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ELICITATION AND ENHANCEMENT OF T AND B CELL RESPONSES

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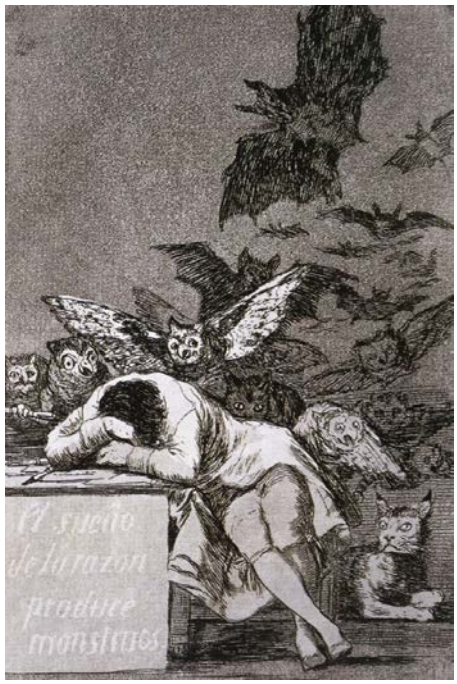

Francisco de Goya, The Sleep of Reason Produces Monsters, 1797

ABSTRACT

The Major histocompatibility complex class I (MHC-I) has been characterized in such great depth that a number of its key properties are well understood and part of its behavior can even be predicted. It is therefore intriguing that the impact of small epitope modifications on immunogenicity and the elicitation of T-cell repertoires remain often unpredictable and full of surprises.

Substitution of the secondary anchor residue at peptide position 3 from a serine to a proline (p3P) significantly increased the stabilization capacity and immunogenicity of the melanoma-associated H-2D^b (D^b)-restricted epitope gp100 (EGS). Despite this strong enhancement the conformation of the modified epitope (EGP) was not altered and vaccination with EGP generated T-cell responses that recognized cells expressing EGS with high functional avidity. Based on these promising results, the p3P modification was applied to the highly immunodominant *Lymphocytic Choriomeningitis Virus* epitope gp33 and the associated escape variants Y4F and Y4A. As for gp100, p3P was found to increase the MHC stabilization capacity and immunogenicity of the modified epitopes V3P, PA and PF, while not altering their structures. Accordingly, T-cell responses were cross-reactive between native and p3P enhanced epitopes and, when used for vaccination of C57BL/6 mice, PF elicited a focused T-cell response against D^b/Y4F.

In parallel, surface plasmon resonance (SPR) measurements revealed that p3P did not only enhance MHC stabilization capacity but also directly increased the affinity of the cognate T-cell receptors (TCRs). To fully characterize the molecular details underlying these two enhancing effects, the thermostability, TCR binding and molecular dynamics (MD) of D^b/EGP were measured in comparison with D^b/EGS. Furthermore, the contribution of Y159, a highly conserved tyrosine that is structurally juxtaposed to p3P, was assessed using a set of soluble D^b-Y159 variants. In conclusion, these measurements clearly demonstrated that specific interactions of p3P with the aromatic ring of Y159 are responsible for the significantly increased MHC stabilization capacity. Surprisingly, the enhanced TCR binding was found to be entirely independent of Y159, suggesting a direct contribution of the buried proline residue to TCR binding. These findings underscore the potential to enhance MHC-I-restricted epitopes at secondary anchor residues, while specifically indicating that proline can directly increase TCR affinity, which could not have been anticipated from our current understanding of the factors shaping TCR recognition.

Not entirely different from T-cell elicitation, the induction of broadly neutralizing antibodies against the *Human Immunodeficiency Virus* type 1 (HIV-1) is to date still an elusive goal despite extensive characterization of the respective antibody-epitope interactions. One of the central challenges is that the virus is highly adapted to immune pressure and the most relevant antibody epitopes on the HIV envelope proteins (Env) are the least immunogenic. Therefore, a highly heterologous prime-boost vaccination strategy was designed in which priming of rabbits with HIV-1 *env* plasmids was followed by a

recombinant *Simian Immunodeficiency Virus* (SIV) Env boost. While the SIV Env trimers were inherently favorable because of their higher stability, the approach was specifically chosen to preferentially boost antibody responses against the few sites that are conserved in HIV and SIV Env. The described approach was generally validated and warrants future investigations as it lead to the elicitation of potent neutralizing antibodies even though it remains to be fully established if the highly heterologous nature of the prime boost strategy was solely responsible.

In summary, the studies presented in this thesis provide the structural and functional platform for a novel and intriguing MHC-I peptide enhancement. Additionally, heterologous immunizations of rabbits offer a promising addition to existing vaccination strategies against HIV.

