F. (2000) Identification of phage antibodies toward the Werner protein by selection on Western blots. *Electrophoresis*, **21**, 509-516.

Realini, C., Rogers, S.W. and Rechsteiner, M. (1994) KEKE motifs. Proposed roles in protein-protein association and presentation of peptides by MHC class I receptors. *FEBS Lett*, **348**, 109-113.

Rebar, E.J. and Pabo, C.O. (1994) Zinc finger phage: affinity selection of fingers with new DNA-binding specificities. *Science*, **263**, 671-673.

Reinartz, S., Wagner, U., Giffels, P., Gruenn, U., Schlebusch, H. and Wallwiener, D. (2000) Immunological properties of a single-chain fragment of the anti-idiotypic antibody ACA125. *Cancer Immunol Immunother*, **49**, 186-192.

Renauld, J.C., Vink, A. and Van Snick, J. (1989) Accessory signals in murine cytolytic T cell responses. Dual requirement for IL-1 and IL-6. *J Immunol*, **143**, 1894-1898.

Richards, J., Le Naour, F., Hanash, S. and Beretta, L. (2002) Integrated genomic and proteomic analysis of signaling pathways in dendritic cell differentiation and maturation. *Ann N Y Acad Sci*, **975**, 91-100.

Rico, A.I., Del Real, G., Soto, M., Quijada, L., Martinez, A.C., Alonso, C. and Requena, J.M. (1998) Characterization of the immunostimulatory properties of Leishmania infantum HSP70 by fusion to the Escherichia coli maltose-binding protein in normal and nu/nu BALB/c mice. *Infect Immun*, **66**, 347-352.

Riechmann, L. and Holliger, P. (1997) The C-terminal domain of TolA is the coreceptor for filamentous phage infection of E. coli. *Cell*, **90**, 351-360.

Rivas, F.V., O'Herrin, S. and Gajewski, T.F. (2001) CD28 is not required for c-Jun N-terminal kinase activation in T cells. *J Immunol*, **167**, 3123-3128.

Rivoltini, L., Barracchini, K.C., Viggiano, V., Kawakami, Y., Smith, A., Mixon, A., Restifo, N.P., Topalian, S.L., Simonis, T.B., Rosenberg, S.A. and et al. (1995) Quantitative correlation between HLA class I allele expression and recognition of melanoma cells by antigen-specific cytotoxic T lymphocytes. *Cancer Res*, **55**, 3149-3157.

Roberts, R.W. and Szostak, J.W. (1997) RNA-peptide fusions for the in vitro selection of peptides and proteins. *Proc Natl Acad Sci U S A*, **94**, 12297-12302.

Robinson, S.P., Saraya, K. and Reid, C.D. (1998) Developmental aspects of dendritic cells in vitro and in vivo. *Leuk Lymphoma*, **29**, 477-490.

Rock, K.L. and Goldberg, A.L. (1999) Degradation of cell proteins and the generation of MHC class I-presented peptides. *Annu Rev Immunol*, **17**, 739-779.

Rode, H.J., Moebius, U. and Little, M. (1999) T cell activation by monoclonal antibodies bound to tumor cells by a cell surface displayed single-chain antibody. *J Immunol Methods*, **224**, 151-160.

Rodriguez, A., Regnault, A., Kleijmeer, M., Ricciardi-Castagnoli, P. and Amigorena, S. (1999) Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat Cell Biol*, **1**, 362-368.

Rohwer, F. and Edwards, R. (2002) The Phage Proteomic Tree: a genome-based taxonomy for phage. *J Bacteriol*, **184**, 4529-4535.

Rondot, S., Koch, J., Breitling, F. and Dubel, S. (2001) A helper phage to improve single-chain antibody presentation in phage display. *Nat Biotechnol*, **19**, 75-78.

Rosenzwajg, M., Jourquin, F., Tailleux, L. and Gluckman, J.C. (2002) CD40 ligation and phagocytosis differently affect the differentiation of monocytes into dendritic cells. *J Leukoc Biol*, **72**, 1180-1189.

Ruckert, R., Brandt, K., Bulanova, E., Mirghomizadeh, F., Paus, R. and Bulfone-Paus, S. (2003) Dendritic cell-derived IL-15 controls the induction of CD8 T cell immune responses. *Eur J Immunol*, **33**, 3493-3503.

Rudd, P.M., Elliott, T., Cresswell, P., Wilson, I.A. and Dwek, R.A. (2001) Glycosylation and the immune system. *Science*, **291**, 2370-2376.

Rudolph, M.G. and Wilson, I.A. (2002) The specificity of TCR/pMHC interaction. *Curr Opin Immunol*, **14**, 52-65.

Rughetti, A., Biffoni, M., Sabbatucci, M., Rahimi, H., Pellicciotta, I., Fattorossi, A., Pierelli, L., Scambia, G., Lavitrano, M., Frati, L. and Nuti, M. (2000) Transfected human dendritic cells to induce antitumor immunity. *Gene Ther*, **7**, 1458-1466.

Ruska, H. (1940) Die Sichtbarmachung der bakteriophagen Lyse im Ubermikroskop. *Naturwissenshaften*, **29**, 367-368.

Russel, M. (1995) Moving through the membrane with filamentous phages. *Trends Microbiol*, **3**, 223-228.

Russel, M., Kidd, S. and Kelley, M.R. (1986) An improved filamentous helper phage for generating single-stranded plasmid DNA. *Gene*, **45**, 333-338.

Russel, M., Linderoth, N.A. and Sali, A. (1997) Filamentous phage assembly: variation on a protein export theme. *Gene*, **192**, 23-32.

Russel, M. and Model, P. (1989) Genetic analysis of the filamentous bacteriophage packaging signal and of the proteins that interact with it. *J Virol*, **63**, 3284-3295.

Russel, M., Whirlow, H., Sun, T.P. and Webster, R.E. (1988) Low-frequency infection of F- bacteria by transducing particles of filamentous bacteriophages. *J Bacteriol*, **170**, 5312-5316.

Russell, J.H. and Ley, T.J. (2002) Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol*, **20**, 323-370.

Saeki, H., Moore, A.M., Brown, M.J. and Hwang, S.T. (1999) Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J Immunol*, **162**, 2472-2475.

Saha, A., Chatterjee, S.K., Foon, K.A., Primus, F.J. and Bhattacharya-Chatterjee, M. (2003) Murine dendritic cells pulsed with an anti-idiotype antibody induce antigen-specific protective antitumor immunity. *Cancer Res*, **63**, 2844-2854.

Salivar, W.O., Tzagoloff, H. and Pratt, D. (1964) Some Physical-Chemical and Biological Properties of the Rod-Shaped Coliphage M13. *Virology*, **24**, 359-371.

Sallusto, F., Cella, M., Danieli, C. and Lanzavecchia, A. (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med*, **182**, 389-400.

Sallusto, F. and Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med*, **179**, 1109-1118.

Salter, R.D., Howell, D.N. and Cresswell, P. (1985) Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics*, **21**, 235-246.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning. A laboratory manual*. Cold Spring Harbor Laboratory Press, New York.

Sanger, F. and Coulson, A.R. (1975) A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J Mol Biol*, **94**, 441-448.

Santini, C., Brennan, D., Mennuni, C., Hoess, R.H., Nicosia, A., Cortese, R. and Luzzago, A. (1998) Efficient display of an HCV cDNA expression library as C-terminal fusion to the capsid protein D of bacteriophage lambda. *J Mol Biol*, **282**, 125-135.

Sanz, L., Garcia-Bermejo, L., Blanco, F.J., Kristensen, P., Feijoo, M., Suarez, E., Blanco, B. and Alvarez-Vallina, L. (2003) A novel cell binding site in the coiled-coil domain of laminin involved in capillary morphogenesis. *Embo J*, **22**, 1508-1517.

Sanz, L., Kristensen, P., Russell, S.J., Ramirez Garcia, J.R. and Alvarez-Vallina, L. (2001) Generation and characterization of recombinant human antibodies specific for native laminin epitopes: potential application in cancer therapy. *Cancer Immunol Immunother*, **50**, 557-565.

Saper, M.A., Bjorkman, P.J. and Wiley, D.C. (1991) Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 A resolution. *J Mol Biol*, **219**, 277-319.

Schier, R., McCall, A., Adams, G.P., Marshall, K.W., Merritt, H., Yim, M., Crawford, R.S., Weiner, L.M., Marks, C. and Marks, J.D. (1996) Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. *J Mol Biol*, **263**, 551-567.

Schjetne, K.W., Thompson, K.M., Nilsen, N., Flo, T.H., Fleckenstein, B., Iversen, J.G., Espevik, T. and Bogen, B. (2003) Cutting edge: link between innate and adaptive immunity: Toll-like receptor 2 internalizes antigen for presentation to CD4+ T cells and could be an efficient vaccine target. *J Immunol*, **171**, 32-36.

Schmitz, R., Baumann, G. and Gram, H. (1996) Catalytic specificity of phosphotyrosine kinases Blk, Lyn, c-Src and Syk as assessed by phage display. *J Mol Biol*, **260**, 664-677.

Schodel, F., Enders, G., Jung, M.C. and Will, H. (1990) Recognition of a hepatitis B virus nucleocapsid T-cell epitope expressed as a fusion protein with the subunit B of Escherichia coli heat labile enterotoxin in attenuated salmonellae. *Vaccine*, **8**, 569-572.

Schodel, F., Peterson, D., Hughes, J., Wirtz, R. and Milich, D. (1996) Hybrid hepatitis B virus core antigen as a vaccine carrier moiety: I. presentation of foreign epitopes. *J Biotechnol*, **44**, 91-96.

Schuler, G., Schuler-Thurner, B. and Steinman, R.M. (2003) The use of dendritic cells in cancer immunotherapy. *Curr Opin Immunol*, **15**, 138-147.

Scott, J.K. and Smith, G.P. (1990) Searching for peptide ligands with an epitope library. *Science*, **249**, 386-390.

Sedwick, C.E., Morgan, M.M., Jusino, L., Cannon, J.L., Miller, J. and Burkhardt, J.K. (1999) TCR, LFA-1, and CD28 play unique and complementary roles in signaling T cell cytoskeletal reorganization. *J Immunol*, **162**, 1367-1375.

Serezani, C.H., Franco, A.R., Wajc, M., Umada Yokoyama-Yasunaka, J.K., Wunderlich, G., Borges, M.M. and Uliana, S.R. (2002) Evaluation of the murine immune response to Leishmania meta 1 antigen delivered as recombinant protein or DNA vaccine. *Vaccine*, **20**, 3755-3763.

Shen, X., Wong, S.B., Buck, C.B., Zhang, J. and Siliciano, R.F. (2002) Direct priming and cross-priming contribute differentially to the induction of CD8+ CTL following exposure to vaccinia virus via different routes. *J Immunol*, **169**, 4222-4229.

Shields, M.J. and Ribaudo, R.K. (1998) Mapping of the monoclonal antibody W6/32: sensitivity to the amino terminus of beta2-microglobulin. *Tissue Antigens*, **51**, 567-570.
Sieber, V., Pluckthun, A. and Schmid, F.X. (1998) Selecting proteins with improved stability by a phage-based method. *Nat Biotechnol*, **16**, 955-960.
Singh, M. and O'Hagan, D. (1999) Advances in vaccine adjuvants. *Nat Biotechnol*, **17**, 1075-1081.
Sjolander, A., Nygren, P.A., Stahl, S., Berzins, K., Uhlen, M., Perlmann, P. and Andersson, R. (1997) The serum albumin-binding region of streptococcal protein G: a bacterial fusion partner with carrier-related properties. *J Immunol Methods*, **201**, 115-123.
Smilowitz, H. (1974) Bacteriophage f1 infection: fate of the parental major coat protein. *J Virol*, **13**, 94-99.
Smith, G.P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*, **228**, 1315-1317.
Smith, G.P. (1988) Filamentous phages as cloning vectors. *Biotechnology*, **10**, 61-83.
Smith, M.M., Shi, L. and Navre, M. (1995) Rapid identification of highly active and selective substrates for stromelysin and matrilysin using bacteriophage peptide display libraries. *J Biol Chem*, **270**, 6440-6449.
Snapper, C.M. and Mond, J.J. (1993) Towards a comprehensive view of immunoglobulin class switching. *Immunol Today*, **14**, 15-17.
Soderlind, E., Duenas, M. and Borrebaeck, C.A. (1995) Chaperonins in phage display of antibody fragments. *Methods Mol Biol*, **51**, 343-353.
Speight, R.E., Hart, D.J., Sutherland, J.D. and Blackburn, J.M. (2001) A new plasmid display technology for the in vitro selection of functional phenotype-genotype linked proteins. *Chem Biol*, **8**, 951-965.
Spellerberg, M.B., Zhu, D., Thompsett, A., King, C.A., Hamblin, T.J. and Stevenson, F.K. (1997) DNA vaccines against lymphoma: promotion of anti-idiotypic antibody responses induced by single chain Fv genes by fusion to tetanus toxin fragment C. *J Immunol*, **159**, 1885-1892.
Springer, G.F. (1997) Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy. *J Mol Med*, **75**, 594-602.
Springer, G.F., Fry, W.A., Desai, P.R., Semerdjian, R.A., Tegtmeyer, H., Neybert, C.G. and Scanlon, E.F. (1985) Further studies on the detection of early lung and breast carcinoma by T antigen. *Cancer Detect Prev*, **8**, 95-100.
Springer, T.A., Kaufman, J.F., Terhorst, C. and Strominger, J.L. (1977) Purification and structural characterisation of human HLA-linked B-cell antigens. *Nature*, **268**, 213-218.
Stausbøl Grøn, B., Wind, T., Kjær, S., Kahns, L., Hansen, N.J., Kristensen, P. and Clark, B.F. (1996) A model phage display subtraction method with potential for analysis of differential gene expression. *FEBS-Lett*, **391**, 71-75 issn: 0014-5793.
Stavnezer, J. (1996) Immunoglobulin class switching. *Curr Opin Immunol*, **8**, 199-205.
Steinman, R.M. and Cohn, Z.A. (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med*, **137**, 1142-1162.
Stern, L.J. and Wiley, D.C. (1994) Antigenic peptide binding by class I and class II histocompatibility proteins. *Structure*, **2**, 245-251.
Stone, R. (2002) Bacteriophage therapy. Stalin's forgotten cure. *Science*, **298**, 728-731.
Summers, W.C. (2001) Bacteriophage therapy. *Annu Rev Microbiol*, **55**, 437-451.
Sylvester-Hvid, C., Nielsen, L.L., Hansen, N.J., Pedersen, L.O. and Buus, S. (1999) A single-chain fusion molecule consisting of peptide, major histocompatibility gene complex class I heavy chain and beta2-microglobulin can fold partially correctly, but binds peptide inefficiently. *Scand J Immunol*, **50**, 355-362.
Tacke, M., Hanke, G., Hanke, T. and Hunig, T. (1997) CD28-mediated induction of proliferation in resting T cells in vitro and in vivo without engagement of the T cell receptor: evidence for functionally distinct forms of CD28. *Eur J Immunol*, **27**, 239-247.
Tafuri, A., Shahinian, A., Bladt, F., Yoshinaga, S.K., Jordana, M., Wakeham, A., Boucher, L.M., Bouchard, D., Chan, V.S., Duncan, G., Odermatt, B., Ho, A., Itie, A., Horan, T., Whoriskey, J.S., Pawson, T., Penninger, J.M., Ohashi, P.S. and Mak, T.W. (2001) ICOS is essential for effective T-helper-cell responses. *Nature*, **409**, 105-109.
Tafuro, S., Meier, U.C., Dunbar, P.R., Jones, E.Y., Layton, G.T., Hunter, M.G., Bell, J.I. and McMichael, A.J. (2001) Reconstitution of antigen presentation in HLA class I-negative cancer cells with peptide-beta2m fusion molecules. *Eur J Immunol*, **31**, 440-449.
Taki, T., Ishikawa, D., Hamasaki, H. and Handa, S. (1997) Preparation of peptides which mimic glycosphingolipids by using phage peptide library and their modulation on beta-galactosidase activity. *FEBS Lett*, **418**, 219-223.
Tang, Y., Jiang, N., Parakh, C. and Hilvert, D. (1996) Selection of linkers for a catalytic single-chain antibody using phage display technology. *J Biol Chem*, **271**, 15682-15686.

Tatsumi, T., Huang, J., Gooding, W.E., Gambotto, A., Robbins, P.D., Vujanovic, N.L., Alber, S.M., Watkins, S.C., Okada, H. and Storkus, W.J. (2003) Intratumoral delivery of dendritic cells engineered to secrete both interleukin (IL)-12 and IL-18 effectively treats local and distant disease in association with broadly reactive Tc1-type immunity. *Cancer Res*, **63**, 6378-6386.

Tawfik, D.S. and Griffiths, A.D. (1998) Man-made cell-like compartments for molecular evolution. *Nat Biotechnol*, **16**, 652-656.

Taylor-Papadimitriou, J., Burchell, J., Miles, D.W. and Dalziel, M. (1999) MUC1 and cancer. *Biochim Biophys Acta*, **1455**, 301-313.

Tew, J.G., DiLosa, R.M., Burton, G.F., Kosco, M.H., Kupp, L.I., Masuda, A. and Szakal, A.K. (1992) Germinal centers and antibody production in bone marrow. *Immunol Rev*, **126**, 99-112.

Tew, J.G., Wu, J., Fakher, M., Szakal, A.K. and Qin, D. (2001) Follicular dendritic cells: beyond the necessity of T-cell help. *Trends Immunol*, **22**, 361-367.

Thery, C. and Amigorena, S. (2001) The cell biology of antigen presentation in dendritic cells. *Curr Opin Immunol*, **13**, 45-51.

Thurner, B., Roder, C., Dieckmann, D., Heuer, M., Kruse, M., Glaser, A., Keikavoussi, P., Kampgen, E., Bender, A. and Schuler, G. (1999) Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. *J Immunol Methods*, **223**, 1-15.

Ting, J.P. and Baldwin, A.S. (1993) Regulation of MHC gene expression. *Curr Opin Immunol*, **5**, 8-16.

Tomlinson, I.M., Cox, J.P., Gherardi, E., Lesk, A.M. and Chothia, C. (1995) The structural repertoire of the human V kappa domain. *Embo J*, **14**, 4628-4638.

Tomlinson, I.M., Walter, G., Jones, P.T., Dear, P.H., Sonnhammer, E.L. and Winter, G. (1996) The imprint of somatic hypermutation on the repertoire of human germline V genes. *J Mol Biol*, **256**, 813-817.

Tomlinson, I.M., Walter, G., Marks, J.D., Llewelyn, M.B. and Winter, G. (1992) The repertoire of human germline VH sequences reveals about fifty groups of VH segments with different hypervariable loops. *J Mol Biol*, **227**, 776-798.

Torres, C.A., Iwasaki, A., Barber, B.H. and Robinson, H.L. (1997) Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. *J Immunol*, **158**, 4529-4532.

Toshitani, K., Braud, V., Browning, M.J., Murray, N., McMichael, A.J. and Bodmer, W.F. (1996) Expression of a single-chain HLA class I molecule in a human cell line: presentation of exogenous peptide and processed antigen to cytotoxic T lymphocytes. *Proc Natl Acad Sci U S A*, **93**, 236-240.

Townsend, A., Ohlen, C., Bastin, J., Ljunggren, H.G., Foster, L. and Karre, K. (1989) Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature*, **340**, 443-448.

Tramontano, A., Bianchi, E., Venturini, S., Martin, F., Pessi, A. and Sollazzo, M. (1994) The making of the minibody: an engineered beta-protein for the display of conformationally constrained peptides. *J Mol Recognit*, **7**, 9-24.

Tripathi, P.K., Qin, H., Bhattacharya-Chatterjee, M., Ceriani, R.L., Foon, K.A. and Chatterjee, S.K. (1999) Construction and characterization of a chimeric fusion protein consisting of an anti-idiotype antibody mimicking a breast cancer-associated antigen and the cytokine GM-CSF. *Hybridoma*, **18**, 193-202.

Tripathi, P.K., Qin, H., Deng, S., Xu, C., Bhattacharya-Chatterjee, M., Foon, K.A. and Chatterjee, S.K. (1998) Antigen mimicry by an anti-idiotypic antibody single chain variable fragment. *Mol Immunol*, **35**, 853-863.

Trompeter, H.I., Weinhold, S., Thiel, C., Wernet, P. and Uhrberg, M. (2003) Rapid and highly efficient gene transfer into natural killer cells by nucleofection. *J Immunol Methods*, **274**, 245-256.

Tsunetsugu-Yokota, Y., Tatsumi, M., Robert, V., Devaux, C., Spire, B., Chermann, J.C. and Hirsch, I. (1991) Expression of an immunogenic region of HIV by a filamentous bacteriophage vector. *Gene*, **99**, 261-265.

Turner, D.J., Ritter, M.A. and George, A.J. (1997) Importance of the linker in expression of single-chain Fv antibody fragments: optimisation of peptide sequence using phage display technology. *J Immunol Methods*, **205**, 43-54.

Ueno, H., Tcherepanova, I., Reygrobellet, O., Laughner, E., Ventura, C., Palucka, A.K. and Banchereau, J. (2004) Dendritic cell subsets generated from CD34(+) hematopoietic progenitors can be transfected with mRNA and induce antigen-specific cytotoxic T cell responses. *J Immunol Methods*, **285**, 171-180.

Uger, R.A. and Barber, B.H. (1998) Creating CTL targets with epitope-linked beta 2-microglobulin constructs. *J Immunol*, **160**, 1598-1605.

Valenzuela, J., Schmidt, C. and Mescher, M. (2002) The roles of IL-12 in providing a third signal for clonal expansion of naive CD8 T cells. *J Immunol*, **169**, 6842-6849.

van Kooten, C. and Banchereau, J. (2000) CD40-CD40 ligand. *J Leukoc Biol*, **67**, 2-17.

Van Tendeloo, V.F., Ponsaerts, P., Lardon, F., Nijs, G., Lenjou, M., Van Broeckhoven, C., Van Bockstaele, D.R. and Berneman, Z.N. (2001) Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. *Blood*, **98**, 49-56.

Van Tendeloo, V.F., Snoeck, H.W., Lardon, F., Vanham, G.L., Nijs, G., Lenjou, M., Hendriks, L., Van Broeckhoven, C., Moulijn, A., Rodrigus, I., Verdonk, P., Van Bockstaele, D.R. and Berneman, Z.N. (1998) Nonviral transfection of distinct types of human dendritic cells: high-efficiency gene transfer by electroporation into hematopoietic progenitor- but not monocyte-derived dendritic cells. *Gene Ther*, **5**, 700-707.

Van Voorhis, W.C., Hair, L.S., Steinman, R.M. and Kaplan, G. (1982) Human dendritic cells. Enrichment and characterization from peripheral blood. *J Exp Med*, **155**, 1172-1187.

van Wezenbeek, P. and Schoenmakers, J.G. (1979) Nucleotide sequence of the genes III, VI and I of bacteriophage M13. *Nucleic Acids Res*, **6**, 2799-2818.

van Wezenbeek, P.M., Hulsebos, T.J. and Schoenmakers, J.G. (1980) Nucleotide sequence of the filamentous bacteriophage M13 DNA genome: comparison with phage fd. *Gene*, **11**, 129-148.

Vandenabeele, S. and Wu, L. (1999) Dendritic cell origins: puzzles and paradoxes. *Immunol Cell Biol*, **77**, 411-419.

Veljkovic, V., Metlas, R., Kohler, H., Urnovitz, H.B., Prljic, J., Veljkovic, N., Johnson, E. and Muller, S. (2001) AIDS epidemic at the beginning of the third millennium: time for a new AIDS vaccine strategy. *Vaccine*, **19**, 1855-1862.

Verhaert, R.M., Van Duin, J. and Quax, W.J. (1999) Processing and functional display of the 86 kDa heterodimeric penicillin G acylase on the surface of phage fd. *Biochem J*, **342 (Pt 2)**, 415-422.

Vermeulen, M., Giordano, M., Trevani, A.S., Sedlik, C., Gamberale, R., Fernandez-Calotti, P., Salamone, G., Raiden, S., Sanjurjo, J. and Geffner, J.R. (2004) Acidosis improves uptake of antigens and MHC class I-restricted presentation by dendritic cells. *J Immunol*, **172**, 3196-3204.

Vest Hansen, N., Ostergaard Pedersen, L., Stryhn, A. and Buus, S. (2001) Phage display of peptide / major histocompatibility class I complexes. *Eur J Immunol*, **31**, 32-38.

Vinner, L., Nielsen, H.V., Bryder, K., Corbet, S., Nielsen, C. and Fomsgaard, A. (1999) Gene gun DNA vaccination with Rev-independent synthetic HIV-1 gp160 envelope gene using mammalian codons. *Vaccine*, **17**, 2166-2175.

Vogel, M., Miescher, S., Biaggi, C. and Stadler, B.M. (1994) Human anti-IgE antibodies by repertoire cloning. *Eur J Immunol*, **24**, 1200-1207.

Wang, J. and Reinherz, E.L. (2000) Structural basis of cell-cell interactions in the immune system. *Curr Opin Struct Biol*, **10**, 656-661.

Warren, A.P. and Thomas, D.B. (1992) Class II (I-Ad) restricted T-cell recognition of the V3 loop region of HIV-1 gp120. *AIDS Res Hum Retroviruses*, **8**, 559-564.

Watts, C. (2001) Antigen processing in the endocytic compartment. *Curr Opin Immunol*, **13**, 26-31.

Webster, R.E. (1991) The tol gene products and the import of macromolecules into Escherichia coli. *Mol Microbiol*, **5**, 1005-1011.

Webster, R.E. (1996) *Biology of the filamentous Bacteriophage. In "Phage display of peptides and proteins - a laboratory manual"*. San Diego: Academic Press, San Diego.

Webster, R.E., Grant, R.A. and Hamilton, L.A. (1981) Orientation of the DNA in the filamentous bacteriophage f1. *J Mol Biol*, **152**, 357-374.

Weiss, G.A. and Sidhu, S.S. (2000) Design and evolution of artificial M13 coat proteins. *J Mol Biol*, **300**, 213-219.

Wentzel, A., Christmann, A., Adams, T. and Kolmar, H. (2001) Display of Passenger Proteins on the Surface of Escherichia coli K-12 by the Enterohemorrhagic E. coli Intimin EaeA. *J Bacteriol*, **183**, 7273-7284.

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

Westerink, M.A., Giardina, P.C., Apicella, M.A. and Kieber-Emmons, T. (1995) Peptide mimicry of the meningococcal group C capsular polysaccharide. *Proc Natl Acad Sci U S A*, **92**, 4021-4025.

White, J., Crawford, F., Fremont, D., Marrack, P. and Kappler, J. (1999) Soluble class I MHC with beta2-microglobulin covalently linked peptides: specific binding to a T cell hybridoma. *J Immunol*, **162**, 2671-2676.

Williams, D.B. and Watts, T.H. (1995) Molecular chaperones in antigen presentation. *Curr Opin Immunol*, **7**, 77-84.

Williams, M.E., Shea, C.M., Lichtman, A.H. and Abbas, A.K. (1992) Antigen receptor-mediated anergy in resting T lymphocytes and T cell clones. Correlation with lymphokine secretion patterns. *J Immunol*, **149**, 1921-1926.

Williams, S.C., Frippiat, J.P., Tomlinson, I.M., Ignatovich, O., Lefranc, M.P. and Winter, G. (1996) Sequence and evolution of the human germline V lambda repertoire. *J Mol Biol*, **264**, 220-232.

Willis, A.E., Perham, R.N. and Wraith, D. (1993) Immunological properties of foreign peptides in multiple display on a filamentous bacteriophage. *Gene*, **128**, 79-83.

Wilson, C.C., Olson, W.C., Tuting, T., Rinaldo, C.R., Lotze, M.T. and Storkus, W.J. (1999) HIV-1-specific CTL responses primed in vitro by blood-derived dendritic cells and Th1-biasing cytokines. *J Immunol*, **162**, 3070-3078.

Wilson, D.S., Keefe, A.D. and Szostak, J.W. (2001) The use of mRNA display to select high-affinity protein-binding peptides. *Proc Natl Acad Sci U S A*, **98**, 3750-3755.

Wind, T., Kjaer, S. and Clark, B.F. (1999) Display of Ras on filamentous phage through cysteine replacement. *Biochimie*, **81**, 1079-1087.

Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A. and Felgner, P.L. (1990) Direct gene transfer into mouse muscle in vivo. *Science*, **247**, 1465-1468.

Worn, A. and Pluckthun, A. (1998) An intrinsically stable antibody scFv fragment can tolerate the loss of both disulfide bonds and fold correctly. *FEBS Lett*, **427**, 357-361.

Wright, S.D. and Griffin, F.M., Jr. (1985) Activation of phagocytic cells' C3 receptors for phagocytosis. *J Leukoc Biol*, **38**, 327-339.

Wright, S.D. and Silverstein, S.C. (1983) Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes. *J Exp Med*, **158**, 2016-2023.

Wu, H., Yang, W.P. and Barbas, C.F., 3rd. (1995) Building zinc fingers by selection: toward a therapeutic application. *Proc Natl Acad Sci U S A*, **92**, 344-348.

Wulfing, C. and Pluckthun, A. (1994) Protein folding in the periplasm of Escherichia coli. *Mol Microbiol*, **12**, 685-692.

Wulfing, C., Purtic, B., Klem, J. and Schatzle, J.D. (2003) Stepwise cytoskeletal polarization as a series of checkpoints in innate but not adaptive cytolytic killing. *Proc Natl Acad Sci U S A*, **100**, 7767-7772.

Xu, Z.L., Mizuguchi, H., Ishii-Watabe, A., Uchida, E., Mayumi, T. and Hayakawa, T. (2001) Optimization of transcriptional regulatory elements for constructing plasmid vectors. *Gene*, **272**, 149-156.

Yewdell, J.W. and Bennink, J.R. (2001) Cut and trim: generating MHC class I peptide ligands. *Curr Opin Immunol*, **13**, 13-18.

Yip, Y.L., Smith, G. and Ward, R.L. (2001) Comparison of phage pIII, pVIII and GST as carrier proteins for peptide immunisation in Balb/c mice. *Immunol Lett*, **79**, 197-202.

York, I.A. and Rock, K.L. (1996) Antigen processing and presentation by the class I major histocompatibility complex. *Annu Rev Immunol*, **14**, 369-396.

Yu, Y.Y., Netuschil, N., Lybarger, L., Connolly, J.M. and Hansen, T.H. (2002) Cutting edge: single-chain trimers of MHC class I molecules form stable structures that potently stimulate antigen-specific T cells and B cells. *J Immunol*, **168**, 3145-3149.

Zwick, M.B., Shen, J. and Scott, J.K. (1998) Phage-displayed peptide libraries. *Curr Opin Biotechnol*, **9**, 427-436.

6 Summary

Generation of efficient protection against pathogens was the historical aim of vaccination. However, the expanding knowledge about immunology combined with a wide spectrum of novel vaccination strategies have expanded the applicability of vaccination from protection against pathogens to direct use in therapy, such as cancer immunotherapy. The efficiency of such vaccines has been optimised through modulation of various parameters, such as choice of antigen, adjuvant, carrier system and administration route. In the presented work we have investigated two adjuvant systems based on 1) the N-terminal domains of the minor coat protein III of the filamentous bacteriophage and 2) a fully functional human dendritic cell line, NemodDC.

The protein III adjuvant system is particularly beneficial for immunisation with anti-idiotypic scFvs selected by phage display, since scFvs seem to retain functionality exceptionally well, when the phage display selection conditions are preserved, in particular the fusion to the N-terminal of the phage coat protein III. This distinct quality led us to designate the adjuvant fusion system – Functionally Fused Antibodies (FuncFAb). The adjuvant system has proven valuable both as protein and DNA vaccines in mice immunisation experiments. It seems to evoke a mixed type 1 and 2 immune response when administered as protein, whereas the DNA vaccine seem to promote a type 1 dominated immune response. Type 1 immune responses are especially well suited for cancer immune therapy, where the induction of a cytotoxic immune response is of major importance. One explanation for the immunomodulatory effect of the FuncFAb system is the intrinsic multivalency, which also potentially enable the administration of vaccines with multiple antigens or intrinsic targeting properties by employing antibodies against specific cell surfaces, such as antigen presenting cells (APCs).

As a supplement to targeting APCs *in vivo* it has become possible to isolate APCs from donors, manipulate them *in vitro* and administer them back into the patients. This technique has been particularly well studied employing dendritic cells (DCs). However, DC therapy has been impeded due to the difficulty of isolating sufficient number of cells. Novel *in vitro* cultivation strategies has surmounted these difficulties, and with the generation of the human dendritic cell line, NemodDC, it is now possible to generate vast amounts of fully functional DCs. In this study we have addressed the potential *in vitro* transfection of NemodDC and the immunological functionality of such transfected cells. We have shown that the novel nucleofection technology transfect NemodDC with

an efficiency of approximately 90%, and we have further shown that NemodDCs nucleofected with a gp120 encoding HIV DNA vaccine are capable of inducing gp120 antigen specific $CD8^+$ T cell activation. We further analysed the potential of NemodDC in tumour therapy by nucleofection with tumour derived (MCF-7) total RNA. This study showed activation of $CD8^+$ T cells specific for a MUC1 derived tumour antigen. The nucleofection technology also seems promising for modulating NemodDC induced immune responses. Indeed transfection of type 1 cytokine IL-12 showed a beneficial effect on $CD8^+$ T cell activation.

We finally addressed a technique theoretically capable of identifying any peptide, which in the context of an MHC class I complex, can bind to a sample of $CD8^+$ T cells. The technique theoretically enable selection of peptides binding to a T cell receptor employing a phage displayed peptide library based on single-chain MHC class I molecules (scMHC) as scaffold. We were however not able to detect binding of a peptide specific T cell clone in a peptide specific manner with the employed setup. Preliminary results however suggest that multivalency of such phage displayed scMHC molecules enable peptide specific activation of T cells. Further optimisation of the technique primarily focusing on the introduction of multivalency will reveal the future applicability of the technique.

7 Abbreviations

a.a.	amino acid
AC	Accessory Cell
AP	Alkaline Phosphatase
APC	Antigen Precenting Cell
β2MG	β2-microglobulin
BCG	*Bacillus Calmette-Guerin mycobacteria*
BCIP	5-bromo,4-chloro,3-indolylphosphate
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CDR	Complement Determining Region
C_H	Constant heavy chain
C_L	Constant light chain
CTL	Cytotoxic T Lymphocyte
DI	Domain I of filamentous bacteriophage coat protein III
DII	Domain II of filamentous bacteriophage coat protein III
DIII	Domain III of filamentous bacteriophage coat protein III
DC	Dendritic Cell
DEX	Dexamethasone
DNA	Deoxynucleic Acid
DTT	Dithio-1,4-threitol
E. coli	*Escherichia coli*
ELISA	Enzyme Linked Immuno-Sorbent Assay
ENV	Envelope gene from HIV-1
ER	Endoplasmic Reticulum
F_{ab}	Fragment antigen binding
F_C	Fragment crystallisable
FCS	Fetale Calf Serum
Fv	Fragment Variable
gIIIp	gene III product of the filamentous bacteriophage
gVIIIp	gene VIII product of the filamentous bacteriophage
GM-CSF	Granulocyte Macrophage - Colony Stimulating Factor
HC	MHC class I Heavy Chain corresponding to HLA (human) or H-2K (mouse)
HLA	Human Lymphocyte Antigen
HRP	Horseradish Peroxidase
HSP	Heat-Shock Protein
IFA	Incomplete Freund's Adjuvant
IFN	Interferon
IL	Interleukin
IMAC	Immobilised Metal Affinity Chromatography
IPTG	Isopropyl-beta-D-thiogalactopyranoside
MOI	Multiplicity of Infection
NBT	Nitroblue Tetrazolium
PCR	Polymerase Chain Reaction
Phage	Filamentous Bacteriophage
Protein III	The minor coat protein III of the filamentous bacteriophage
Protein VIII	The major coat protein VIII of the filamentous bacteriophage

RT-PCR	Reverse Transcriptase PCR
V_H	Variable heavy chain
V_L	Variable light chain
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
MPBS	Phosphate buffered saline supplemented with milk powder
NK cells	Natural Killer cells
ON	Over Night
PBS	Phosphate buffered saline
PhAb	Phage displayed antibody fragment or phage antibody
PPSC	Pluripotent Stem Cell
Rev	Rev gene from HIV-1
RT	Room Temperature
scMHC	single-chain MHC
scpMHC	single-chain peptide-MHC
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
ssDNA	single-stranded DNA
SSP	Sequence Specific Primer
TCR	T cell Receptor
TF	Thomsen-Friedenreich disaccharide
TGF	Tumour Growth Factor
TM	Trans Membrane
TNF	Tumour Necrosis Factor

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„The secret to creativity is knowing how to hide your sources."

Albert Einstein

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Appendices

Appendix A

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Functional improvement of antibody fragments using a novel phage coat protein III fusion system

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Abstract

Functional expressions of proteins often depend on the presence of host specific factors. Frequently recombinant expression strategies of proteins in foreign hosts, such as bacteria, have been associated with poor yields or significant loss of functionality. Improvements in the performance of heterologous expression systems will benefit present-day quests in structural and functional genomics where high amounts of active protein are required. One example, which has been the subject of considerable interest, is recombinant antibodies or fragments thereof as expressions of these in bacteria constitute an easy and inexpensive method compared to hybridoma cultures. Such approaches have, however, often suffered from low yields and poor functionality. A general method is described here which enables expressions of functional antibody fragments when fused to the amino-terminal domain(s) of the filamentous phage coat protein III. Furthermore, it will be shown that the observed effect is neither due to improved stability nor increased avidity.

Keywords: Antibodies; scFv; Expression; Fusion; Phage display; Antibody; Stability; Protein III; Multimerisation

Expressions of heterologous genes in bacteria have been the subject of considerable interest as this is by far the simplest and most inexpensive system for obtaining vast amounts of a desired polypeptide. Although expression is straightforward, problems are often encountered when recombinant proteins are to be expressed in bacteria. The most prominent of these seems to be loss of function. This is probably caused by poor folding efficiency in the bacteria or by insufficient amounts of folding factors upon overexpression, leading to degradation of unfolded protein and/or aggregation and concomitant formation of inclusion bodies [1]. Following the development of phage display antibody repertoires and the use thereof, much attention has been devoted to the heterologous expressions of antibody fragments in various hosts and especially to the conditions under which functional protein can be obtained from *E. coli* [2–4].

Antibodies are traditionally considered to be molecules that consist of two heavy and two light chains, which comprise both constant and variable domains. The antigen-binding site resides in the variable domains and can be expressed separately without loss of the binding properties [5]. Furthermore, a single chain fragment variable (scFv) can be formed with a flexible linker to join the variable heavy and light chains [6,7]. Due to its smaller size (25 kDa) and reduced complexity compared to whole antibodies, scFvs are among the

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most widely expressed antibody formats in *E.coli* [8]. These properties are exploited in the generation of recombinant antibodies by, e.g., phage display, where repertoires of scFvs, functionally displayed on the surface of the filamentous phage by fusion to coat protein III, are affinity selected for required specificities (reviewed in [9,10]). Loss of functionality has been a general observation when phage-display derived scFvs are expressed as soluble scFv. This loss of functionality has been attributed to two phenomena, namely the loss of avidity and stability. With regard to the avidity component, the filamentous bacteriophage contains up to five copies of protein III to which the scFv can be fused [11]. However, the display format often used favours monovalent display on the phage particle. Therefore it seems likely that the stability component deserves more attention.

Many approaches have been used to deal with the stability issue reviewed by Wörn and Plückthun [8]. The overall stability of scFvs can be increased either by insertion of disulphide bridges between the two chains [12], or by the introduction of mutations in the framework using rational or evolutionary strategies [13,14]. The peptide linker that connects the heavy and light chains is another target for optimisation as it influences the stability and both linker length [15] and sequence [16] have been investigated. Likewise, the succession of the variable domains in the scFv has been examined (V_HV_L and V_LV_H constructions), but no general rule has been found and the optimal order has to be determined for each individual scFv [15]. Additionally, complementarity determining regions (CDRs) from non-stable frameworks have been grafted to stable ones [17]. This approach has been implemented in the construction of phage displayed antibody repertoires using stable frameworks as building blocks [18–20].

A general method to increase expression yield, solubility, and stability is to fuse the protein in question to peptides or proteins with beneficial properties [21]. Such approaches have been very attractive with respect to antibody fragments as generic improvements can be obtained avoiding laborious analysis of each scFv separately. A panel of fusion partners has been reported, e.g., maltose-binding protein [22,23], alkaline phosphatase [24], green fluorescent protein [25], lambda head protein D [26], and human interleukin 2 fragment [27]. All of these have improved either the expression yield, increased the stability or added functions to the scFv. In addition to this, co-expression of recombinant antibody fragments with chaperones seems to increase the yield of functional scFv [4,28].

In the present study we have investigated the functional improvement of scFvs through fusion to filamentous phage protein III (Protein Accession Code: CAA23862.1 and http://www.mrc-cpe.cam.ac.uk/~/g1p.html). Protein III is responsible for the interaction with the bacterial receptors, F-pilus and TolA, and filamentous bacteriophage infection. It is structurally divided in three domains: domain I and domain II interact with the bacterial receptors, whereas domain III integrates protein III in the phage capsid [29]. We observed that some scFvs, which bound their cognate antigen when expressed on the surface of the filamentous bacteriophage, lost their binding properties when expressed as scFv alone. Thus suggesting that protein III is partly or totally responsible for inducing the active fold of the scFv, and as such, it is a reasonable fusion partner. In the following we will report that an inactive antibody fragment can be functionally rescued by fusion to the N-terminal domains of the original phage display fusion partner—protein III—in a novel expression system designated FuncFAb—Functionally Fused Antibody.