PUBLICATIONS

This thesis is based on the following publications and manuscripts

I. **Uchtenhagen H**, Abualrous ET, Stahl E, Loschinski K, Sluijter M, van Hall T, Nygren PÅ, Springer S & Achour A **Molecular basis underlying enhanced immunogenicity of optimized H-2D^b-restricted melanoma-associated epitopes** *Manuscript*

II. Allerbring EB*, Duru AD*, **Uchtenhagen H**, Madhurantakam C, Tomek MB, Grimm S, Mazumdar PA, Friemann R, Uhlin M, Sandalova T, Nygren PÅ* & Achour A.* **The unexpected T-cell recognition of an altered peptide ligand is driven by reversed thermodynamics** *Eur J Immunol. 2012 Jul 26. doi: 10.1002/eji.201242588.*

III. Duru AD*, Allerbring EB*, **Uchtenhagen H**, Gonzalez FE, Mazumdar PA, Badia-Martinez D, Madhurantakam C, Sandalova T, Nygren PÅ & Achour A. **Induction of efficient CTL responses against a viral escape mutant through an unconventional peptide optimization** *Manuscript*

IV. **Uchtenhagen H**, Sourial S, Friemann R, Ehnlund M, Spetz AL, Harris RA, Madhurantakam C & Achour A. **Production, purification, crystallization and preliminary X-ray diffraction analysis of the HIV-2-neutralizing V3 loop-specific Fab fragment 7C8** *Acta Crystallogr Sect F Struct Biol Cryst Commun. 2009 Jul 1;65(Pt 7):705-8*

V. **Uchtenhagen H**, Friemann R, Raszewski G, Spetz AL, Nilsson L & Achour A. **Crystal structure of the HIV-2 neutralizing Fab fragment 7C8 with high specificity to the V3 region of gp125** *PLoS One. 2011 Apr 26;6(4):e18767.*

VI. **Uchtenhagen H***, Schiffner T*, Bowles EJ, Heyndrickx L, LaBranche C, Applequist S, Jansson M, Achour A, Fomsgaard A, Montefiori D, Stewart-Jones GB* & Spetz AL*. **SIVmac239 gp140 trimers induce potent cross-reactive HIV-1 neutralization when primed with HIV-1 Envelope DNA mixture** *Manuscript*

VII. Ozkaya Sahin G, Bowles EJ, Parker J, **Uchtenhagen H**, Sheik-Khalil E, Taylor S, Pybus OG, Mäkitalo B, Walther-Jallow L, Spångberg M, Thorstensson R, Achour A, Fenyö EM, Stewart-Jones GB & Spetz AL. **Generation of neutralizing antibodies and divergence of SIVmac239 in cynomolgus macaques following short-term early antiretroviral therapy.** *PLoS Pathog. 2010 Sep 2;6(9):e1001084.*

VIII. Borggren M, Repits J, Sterjovski J, **Uchtenhagen H**, Churchill MJ, Karlsson A, Albert J, Achour A, Gorry PR, Fenyö EM & Jansson M. **Increased sensitivity to broadly neutralizing antibodies of end-stage disease R5 HIV-1 correlates with evolution in Env glycosylation and charge**. *PLoS One. 2011;6(6):e20135.*

The following publications and manuscripts were obtained during the course of the education but are outside the scope of the thesis

I. Li NL, Fu L, **Uchtenhagen H**, Achour A & Burshtyn DN. **Cis association of leukocyte Ig-like receptor 1 with MHC class I modulates accessibility to antibodies and HCMV UL18**. *Manuscript*

II. Boulanger B*, Hein Z*, **Uchtenhagen H**, Abualrous ET, Achour A, Zacharias M, Werner J, Elliott T, & Springer S. **Restricted movement of the F pocket region triggers the cell surface transport of MHC class I molecules**. *Manuscript*

III. Nilsson OB, Bronge M, Grundström J, Sarma R, **Uchtenhagen H**, Kikhney A, Sandalova T, Holmgren E, Svergun D, Achour A, van Hage M & Grönlund H. **A recombinant tetramerical dog allergen vaccine molecule – a viable alternative for diagnosis and treatment of dog allergic patients.** *Manuscript*

IV. Brinckmann S, da Costa K, van Gils MJ, Hallengärd D, Klein K, Madeira L, Mainetti L, Palma P, Raue K, Reinhart D, Reudelsterz M, Ruffin N, Seifried J, Schäfer K, Sheik-Khalil E, Sköld A, **Uchtenhagen H**, Vabret N, Ziglio S, Scarlatti G, Shattock R, Wahren B & Gotch F. **Rational design of HIV vaccines and microbicides: report of the EUROPRISE network annual conference 2010.** *J Transl Med. 2011 Apr 12;9:40.*

V. Wahren B, Biswas P, Borggren M, Coleman A, Da Costa K, De Haes W, Dieltjens T, Dispinseri S, Grupping K, Hallengärd D, Hornig J, Klein K, Mainetti L, Palma P, Reudelsterz M, Seifried J, Selhorst P, Sköld A, **Uchtenhagen H**, van Gils MJ, Weber C, Shattock R & Scarlatti G. **Rational design of HIV vaccine and microbicides: report of the EUROPRISE annual conference.** *J Transl Med. Jul 26;8:72. (correction in J Transl Med. 2010 Sep 3;8(1):82).*

VI. Madhurantakam C, Nilsson OB, **Uchtenhagen H**, Konradsen J, Saarne T, Högbom E, Sandalova T, Grönlund H & Achour A. **Crystal structure of the dog lipocalin allergen Can f 2: implications for cross-reactivity to the cat allergen Fel d 4.** *J Mol Biol. 2010 Aug 6;401(1):68-83*