Materials and methods

Construction of pKBJ vectors. A scFv was initially subcloned from pHEN2 (http://www.mrc-cpe.cam.ac.uk/~/g1p.html) into the HindIII and *Not*I sites of the pUC119 His6MycXbaI [30] plasmid. This modified vector was used as founder vector for the pKBJ vectors. Gene III (Nucleotide Accession Code: V00604) of pHEN2 (http://www.mrc-cpe.cam.ac.uk/~/g1p.html) was PCR amplified using primers appropriate for each vector. pKBJ1 was constructed by PCR amplification with primers gIII N-term *Not*I and DII-III *Eag*I and subsequent cloning of the *Eag*I digested PCR product into the *Not*I site of the founder vector. For pKBJ2, gene III was PCR amplified with primers VL-link and DII-III Opal-*Eco*RI, digested with *Not*I and *Eco*RI and cloned into these sites of the founder vector. Quick-Change (Stratagene) with primers gIII N-term QC Amber-back and gIII N-term QC Amber-fw was subsequently used to remove the amber stop codon between the scFv and gene III in pHEN2. The last construct, pKBJ3, was constructed essentially as pKBJ1, using primers gIII N-term *Not*I and DI-DII *Eag*I. All constructs were subsequently sequenced with primers M13-Rev, VL-link, and M13-20 using an Applied Biosystems 373A sequencer and the ABI Prism Dye terminator cycle sequencing kit (Perkin–Elmer). All enzymes were purchased from New England Biolabs unless otherwise stated and used according to manufacturer's instructions.

Expression of antibodies. Clones were picked from TYE-plates, grown overnight at 37 °C in 2xTY [31] supplemented with 100 μg/mL ampicillin and 1% glucose, before 1:100 dilution in 2xTY with 100 μg/mL ampicillin and 0.1% glucose, and incubated for four hours at 37 °C shaking. The cultures were induced by addition of 1 mM IPTG and grown overnight at room temperature. Cells were pelleted at 6000*g* and resuspended in 50 mM $Na_xH_yPO_4$, pH 8.0, before lysis in French Press (American Instruments, Silver Spring, MD, USA). The suspension was subsequently cleared by centrifugation (26,000*g*) and the supernatant was being supplemented with 30 mM imidazole and 300 mM NaCl prior to immobilised metal affinity chromatography (IMAC). Ni-NTA was incubated with the supernatant for 2 h at 4 °C and subsequently washed with a minimum of 100 mL wash buffer (50 mM $Na_xH_yPO_4$ pH 8.0, 300 mM NaCl, and 30 mM imidazole) followed by 50 mL high saline wash buffer (50 mM $Na_xH_yPO_4$, pH 8.0, 750 mM NaCl, and 30 mM imidazole). Protein was eluted with wash buffer supplemented with Imidazole to 300 mM. The concentration was determined according to Bradford [32] and the purity was analysed by SDS–PAGE [33].

Activity measurements using ELISA. ELISA using the pKBJ derivatives of the scFv antibodies L36 [34] and D4 (K.B. Jensen et al.,

submitted for publication) was performed by coating of either Laminin-1 (Sigma) or Fibronectin (Sigma), respectively, overnight at 4 °C in 50 mM $NaHCO_3$, pH 9.6, at a concentration of 0.5 μg/well in ELISA-plates (MAXI-sorp, NUNC, Roskilde, Denmark). Residual binding to the plastic surface was blocked using 2% MPBS (PBS supplemented with 2% weight/volume low-fat milk powder) for 2 h and antibody derivatives were added at varying concentrations for 1 h. Plates were subsequently washed six times in PBS and bound antibody was detected with a murine antibody 9E10, which recognises the c-myc tag of the expressed scFv derivatives (provided by the European collection of animal cell cultures, ECACCs) at a concentration of 0.5 μg/mL. After washing six times in PBS the plates were incubated with a 1:1000 dilution of a HRP-conjugated rabbit anti mouse antibody (DAKO, Denmark) in 2% MPBS. The reaction was developed with *o*-phenylenediamine (OPD)-tablets (DAKO, Denmark) according to manufacturer's instructions after six additional washes in PBS.

To test activity of the scFv antibody R5 and its derivatives, Anti-Thomsen-Friedenreich/MUC-1 antibody (A76 A/C7) [35] was coated at a concentration of 0,1 μg/well overnight at 4 °C in PBS. Residual binding was blocked with 2% BSA (Sigma) in PBS for 2 h and the plates were subsequently incubated with antibody derivatives for 2 hours. The R5 antibody was detected with a polyclonal rabbit anti-c-myc (A-14) antibody (Santa Cruz Biotechnology) and subsequently a HRP-conjugated swine anti-rabbit antibody (DAKO, Denmark). Between each incubation step the plates were washed five times in PBS and developed as described above.

Stability measurements. Purified L36 fusion scFv was diluted to a final concentration of 1 μM in different concentrations of Guanidinium Chloride (GdmCl) in 10 mM Tris–HCl, pH 8, and transferred to a 3 mm path length quartz cuvette. The cuvette was placed in a RTC2000 fluorimeter with a 75 W Xenon arc lamp (Photon Technology International, Lawrenceville, NJ) and the emission scan from 300 to 400 nm was recorded (λ_{ex} 285 nm, excitation slit width 2 nm and emission slit width 6 nm). Folded scFv had emission maximum at 330 nm while maximum for unfolded was at 355 nm, thus the ratio between fluorescence at these wavelengths was used as signal to determine $[GdmCl]_{50\%}$ [36].

Gel filtration analysis. Gel filtration analysis was performed to determine whether antibody fusions multimerised. Gel filtration was performed on a TSK-gel G3000 SW column with a precolumn (ToSoHaas) using HPLC (Biotek Instruments). For correlation of retention times with globular molecular mass, bovine plasma fibronectin, murine IgG, BSA, and GST were applied onto the column and protein detected with a Diode Array Detector 540+ (Biotek Instruments). At least 1 mg scFv-fusion protein was applied to the column of each of the constructs and fractions were collected for subsequent analysis in ELISA.

Results

Construction of pKBJ family of vectors

Plasmids enabling fusion of scFv to various fragments of gene III were constructed by amplification of gene III domain I (pKBJ3) and gene III domain I-II (pKBJ1 and pKBJ2) (Fig. 1). A construct for domain I fusions, which encodes the first 80 amino acids of protein III plasmid, was first constructed (pKBJ3) enabling amino-terminal fusions to domain I of selected scFvs. Furthermore domain I is followed by a mycHis6-tag for immunodetection and purification. A pelB leader precedes the fusion protein directing expression of fusion protein to the periplasmic space of *E. coli* (Fig. 1).

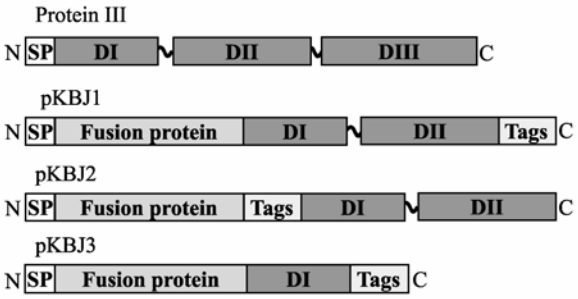

Fig. 1. Illustration of filamentous bacteriophage protein III and linear structure of the novel fusion proteins. A schematic representation of the domain structure of protein III and the constructed fusion proteins using the following abbreviations: DI, domain I, DII, domain II, DIII domain III, SP, signal peptide—pelB leader. The pKBJ2 has the native tag structure from the phage display format, whereas pKBJ1 and pKBJ3 have carboxy-terminal positioning of the tags.

pKBJ1 was constructed analogously (see Materials and methods) allowing scFvs to be fused to domains I and II of gene III. In the last construct, pKBJ2, the tags were positioned as in the phagemid vector and separate the scFv from domains I and II of gene III (see Table 1).

Expression and activity analysis of recombinant scFv

Three different phage display selected scFv antibodies, L36, D4, and R5, were sub-cloned into the three pKBJ vectors and the pUC119 vector in order to analyse the level of expression and the activity of the antibodies. L36 recognises Laminin-1 and is expressed in reasonable amounts as soluble active antibody [34]. The antigen of D4 has recently been identified as Fibronectin (K.B. Jensen et al., submitted for publication) and it is inactive when expressed as soluble scFv, though still expressed in fair amounts. R5 mimics a combined conformational epitope of MUC-1 and Thomsen-Friedenreich carbohydrate structure recognised by a murine antibody (unpublished results). This antibody is likewise expressed as active soluble scFv in decent amounts. Expression of these antibodies from the three novel vectors gave expression yields of the recombinant protein comparable to that of a traditional scFv expression system and of similarly high purity after IMAC purification (Table 2 and Fig. 2). In general, a slight increase in yield was observed by fusion, except for D4. It is well established that the amino-terminal part of a protein is most important for expression yields, thus similar expression yields were expected for the scFv and their respective derivatives [23]. In addition, the solubility of the scFvs was found unaltered upon fusion as determined by Western blotting of the supernatant and pellet after cell lysis (data not shown). The difference in yields for D4 scFv and derivatives could be caused by accumulation and aggregation of poorly folded inactive protein in inclusion bodies, a phenomenon often associated with

Table 1
Oligonucleotide sequences

Name	Sequence
GIII N-term *Not*I	5′AAGGAAAAAAGCGGCCGCCGGGGCCGCAACTGTTGAAAGTTGTTTAGC 3′
DII-III *Eag*I	5′AAGCCGGCCGAGCCGCCAGCATTGACAGG 3′
DI-DII *Eag*I	5′AAGCCGGCCGAACCGCCACCCTCAGAACC 3′
VL-link	5′ACCGCCAGAGCCACCTCCGCC 3′
DII-IIIOpal-*Eco*RI	5′CGGAATTCTCAGCCGCCAGCATTGACAGG 3′
GIII N-term QC Amber-back	5′CTTTCAACAGTCTGTGCGGCCCC 3′
GIII N-term QC Amber-fw	5′GGGGCCGCACAGACTGTTGAAAG 3′
M13-Rev	5′AAACAGCTATGACCATG 3′
M13–20	5′GTAAAACGACGGCCAGT 3′

Sequences of the primers applied for construction of vectors and subsequent verification of vectors sequences.

Table 2
Expression yields for scFv and their derivatives

Antibody	scFv	pKBJ1	pKBJ2	pKBJ3
L36	0.8	3.4	1.5	2.6
D4	13.7	3.3	6.2	4.0
R5	5	7	11	1.5

Expression yield in mg purified protein pr. L culture.

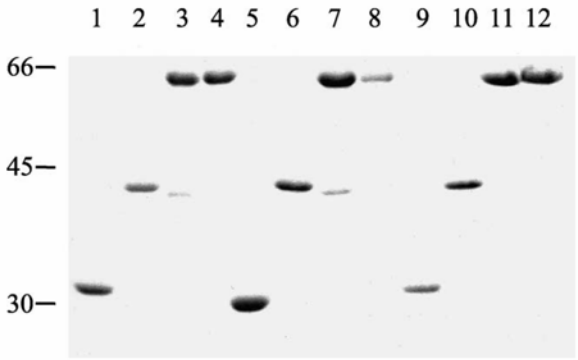

Fig. 2. IMAC purified antibodies and antibody derivatives. SDS–PAGE showing IMAC purified protein of the four different constructs for each of the three antibodies—D4 (lanes 1–4), L36 (lanes 5–8), and R5 (lanes 9–12) in pUC119 (lanes 1, 5, and 9), pKBJ3 (lanes 2, 6, and 10), pKBJ1 (lanes 3, 7, and 11), and pKBJ2 (lanes 4, 8, and 12).

significant increase in expression of recombinant proteins [1]. This is not very likely since the amount of D4 accumulating in the insoluble fraction was unaltered by fusion to protein III.

ELISA was performed to test the effect of fusing fragments of protein III to the scFvs. All preparations of protein expressed in the FuncFAb-system appeared to be active, and a higher reactivity was observed for some when compared to antibodies expressed in the traditional expression system (Fig. 3). D4, which has only been active when displayed on the surface of the filamentous bacteriophage, was found to be active as fusion to either domain I or domain I-II (Fig. 3A), demonstrating that protein III exhibits a positive effect on the functionality of the antibody. In addition, the activities of the domain I fusions were higher for all antibodies than those of both the domain I-II fusion and scFv alone. Likewise the position of the c-myc and his-tags either internally or terminally did not have any influence on the antibody activity. Subsequently, a panel of 14 antibodies has been cloned into the vectors and restoration of the activity was seen in 9 out of 10 antibodies, whereas 4 were active both before and after fusion (Unpublished results). Thus, it has been found that inactive scFv selected from phage displayed antibody repertoires can be activated when expressed in the molecular context, in which it was selected.

Activity measurements were performed after incubation for varying times and at different temperatures to test the effect of fusion on the stability of antibody derivatives. For all derivatives of L36 and R5 no apparent effect was observed after incubation at room temperature, 30 °C and 37 °C for up to four days (data not shown). The activity of D4 derivatives was unaffected by incubation at room temperature. However, after exposure to thirty degrees the activity decreased slightly and after one day at thirty-seven degrees inactivation was observed (data not shown). To further examine the stabilities of the different fusion proteins with that of the scFv, L36 and derivatives thereof were subjected to GdmCl induced unfolding measured by steady state fluorescence spectroscopy. The concentrations needed to half denature scFv-L36, pKBJ1-L36, pKBJ2-L36, and pKBJ3-L36 were 2.73 ± 0.11, 2.70 ± 0.07, 2.67 ± 0.03, and 2.41 ± 0.09 M ($[\mathrm{GdmCl}]_{50\%}$) respectively. Consequently, the increase in activity was not a consequence of increased stability. Furthermore, given the fact that the pKBJ1 and pKBJ2 expression systems behaved similarly in the initial activity assays, pKBJ1 was chosen for further characterisation, since the tags are located terminally.

Gel filtration of scFv fusions

The crystal structure of protein III domain I-II shows intramolecular domain interactions [37] and therefore it

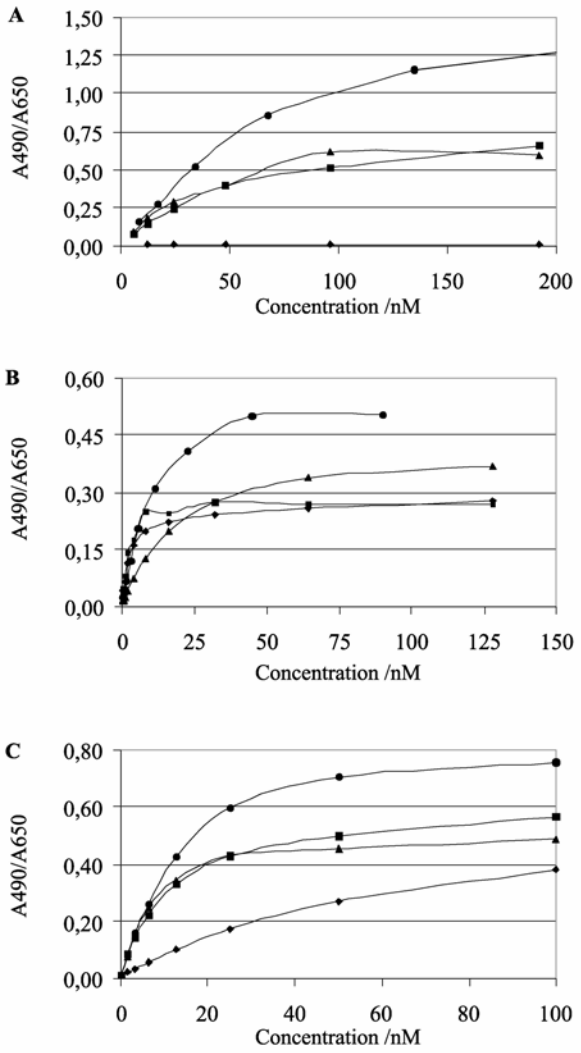

Fig. 3. Activity assays of the antibodies and derivatives in ELISA. Activity assays of the three scFvs, which show binding of D4 to fibronectin (A), L36 to Laminin-1 (B), and R5 to A/C7 monoclonal mouse antibody (C). The four different constructs from each antibody were tested in dilution series in duplicate and the absorbance was read at 490 nm with the absorbance at 650 nm as reference (A490/A650). Generally the domain I fusion, pKBJ3 (●), is more active than the domain I-II fusions, pKBJ1 (■) and pKBJ2 (▲), which are again more active than the non-fused scFv, pUC119 (◆).

can be speculated that the increased activity of the antibody fusions is an avidity effect caused by multimerisation. Consequently, gel filtration analysis was performed. The three antibodies did indeed multimerise into varying amounts of monomer, dimer, and higher order multimer (Table 3). The multimer eluted in void volume, which corresponds to a globular size above approximately 500 kDa as this is the exclusion limit for the column applied (data not shown). Aggregation was not observed in any of the preparations, which indicates that the proteins formed discrete multimer. In addition, when multimer was resampled monomer and dimer appeared, indicating reversibility in the formation of the high molecular weight complex. It was therefore concluded that the formation of multimer is an ordered process and not a result of aggregation. Multimerisation has been reported previously for other filamentous phage proteins such as protein IV, which forms discrete 14 mers in the outer membrane of *E. coli* [38].

It was contemplated which of the complexes corresponded to active fusion protein. Fractions were consequently collected from the gel filtration column and tested for activity (Fig. 4). The concentration of protein was normalised according to an arbitrary absorbance at 220 nm to ensure that approximately the same amount of protein was used in each activity experiment and that the observed activities were comparable. The domain I fusions are the only ones active as multimer; however, no general conclusions could be made, since the three tested antibodies behaved differently under the conditions chosen. This variation could be an effect of the different antigens applied as well as distinct behaviour of these upon coating, thus making comparisons difficult. D4 fused to domain I-II is active as monomer and inactive as multimer, but when fused to domain I no monomer is found, and in this case, the multimer is active. L36 is most active as dimer when fused to both domain I and domain I-II. In the case of R5 domain I-II fusion, multimer and monomer are equally active, whereas fusion to domain I makes the multimer most active. Based on these results, protein III domain I seems to be capable of inducing multimerisation, and when domain II is present the antigen-binding sites of some antibodies are shielded in the multimers, because these are inactive.

Discussion

Previous studies on expression of scFv isolated from phage displayed antibody repertoires and optimisation thereof have either focused on the antibody framework [17], co-expression of bacterial chaperones increasing the yield of functional scFv [4,28], or on fusion to proteins increasing the solubility of the fusion product [22].

Here an analogous system is described, where scFvs isolated from different phage displayed antibody repertoires, the Tomlinson I (D4), Griffin (L36), and Tomlinson J (R5) (http://www.mrc-cpe.cam.ac.uk/~/g1p.html) [20], are expressed together with the entity with which they were selected, that is protein III. The three antibodies used for proof of concept had very homologous primary sequences, R5 and D4 were both encoded by VH3 gene family member DP-47, and VK1 family member DPK9, and differ on one residue only (residue 5 in the variable heavy chain) besides the variability in the

Table 3
Antibody and derivatives analysed by gel filtration

		Monomer (%)	Dimer (%)	Multimer (%)
R5	pUC119	100	0	0
	pKBJ1	51	47	3
	pKBJ3	49	16	35
L36	pUC119	93	7	0
	pKBJ1	60	14	25
	pKBJ3	30	2	68
D4	pUC119	n.d	n.d.	n.d.
	pKBJ1	3	0	97
	pKBJ3	0	0	100

The amount of each species is calculated as the area beneath the gel filtration curve, and the distribution between monomer, dimer, and multimer varies according to the constructs. Three different constructs for each scFv are compared—pUC119, pKBJ1, and pKBJ3. n.d.: not determined.

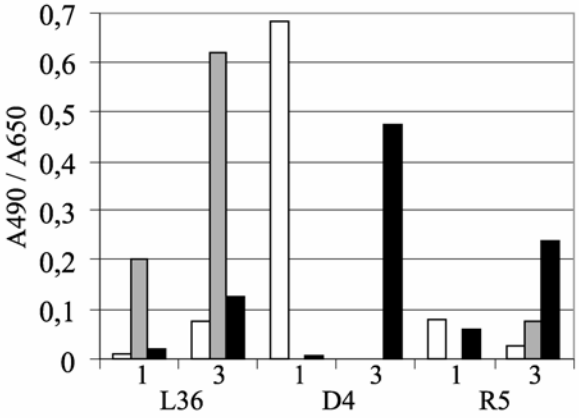

Fig. 4. Activity of monomer, dimer, and multimer collected from gel filtration. The activity of the different multimerisation states of pKBJ1 and pKBJ3 constructs is compared using equal amounts of monomer (empty), dimer (grey), and multimer (filled) for each antibody construct. Arbitrary absorbance units at 220 nm obtained from gel filtration analysis were applied in order to use equivalent amounts of monomer, dimer, and multimer. The different constructs were assayed in duplicate and the absorbance was read at 490 nm with the absorbance at 650 nm as reference (A490/A650).