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LIST OF ABBREVIATIONS

MHC-I/II	Major histocompatibility complex class I/II
pMHC	MHC-I peptide complex
p3P	Proline at position 3 of MHC-I restricted epitopes
D^b	H-2D^b
HIV-1	*Human Immunodeficiency Virus* type 1
SIV	*Simian Immunodeficiency Virus*
Env	SIV/HIV envelope protein complex
NAb	Neutralizing antibody
ART	Antiretroviral therapy
APCs	Antigen presenting cells
pAPCs	Professional APCs
DCs	Dendritic Cells
CTLs	$CD8^+$ cytotoxic T-cells
BCR	B-cell receptor
TCR	T-cell receptor
β_2m	β2-microglobulin
CD4bs	CD4 binding site
CDRs	Complementarity determining regions
TRBV	TCR β variable chain
MD	Molecular dynamics
NMR	Nuclear magnetic resonance
SPR	Surface plasmon resonance
ITC	Isothermal titration calorimetry
APL	Altered peptide ligand
AIDS	Acquired immunodeficiency syndrome
HAART	Highly active antiretroviral therapy
cryo-EM	Cryo-electron microscopy
Ig	Immunoglobulin
pdb	Protein Data Bank
CD	Circular dichroism

1 INTRODUCTION

1.1 Some fundamental principles of adaptive immune recognition

The principle features of our adaptive immune system represent a beautiful solution to an incredibly complicated question. This is represented by the need to recognize yet unknown antigens in near unimaginable variations while not damaging the host. In an intriguing way the immune system meets the challenges through an intrinsic ability to 'simply' react against everything, while being specifically instructed only to avoid self.

1.1.1 Antigen presentation with an emphasis on MHC-I

Independently from the division between its adaptive and innate arms, the functions of the immune system can also be described as the abilities to recognize antigens and to mount effector responses against the associated pathogens. Presentation of antigens to the immune system is a crucial part of the actual T-cell recognition and the rules of the latter are significantly shaped by the properties of the former. The 'unit of presentation' in antigen recognition is defined by the class I and II major histocompatibility complexes (MHC-I and –II, respectively), which present peptide antigens on the cell surface to T cells and natural killer (NK) cells. The ubiquitous MHC-I molecules present intracellularly processed peptide antigens on the surface of practically all cells in the body, turning them into antigen presenting cells (APCs) [6,7], ultimately allowing T-cells to recognize and eliminate cells that harbor intracellular pathogens. In contrast, MHC-II molecules, which present mainly extracellular peptide antigens, are restricted to professional APCs (pAPCs) including dendritic cells (DCs), B-cells and Macrophages. MHC-II/peptide complexes are recognized by $CD4^+$ T helper cells and therefore they play a central role in stimulation of full immune responses [8]. A main focus of this thesis is the MHC-I-restricted antigen presentation and antigen recognition by $CD8^+$ cytotoxic T-cells (CTLs).

The MHCs owe their original description and name to their key role in determining rejection or acceptance of (tumor) transplants [9]. This in turn is based on the high polymorphism of the MHC, which provides this unique unit of presentation with the capacity to present a very wide range of different peptide antigens in different individuals [10,11]. This polymorphism defines that the 'self' in adaptive immunity is really the individual and not for example the species. While MHC restriction and peptide presentation will be discussed in detail below, it should be mentioned already here that a defining feature of peptide presentation and of the entire adaptive immune system is that the presented peptide antigens are not specifically pathogenic or 'foreign' but rather constitute a more or less representative sample of intra- and extracellular antigens [12,13]. This appears to be a sensible characteristic, given the degree to which viruses rely on the host machinery for biosynthesis. Although highly hypothetical, it could also be speculated

that this allows for a more efficient reaction against members of the same species (alloreactivity) as well as against antigens associated with developing malignancies.

1.1.2 "Nothing in biology makes sense except in the light of…" Lymphocyte selection in T-cell immunity

Attempting to explain the properties of our T- and B-cell repertoires, one must account for their ability to (**I**) not react against itself (tolerance), (**II**) recognize a virtually unlimited variation of antigens, (**III**) be able to do so even before their appearance in the host and (**IV**) alloreactivity determined by MHC disparity. Awareness of alloreactivity in combination with **I** and **II** was an early and principal driving force for basic research into the fundamentals of the immune system [9,14-16]. A huge leap forward in a conceptual understanding of this system was derived from the theory of clonal selection and the formal recognition that **III** essentially ruled out any form of *de novo*, antigen-induced selection for the ability of the immune system to react to foreign antigens [17,18].

The cellular solution to this is somewhat indirect in that a foreign antigen is not recognized on the basis of a specific structural property distinguishing it from 'self', but on the basis of a higher avidity compared to 'self'. Antigen recognition is mediated by a vast repertoire of B- and T-cells of which each member expresses one variant of an equally huge, randomly assembled, diverse and cross-reactive repertoire of B- and T- cell receptors (BCR and TCR, respectively) [17-19]. This fixed link between individual B- and T-cells and their antigen-specificity is the basis of a central and constitutive positive and negative selection in which cells are eliminated or stimulated to survive based on their specificity, and which takes place in the thymus and bone marrow during T- and B-cell development, respectively.

Positive selection assures that all surviving B- and T-cells display BCRs that are properly folded, or TCRs with the capacity to recognize MHC/peptide complexes (pMHCs) [20,21]. Tolerance is subsequently acquired through negative selection, which eliminates all B- and T-cells that recognize 'self' antigens with too high avidity [15,16,18,22]. This two-step selection results in a repertoire of B- and T-cells with the *a priori* capacity to recognize a virtually unlimited variation of antigens while remaining tolerant to 'self'. Furthermore, it also means that the meaning of 'self' could be translated to *'whatever is presented in the thymus/bone marrow during negative selection'* rather than being taken literally. Furthermore, the presence of infection-related signals is a necessary condition for T-cell activation providing an additional mechanism that assures tolerance to 'self' [23]. Sometimes described as contrasting [24], one could combine central and peripheral tolerance by stating that the immune system achieves self/non-self specificity by recognizing as 'self' *'everything that is presented in the thymus/bone marrow during negative selection or recognized without simultaneous signs of infection'*.

1.1.3 TCR-pMHC interactions and T-cell activation

The positive selection of T-cells is based on their capacity to recognize pMHCs rather than controlling only for the proper folding of the receptor as in the case of the BCRs [20,25]. This is explained by the strict MHC-restriction of T-cell responses, in which TCRs recognize a mixed surface composed by the MHC peptide-binding cleft and the presented peptide [1,11,26,27]. In order to be recognized, a presented 'foreign' peptide must therefore sufficiently transform the mixed surfaces encountered during negative selection – a phenomenon aptly described as 'altered self' before many of the structural details were established [28]. In the case of alloreactivity, T-cells encounter MHC-peptide interfaces that are sufficiently different from the ones encountered during negative selection but sufficiently similar to the ones encountered during positive selection [29].

It is interesting to note that alloreactivity has been implicated in the evolution of the adaptive immune system, e.g. [30-32], as it could be argued to be one of the characteristics that most critically depends on MHC restriction and its individualization of the unit of presentation.

The exact binding requirements and nature of the outside-in signaling of TCRs are still not exactly understood (*e.g.* how the T-cell is triggered). Regardless, productive recognition of pMHCs by a naïve T-cell is required for activation, leading to clonal expansion and the killing of target cells, which carry appropriate pMHCs by primed CTLs [33,34]. Notably, this process is not binary and different degrees of T-cell activation can result from differences in ligand density and/or TCR-pMHC interactions [35-38] (See 1.3.2ff).

1.2 More details about selected features of MHC-I antigen presentation

1.2.1 Molecular details of peptide binding by MHC-I

Although this section intends to focus on the details of MHC class I-restricted peptide presentation, it should be noted that several of the structural and biophysical features that define peptide binding and presentation by MHC-I also apply to MHC-II [39]. X-ray crystallography has played, and still plays, a crucial role in the understanding of the molecular details underlying MHC antigen presentation and TCR-pMHC interactions. A number of remarkable contributions have provided detailed understanding of prevailing and/or competing hypotheses (see refs [40,41] for a short perspective on the first pMHC and TCR-pMHC structures). The first three-dimensional X-ray structure of an MHC-I molecule revealed the molecular architecture of HLA-A0201 and its novel peptide-binding domain [4] (Figure 1a). Most importantly, and despite the fact that it was not yet possible to determine the structure of individually bound peptides, this pioneering work by Pamela Bjorkman and Don Wiley provided the first structural description of peptide binding and a striking rationalization of MHC-restriction as well as the phenomenon of 'altered self' (Figure 1b). The crystal structure demonstrated that the pMHC complex

present a mixed interface, composed of the MHC heavy chain ('self') combined with the potentially foreign peptide, to the TCR [4,11]. Additionally, it clarified the role of the β2-microglobulin light chain (β_2m) as a non-covalently bound support molecule for the heavy-chain β sheets, which provide structural support to the peptide-binding cleft. The Bjorkman study demonstrated that polymorphisms between MHC alleles and isotypes could largely be mapped to the peptide-binding cleft. Consequentially, it was inferred that different MHC molecules have different peptide-binding preferences, presenting different peptide repertoires [4,11]. As differences in the peptide repertoire are expected to result in a distinct 'self' repertoire presented during the acquisition of tolerance, this study provided a compelling structural explanation for the observed correlation between MHC heterogeneity and alloreactivity in transplantation success [4,9,11,42]. A fascinating twist to this theme was provided by two recent studies, both of which demonstrated how a drug can cause autoimmunity through direct interaction with the peptide-binding cleft of HLA-B*57:01**,** thus altering the peptide repertoire presented in the periphery [43,44].

Structurally, the membrane-bound polymorphic heavy chain of MHC-I forms a heterodimer with the relatively conserved β_2m. The MHC-I heavy chain consists of the three extracellular domains α1, α2 and α3. The α1 and α2 domains form the peptide-binding groove through the assembly of eight antiparallel β-sheets whose edges are lined by two broken α-helixes. The formed groove, correctly assumed as the peptide-binding cleft [11], is oriented away from the cell membrane and thus perfectly suited for the presentation of peptide antigens to the immune system. The relatively more conserved α3 domain and the β_2m subunit form the base of the MHC heterodimer, connecting the peptide-binding groove to the transmembrane domain of the heavy chain (Figure 1a,b).