CDRs. L36 is encoded by a different VH3 family member (DP46) and differs on two residues in the heavy chain compared to R5 and D4. Greater difference was found in the light chain, which belongs to the VL1 variable gene family (V-base—http://www.mrc-cpe. cam.ac.uk/imt-doc/). Consequently small sequence differences are important for successful expression of recombinant antibody fragments in vitro, as reported by others [8,13].

The rationale for our study originated from the observation that some antibodies isolated from antibody repertoires are active when displayed on the filamentous phage; nevertheless, all attempts to produce soluble antibody have failed. Thus, it could be speculated that protein III has some sort of beneficial effect, like those observed with proteins such as the periplasmic substrate binding family of proteins [39]. Filamentous phage coat protein III is a globular protein consisting of three domains separated by flexible linker regions [29]. The structures of the two amino-terminal domains have been solved by X-ray crystallography for the two homologous filamentous phages, M13 and Fd, and show interactions between the two domains [37,40]. In order to test the effect of the filamentous phage coat protein, three different constructs were created which enable antibody fusion to truncated versions of protein III. Tags were either located as in the selected molecule or carboxy-terminally (Fig. 1). It is not likely that any gain of function would be due to the classical increase in solubility, since the probability for expression as soluble protein did not increase significantly: 11%, 17%, 18%, and 19% for L36 in pUC119, pKBJ1, pKBJ2, and pKBJ3, respectively, according to the Wilkinson-Harrison solubility model [41].

After fusion to the coat protein, antibodies could be expressed in amounts similar to that of the unfused antibody (Table 2). The solubility was likewise unaltered as determined by Western blotting experiments (data not shown) supporting the calculated probabilities for soluble expression using the solubility model. However, the activity of the antibodies, when fused to domain I and to domain I-II, was increased, in particular that of the domain I fusion.

The selection pressure applied in antibody selections favours an increase in antibody affinity. This can be obtained by optimising the surface interactions between antibody and antigen, but also indirectly by increasing the stability and thereby the functional fraction of antibody fragments [14]. Phage displayed antibodies can be considered as either antibodies fused to an irrelevant protein or an entity of both antibody and phage protein III. Therefore, the selection pressure applied can either stabilise the antibody or the fusion entity. As a result, antibodies, where selection has stabilised the scFv, will not necessarily gain activity by fusion neither will they lose activity, since this is the context in which they were actively selected as for L36 (Fig. 3B). The most extreme

effect was observed for antibody D4, which was not active as scFv (Fig. 3A).

Two different phenomena could cause increased activity. First, the fraction of folded antibody is increased, which ensures a higher reactivity in the sample. Second, the increased activity is due to the formation of multimer, which increases the avidity of the antibody. A clear formation of multimer was observed when the domain I-II fusion proteins were analysed using gel filtration; nonetheless, active fractions of antibodies were found in the monomer or dimer fraction (Fig. 4). This indicates that the increased activity compared to scFv observed for D4 is due to a higher percentage of correctly folded protein (Fig. 3A). In the case of L36 and partly R5 the activity of scFv and domain I-II fusion is almost identical (Figs. 3B and C), which points to the fact that they are already stable as scFvs. The further increase in activity observed for these antibodies when fused to domain I could be caused by a combined effect of stability and avidity as both monomer and multimer are found active (Fig. 4). Thus, it could be argued that fusion increases the activity by improving antibody folding and that the phage protein therefore could function as a chaperone. Alternatively, fusion to protein III could optimise the surface interactions between antigen and antibody.

This is to our knowledge the first report which shows the potential of fusion to the phage coat protein III. It has not yet been established whether the coat protein has intrinsic chaperone activity, contains elements responsible for recruiting folding components in the bacterial periplasm or improves the antigen-antibody interface. Chaperone properties have been reported for other fusion partners such as maltose-binding protein [23] and lambda head protein D [26]. However, the effects have never been as dramatic as those observed with protein III, where the reactivity of an antibody was restored upon fusion. Whether the function of protein III holds true for all proteins or only those selected by phage display has so far not been determined.

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References

[1] G. Georgiou, P. Valax, Expression of correctly folded proteins in *Escherichia coli*, Curr. Opin. Biotechnol. 7 (1996) 190–197.

[2] R. Verma, E. Boleti, A.J. George, Antibody engineering: comparison of bacterial, yeast, insect and mammalian expression systems, J. Immunol. Methods 216 (1998) 165–181.

[3] S.M. Kipriyanov, G. Moldenhauer, M. Little, High level production of soluble single chain antibodies in small-scale *Escherichia coli* cultures, J. Immunol. Methods 200 (1997) 69–77.

[4] A. Knappik, C. Krebber, A. Plückthun, The effect of folding catalysts on the in vivo folding process of different antibody fragments expressed in *Escherichia coli*, Biotechnology (NY) 11 (1993) 77–83.

[5] A. Skerra, A. Plückthun, Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*, Science 240 (1988) 1038–1041.

[6] R.E. Bird, K.D. Hardman, J.W. Jacobson, S. Johnson, B.M. Kaufman, S.M. Lee, T. Lee, S.H. Pope, G.S. Riordan, M. Whitlow, Single-chain antigen-binding proteins, Science 242 (1988) 423–426.

[7] J.S. Huston, D. Levinson, M. Mudgett-Hunter, M.S. Tai, J. Novotný, M.N. Margolies, R.J. Ridge, R.E. Bruccoleri, E. Haber, R. Crea, Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*, Proc. Natl. Acad. Sci. USA 85 (1988) 5879–5883.

[8] A. Wörn, A. Plückthun, Stability engineering of antibody single-chain Fv fragments, J. Mol. Biol. 305 (2001) 989–1010.

[9] P. Kristensen, P. Ravn, K.B. Jensen, K.H. Jensen, Applying phage display technology in aging research, Biogerontology 1 (2000) 67–78.

[10] G. Winter, A.D. Griffiths, R.E. Hawkins, H.R. Hoogenboom, Making antibodies by phage display technology, Annu. Rev. Immunol. 12 (1994) 433–455.

[11] D.A. Marvin, Filamentous phage structure, infection and assembly, Curr. Opin. Struct. Biol. 8 (1998) 150–158.

[12] R. Glockshuber, M. Malia, I. Pfitzinger, A. Pluckthun, A comparison of strategies to stabilize immunoglobulin Fv-fragments, Biochemistry 29 (1990) 1362–1367.

[13] A. Knappik, A. Pluckthun, Engineered turns of a recombinant antibody improve its in vivo folding, Protein Eng. 8 (1995) 81–89.

[14] P. Forrer, S. Jung, A. Plückthun, Beyond binding: using phage display to select for structure, folding and enzymatic activity in proteins, Curr. Opin. Struct. Biol. 9 (1999) 514–520.

[15] D. Desplancq, D.J. King, A.D. Lawson, A. Mountain, Multimerization behaviour of single chain Fv variants for the tumour-binding antibody B72.3, Protein Eng. 7 (1994) 1027–1033.

[16] Y. Tang, N. Jiang, C. Parakh, D. Hilvert, Selection of linkers for a catalytic single-chain antibody using phage display technology, J. Biol. Chem. 271 (1996) 15682–15686.

[17] S. Jung, A. Plückthun, Improving in vivo folding and stability of a single-chain Fv antibody fragment by loop grafting, Protein Eng. 10 (1997) 959–966.

[18] A. Pini, F. Viti, A. Santucci, B. Carnemolla, L. Zardi, P. Neri, D. Neri, Design and use of a phage display library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel, J. Biol. Chem. 273 (1998) 21769–21776.

[19] A. Knappik, L. Ge, A. Honegger, P. Pack, M. Fischer, G. Wellnhofer, A. Hoess, J. Wölle, A. Plückthun, B. Virnekäs, Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides, J. Mol. Biol. 296 (2000) 57–86.

[20] R.M. de Wildt, C.R. Mundy, B.D. Gorick, I.M. Tomlinson, Antibody arrays for high-throughput screening of antibody–antigen interactions, Nat. Biotechnol. 18 (2000) 989–994.

[21] C. di Guan, P. Li, P.D. Riggs, H. Inouye, Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein, Gene 67 (1988) 21–30.

[22] A. Hayhurst, Improved expression characteristics of single-chain Fv fragments when fused downstream of the *Escherichia coli* maltose-binding protein or upstream of a single immunoglobulin-constant domain, Protein Expr. Purif. 18 (2000) 1–10.
[23] H. Bach, Y. Mazor, S. Shaky, A. Shoham-Lev, Y. Berdichevsky, D.L. Gutnick, I. Benhar, *Escherichia coli* maltose-binding protein as a molecular chaperone for recombinant intracellular cytoplasmic single-chain antibodies, J. Mol. Biol. 312 (2001) 79–93.
[24] R.A. Griep, C. van Twisk, R.J. Kerschbaumer, K. Harper, L. Torrance, G. Himmler, J.M. van der Wolf, A. Schots, pSKAP/S: an expression vector for the production of single-chain Fv alkaline phosphatase fusion proteins, Protein Expr. Purif. 16 (1999) 63–69.
[25] R.A. Griep, C. van Twisk, J.M. van der Wolf, A. Schots, Fluobodies: green fluorescent single-chain Fv fusion proteins, J. Immunol. Methods 230 (1999) 121–130.
[26] P. Forrer, R. Jaussi, High-level expression of soluble heterologous proteins in the cytoplasm of *Escherichia coli* by fusion to the bacteriophage lambda head protein D, Gene 224 (1998) 45–52.
[27] L. Sanchez, M. Ayala, F. Freyre, I. Pedroso, H. Bell, V. Falcon, J.V. Gavilondo, High cytoplasmic expression in *E. coli*, purification, and in vitro refolding of a single chain Fv antibody fragment against the hepatitis B surface antigen, J. Biotechnol. 72 (1999) 13–20.
[28] E. Söderlind, M. Duenas, C.A. Borrebaeck, Chaperonins in phage display of antibody fragments, Methods Mol. Biol. 51 (1995) 343–353.
[29] L. Riechmann, P. Holliger, The C-terminal domain of TolA is the coreceptor for filamentous phage infection of *E. coli*, Cell 90 (1997) 351–360.
[30] A.D. Griffiths, S.C. Williams, O. Hartley, I.M. Tomlinson, P. Waterhouse, W.L. Crosby, R.E. Kontermann, P.T. Jones, N.M. Low, T.J. Allison, Isolation of high affinity human antibodies directly from large synthetic repertoires, EMBO J. 13 (1994) 3245–3260.
[31] J. Sambrook, E.F. Fritsch, T. Miniatis, Molecular Cloning, second ed., Cold Spring Harbor Laboratory Press, New York, 1989.
[32] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[33] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[34] L. Sanz, P. Kristensen, S.J. Russell, J.R. Ramirez Garcia, L. Alvarez-Vallina, Generation and characterization of recombinant human antibodies specific for native laminin epitopes: potential application in cancer therapy, Cancer Immunol. Immunother. 50 (2001) 557–565.
[35] M.R. Price, P.D. Rye, E. Petrakou, A. Murray, K. Brady, S. Imai, S. Haga, Y. Kiyozuka, D. Schol, M.F. Meulenbroek, F.G. Snijdewint, S. von Mensdorff-Pouilly, R.A. Verstraeten, P. Kenemans, A. Blockzjil, K. Nilsson, O. Nilsson, M. Reddish, M.R. Suresh, R.R. Koganty, S. Fortier, L. Baronic, A. Berg, M.B. Longenecker, J. Hilkens, M. Boer, V. Karanikas, I.F.C. McKenzie, O.E. Galanina, L.A. Simeoni, A.G. Ter-Grigoryan, I.M. Belyanchikov, N.V. Bovin, Y. Cao, U. Karsten, J. Dai, W.J. Allard, G. Davis, K.K. Yeung, F.-G. Hanisch, K.O. Lloyd, V. Kudryashov, R. Sikut, A. Sikut, K. Zhang, D. Baeckström, G.C. Hansson, C.A. Reis, H. Hassan, E.P. Bennett, H. Claussen, L. Norum, T. Varaas, B. Kierulf, K. Nustad, P. Ciborowski, W.M. Konitzki, J. Magarian-Blander, O.J. Finn, J. Hilgers, Summary report on the ISOBM TD-4 Workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin. San Diego, CA, November 17–23, 1996, Tumour Biol. 19 (Suppl. 1) (1998) 1–20.
[36] M.M. Santoro, D.W. Bolen, Unfolding free energy changes determined by the linear extrapolation method. 1. Unfolding of phenylmethanesulfonyl α-chymotrypsin using different denaturants, Biochemistry 27 (1988) 8063–8068.
[37] J. Lubkowski, F. Hennecke, A. Plückthun, A. Wlodawer, The structural basis of phage display elucidated by the crystal structure of the N-terminal domains of g3p, Nat. Struct. Biol. 5 (1998) 140–147.
[38] N.A. Linderoth, M.N. Simon, M. Russel, The filamentous phage pIV multimer visualized by scanning transmission electron microscopy, Science 278 (1997) 1635–1638.
[39] G. Richarme, T.D. Caldas, Chaperone properties of the bacterial periplasmic substrate-binding proteins, J. Biol. Chem. 272 (1997) 15607–15612.
[40] P. Holliger, L. Riechmann, R.L. Williams, Crystal structure of the two N-terminal domains of g3p from filamentous phage fd at 1.9 Å: evidence for conformational lability, J. Mol. Biol. 288 (1999) 649–657.
[41] D.L. Wilkinson, R.G. Harrison, Predicting the solubility of recombinant proteins in *Escherichia coli*, Biotechnology (NY) 9 (1991) 443–448.

Appendix B

Functionally Fused Antibodies – a novel adjuvant fusion system[1]

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Abstract

The pursuit for new and improved therapies for prevalent human diseases often involves the phage display technique for isolation of useful binders capable of recognizing key molecular targets. These approaches often aim at non-immunogenic antibody fragments, for example in tumour therapy, in order to achieve specific toxin-, radioactivity- or effector-domain delivery, or to induce signalling cascades with minimal neutralizing immune reactions. On the other hand, increasing interest emerges in using antibodies, in form of anti-idiotypic antibodies, molecular mimicry molecules, or other non-antibody polypeptides for immunization aimed to induce potent immune responses against an imitated or shared target structure. In order to improve immunogenecity of anti-idiotypic antibodies and mimicry structures we investigated a new fusion system for vaccination purposes. We demonstrate the adjuvant effects generated by preserving the fusion of antibody fragment to portions of the filamentous bacteriophage coat protein 3. By linking the antibody fragment to the N-terminal domain of coat protein 3, we induce multimerisation of the antibody fragments, improving their therapeutic potential possibly due to epitope clustering, slower release and increased survival in the blood stream. Therefore, an effective immune response towards antibody fragments can be obtained using a defined immunogenic portion of a phage coat protein without the use of traditional adjuvants.

Introduction

Combating human diseases by vaccination can be achieved by the administration of live attenuated pathogens, whole inactivated organisms or inactivated toxins. Such vaccines act by presenting a plethora of epitopes to the human immune system, which subsequently activate an array of biological mechanisms aiding the clearance of the disease causing agent.

Unfortunately, in a large number of human diseases the disease causing agent may not be capable of eliciting an effective immune reaction. This is particularly true for tumor vaccines, where specific tumor associated antigens (TAA) often produce poor immunological reactions. Furthermore, the application of vaccines relying on e.g. live attenuated pathogens, whole inactivated organisms or inactivated toxins may pose a risk factor to weak patients, and the productions of such vaccines may also prove difficult. Several studies have focused on alternative vaccine models, such as peptide vaccines, subunit vaccines and chimeric live vector vaccines that frequently rely on the addition of an adjuvant. Adjuvants can improve immune response towards an antigen in several different ways: (1) increase immunogenecity towards weak antigens; (2) improve blood clearance and transportation; (3) modulate antibody avidity, specificity, isotype or subclass distribution; (4) stimulate cell mediated immunity, (5) enable beneficial routes of administration and (6) decrease the dose of antigen. (1)

However, severe difficulties arise in the quest for the best vaccine adjuvants, due to e.g. toxicity and auto-immunity (2-5), and today the only FDA approved adjuvants for human therapy are formulations based on aluminum salts. Different alternatives to classical adjuvants have been explored, including protein fusions of immunostimulatory cytokines to antigens like scFv or peptides (6-9). Also a few reports on antigens fused to heat-shock proteins (HSP) and pathogenic proteins are available: *Mycobacterium bovis* strain BCG HSP65 (10), HSP70 (11), HSP71 (10),

tetanus toxin (12), serum albumin-binding region of streptococcal protein G (13), Hepatitis B virus core antigen (14), *E. coli* heat-labile enterotoxin B subunit (15) and B-subunit of cholera toxin (16).

Apart from the adjuvant also a disease specific component is essential for a vaccine. Subunit vaccines consist of a fragment of the disease causing agent, selected based on properties such as immunogenecity, specificity, toxicity and stability. Several carbohydrate TAA are very weak immunogens and may also fail to fulfill other of the above requirements, examples being Thomsen-Friedenreich (TF), Tn-antigen (17) and (sialylated)-Lewis Y/X/a (18). Molecular mimicry by peptides or anti-idiotypic antibodies has been suggested as a solution to the immunogenecity problem of such carbohydrates. To further increase the effect of such antigens the simultaneous use of several different peptides or anti-idiotypic antibodies as well as specific targeting may be effective – this could be obtained through for instance heteromultimerisation.

Previous studies in rabbit and mice have shown that the filamentous bacteriophage exhibits an adjuvant-like effect when applied in vaccination strategies. (19-21). Multivalent display in vaccination strategies as a beneficial carrier property has been investigated in several studies. These works have primarily focused on display of antigens on cell surfaces (22-24) and virus particles (25-27), although a few fusion protein systems have been investigated, such as the pentameric cholera toxin B subunit (28) as well as the closely related pentameric E. coli heat-labile enterotoxin B subunit (15). The two latter examples both perform well in oral administration, although Complete or Incomplete Freunds Adjuvant is needed to evoke the immune response. Other multivalent display systems have been applied in immunizations without directly investigating the possible beneficial effect of multimerisation e.g. dimeric IFN-γ (6) and dimeric glutathione S-transferase (10, 29)

In this work we have investigated the adjuvant and carrier properties of the FuncFAb (**Func**tionally **F**used **A**nti**b**ody) system (30). Here, we demonstrate that the immune response to scFv fragments is significantly improved by fusion or co-administration of portions of the coat protein 3 of the filamentous bacteriophage. Additionally multimerisation of the scFv fusions to coat protein 3 leads to potential heteromultimerisation, thus enabling adjuvant targeting.

Materials and Methods

Construction of pKBJ vectors and expression of protein

Vectors were constructed as described in Jensen, K.B. et al. 2002. Briefly, filamentous phage gene III fragments from pHEN2 (http://www.mrc-cpe.cam.ac.uk/g1p.php?page=1808) were subcloned into pUC119 His6MycXbaI and a chloramphenicol resistant version of pHEN2 in order to facilitate expression of antibody fragments fused to DI (pKBJ3) and DI-DII (pKBJ1). Furthermore, a DI vector was constructed which enables expression of DI without a fused antibody fragment. The oligos 5'-CAT GGC CGG GGC-'3 and 5'-GGC CGC CCC GGC-'3 were annealed by mixing 1 µM of each oligo, heating to 100° C and subsequently cooling to 4° C. The annealed oligo was ligated into the *NcoI/NotI* cut pKBJ3 vector. Sequencing was done with primer (M13 rev Sequence) by SeqLab Sequence Laboratories Göttingen GmbH (Göttingen, Germany).

All expressions and immobilized metal ion affinity chromatography (IMAC) purifications of FuncFAb antibodies, non fused antibodies and non-fused DI were performed as described in Jensen, K.B. et al. 2002. Protein concentrations were subsequently determined according to Bradford (31) and purity by SDS-PAGE according to Sambrook et al. (32).

Activity measurements using ELISA

Activity of the R5/D4 pKBJ3 heteromultimer was measured by sandwich ELISA. The ELISA was performed by coating of fibronectin (Sigma) or A76-A/C7 overnight at 4ºC in PBS at a concentration of 0.1 µg/well in ELISA-plates (MAXI-sorp™, NUNC, Roskilde, Denmark). Next, residual non-specific binding to the wells were blocked by incubation with PBS containing 2% w/v low-fat milk powder (2% MPBS) for 2 hours. Following, the blocking step heteromultimeric antibody fusion protein was added at varying concentrations in 2% MPBS for 1 hour. Plates were subsequently washed 6 times in PBS, and bound antibody was detected with either A76-A/C7 when

fibronectin was coated or fibronectin and fibrinogen (DAKO, Denmark) when A76-A/C7 was coated. Each protein was added at a concentration of 0.5 μg/ml. After washing six times in PBS the plates were incubated with a 1:1000 dilution of an HRP-conjugated rabbit anti mouse antibody (DAKO, Denmark) or an HRP-conjugated goat anti rabbit antibody (DAKO, Denmark) in 2%MPBS, respectively. The reaction was developed with *o*-phenylenediamine (OPD) -tablets (DAKO, Denmark) according to manufacturer's instructions after additional six washes in PBS, and read at 490 nm with an ELISA reader (Bio-Rad, USA).