The subsequent determination of the crystal structures of different MHC-I alleles in complex with an array of immunodominant peptides [5,45-52] (Figure 1c) combined with the increased understanding obtained from the elution and sequencing of MHC-I bound peptides [53,54], allowed for a more detailed description of the rules that govern peptide binding by different MHC-I alleles as well as some general principles underlying TCR-recognition [55-60]. The peptides are tightly anchored to the MHC binding-groove through interactions between specific peptide side chains and MHC residues that co-localize in more or less deep pockets [42] (Figure 1c). The pockets have different sizes and chemical properties in different allelic variants of MHC class I molecules. Thereby they impose distinct sequence constraints on the presented peptides and determine that class I binding peptides are characterized by allele-specific sequence motifs. At least two of these peptide-binding pockets, of which one always localized at the C-terminus of the peptide, define the main anchor motif of the alleles and are highly selective for specific side-chains [39,61] (Figure 1c). While the main anchor residues contribute disproportionally to the strength of MHC-peptide interactions [62], it is important to point out that the entire length of the peptide makes extensive contacts with the peptide-binding cleft and that secondary anchor residues play

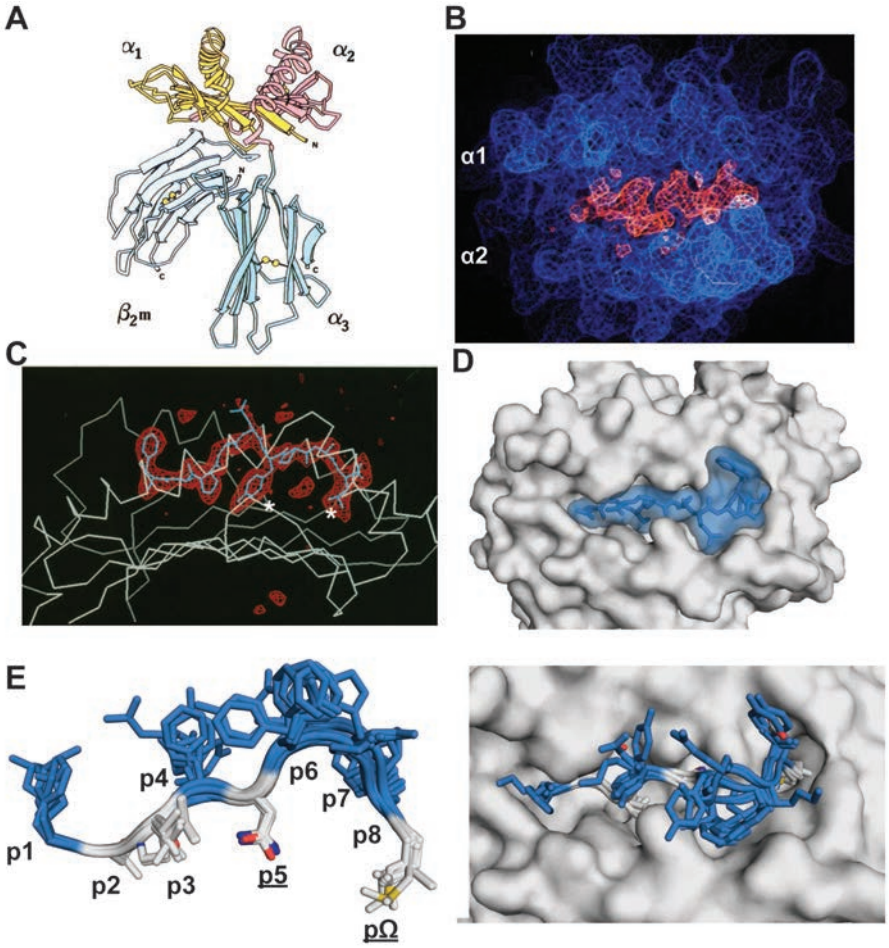

Figure 1: Peptide presentation by the MHC-I

A) Representation of the first MHC-I crystal structure revealing its unique peptide-binding cleft, the α_1, α_2, α_3 domains (yellow, salmon and blue, respectively) and the β_2m light chain (white). The molecule is displayed from the side with the truncated transmembrane domain oriented downwards. B) The electron density of the peptide binding cleft from the same structure is displayed in blue and the MHC shown from the perspective of the TCR. The electron density that was correctly assumed to represent bound peptides (red) provides a visualization of the concept of 'altered self'. C) H-2K^b/FAPGNYPAL, one of the first structures of an MHC-I molecule in which an individual peptide could be modeled. The peptide-binding cleft is displayed from the side, visualizing how the peptide (blue; electron density in red) is anchored to the MHC through specific side chains (white stars) while other side chains point towards the TCR. D) Surface representation of a pMHC (same orientation as in B), demonstrating how the entire peptide (blue) makes extensive contacts with the peptide-binding cleft (grey). E) Left panel: Superposition of a selection of peptides determined in complex with D^b that highlights the conserved position of the buried side chains (grey; main anchor residues underlined) as well the significant flexibility of the rest of the peptide (blue). Right panel: Same complexes shown from the perspective of the TCR to illustrate how the exposed side chain generate a unique antigenic identity for each pMHC complex – the altered self. Figures A, B and C have been reproduced with permission from [4] and [5], respectively.

an important role in tuning MHC-peptide interactions (e.g. refs. [58,60,63,64]) (Figure 1d). Consequently, it is important to note that the MHC-I binding affinity and stabilization capacity of peptides containing the preferred main anchor residues may range from extreme binding to very weak or no binding at all depending on their overall sequence.

In contrast to the buried anchor residues, large parts of the bound peptides display significant conformational freedom, with some of the peptide side chains pointing away from the MHC binding groove (Figure 1e). These residues generally contribute less to the allele-specific binding motif and are thus variable in both sequence and conformation. As a result, each peptide provides a unique chemical and conformational antigenic identity [45] to the mixed interface of MHC and bound peptide (Figure 1e). In conclusion, each presented peptide has side chains that point towards the peptide-binding grove, defining MHC-binding and residues pointing away, defining the antigenic identity. Additionally, several of the secondary anchor residues are sufficiently exposed to be able to contribute to the antigenic identity of the peptide and *vice versa*, stressing that there is no defined separation between the two roles (see 1.3.1).

1.2.2 Short introduction into MHC-I restricted antigen processing

Although, not the focus of this thesis, some principles of MHC-I antigen processing will be summarized here below, given its central role in antigen presentation and CTL priming *in vivo* (detailed descriptions and discussions of antigen processing are provided in *e.g.* [12,13,65]). In order to act as a 'window into the cell', MHC-I presents endogenous peptides derived for example from proteasomal and defective ribosomal translation products (DRiPs, defective ribosomal products) in a process that is dependent on TAP (transporter associated with antigen processing) and a whole range of proteasomes and chaperones, finally resulting in the assembly of pMHCs in the endoplasmic reticulum. Additionally, uninfected pAPCs have the capacity to cross-present exogenous antigens on MHC-I molecules thereby cross-priming CTLs with antigens delivered to the lymph node [66-69]. This seems to play a crucial role in the fast activation of T-cells without prior infection of pAPCs in the lymph nodes and allows the priming of CTLs specific to TAAs [70]. Additionally, and although the *in vivo* relevance of this process is only beginning to be established [71,72], pAPCs can directly acquire pMHCs from infected cells [73], a phenomenon termed cross-dressing [70].

All together, the details of cross-dressing, cross-presentation and of other aspects of antigen processing, such as immunoproteasome activity and intracellular compartmentalization, have the potential to further modulate T-cell activation and introduce some degree of foreign antigen bias to antigen presentation, which has been generally (including above) described as non-specific [12,74-77]. Furthermore, gap-junctions between cells have been demonstrated to be a source for MHC-restricted

antigens in target killing or cross-priming [78]. Finally, a number of studies have also reported and provided increasing examples of TAP-independent presentation of viral and tumor antigens, further increasing the complexity of endogenous class I antigen processing [79-83]. The ability of MHC-I molecules localized on the cell surface to bind exogenous antigens [84] is probably of limited relevance *in vivo* because of their low concentration, but should nevertheless be mentioned as this property forms the basis of the cellular MHC-I binding affinity and stability assays applied in Paper **II**.

1.2.3 Stability and density of pMHCs on the cell surface

As only a very small portion of the pMHCs can be expected to carry 'foreign' peptides, T-cells require the ability to quickly scan a large number of pMHCs and the sensitivity to become activated by a highly limited fraction of cognate antigens (see 1.3.2) [85,86]. A defining feature of pMHC-I complexes is that the bound peptides are required for complex stability [84] and peptide-deficient MHCs rapidly disappear from the cell surface [87,88]. Therefore, the binding affinity of peptides, and most importantly, their MHC stabilization capacity determines the density and the half-life of pMHCs on the cell surface [87-89]. pMHC density and half-life in turn are important parameters that tune the activation of T-cells and thus MHC-I stabilization capacity contributes significantly to the immunogenicity and the immunodominance hierarchies of MHC-I restricted peptides antigens (see 1.3.3) e.g. [36-38,75,90-94]. As a rersult, peptides that are presented by a specific MHC but have very poor stabilization capacity can be expected to cause only limited deletion of T-cells during negative selection tolerance even if they are 'self' and in principle presented on MHCs. This is believed to contribute to the fact that many promising TAAs (defined through the identification of respective CTL clones) have low MHC stabilization capacity and directly related to the work presented in Manuscript **I**, which characterizes the enhancement of a poorly binding TAA that can be recognized by high-avidity CTLs [95-99].

1.3 CTL activation with an emphasis on altered peptide ligands and TCR-pMHC interactions

1.3.1 Structural details of TCR-pMHC interactions

The first X-ray crystal structures of ternary TCR-pMHC complexes provided the ultimate validation of the 'altered self' hypothesis for T-cell recognition [1,26] (Figure 2) and, together with subsequent studies, delivered detailed structural characterization of how TCRs recognize mixed MHC/peptide interfaces (see refs. [100-102] for comprehensive reviews). Limited buried surface area and shape complementarity generally characterizes the low-affinity TCR-pMHC complexes (Figure 2c). In general, TCRs bind MHC/peptide interfaces with a relatively conserved diagonal orientation centered on the middle part of the presented peptides. The diagonal binding mode of TCRs leads to a predominant, but

not exclusive interaction of the most variable, recombined third complementarity determining regions (CDRs) with the presented peptides. Conversely the germline-encoded CDRs 1 and 2 generally interact more but not exclusively with the MHC commonly involving a set of specific exposed MHC residues, which are more or less conserved between allelic variants [103,104] (Figure 2). Accordingly, several 'hot-spots' on that contribute significantly to TCR-pMHC interactions have been identified on the MHCs [101] (Figure 2b). However, comparative structural analysis also demonstrated that CDRs display limited but important flexibility providing them with the capacity to adapt to pMHCs [105]. A beautiful study by Stephanie Gras *et al.* demonstrated how the capacity of TCRs to recognize a specific pMHC complex could be constrained by negative selection providing a structural visualization of the consequence of acquired tolerance [106]. Fittingly, a number of ternary X-ray structures of allo- and autoreactive TCRs suggested that their often-unusual binding modes are driven by thymic selection and by the deletion of conventionally binding TCRs [107]. Finally, a series of studies determined the X-ray structures of TCR-pMHC complexes in which functional differences were associated to limited structural changes and could not be directly correlated (see 1.3.3) [108-110].