Gel filtration analysis of higher order multimers

The heteromultimer consisting of R5 scFv-DI and D4 scFv-DI was analyzed with a TSK-gel G2000 SW column with a precolumn (ToSoHaas) at a flow rate of 0.8 mL/minute with PBS. A low molecular weight protein marker (Amersham Biosciences) was used for standardization and protein was detected using a Diode array detector 540+ (Biotek Instruments).
Fusion protein was applied in as high amounts as possible.

Animals and immunizations

The immunological properties of the scFvs and DI were examined in 10-12 weeks old Balb/C female mice, which were immunized three times with: L36 (50 μg), L36 (50 μg)+ Incomplete Freund's Adjuvant (IFA), L36-DI (50 μg), L36-DI-DII (50 μg), PACA17 (50 μg), PACA17 (50 μg) + IFA, PACA17-DI (75 μg), PACA17 + DI (50 μg + 25 μg), and DI alone (25 μg). Each group consisted of 3 identically treated mice. Injections were given intraperitoneal (i.p.) at Day 0 and Day 14, and blood samples were collected at Day –1 and Day 28. Immunizations were performed by EPO GmbH, Berlin, Germany.

IgG1 and IgG2a antibody immune responses

The immunoassays were performed in Maxi-sorp™ plates (Nunc, Roskilde, Denmark) coated overnight at 4° C with 250 µg/well scFv in PBS. After washing three times in PBS and blocking for 30 min RT with 2% BPBS, diluted sera in 2% BPBS were added to the wells. After 3 washes the IgG subclasses were detected by incubation with 1:1000 dilution of polyclonal Rabbit IgG1 and IgG2a (Abcam, Cambridge,UK) respectively. After additional washing, a POD-conjugated goat anti-rabbit antibody (DAKO, Glostrup, Denmark) was used for detection. The ELISAs were developed using TMB-substrate, and subsequently quenched with 2.5M H_2SO_4. Absorbance of light with wavelength between 450nm and 650nm were measured.

Inhibition of L36 binding to Laminin-1

The ability of diluted sera to prevent L36 scFv binding to Laminin-1 (Becton Dickinson) was studied by competition ELISA using Maxi-sorp™ plates coated ON at 4ºC with Laminin-1 (1 µg/well). After blocking for 2 h at RT with PBS supplemented with 4% BSA (BPBS), a mixture of L36 at 1.5µg/ml preincubated with different dilutions of each serum in 1% BPBS supplemented with 0.05% Tween-20 was added for 1 h. Plates were washed three times and bound scFv was detected with HRP-conjugated anti-myc antibody (Invitrogen). After washing, the chromogenic substrate TMB (3'.3'.5'.5'.-tetramethylbenzidine) was added and the absorbance was measured at 450 nm.

Determination of anti-idiotypic responses

To study the presence of anti-idiotypic antibodies in the different sera, an ELISA assay was carried out by coating Maxi-sorp™ plates with rabbit anti-EHS Laminin polyclonal antibody (Sigma) ON at 4ºC in PBS (0.3 µg/well). Plates were blocked with 4% BPBS for 2 h at RT and then incubated

with serum diluted 1:25 in 1% BPBS supplemented with 0.05% Tween-20 for 1 h. After washing, anti-idiotypic antibodies were detected using HRP-conjugated goat-anti-mouse IgG antibody (Sigma).

Results

Construction of pKBJ vectors and derivatives thereof

The previously described family of pKBJ vectors (30) enables fusion of scFvs to domains of the filamentous bacteriophage gene III (Fig. I). In order to study the ability of domain I (DI) fusion proteins to heteromultimerize we constructed two pKBJ3 vectors having distinct antibiotic resistance markers. Accordingly, the fusion gene scFv-DI was subcloned from the original pKBJ3 vector encoding ampicillin resistance into an analogous vector in which the ampicillin resistance gene has been exchanged by chloramphenicol resistance.

Expression and activity analysis of recombinant scFv

Four different scFv antibodies, R5, D4, L36 and PACA17, were cloned into the FuncFAb fusion vectors and a non-fusion expression plasmid. R5 and PACA17 (obtained by proteolytically selective phage display and specific elution as described in (33)) bind in a competitive manner to the antigen combining site of the murine antibodies A76-A/C7 (34, 35) and A46-B/B10 (36) respectively. A76-A/C7 recognizes a MUC1 conformational peptide epitope of the immunodominant tandem repeat region of MUC1 which is induced by a Tn or Thomsen-Friedenreich glycosylation of the DTR motif, whereas A46-B/B10 recognizes the H type 2 trisaccharide (Fucα1-2Galß2-4GlcNAcß-). R5 and PACA17 might be structural mimicking anti-idiotypic scFvs, displaying the internal image of the combining sites of the murine antibodies against which they were selected.. D4 scFv and L36 scFv recognizes fibronectin (37) and laminin-1 (38), respectively.

Yields and purities of expressions and purifications of R5 and L36 non-fused and fused to either domain I (DI) or domain I-II (DI-II) are previously reported, together with ELISA data showing the

activity of the different constructs (30). Co-expression and IMAC purification of R5 and D4 both fused to DI gave similar yields and purity as compared to the expression and purification of the respective scFvs fused to DI (data not shown). DI of protein III alone and PACA17 as scFv and in fusion with DI all gave similar expression yields and purity as compared to earlier reported FuncFAb proteins.

Multimerisation analysis by gel filtration

Homomultimers

As observed previously, FuncFAbs expressed in *E.coli* form dimers and higher multimers, 2% dimer and 68% higher multimers in the case of L36-DI (30) which could have immense impact on their immunological properties. In order to study the stability and reversibility of multimers, fractions containing monomers, dimers and higher multimers were incubated for 36 hours at 37°C followed by gel filtration analysis. The monomer remained as monomer, the dimers dissociated into monomers, and about 50% of the higher multimers dissociated into monomers. No dimers were observed upon multimer dissociation at 37°C, indicating a leakage of monomers. These data suggest that the multimerisation state equilibrium is shifted towards the monomeric state at body temperatures, however, about 50% of the higher multimers are still present after 36 h.

The multimers are much more stable at low temperatures as observed for the L36-DI fusion stored at 4°C, which seems to retain multimerisation at least for several weeks, reflecting the slower equilibration dynamics at low temperatures (data not shown). In order to test the ability to generate multimers in vitro, the monomers were concentrated from 0.3 mg/ml to 3 mg/ml at room temperature leading to the formation of dimers (7%) but no higher multimers. These data suggests that in vivo expression and native purification of DI fusion proteins are essential for obtaining highly multimerized protein.

The generation of higher order multimers during cellular expression of protein III DI fusion scFvs in E.coli remains unclear. Possible explanations might be higher intracellular protein concentrations, structural regions of domain I and domain II, which has been revealed by structural studies to interact (39, 40) or intermolecular interactions within non-exposed regions of the polypeptide chain initiated before or during the folding process.

Heteromultimers

Since previous data not only suggest the formation of multimers but also that these multimers are active (30), the possible heteromultimeric assembly of co-expressed R5 and D4 antibody fragments both fused to DI of protein III was investigated. R5 DI fusion from pKBJ3 was subcloned into a similar vector carrying a different antibiotic resistance marker and co-expression was performed from double transfected *E.coli* with plasmids encoding D4 and R5 DI fusion proteins. After IMAC purification of the expressed protein it was analyzed in gel filtration analysis and sandwich ELISA. The gel filtration analysis showed clear formation of multimer (Fig. II.A). Moreover, ELISA indicated that the co-expressed protein preparation contained active R5 and D4 scFvs heteromultimer (Fig. II.B). This supports the formation of active heteromultimer upon co-expression of DI fused scFvs.

Immunization with FuncFAb protein

Balb/c mice were immunized with L36 and PACA17 scFvs alone, mixed with IFA or fused toDI of protein III using the FuncFAb system. PACA17 was furthermore used for immunization in a mixture with DI (non-fused); DI alone was used as control. ELISA data are shown in Figure III. They show that the IgG responses towards L36 scFv and PACA17 scFv were increased by fusion to DI compared to the scFv's alone. The observed increases were either slightly lower (L36) or

comparable (PACA17) to the response of L36 scFv and PACA17 scFv injected with the adjuvant IFA. The response against PACA17 scFv administered in a mixture with DI was interestingly higher than the one obtained with PACA17 fused to DI or PACA17 scFv supplemented with IFA. L36 DI-II fusion demonstrated a slightly weaker response (data not shown) than the L36 DI fusion response.

The differentiation of the antibody responses into IgG1 and IgG2a responses shows generally somewhat higher IgG1 levels than IgG2a, however both subclasses were prominent as determined by the observed ELISA signals. Despite that the two IgG subclasses are detected with two different antibodies**,** the ELISA signals are comparable, since the two antibodies show equivalent activity on the same amount of IgG1 and IgG2a respectively (data not shown). The presence of both IgG1 and IgG2a antibodies indicates that both Th1 and Th2 type T cells are responsible for the immune response.

Anti-idiotypic response after L36 immunization

The specificity of the sera obtained from mice immunized with scFv L36 in the various formulations was further examined by testing their ability to inhibit the binding of L36 to its ligand, laminin-1. L36 and sera were co-incubated and screened for L36 binding to coated laminin-1 by ELISA (Fig. IV.A). This revealed that in some of the sera, components are induced, which were able to compete with L36 binding to laminin-1. Moreover, the observed effect was directly correlated to the observed level of humoral immune response against L36 scFv. Therefore, the L36 fusion to the N-terminal domain of protein III seems to present the scFv binding site as immunological accessible as the non-fused scFv.

Anti-idiotypic antibodies competing for L36 binding to laminin-1 may be anti-idiotypic antibodies type Ab2γ (idiotopes close to the antigen binding site) or Ab2β (idiotopes in the antigen binding

site, resembling the epitope recognized by L36). To test whether any anti-idiotypic antibodies mimicking laminin-1 was present in the sera, we studied sera binding to a polyclonal rabbit antibody against laminin-1. (Fig. IV.B). Immunization with either L36 scFv supplemented with IFA or L36 fused to the N-terminal domains of protein III induced significant anti-idiotypic Ab2β immune response, whereas mice immunized with L36-scFv alone generated no anti-idiotypic Ab2β immune response. Comparing figure III and IV.B imply that the diverging strength and nature of the anti-idiotypic immune response seems not to be dependent on the overall antibody immune response. Presumable the underlying difference can be found in the difference between the inherent immune system of the mice.

Discussion

Although the use of filamentous bacteriophage particles as adjuvant with the antigen displayed on either protein III (19) or protein VIII (20, 29, 41-43) has been reported, this is to our knowledge the first time that fusion proteins with N-terminal fragments of protein III have been applied for this purpose. Previous studies also assessed that protein III is indeed immunogenic (43, 44); in this work we demonstrate that DI of protein III is sufficient to confer immunogenecity.

Our experiments document adjuvant property of the N-terminal fragment of protein III of the filamentous bacteriophage, since immunizations with otherwise non-immunogenic scFvs fused to the N-terminal domain(s) of protein III induce a thymus-dependent humoral immune response towards the scFv. Taking into account the overall humoral immune response, no significant difference between immunizations with scFv fused to DI or to DI-II of protein III could be observed (data not shown). Interestingly, we have observed differences in the specific anti-idiotypic response indicating that DI fusions give a higher response than fusion to DI-II. This suggests that the DI fusion's increased multimerisation and/or active folding compared to DI-II fusion benefits the specificity of the immune response towards the scFv's combining site (30). We also determined the Th1/Th2 profile via comparison of the antibody isotype responses IgG1 vs. IgG2a in mice showing that immunizations using IFA or DI fusion as adjuvant both give mixed IgG1 and IgG2a antibody responses with slight tendency towards Th2. Interestingly the cytokine expression patterns of the two types of T helper cells has been proposed to be mutually antagonistic although this is still a controversial subject (1, 45, 46). Since we observed a mixed Th1/Th2 profile our results are in better agreement with recent similar observations by O'Hagan et al. Taken together with the companion paper by Suarez et al. the administration route and vaccination format are of great importance in regard to the Th1/Th2 profile concordant with earlier studies (47-49). The i.m.

administration of the L36 domain I fusion protein as a DNA vaccine gives a Th1 shifted mixed Th1/Th2 profile (Suarez E et al. Journal of Immunology, companion article) compared to the Th2 shifted mixed profile for i.p. immunization with L36-DI protein reported in the present study.

Generally multimerisation leads to longer *in vivo* half-life due to the reciprocal proportional relation between the size of the molecule and its half life in blood (50, 51). Theoretically this should result in prolonged contact between the antigen containing multimeric compound and the immune system. This fact is used by a series of other vaccine studies primarily focusing on multivalent display of antigens on cell surfaces (22-24) and virus particles (25-27). The few fusion protein systems which have been investigated, such as the pentameric cholera toxin B subunit and related structures (28) (15), needed Complete or Incomplete Freunds Adjuvant to evoke potent immune response. In contrast, the scFv-DI fusion proteins with their potential to multimerize do not need Freunds Adjuvants in order to induce strong IgG immune responses. Furthermore, the introduction of heteromultimerisation potentially enhances the immunological properties of the FuncFAb system by enabling (1) co-administration of both CTL and T-helper epitopes, when they are not both present in the monomeric multimer (52) and (2) dual display of two different scFvs to target an epitope of interest to a specific cell or tissue type, e.g. APCs within lymph nodes or peripheral tissues (1, 53). Moreover, as slow dissociation from the multimer occurs continuously FuncFAbs could potentially be applied as a “magic bullet” for tumor targeting. Under these circumstances FuncFAb would benefit from decreased blood clearance of multimers, leading to better tumor localization, and subsequently also from better tumor penetrating abilities of the constantly leaking monomers. In this case the heteromultimer could consist of a tumor specific antibody and a toxic agent. However the immunogenic properties of the FuncFAb may in this case show to be the major obstacle, since the immunological response would decrease the efficacy of repetitive drug administration (54).

Based on the above studies in mice, we suggest that the FuncFAb system shows great promise as an adjuvant for vaccine purposes, especially for anti-idiotype vaccines which recently came more into practical focus due to novel potent technologies of generating anti-idiotypes (33). We speculate that the potent effect of the FuncFab expression system is due to the multimerization and increased active folding of the antibody fragments. The FuncFab system is especially valuable for scFvs, which are difficult to obtain in an active form (30). Therefore the FuncFab system is likely to be not only suitable for antibody fragments but also for other antigenic structures to be expressed in *E.coli*. The ability to build multivalent vaccines with combined specificities is another important feature, e.g. combination of CTL and B-cell epitopes..

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Reference

1. Singh, M., and D. O'Hagan. 1999. Advances in vaccine adjuvants. *Nat Biotechnol 17:1075*.
2. Shi, Y., H. HogenEsch, F. E. Regnier, and S. L. Hem. 2001. Detoxification of endotoxin by aluminum hydroxide adjuvant. *Vaccine 19:1747*.
3. Moingeon, P. 2001. Cancer vaccines. *Vaccine 19:1305*.
4. O'Hagan, D. T., M. L. MacKichan, and M. Singh. 2001. Recent developments in adjuvants for vaccines against infectious diseases. *Biomol Eng 18:69*.
5. Olszewska, W., C. D. Partidos, and M. W. Steward. 2000. Antipeptide antibody responses following intranasal immunization: effectiveness of mucosal adjuvants. *Infect Immun 68:4923*.
6. McCormick, A. L., M. S. Thomas, and A. W. Heath. 2001. Immunization with an interferon-gamma-gp120 fusion protein induces enhanced immune responses to human immunodeficiency virus gp120. *J Infect Dis 184:1423*.
7. Hakim, I., S. Levy, and R. Levy. 1996. A nine-amino acid peptide from IL-1beta augments antitumor immune responses induced by protein and DNA vaccines. *J Immunol 157:5503*.
8. Penichet, M. L., and S. L. Morrison. 2001. Antibody-cytokine fusion proteins for the therapy of cancer. *J Immunol Methods 248:91*.
9. Batova, A., A. Kamps, S. D. Gillies, R. A. Reisfeld, and A. L. Yu. 1999. The Ch14.18-GM-CSF fusion protein is effective at mediating antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity in vitro. *Clin Cancer Res 5:4259*.
10. Anthony, L. S., H. Wu, H. Sweet, C. Turnnir, L. J. Boux, and L. A. Mizzen. 1999. Priming of CD8+ CTL effector cells in mice by immunization with a stress protein-influenza virus nucleoprotein fusion molecule. *Vaccine 17:373*.
11. Rico, A. I., G. Del Real, M. Soto, L. Quijada, A. C. Martinez, C. Alonso, and J. M. Requena. 1998. Characterization of the immunostimulatory properties of Leishmania infantum HSP70 by fusion to the Escherichia coli maltose-binding protein in normal and nu/nu BALB/c mice. *Infect Immun 66:347*.
12. Spellerberg, M. B., D. Zhu, A. Thompsett, C. A. King, T. J. Hamblin, and F. K. Stevenson. 1997. DNA vaccines against lymphoma: promotion of anti-idiotypic antibody responses induced by single chain Fv genes by fusion to tetanus toxin fragment C. *J Immunol 159:1885*.
13. Sjolander, A., P. A. Nygren, S. Stahl, K. Berzins, M. Uhlen, P. Perlmann, and R. Andersson. 1997. The serum albumin-binding region of streptococcal protein G: a bacterial fusion partner with carrier-related properties. *J Immunol Methods 201:115*.
14. Schodel, F., D. Peterson, J. Hughes, R. Wirtz, and D. Milich. 1996. Hybrid hepatitis B virus core antigen as a vaccine carrier moiety: I. presentation of foreign epitopes. *J Biotechnol 44:91*.
15. Schodel, F., G. Enders, M. C. Jung, and H. Will. 1990. Recognition of a hepatitis B virus nucleocapsid T-cell epitope expressed as a fusion protein with the subunit B of Escherichia coli heat labile enterotoxin in attenuated salmonellae. *Vaccine 8:569*.
16. Lebens, M., J. B. Sun, H. Sadeghi, M. Backstrom, I. Olsson, N. Mielcarek, B. L. Li, A. Capron, C. Czerkinsky, and J. Holmgren. 2003. A mucosally administered recombinant fusion protein vaccine against schistosomiasis protecting against immunopathology and infection. *Vaccine 21:514*.
17. Springer, G. F. 1997. Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy. *J Mol Med 75:594*.
18. Le Pendu, J., S. Marionneau, A. Cailleau-Thomas, J. Rocher, B. Le Moullac-Vaidye, and M. Clement. 2001. ABH and Lewis histo-blood group antigens in cancer. *Apmis 109:9*.
19. de la Cruz, V. F., A. A. Lal, and T. F. McCutchan. 1988. Immunogenicity and epitope mapping of foreign sequences via genetically engineered filamentous phage. *J Biol Chem 263:4318*.
20. Greenwood, J., A. E. Willis, and R. N. Perham. 1991. Multiple display of foreign peptides on a filamentous bacteriophage. Peptides from Plasmodium falciparum circumsporozoite protein as antigens. *J Mol Biol 220:821*.
21. Frenkel, D., I. Dewachter, F. Van Leuven, and B. Solomon. 2003. Reduction of beta-amyloid plaques in brain of transgenic mouse model of Alzheimer's disease by EFRH-phage immunization. *Vaccine 21:1060*.
22. de Ines, C., B. Cochlovius, S. Schmidt, and M. Little. 2000. A cell surface-displayed anti-c-myc single-chain antibody: new perspectives for the genetic improvement of cellular tumor vaccines. *Cancer Gene Ther 7:1257*.
23. Paul, S., D. Snary, J. Hoebeke, D. Allen, J. M. Balloul, N. Bizouarne, K. Dott, M. Geist, J. Hilgers, M. P. Kieny, J. Burchell, J. Taylor-Papadimitriou, and R. B. Acres. 2000. Targeted macrophage cytotoxicity using a nonreplicative live vector expressing a tumor-specific single-chain variable region fragment. *Hum Gene Ther 11:1417*.

24. Rode, H. J., U. Moebius, and M. Little. 1999. T cell activation by monoclonal antibodies bound to tumor cells by a cell surface displayed single-chain antibody. *J Immunol Methods 224:151*.
25. Mottershead, D. G., K. Alfthan, K. Ojala, K. Takkinen, and C. Oker-Blom. 2000. Baculoviral display of functional scFv and synthetic IgG-binding domains. *Biochem Biophys Res Commun 275:84*.
26. McInerney, T. L., F. R. Brennan, T. D. Jones, and N. J. Dimmock. 1999. Analysis of the ability of five adjuvants to enhance immune responses to a chimeric plant virus displaying an HIV-1 peptide. *Vaccine 17:1359*.
27. Jiang, J., L. Abu-Shilbayeh, and V. B. Rao. 1997. Display of a PorA peptide from Neisseria meningitidis on the bacteriophage T4 capsid surface. *Infect Immun 65:4770*.
28. Liljeqvist, S., S. Stahl, C. Andreoni, H. Binz, M. Uhlen, and M. Murby. 1997. Fusions to the cholera toxin B subunit: influence on pentamerization and GM1 binding. *J Immunol Methods 210:125*.
29. Yip, Y. L., G. Smith, and R. L. Ward. 2001. Comparison of phage pIII, pVIII and GST as carrier proteins for peptide immunisation in Balb/c mice. *Immunol Lett 79:197*.
30. Jensen, K. B., M. Larsen, J. S. Pedersen, P. A. Christensen, L. Alvarez-Vallina, S. Goletz, B. F. Clark, and P. Kristensen. 2002. Functional improvement of antibody fragments using a novel phage coat protein III fusion system. *Biochem Biophys Res Commun 298:566*.
31. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem 72:248*.
32. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning. A laboratory manual*. Cold Spring Harbor Laboratory Press, New York.
33. Goletz, S., P. A. Christensen, P. Kristensen, D. Blohm, I. Tomlinson, G. Winter, and U. Karsten. 2002. Selection of large diversities of antiidiotypic antibody fragments by phage display. *J Mol Biol 315:1087*.
34. Karsten, U., C. Diotel, G. Klich, H. Paulsen, S. Goletz, S. Muller, and F. G. Hanisch. 1998. Enhanced binding of antibodies to the DTR motif of MUC1 tandem repeat peptide is mediated by site-specific glycosylation. *Cancer Res 58:2541*.
35. Price, M. R., P. D. Rye, E. Petrakou, A. Murray, K. Brady, S. Imai, S. Haga, Y. Kiyozuka, D. Schol, M. F. Meulenbroek, F. G. Snijdewint, S. von Mensdorff-Pouilly, R. A. Verstraeten, P. Kenemans, A. Blockzjil, K. Nilsson, O. Nilsson, M. Reddish, M. R. Suresh, R. R. Koganty, S. Fortier, L. Baronic, A. Berg, M. B. Longenecker, J. Hilgers, and et al. 1998. Summary report on the ISOBM TD-4 Workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin. San Diego, Calif., November 17-23, 1996. *Tumour Biol 19 Suppl 1:1*.
36. Karsten, U., G. Pilgrim, F. G. Hanisch, G. Uhlenbruck, M. Kasper, P. Stosiek, G. Papsdorf, and G. Pasternak. 1988. A new monoclonal antibody (A46-B/B10) highly specific for the blood group H type 2 epitope: generation, epitope analysis, serological and histological evaluation. *Br J Cancer 58:176*.
37. Jensen, K. B., O. N. Jensen, P. Ravn, B. F. Clark, and P. Kristensen. 2003. Identification of keratinocyte specific markers using phage display and mass spectrometry. *Mol Cell Proteomics*.
38. Sanz, L., P. Kristensen, S. J. Russell, J. R. Ramirez Garcia, and L. Alvarez-Vallina. 2001. Generation and characterization of recombinant human antibodies specific for native laminin epitopes: potential application in cancer therapy. *Cancer Immunol Immunother 50:557*.
39. Holliger, P., L. Riechmann, and R. L. Williams. 1999. Crystal structure of the two N-terminal domains of g3p from filamentous phage fd at 1.9 A: evidence for conformational lability. *J Mol Biol 288:649*.
40. Lubkowski, J., F. Hennecke, A. Pluckthun, and A. Wlodawer. 1999. Filamentous phage infection: crystal structure of g3p in complex with its coreceptor, the C-terminal domain of TolA [In Process Citation]. *Structure Fold Des 7:711*.
41. Willis, A. E., R. N. Perham, and D. Wraith. 1993. Immunological properties of foreign peptides in multiple display on a filamentous bacteriophage. *Gene 128:79*.
42. Felici, F., A. Luzzago, A. Folgori, and R. Cortese. 1993. Mimicking of discontinuous epitopes by phage-displayed peptides, II. Selection of clones recognized by a protective monoclonal antibody against the Bordetella pertussis toxin from phage peptide libraries. *Gene 128:21*.
43. Minenkova, O. O., A. A. Ilyichev, G. P. Kishchenko, and V. A. Petrenko. 1993. Design of specific immunogens using filamentous phage as the carrier. *Gene 128:85*.
44. Tsunetsugu-Yokota, Y., M. Tatsumi, V. Robert, C. Devaux, B. Spire, J. C. Chermann, and I. Hirsch. 1991. Expression of an immunogenic region of HIV by a filamentous bacteriophage vector. *Gene 99:261*.
45. O'Hagan, D. T., M. Singh, J. Kazzaz, M. Ugozzoli, M. Briones, J. Donnelly, and G. Ott. 2002. Synergistic adjuvant activity of immunostimulatory DNA and oil/water emulsions for immunization with HIV p55 gag antigen. *Vaccine 20:3389*.

46. O'Hagan, D. T., M. Ugozzoli, J. Barackman, M. Singh, J. Kazzaz, K. Higgins, T. C. Vancott, and G. Ott. 2000. Microparticles in MF59, a potent adjuvant combination for a recombinant protein vaccine against HIV-1. *Vaccine 18:1793*.
47. Serezani, C. H., A. R. Franco, M. Wajc, J. K. Umada Yokoyama-Yasunaka, G. Wunderlich, M. M. Borges, and S. R. Uliana. 2002. Evaluation of the murine immune response to Leishmania meta 1 antigen delivered as recombinant protein or DNA vaccine. *Vaccine 20:3755*.
48. McCluskie, M. J., R. D. Weeratna, and H. L. Davis. 2000. Intranasal immunization of mice with CpG DNA induces strong systemic and mucosal responses that are influenced by other mucosal adjuvants and antigen distribution. *Mol Med 6:867*.
49. Herrick, C. A., L. Xu, A. N. McKenzie, R. E. Tigelaar, and K. Bottomly. 2003. IL-13 is necessary, not simply sufficient, for epicutaneously induced Th2 responses to soluble protein antigen. *J Immunol 170:2488*.
50. Willuda, J., A. Honegger, R. Waibel, P. A. Schubiger, R. Stahel, U. Zangemeister-Wittke, and A. Pluckthun. 1999. High thermal stability is essential for tumor targeting of antibody fragments: engineering of a humanized anti-epithelial glycoprotein-2 (epithelial cell adhesion molecule) single-chain Fv fragment. *Cancer Res 59:5758*.
51. Powers, D. B., P. Amersdorfer, M. Poul, U. B. Nielsen, M. R. Shalaby, G. P. Adams, L. M. Weiner, and J. D. Marks. 2001. Expression of single-chain Fv-Fc fusions in Pichia pastoris. *J Immunol Methods 251:123*.
52. De Berardinis, P., R. Sartorius, C. Fanutti, R. N. Perham, G. Del Pozzo, and J. Guardiola. 2000. Phage display of peptide epitopes from HIV-1 elicits strong cytolytic responses. *Nat Biotechnol 18:873*.
53. Schoonjans, R., A. Willems, S. Schoonooghe, W. Fiers, J. Grooten, and N. Mertens. 2000. Fab chains as an efficient heterodimerization scaffold for the production of recombinant bispecific and trispecific antibody derivatives. *J Immunol 165:7050*.
54. Schmidt, M., M. Maurer-Gebhard, B. Groner, G. Kohler, G. Brochmann-Santos, and W. Wels. 1999. Suppression of metastasis formation by a recombinant single chain antibody-toxin targeted to full-length and oncogenic variant EGF receptors. *Oncogene 18:1711*.

Figure Legends

FIGURE I – Schematic representation of FuncFAb domain structure.
Illustration of filamentous bacteriophage protein III and linear structure of the FuncFAb fusion proteins. A schematic representation of the domain structure of protein III and the constructed fusion proteins using the following abbreviations: DI, domain I, DII, domain II, DIII domain III, SP, signal peptide – pelB leader.

FIGURE II – Heteromultimerisation of R5-DI and D4-DI.
Heteromultimeric R5-DI and D4-DI IMAC purified protein was analyzed for multimerisation on an HPLC gel filtration column with theoretical exclusion limit at 120 kDa (A) and for bispecific activity in a sandwich ELISA experiment (B). The sandwich ELISA was performed both with fibronectin and with A76-A/C7 coated to the wells as indicated in the figure key. The bispecific heteromultimer was subsequently recognized with the reverse binding partner, A76-A/C7 and fibronectin, respectively. Abbreviation: DI, domain I.

FIGURE III – IgG titer of mice immunized with FuncFAb protein.
The bar diagram shows the immune response for different mice immunized with L36 scFv, PACA17 scFv, DI and derivatives thereof. The immune response was for each mouse determined specifically against the non-fused scFv from which the immunized protein originated. The immune responses were furthermore analyzed for their IgG1 and IgG2a subclass distribution. As controls pre-immune sera and sera from mice immunized with DI are shown. All pre-immune sera as well as sera from DI immunized mice show equally low signal intensity respectively and each is therefore only represented with sera from one mouse. Recognition of both L36 scFv (*) and PACA17 scFv (**) was tested with both pre-immune sera and sera from DI immunized mice. The error bars represent the standard deviation. Abbreviation: DI, domain I.

FIGURE IV – Anti-idiotypic response towards L36 scFv and derivatives.
Diagram of a sera competition assay (A) and an assay determining the presence of structural mimics of Laminin-I in the sera (B). The ELISA competition assay is pictured as a bar diagram, showing the ability of different sera to compete for the binding of L36 to Laminin-I. Optimized sera concentrations were found to be 1:15 dilution. Structural mimics of Laminin-I among the antibodies present in the sera, were recognized in ELISA by the rabbit anti-EHS Laminin-I polyclonal antibody. Abbreviation: DI, domain I.

Figure I

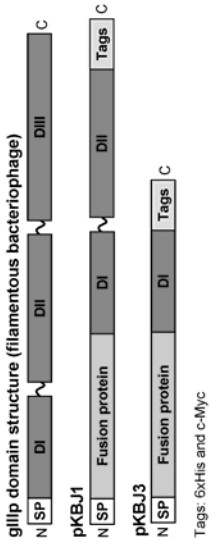

gIIIp domain structure (filamentous bacteriophage)
N
SP
DI
DII
DIII
C
pKBJ1
N
SP
Fusion protein
DI
DII
Tags
C
pKBJ3
N
SP
Fusion protein
DI
Tags
C
Tags: 6xHis and c-Myc

Figure II

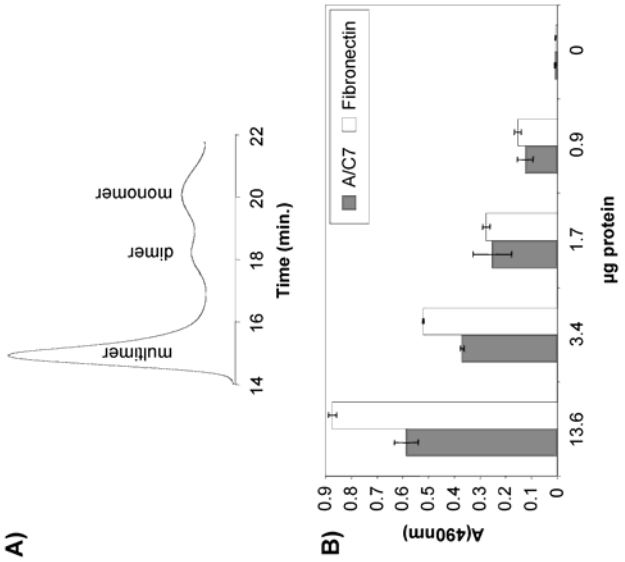

A)
multimer
dimer
monomer
14
16
18
20
22
Time (min.)
B)
A(490nm)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
A/C7
Fibronectin
13.6
3.4
1.7
0.9
0
µg protein

Figure III

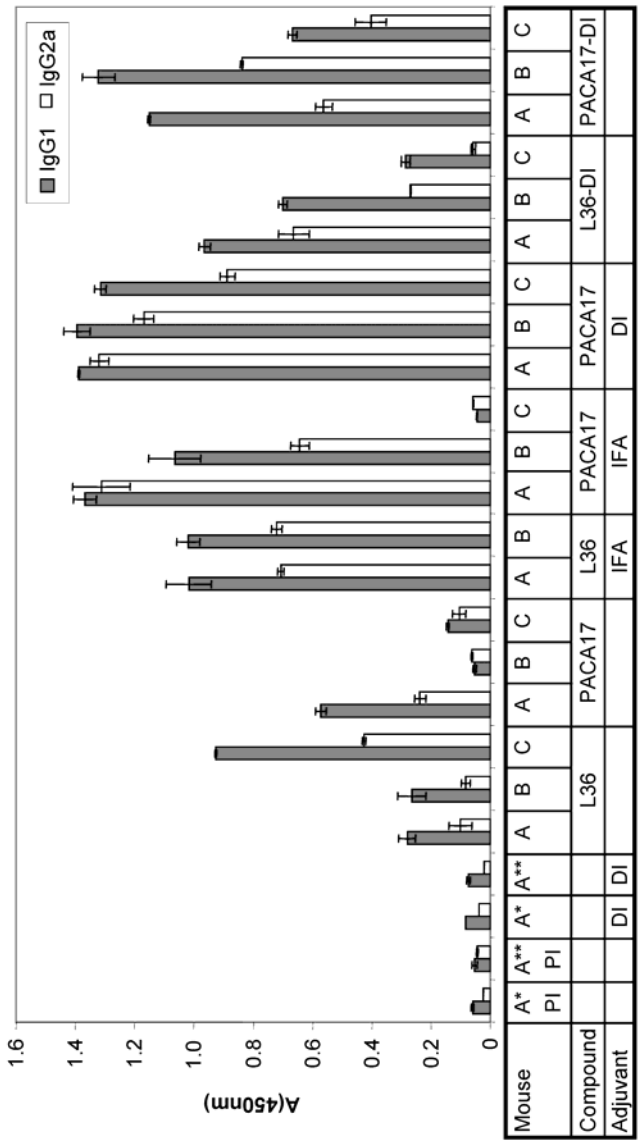
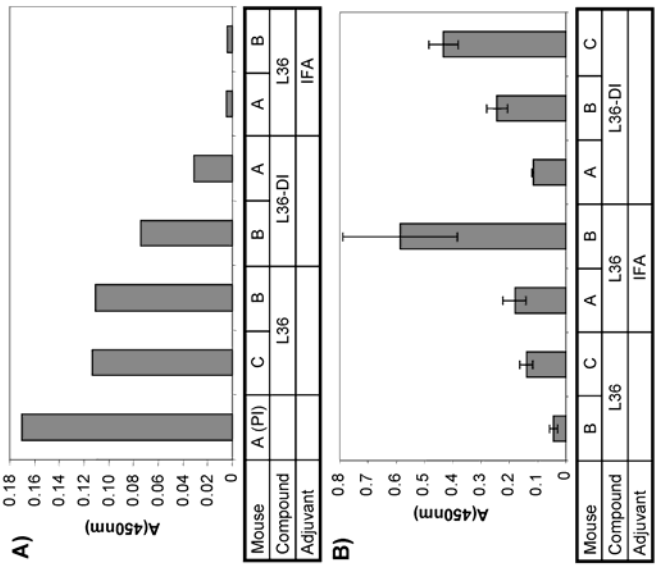

IgG1
IgG2a
A(450nm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Mouse
Compound
Adjuvant
A* PI
A** PI
A*
A**
A
B
C
L36
DI
PACA17
IFA
L36-DI
PACA17-DI

Figure IV


A)
A(450nm)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Mouse
A (PI)
C
B
B
A
A
B
Compound
L36
L36-DI
L36
Adjuvant
IFA
B)
A(450nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Mouse
B
C
A
B
A
B
C
Compound
L36
L36
L36-DI
Adjuvant
IFA

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[3] DI, Domain I; DI-II, Domain I-II; scFv, single-chain Fragment Variable, FDA, The Food and Drug Administration; FuncFAb, Functionally Fused Antibody.

Appendix C

Filamentous bacteriophage coat protein III domain I in DNA vaccines promotes a Th1-type dominated immune response

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Running title: Coat protein III–based DNA vaccine promotes Th1 response

Keywords: adjuvant, bacteriophage, scFv, genetic fusion and idiotypic vaccine

INTRODUCTION

Improvement of vaccine efficacy has become a critical goal in the development of DNA vaccination as an antitumor therapy. A prerequisite for successful antitumor vaccination is breaking the tolerance to tumor-associated antigens, which represent 'self antigens' and are, therefore, poorly immunogenic. Various DNA fusion vaccine strategies, whereby antigens are genetically linked to immuno-enhancing molecules, have been explored (1). These immuno-activating genes include: cytokines (2), chemokines (3), costimulatory molecules (4) and pathogen-derived sequences (5-7).

Bacterial virus particles, such as filamentous bacteriophage, have been found to exhibit an adjuvant-like effect when applied in vaccination strategies (8). Furthermore, it has been shown that the immune response to scFv antibody fragments is significantly improved by fusion or co-administration of fragments of coat protein III of the filamentous bacteriophage (Larsen et al., companion article). The adjuvant and carrier properties of the FuncFAb (Functionally Fused Antibody) system (9) are due to multimerization of the scFv fusions to coat protein III, leading also to potential heteromultimerization (Larsen et al., companion article).

Here, we show that fragments of coat protein III of the filamentous bacteriophage can likewise induce immunity when delivered as a fusion gene with a model single-chain antibody fragment (scFv). Moreover, the expressed fusion protein promotes anti-idiotypic antibodies of the IgG2a isotype, indicative of a Th1 response.

MATERIALS AND METHODS

Construction of vectors

Domain I of the filamentous bacteriophage gene III (DI) was amplified from plasmid pKBJ3 (9) with primer pair pKBJ3NotI (5´-AGG TGC GGC CGC CGG GGC CGC AAC TGT TGA-3´) and pKBJ3XbaI (5´-AAA ATC TAG ACC AGT GAA TTC TTA TTA ATG-3´). The NotI/XbaI-cleaved PCR fragment was ligated into the NotI/XbaI digested backbone of plasmid pCR3.1-L36 (10), resulting in pCR3.1-L36-DI. The sequence was verified using primer BGHreverse (5´- TAG AAG GCA CAG TCG AGG- 3´). Plasmid DNA was purified with the Endofree plasmid megakit (Qiagen, Hilden, Germany).

Expression and purification of recombinant antibodies

The expression and purification of non-fused and DI-fused scFv antibodies were performed as described (9). The purified antibodies were verified by SDS-PAGE.

Culture conditions and cell transfections

Human embryonic kidney 293T cells (ATCC CRL-1573) were grown in DMEM supplemented with 10% FCS, (Invitrogen Life Technologies, Carlsbad, CA, USA). 293T cells were transfected with plasmids pCR3.1 (Invitrogen Life Technologies), pCR3.1-L36 or pCR3.1 L36-DI using Lipofectamine (Invitrogen Life Technologies). Supernatants were collected 48 h post-transfection and secretion of functional scFv was demonstrated by specificity ELISA (10) and SDS-PAGE.

Animals and immunizations

Two groups of BALB/c mice (6-8-week-old females; Harlan Ibérica, Barcelona, Spain) were immunized with PBS (n=2) or recombinant L36 (50 μg) emulsified in 100 μl of Incomplete Freund´s Adjuvant (n=4). Injections were given intraperitoneally (i.p.) at days 0 and 14. Two groups of mice (n=4) were injected with 50 μg of plasmid DNA (pCR3.1-L36 or pCR3.1-L36-DI). Mice received bilateral injections (25 μg each) into left and right posterior thigh muscles at days 0, 7 and 14. Two mice from each group received an additional injection (50 μg) of plasmid DNA (pCR3.1-L36 or pCR3.1-L36-DI) at day 21 (11). All animals were bled by retro-orbital puncture at regular time intervals and plasma was stored at -20°C until assayed.