Despite the prominence of the central part of the peptide, it has to be stressed that potentially all peptide residues can contribute to the antigenic identity of the MHC-restricted epitope and that it is still notoriously difficult to predict the consequences of peptide modification on TCR interaction unless done with a specific and structurally characterized TCR/MHC pair. Finally, it is also important to highlight that our current structural understanding of TCR-pMHC interactions is probably biased and incomplete. A few TRBV (TCR β variable chain) genes are strongly overrepresented in the relatively short list of determined TCR-pMHC structures as they are seemingly more amendable to protein crystallography. Namely, of the roughly 37 unique TCR-pMHC complexes, more than half of the TCRs contain either the murine TRBV13 V_β domain or the highly related human TRBV6 V_β domain (http://imgt.cines.fr [111]). Further studies that make use of molecular dynamic simulations (MD simulation), NMR (nuclear magnetic resonance), spectroscopy and several other methods generate important data that complements the largely static information provided by X-ray crystallography. These approaches have been used to characterize the dynamic aspects of MHC-bound peptides and TCR-pMHC interactions, which have been difficult to explain by X-ray crystallography. The research group of Brian Baker has provided several examples of such studies including the use of Deuterium exchange and MD simulations to analyze the mechanism for signaling in which TCRs lose flexibility upon binding to pMHCs [112]. Additionally, NMR, anisotropic fluorescence measurements and MD simulations have been successfully used to explain how the structurally conserved enhancement of a main anchor residue resulted in increased peptide flexibility, leading to disturbed rather than increased TCR binding [113,114].