Evaluation of immune response

L36 specific antibodies were detected by ELISA, using Maxisorp® 96-well plates (Nunc, Roskilde, Denmark) coated ON at 4ºC with recombinant scFv (0.25 μg/well). After three washes, plates were blocked for 30 min at RT with 3% BSA in PBS (BPBS), then diluted sera in 3% BPBS were added and the plates were incubated for 1 h at RT. After three washes, IgG1 and IgG2a isotypes were detected by incubation with anti-mouse IgG1 and IgG2a polyclonal rabbit antibodies (Abcam, Cambridge, UK), respectively. After additional washing, a POD-conjugated goat anti-rabbit antibody (DAKO, Glostrup, Denmark) was used for detection. To study the presence of anti-idiotypic antibodies, an ELISA assay was carried out by coating Maxisorp® 96-well plates with rabbit anti-EHS laminin polyclonal antibody (Sigma Biosciences) ON at 4ºC in PBS (0.3 μg/well). Plates were blocked with 4% BPBS for 2 h at RT and then incubated with serum diluted 1:25 in 1% BPBS supplemented with 0.05% Tween-20 for

1 h. After washing, anti-idiotypic antibodies were detected using HRP-conjugated goat-anti-mouse IgG antibody (Sigma Biosciences).

RESULTS

Construction of a DI-fused L36-encoding mammalian expression vector

We have previously described and characterized the hCMV-driven mammalian expression vector pCR3.1-L36 containing the human anti-laminin-1 L36 scFv antibody (10). In order to study the ability of mammalian cells to assemble and secrete a filamentous phage gene III domain I-fused scFv antibody, we constructed the plasmid pCR3.1-L36-DI, containing the human oncostatin M leader sequence followed by L36 scFv fused to the first 80 translated non-leader sequence codons of gIIIp (Fig. 1A). Transfection of human 293T cells with plasmid pCR3.1-L36 or pCR3.1-L36-DI resulted in the secretion of functional antibodies. The secreted non-fused and DI-fused scFvs bound specifically to their cognate antigen (laminin-1), as determined by ELISA (Fig. 1B). Antibody was not detected in the cell culture supernatant of 293T cells transfected with pCR3.1 control plasmid (Figs. 1B and 1C). SDS-PAGE analysis of culture medium from 293T cells transfected with pCR3.1-L36 or pCR3.1-L36-DI plasmids demonstrated that the migration pattern of the secreted antibodies was consistent with the predicted molecular weight (Fig. 1C). Under reducing conditions, the migration patterns of both mammalian- and bacterial-produced non-fused L36 antibodies were similar, whereas the mammalian-produced DI-fused L36 scFv was slightly larger than the bacterial-produced DI fusion, probably due to differences in the glycosylation pattern. In fact, protein III domain I has a predicted N-glycosylated site (residues 57–60) (12).

Vaccination and immune response

Mice were immunized with plasmids encoding non-fused and DI-fused L36 scFv, and their humoral responses were compared with the mice injected with purified non-fused L36 scFv mixed with the IFA. Anti-L36 IgG antibodies were detected in mice immunized with purified L36 scFv or with plasmid pCR3.1-L36-DI (Fig. 2). Notably, only those animals receiving an additional injection of plasmid pCR3.1-L36-DI on day 21 showed significant and reproducible titers of IgG antibodies (Fig. 2, mice C and D). Constructs containing L36 scFv alone failed to induce significant levels of anti-L36 scFv antibodies (Fig. 2). In mice immunized with purified L36 scFv mixed with IFA, the subclass distribution of IgG1 and IgG2a showed higher IgG1 levels than IgG2a, although both subclasses were prominent, as determined by observed ELISA signals and similar reported data (Larsen et al., companion article). Mice immunized with pCR3.1-L36-DI plasmid showed dominance of IgG2a (Fig. 2).

Mice immunized with purified L36, either non-fused or fused to the N-terminal domains of protein III induced significant anti-idiotypic responses (*Larsen et al., companion article*). To test whether anti-idiotypic antibodies mimicking the laminin-1 domain recognized by L36 scFv (anti-idiotypic Ab2β antibodies) were present in mice immunized with the L36-encoding constructs, we studied sera binding to a polyclonal rabbit anti-laminin-1 antibody. Immunization with either purified L36 scFv (+IFA) or plasmid pCR3.1-L36-DI induced significant anti-idiotypic Ab2β immune responses, while mice immunized with plasmid pCR3.1-L36 generated no anti-idiotypic Ab2β immune response (Fig. 3).

DISCUSSION

We have demonstrated the feasibility of using the amino-terminal domain of filamentous bacteriophage protein III as an adjuvant molecule in a genetic vaccination protocol. Mammalian-produced DI-scFv fusion protein was correctly folded, and the antibody retained its full biological activity. With the appropriate immunization schedule, a naked DNA plasmid vaccine encoding a scFv-DI fusion induced significant titers of anti-idiotypic antibodies of the IgG2a subclass, characteristic of a Th1-type response, whereas protein vaccination generated predominant IgG1 antibody isotypes, related to a Th2-type response. Th1 immunity has classically been considered more attractive in a therapeutic setting, given that the associated high CTL activity plays a key role in host defence against tumors and viral infections.

The differences observed between the two protocols could be explained by the fact that plasmid DNA uptake results in an endogenous synthesis of the encoded protein and its subsequent association with MHC class I molecules; in contrast, administered soluble proteins are processed predominantly through the exogenous pathway. Activation of the immune response is also known to be influenced by both the nature and molecular form of the antigen, with large aggregates being particularly immunogenic (13). Previous results suggest that aggregated antigen amplifies a Th1-type response, already activated by the cytokine environment generated by injection of DNA (14). Induction of IgG2a-dominant responses has also been noted, using genetic fusions of aggregating plant viral coat proteins to both peptides and scFv fragments (7, 15). In our model, multimerization of the scFv is accomplished by linking the antibody fragment to domain I of coat protein III, (*Larsen et al., companion article*).

The fact that immunization with a plasmid encoding scFv-DI induced a significant anti-idiotypic Th1-type response has interesting therapeutic implications. The clone-

specific idiotypic Ig of B-cell malignancies can be engineered into a scFv format and fused to DI to elicit anti-tumoral responses. DNA idiotypic vaccines have been successfully developed using sequences from tetanus toxin or potato virus X coat protein as adjuvants (5, 7 and 16). The advantage of the model presented here is the availability of a 'built-in' adjuvant, the domain I of phage coat protein III, when scFv are generated in a phage context. Furthermore, our results raise the possibility of combining DNA and purified protein in DI-mediated vaccination protocols. In some cases, this approach has been shown to considerably increase antitumor activity (17).

ACKNOWLEDGEMENTS

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FOOTNOTES

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e-mail: lalvarezv.hpth@salud.madrid.org

REFERENCES

1. Zhu, D., and F. K. Stevenson. 2002. DNA gene fusion vaccines against cancer. *Curr Opin Mol Ther 4:41*.
2. Syrengelas, A. D., T. T. Chen, and R. Levy. 1996. DNA immunization induces protective immunity against B-cell lymphoma. *Nat Med 2:1038*.
3. Biragyn, A., K. Tani, M. C. Grimm, S. Weeks, and L. W. Kwak. 1999. Genetic fusion of chemokines to a self tumor antigen induces protective, T-cell dependent antitumor immunity. *Nat Biotechnol 17:253*.
4. Conry, R. M., G. Widera, A. F. LoBuglio, J. T. Fuller, S. E. Moore, D. L. Barlow, J. Turner, N. S. Yang, and D. T. Curiel. 1996. Selected strategies to augment polynucleotide immunization. *Gene Ther 3:67*.
5. Spellerberg, M. B., D. Zhu, A. Thompsett, C. A. King, T. J. Hamblin, and F. K. Stevenson. 1997. DNA vaccines against lymphoma: promotion of anti-idiotypic antibody responses induced by single chain Fv genes by fusion to tetanus toxin fragment C. *J Immunol 159:1885*.
6. Chen, C. H., T. L. Wang, C. F. Hung, Y. Yang, R. A. Young, D. M. Pardoll, and T. C. Wu. 2000. Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP70 gene. *Cancer Res 60:1035*.
7. Savelyeva, N., R. Munday, M. B. Spellerberg, G. P. Lomonossoff, and F. K. Stevenson. 2001. Plant viral genes in DNA idiotypic vaccines activate linked CD4+ T-cell mediated immunity against B-cell malignancies. *Nat Biotechnol 19:760*.
8. Willis, A. E., R. N. Perham, and D. Wraith. 1993. Immunological properties of foreign peptides in multiple display on a filamentous bacteriophage. *Gene 128:79*.
9. Jensen, K. B., M. Larsen, J. S. Pedersen, P. A. Christensen, L. Alvarez-Vallina, S. Goletz, B. F. Clark, and P. Kristensen. 2002. Functional improvement of antibody fragments using a novel phage coat protein III fusion system. *Biochem Biophys Res Commun 298:566*.
10. Sanz, L., P. Kristensen, B. Blanco, S. Facteau, S. J. Russell, G. Winter, and L. Alvarez-Vallina. 2002. Single-chain antibody-based gene therapy: inhibition of tumor growth by in situ production of phage-derived human antibody fragments blocking functionally active sites of cell-associated matrices. *Gene Ther 9:1049*.
11. Stevenson, F. K., D. Zhu, C. A. King, L. J. Ashworth, S. Kumar, and R. E. Hawkins. 1995. Idiotypic DNA vaccines against B-cell lymphoma. *Immunol Rev 145:211*.
12. Hill, D. F., and G. B. Petersen. 1982. Nucleotide sequence of bacteriophage f1 DNA. *J Virol 44:32*.
13. Jagadish, M. N., S. J. Edwards, M. B. Hayden, J. Grusovin, K. Vandenberg, P. Schoofs, R. C. Hamilton, D. D. Shukla, H. Kalnins, M. McNamara, J. Haynes, I. T. Nisbet, C. W. Ward, and D. Pye. 1996. Chimeric potyvirus-like particles as vaccine carriers. *Intervirology 39:85*.
14. Roman, M., E. Martin-Orozco, J. S. Goodman, M. D. Nguyen, Y. Sato, A. Ronaghy, R. S. Kornbluth, D. D. Richman, D. A. Carson, and E. Raz. 1997. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat Med 3:849*.
15. McInerney, T. L., F. R. Brennan, T. D. Jones, and N. J. Dimmock. 1999. Analysis of the ability of five adjuvants to enhance immune responses to a chimeric plant virus displaying an HIV-1 peptide. *Vaccine 17:1359*.
16. King, C. A., M. B. Spellerberg, D. Zhu, J. Rice, S. S. Sahota, A. R. Thompsett, T. J. Hamblin, J. Radl, and F. K. Stevenson. 1998. DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. *Nat Med 4:1281*.
17. Zoller, M., and O. Christ. 2001. Prophylactic tumor vaccination: comparison of effector mechanisms initiated by protein versus DNA vaccination. *J Immunol 166:3440*.

FIGURE LEGENDS

Figure 1. (A) Schematic representation of scFv gene constructs. The direction of transcription is indicated by arrows. His6myc tag appended is for immunodetection. Abbreviation: L, Oncostatin M leader sequence. (B) Secretion of functional L36 scFv into the cell culture supernatant by human 293T cells transfected with pCR3.1, pCR3.1-L36 or pCR3.1-L36-DI plasmids. The functionality of scFv antibodies was demonstrated in ELISA against plastic immobilized human BSA (■) and laminin-1 (□). (C) SDS-PAGE analysis of secreted scFv antibodies into the cell culture supernatant by 293T cells transfected with either plasmid pCR3.1-L36 (2), pCR3.1-L36-DI (4) or pCR3.1 (5). Recombinant L36 non-fused (1) and fused to DI (3) purified from bacteria.

Figure 2. Antibody isotypes (IgG1 and IgG2a) in BALB/c mice 4 weeks after immunization with recombinant non-fused L36 scFv +IFA (days 0 and 14), pCR3.1-L36 or pCR3.1-L36-DI plasmids (days 0, 7 and 14)**.** Two mice (C and D) from each group received an additional injection of plasmid DNA (pCR3.1-L36 or pCR3.1-L36-DI) at day 21. As controls, pre-immune (PI) sera and sera from mice immunized with PBS are shown. Error bars represent standard deviations. Abbreviation: DI, domain I.

Figure 3. Anti-idiotypic response towards L36 scFv. Structural mimics of laminin-1 among antibodies present in the sera were recognised in ELISA by an anti-laminin polyclonal antibody. As controls, pre-immune (PI) sera and sera from mice immunized with PBS are shown. Error bars represent standard deviations. Abbreviation: DI, domain I.

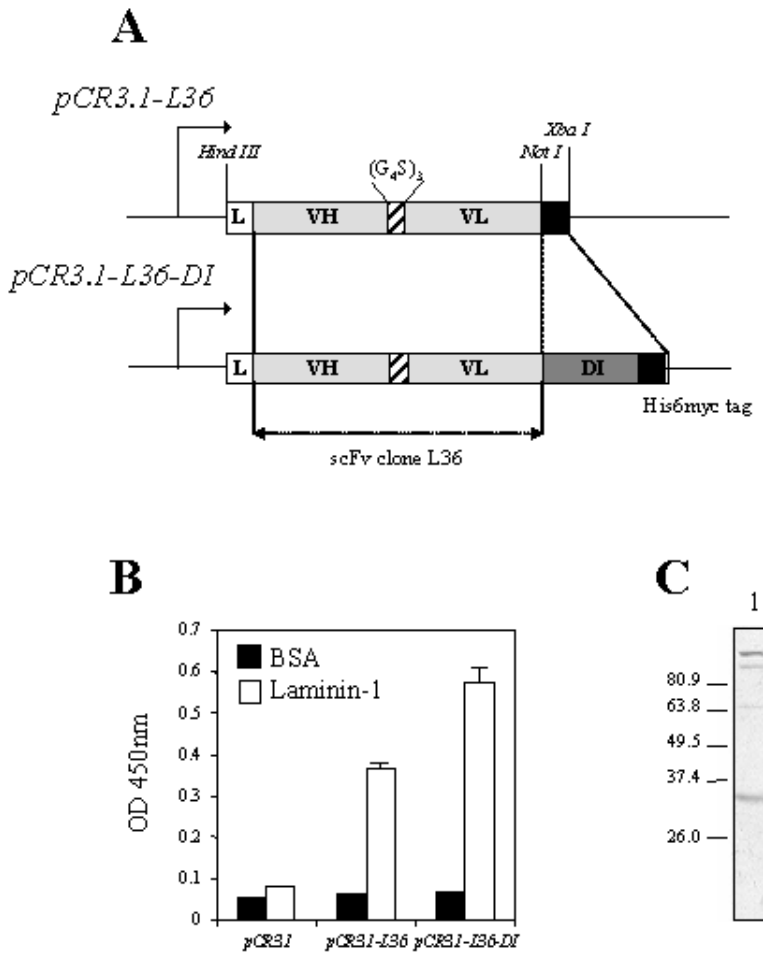

A
pCR3.1-L36
Hind III
(G4S)3
Xba I
Not I
L
VH
VL
pCR3.1-L36-DI
L
VH
VL
DI
His6myc tag
scFv clone L36
B
BSA
Laminin-1
OD 450nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
pCR3.1
pCR3.1-L36
pCR3.1-L36-DI
C
1 2 3 4 5
80.9
63.8
49.5
37.4
26.0

Figure 1

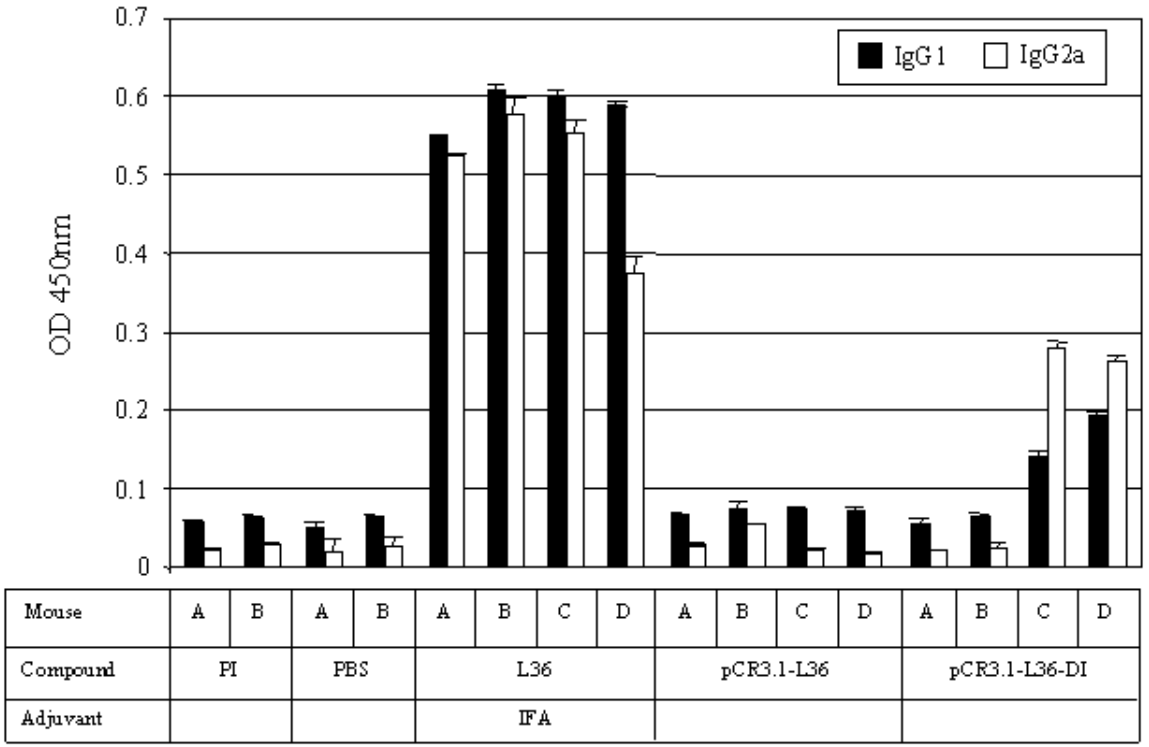
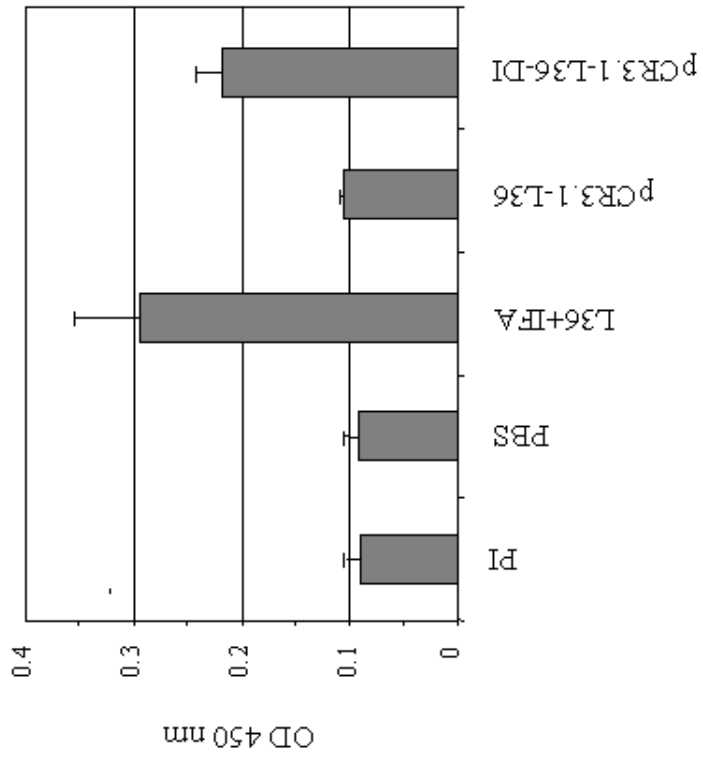

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD 450nm
IgG1
IgG2a
Mouse A B A B A B C D A B C D A B C D
Compound PI PBS L36 pCR3.1-L36 pCR3.1-L36-DI
Adjuvant IFA

Figure 2

Figure 3



Appendix D
Sequences of pKBJ Constructs

pKBJ1

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1                                                                                                   100
MKYLLPTAAAGLLLLAAQPAMAQVQLVQSGAEVKKPGATVKISCKVSGYTFTDYYMHWVQQAPGKGLEWMGLVDPEDGETIYAEKFQGRVTITADTSTDT

101                                                                                                 200
AYMELSSLRSEDTAVYYCARKNPRASWYWGQGTLVTVSSGGGGSGGGGSGGSALQSVLTQPPSVFGGGTKLTVLGAAAGAATVESCLAKPHTENSFTNVW

201                                                                                                 300
KDDKTLDRYANYEGCLWNATGVVVCTGDETQCYGTWVPIGLAIPENEGGGSEGGGSEGGGSEGGGTKPPEYGDTPIPGYTYINPLDGTYPPGTEQNPANP

301                                                                                                 400
NPSLEESQPLNTFMFQNNRFRNRQGALTVYTGTVTQGTDPVKTYYQYTPVSSKAMYDAYWNGKFRDCAFHSGFNEDPFVCEYQGQSSDLPQPPVNAGGSA

401                 422
AEQKLISEEDLNGAAHHHHHH-
```

pKBJ2

```
1                                                                                                   100
MKYLLPTAAAGLLLLAAQPAMAQVQLVQSGAEVKKPGATVKISCKVSGYTFTDYYMHWVQQAPGKGLEWMGLVDPEDGETIYAEKFQGRVTITADTSTDT

101                                                                                                 200
AYMELSSLRSEDTAVYYCARKNPRASWYWGQGTLVTVSSGGGGSGGGGSGGSALQSVLTQPPSVFGGGTKLTVLGAAAHHHHHHGAAEQKLISEEDLNGA

201                                                                                                 300
AQTVESCLAKPHTENSFTNVWKDDKTLDRYANYEGCLWNATGVVVCTGDETQCYGTWVPIGLAIPENEGGGSEGGGSEGGGSEGGGTKPPEYGDTPIPGY

301                                                                                                 400
TYINPLDGTYPPGTEQNPANPNPSLEESQPLNTFMFQNNRFRNRQGALTVYTGTVTQGTDPVKTYYQYTPVSSKAMYDAYWNGKFRDCAFHSGFNEDPFV

401              420
CEYQGQSSDLPQPPVNAGG-
```

pKBJ3

```
1                                                                                                   100
MKYLLPTAAAGLLLLAAQPAMAQVQLVQSGAEVKKPGATVKISCKVSGYTFTDYYMHWVQQAPGKGLEWMGLVDPEDGETIYAEKFQGRVTITADTSTDT

101                                                                                                 200
AYMELSSLRSEDTAVYYCARKNPRASWYWGQGTLVTVSSGGGGSGGGGSGGSALQSVLTQPPSVFGGGTKLTVLGAAAGAATVESCLAKPHTENSFTNVW

201                                                                                         284
KDDKTLDRYANYEGCLWNATGVVVCTGDETQCYGTWVPIGLAIPENEGGGSEGGGSEGGGSAAEQKLISEEDLNGAAHHHHHH-
```

PelB leader 

HIS and c-Myc tag 

Appendix E
Protein Sequences of scMHC Constructs

scMHC peptide - HLA-A2*02011 – 10mer linker – β2MG - gIIIp

```
1                                                                                                   100
AAGIGILTVGGGGSSGGGSMGSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRMEPRAPWIEQEGPEYWDGETRKVKAHSQTHRVDLGT

101                                                                                                 200
LRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAYDGKDYIALKEDLRSWTAADMAAQTTKHKWEAAHVAEQLRAYLEGTCVEWLRRYLENGKETLQ

201                                                                                                 300
RTDAPKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGTFQKWAAVVVPSGQEQRYTCHVQHEGLPKPLTGGIGSGGGG

301                                                                                                 400
SGGGGSIQRTPKIQVYSRHPAENGKSNFLNCYVSGFHPSDIEVDLLKNGERIEKVEHSDLSFSKDWSFYLLYYTEFTPTEKDEYACRVNHVTLSQPKIVK

401                               434
WDRDMAAAHHHHHHGAAEQKLISEEDLNGAA-TV……gIIIp……
```

scMHC peptide - β2MG – 10mer linker - HLA-A2*02011 - gIIIp

```
1                                                                                                   100
AAGIGILTVGGGGSSGGGSMAIQRTPKIQVYSRHPAENGKSNFLNCYVSGFHPSDIEVDLLKNGERIEKVEHSDLSFSKDWSFYLLYYTEFTPTEKDEYA

101                                                                                                 200
CRVNHVTLSQPKIVKWDRDMGGIGSGGGGSGGGGSGGSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRMEPRAPWIEQEGPEYWDGETR

201                                                                                                 300
KVKAHSQTHRVDLGTLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQYAYDGKDYIALKEDLRSWTAADMAAQTTKHKWEAAHVAEQLRAYLEGTCV

301                                                                                                 400
EWLRRYLENGKETLQRTDAPKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGTFQKWAAVVVPSGQEQRYTCHVQHEG

401                               435
LPKPLTAAAHHHHHHGAAEQKLISEEDLNGAA-TV……gIIIp……
```

Peptide 

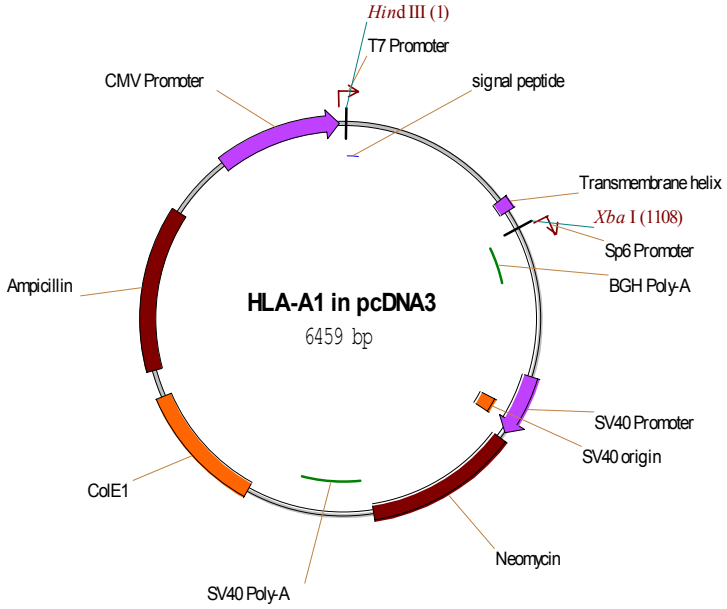
HLA-A2*02011 

β2MG 

Peptide : AAGIGILTV
Superpeptide : ELAGIGILTV

Linker10 : GGGGSSGGGS
Linker15 : GGGGSSGGGSGGGGS

Appendix F
Protein Sequences of HLA-A1 Construct




```
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     HindIII
     ~~~~~
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      G   A   L   A   L   T   Q   T   W   A   G   S   H   S   M   R   Y
  51  GGGGCCCTGG CCCTGACCCA GACCTGGGCG GGCTCCCACT CCATGAGGTA

        F   F   T   S   V   S   R   P   G   R   G   E   P   R   F   I
 101  TTTCTTCACA TCCGTGTCCC GGCCCGGCCG CGGGGAGCCC CGCTTCATCG

     A   V   G   Y   V   D   D   T   Q   F   V   R   F   D   S   D   A
 151 CCGTGGGCTA CGTGGACGAC ACGCAGTTCG TGCGGTTCGA CAGCGACGCC

      A   S   Q   K   M   E   P   R   A   P   W   I   E   Q   E   G   P
 201 GCGAGCCAGA AGATGGAGCC GCGGGCGCCG TGGATAGAGC AGGAGGGGCC

        E   Y   W   D   Q   E   T   R   N   M   K   A   H   S   Q   T
 251 GGAGTATTGG GACCAGGAGA CACGGAATAT GAAGGCCCAC TCACAGACTG

     D   R   A   N   L   G   T   L   R   G   Y   Y   N   Q   S   E   D
 301 ACCGAGCGAA CCTGGGGACC CTGCGCGGCT ACTACAACCA GAGCGAGGAC

      G   S   H   T   I   Q   I   M   Y   G   C   D   V   G   P   D   G
 351 GGTTCTCACA CCATCCAGAT AATGTATGGC TGCGACGTGG GGCCGGACGG
```

```
       R  F  L   R  G  Y  R   Q  D  A   Y  D  G   K  D  Y
401  GCGCTTCCTC CGCGGGTACC GGCAGGACGC CTACGACGGC AAGGATTACA

     I  A  L  N   E  D  L   R  S  W  T   A  A  D   M  A  A
451  TCGCCCTGAA CGAGGACCTG CGCTCTTGGA CCGCGGCGGA CATGGCAGCT

      Q  I  T  K   R  K  W   E  A  V   H  A  A  E   Q  R  R
501  CAGATCACCA AGCGCAAGTG GGAGGCGGTC CATGCGGCGG AGCAGCGGAG

       V  Y  L   E  G  R  C   V  D  G   L  R  R   Y  L  E
551  AGTCTACCTG GAGGGCCGGT GCGTGGACGG GCTCCGCAGA TACCTGGAGA

     N  G  K  E   T  L  Q   R  T  D  P   P  K  T   H  M  T
601  ACGGGAAGGA GACGCTGCAG CGCACGGACC CCCCCAAGAC ACATATGACC

      H  H  P  I   S  D  H   E  A  T   L  R  C  W   A  L  G
651  CACCACCCCA TCTCTGACCA TGAGGCCACC CTGAGGTGCT GGGCCCTGGG

       F  Y  P   A  E  I  T   L  T  W   Q  R  D   G  E  D
701  CTTCTACCCT GCGGAGATCA CACTGACCTG GCAGCGGGAT GGGGAGGACC

     Q  T  Q  D   T  E  L   V  E  T  R   P  A  G   D  G  T
751  AGACCCAGGA CACGGAGCTC GTGGAGACCA GGCCTGCAGG GGATGGAACC

      F  Q  K  W   A  A  V   V  V  P   S  G  E  E   Q  R  Y
801  TTCCAGAAGT GGGCGGCTGT GGTGGTGCCT TCTGGAGAGG AGCAGAGATA

       T  C  H   V  Q  H  E   G  L  P   K  P  L   T  L  R
851  CACCTGCCAT GTGCAGCATG AGGGTCTGCC CAAGCCCCTC ACCCTGAGAT

     W  E  L  S   S  Q  P   T  I  P  I   V  G  I   I  A  G
901  GGGAGCTGTC TTCCCAGCCC ACCATCCCCA TCGTGGGCAT CATTGCTGGC

      L  V  L  L   G  A  V   I  T  G   A  V  V  A   A  V  M
951  CTGGTTCTCC TTGGAGCTGT GATCACTGGA GCTGTGGTCG CTGCCGTGAT

       W  R  R   K  S  S  D   R  K  G   G  S  Y   T  Q  A
1001 GTGGAGGAGG AAGAGCTCAG ATAGAAAAGG AGGGAGTTAC ACTCAGGCTG

     A  S  S  D   S  A  Q   G  S  D  V   S  L  T   A  C  K
1051 CAAGCAGTGA CAGTGCCCAG GGCTCTGATG TGTCTCTCAC AGCTTGTAAA

      V  *  S  R
            XbaI
           ~~~~~~~
1101 GTGTGATCTA GA
```

Appendix G
Protein Sequences of HIV Vaccines

```
          EcoRI
          ~~~~~~
 751      GAATTCGC CAAGTTTGTT TCATGACAAA AGCCTTAGGC ATCTCCTATG

 801   GCAGGAAGAA GCGGAGACAG CGACGAAGAG CTCATCAGAA CAGTCAGACT

 851   CATCAAGCTT CTCTATCAAA GCAGTAAGTA GTACATGTAA TGCAACCTAT

 901   AATAGTAGCA ATAGTAGCAT TAGTAGTAGC AATAATAATA GCAATAGTTG

 951   TGTGGTCCAT AGTAATCATA GAATATAGGA AAATATTAAG ACAAAGAAAA

1001   ATAGACAGGT TAATTGATAG ACTAATAGAA AGAGCAGAAG ACAGTGGCAA

1051   TGAGAGTGAA GGAGAAGTAT CAGCACTTGT GGAGATGGGG GTGGAAATGG

   +3                                      S  A  T  E  K  L
1101   GGCACCATGC TCCTTGGGAT ATTGATGATC TGTAGTGCTA CAGAAAAATT

   +3   W  V  T  V  Y  Y  G  V  P  V  W  K  E  A  T  T
1151   GTGGGTCACA GTCTATTATG GGGTACCTGT GTGGAAGGAA GCAACCACCA

   +3 T  L  F  C  A  S  D  A  K  A  Y  D  T  E  V  H  N
1201   CTCTATTTTG TGCATCAGAT GCTAAAGCAT ATGATACAGA GGTACATAAT

   +3  V  W  A  T  H  A  C  V  P  T  D  P  N  P  Q  E  V
1251   GTTTGGGCCA CACATGCCTG TGTACCCACA GACCCCAACC CACAAGAAGT

   +3   V  L  V  N  V  T  E  N  F  N  M  W  K  N  D  M
1301   AGTATTGGTA AATGTGACAG AAAATTTTAA CATGTGGAAA AATGACATGG

   +3 V  E  Q  M  H  E  D  I  I  S  L  W  D  Q  S  L  K
1351   TAGAACAGAT GCATGAGGAT ATAATCAGTT TATGGGATCA AAGCCTAAAG

   +3  P  C  V  K  L  T  P  L  C  V  S  L  K  C  T  D  L
1401   CCATGTGTAA AATTAACCCC ACTCTGTGTT AGTTTAAAGT GCACTGATTT

   +3   K  N  D  T  N  T  N  S  S  S  G  R  M  I  M  E
1451   GAAGAATGAT ACTAATACCA ATAGTAGTAG CGGGAGAATG ATAATGGAGA

   +3 K  G  E  I  K  N  C  S  F  N  I  S  T  S  I  R  D
1501   AAGGAGAGAT AAAAAACTGC TCTTTCAATA TCAGCACAAG CATAAGAGAT

   +3  K  V  Q  K  E  Y  A  F  F  Y  K  L  D  I  V  P  I
1551   AAGGTGCAGA AAGAATATGC ATTCTTTTAT AAACTTGATA TAGTACCAAT
```

```
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   +3 Q A C P   K V S   F E P I   P I H   Y C A
1651  AGGCCTGTCC AAAGGTATCC TTTGAGCCAA TTCCCATACA TTATTGTGCC

   +3  P A G F   A I L   K C N   N K T F   N G T
1701  CCGGCTGGTT TTGCGATTCT AAAATGTAAT AATAAGACGT TCAATGGAAC

   +3   G P C   T N V S   T V Q   C T H   G I R
1751  AGGACCATGT ACAAATGTCA GCACAGTACA ATGTACACAT GGAATCAGGC

   +3 P V V S   T Q L   L L N G   S L A   E E D
1801  CAGTAGTATC AACTCAACTG CTGTTAAATG GCAGTCTAGC AGAAGAAGAT

   +3  V V I R   S A N   F T D   N A K T   I I V
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   +3   Q L N   T S V E   I N C   T R P   N N N
1901  ACAGCTGAAC ACATCCGTAG AAATTAATTG TACAAGACCC AACAACAATA

   +3 T R K S   I R I   Q R G P   G R A   F V T
1951  CAAGAAAAAG TATCCGTATC CAGAGGGGAC CAGGGAGAGC ATTTGTTACA

   +3  I G K I   G N M   R Q A   H C N I   S R A
2001  ATAGGAAAAA TAGGAAATAT GAGACAAGCA CATTGTAACA TTTCTAGAGC

   +3   K W N   A T L K   Q I A   S K L   R E Q
2051  AAAATGGAAT GCCACTTTAA AACAGATAGC TAGCAAATTA AGAGAACAAT

   +3 F G N N   K T I   I F K Q   S S G   G D P
2101  TTGGAAATAA TAAAACAATA ATCTTTAAGC AATCCTCAGG AGGGGACCCA

   +3  E I V T   H S F   N C G   G E F F   Y C N
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   +3   S T Q   L F N S   T W F   N S T   W S S
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   +3 E G S N   N T E   G S D T   I T L   P C R
2251  AAGGGTCAAA TAACACTGAA GGAAGTGACA CAATCACACT CCCATGCAGA

   +3  I K Q F   I N M   W Q E   V G K A   M Y A
2301  ATAAAACAAT TTATAAACAT GTGGCAGGAA GTAGGAAAAG CAATGTATGC

   +3   P P I   S G Q I   R C S   S N I   T G L
2351  CCCTCCCATC AGTGGACAAA TTAGATGTTC ATCAAATATT ACTGGGCTGC

   +3 L L T R   D G G   N N N N   G S E   I F R
2401  TATTAACAAG AGATGGTGGT AATAACAACA ATGGGTCCGA GATTTTCAGA
```

```
   +3  P  G  G  G   D  M  R   D  N  W   R  S  E  L   Y  K  Y
2451  CCTGGAGGAG GCGATATGAG GGATAATTGG AGAAGTGAAT TATATAAATA

   +3   K  V  V   K  I  E  P   L  G  V   A  P  T   K  A  K
2501  TAAAGTAGTA AAAATTGAAC CATTAGGAGT AGCACCCACC AAGGCAAAGA

   +3 R  R  V  V   Q  R  E   K  R  A  V  G/*  I  G   A  L  F
                                    ENV-STOP primer
                           ===============TGA================
                               BssHII
                               ~~~~~~
2551  GACGCGTGGT GCAGAGAGAA AAGCGCGCAG TGGGAATAGG AGCTCTGTTC

   +3  L  G  F  L   G  A  A   G  S  T   M  G  A  A   S  M  T
       ======
2601  CTTGGGTTCT TGGGAGCAGC AGGAAGCACT ATGGGCGCAG CGTCAATGAC

   +3   L  T  V   Q  A  R  Q   L  L  S   D  I  V   Q  Q  Q
2651  GCTGACGGTA CAGGCCAGAC AATTATTGTC TGATATAGTG CAGCAGCAGA

   +3 N  N  L  L   R  A  I   E  A  Q  Q   H  L  L   Q  L  T
2701  ACAATTTGCT GAGGGCTATT GAGGCGCAAC AGCATCTGTT GCAACTCACA

   +3  V  W  G  I   K  Q  L   Q  A  R   I  L  A  V   E  R  Y
2751  GTCTGGGGCA TCAAACAGCT CCAGGCAAGA ATCCTGGCTG TGGAAAGATA

   +3   L  K  D   Q  Q  L  L   G  I  W   G  C  S   G  K  L
2801  CCTAAAGGAT CAACAGCTCC TGGGGATTTG GGGTTGCTCT GGAAAACTCA

   +3 I  C  T  T   A  V  P   W  N  A  S   W  S  N   K  S  L
2851  TTTGCACCAC TGCTGTGCCT TGGAATGCTA GTTGGAGTAA TAAATCTCTG

   +3  E  Q  I  W   N  N  M   T  W  M   E  W  D  R   E  I  N
2901  GAACAGATTT GGAATAACAT GACCTGGATG GAGTGGGACA GAGAAATTAA

   +3   N  Y  T   S  L  I  H   S  L  I   E  E  S   Q  N  Q
2951  CAATTACACA AGCTTAATAC ACTCCTTAAT TGAAGAATCG CAAAACCAGC

   +3 Q  E  K  N   E  Q  E   L  L  E  L   D  K  W   A  S  L
3001  AAGAAAAGAA TGAACAAGAA TTATTGGAAT TAGATAAATG GGCAAGTTTG

   +3  W  N  W  F   N  I  T   N  W  L   W  Y  I  K   L  F  I
3051  TGGAATTGGT TTAACATAAC AAATTGGCTG TGGTATATAA AATTATTCAT

   +3   M  I  V   G  G  L  V   G  L  R   I  V  F   A  V  L
3101  AATGATAGTA GGAGGCTTGG TAGGTTTAAG AATAGTTTTT GCTGTACTTT

   +3 S  I  V  N   R  V  R   Q  G  Y  S   P  L  S   F  Q  T
3151  CTATAGTGAA TAGAGTTAGG CAGGGATATT CACCATTATC GTTTCAGACC
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   +3  H  L  P  I   P  R  G   P  D  R   P  E  G  I   E  E  E
                  AvaI
                 ~~~~~~
3201  CACCTCCCAA TCCCGAGGGG ACCCGACAGG CCCGAAGGAA TAGAAGAAGA

   +3   G  G  E   R  D  R  D   R  S  I   R  L  V   N  G  S
3251  AGGTGGAGAG AGAGACAGAG ACAGATCCAT TCGATTAGTG AACGGATCCT

   +3 L  A  L  I   W  D  D   L  R  S  L   C  L  F   S  Y  H
3301  TAGCACTTAT CTGGGACGAT CTGCGGAGCC TGTGCCTCTT CAGCTACCAC

   +3  R  L  R  D   L  L  L   I  V  T   R  I  V  E   L  L  G
3351  CGCTTGAGAG ACTTACTCTT GATTGTAACG AGGATTGTGG AACTTCTGGG

   +3   R  R  G   W  E  A  L   K  Y  W   W  N  L   L  Q  Y
3401  ACGCAGGGGG TGGGAAGCCC TCAAATATTG GTGGAATCTC CTACAGTATT

   +3 W  G  Q  E   L  K  N   S  A  V  N   L  L  N   A  T  A
3451  GGGGTCAGGA ACTAAAGAAT AGTGCTGTTA ACTTGCTCAA TGCCACAGCC

   +3  I  A  V  A   E  G  T   D  R  V   I  E  V  L   Q  A  A
3501  ATAGCAGTAG CTGAGGGGAC AGATAGGGTT ATAGAAGTAT TACAAGCAGC

   +3   Y  R  A   I  R  H  I   P  R  R   I  R  Q   G  L  E
3551  TTATAGAGCT ATTCGCCACA TACCTAGAAG AATAAGACAG GGCTTGGAAA

   +3 R  I  L  L   *
                   ApaI
                  ~~~~~~
3601  GGATTTTGCT ATAAGGGCCC GTTTAAACCC GCTGATCAG
```