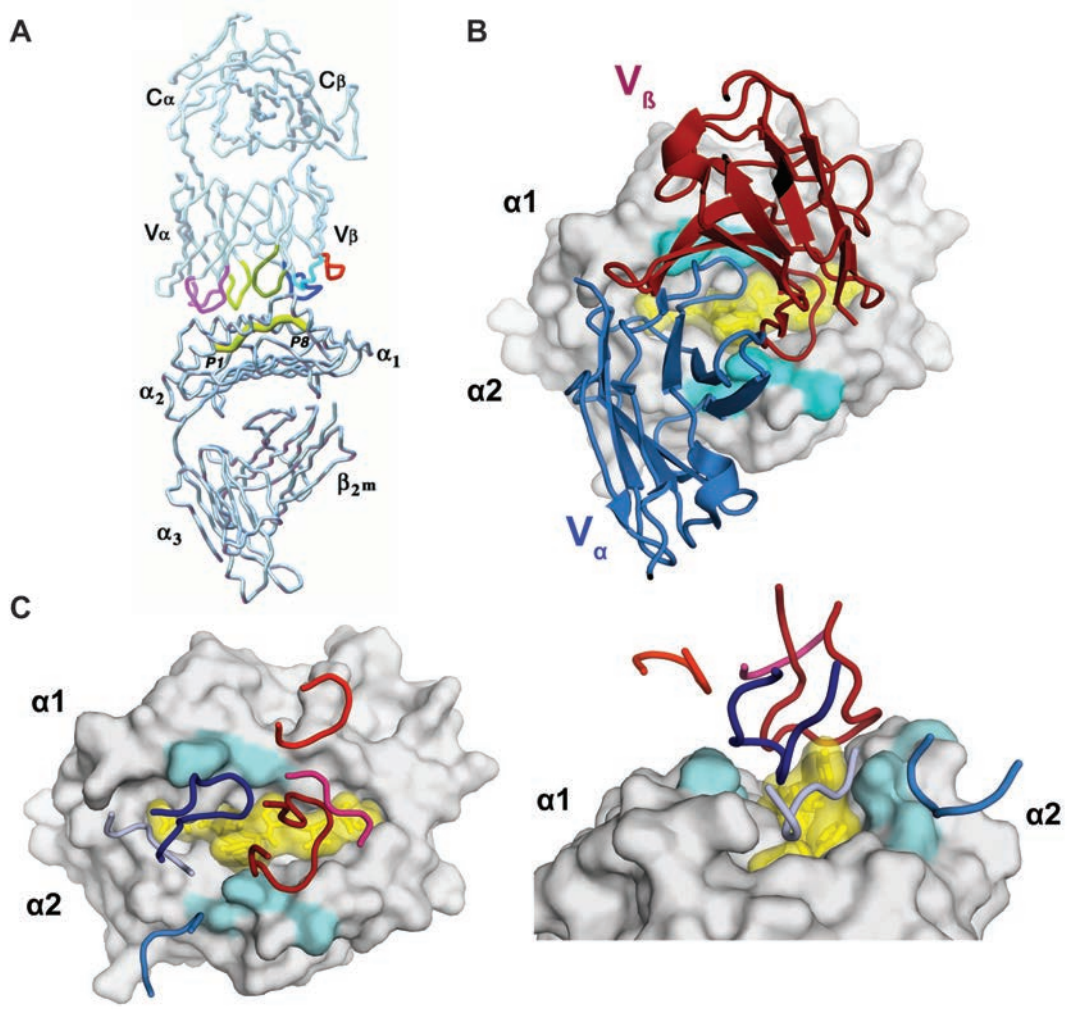

Figure 2: Classical TCR binding of pMHCs

A) The three-dimensional ternary structure of one of the two first TCR-pMHC complexes to be determined reveals how the TCR interacts with its six CDRs (colored individually) with the MHC-peptide interface. B) Representation of the TCR-pMHC complex from the perspective of the TCR. The variable domains of the TCR are displayed (V_α in blue, V_β in red) and bind to the pMHC (MHC in grey, peptide in yellow) in the generally conserved diagonal docking mode, which is centered around on conserved MHC contact residues (cyan) and the highly exposed peptide side-chain in the middle of the peptide. C) Representation of how the six CDRs (CDRs α1-3 in light blue, blue and dark blue, respectively; CDRs β1-3 in salmon, red and dark red, respectively) of the TCR interact with the pMHC shown from the top (left panel) and from the side (right panel). This figure highlights how the most variable third CDRs are focused on the exposed central part of the peptide, while also providing an understanding of the limited surface complementarity between the TCR and pMHC as particularly the CDRs β1 and β2 make almost no interaction with the pMHC complex in this specific ternary complex. Figure A is reproduced with permission from [1]

1.3.2 Kinetics, energetics and specificity of the TCR-pMHC interaction

In addition to structural characterizations, the affinity, kinetics and thermodynamics of TCR-pMHC interactions have been studied extensively on soluble molecules [117-119] and increasingly using systems that mimic aspects of the cellular context of *in situ* TCR-pMHC interactions [120-123]. Invariably, measurements with soluble molecules capture only some aspects of the multi-facetted interactions between T-cells and APCs, perhabs explaining why extensive characterizations have not yet provided a universal relationship between T-cell activation and the kinetic and thermodynamic parameters underlying TCR-pMHC interactions [118,119,124]. Nevertheless, crucial insights have been gained from these *in vitro* studies, which remain tremendously useful in order to characterize differences within the TCR recognition of specific sets of MHC-restricted peptides. Accordingly, surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) was an important part of the characterization of TCR recognition of modified peptides performed in Paper **II** and Manuscripts **I and III**.

SPR and ITC studies revealed that the binding of TCRs to pMHCs is characterized by relatively weak affinities and fast kinetics with K_D values stretching from 1 μM to about 30-70 μM, for strongly and weakly activating interactions, respectively [117]. Awareness of these small differences highlights the extreme specificity of T-cell recognition and the fact that TCR-pMHC interactions are tuned to translate extremely subtle changes in free binding energy and kinetics into an entire spectrum of functional outcomes e.g. [108,119,125-127]. Fittingly, the study presented in Paper **II** provides a thermodynamic characterization of a TCR-pMHC interaction in which the removal of a hydroxyl group at the tip of the main TCR-interacting peptide residue abolished functional recognition. Additionally, this study and Manuscript **III** describe peptide variants in which very small affinity differences correlate with significant differences in CTL activation.

This extreme specificity is particularly striking given that TCRs are also considerably cross-reactive, with the ability to structurally adapt to different pMHC complexes (see recent reviews [101,128] and references therein). These two features represent two central requirements of the adaptive immune system in which cross-reactivity seems to be necessary in order to allow a limited repertoire of TCRs to recognize a virtually unlimited variety of antigens [128,129]. Conversely, extreme specificity is an absolute requirement, given that the difference between 'self' and a vast number of 'foreign' antigens is only defined through very small differences in the significantly conserved MHC/peptide interface. This specificity together with the absence of affinity maturation also seems to explain the relatively low affinity of the TCR-pMHC interaction. In addition the requirement of TCRs to transiently interact with a large number of pMHCs demand that the TCR-pMHC interaction is governed by fast kinetics, which might have also played a role in preventing higher affinities of TCRs towards pMHCs.

The apparent contradiction between specificity and cross-reactivity can probably be resolved by suggesting that TCRs are extremely sensitive to very small changes in exposed peptide residues in the context of one specific pMHC-TCR complex, whereas conservation of the MHC surface and characteristics of the TCR paratopes following thymic selection allows the same TCRs to also specifically recognize other, possibly very different, pMHCs [101,105,128,130-136]. Hence, the term 'polyspecificity' has been suggested as more appropriate than 'promiscuity' or 'cross-reactivity' [137].

1.3.3 From pMHC recognition to CTL activation

With the perplexing results of Ding *et al.*, and similar studies, which essentially demonstrated that the tenary structures of several TCR-pMHC could not explain their functional differences, it became clear that the molecular basis of outside-in TCR signaling was not straightforward [108-110]. While still not completely understood, T-cell triggering is an extremely lively area of research and a number of recent publications have shed more light on this process and convincingly implicated direct signaling [112,138], clustering of co-stimulatory molecules CD4 and CD8 [139,140], the geometry of pMHC-TCR interaction [141], the cytoskeleton [121,142] as well as adhesion- and other cell-surface proteins [120,143]. Aside from the actual triggering, it should be noted that a whole range of additional factors, including glycosylation [144] as well as cell surface clustering of TCRs and pMHCs [74,145,146] are expected to tune T-cell activation *in vivo* [147].

CTL activation is highly dependent on cognate pMHC ligand density. With that in mind and facing the enormous complexity described above when defining TCR-pMHC interactions *in vivo*, the somewhat fuzzy terms 'functional avidity' or 'recognition efficiency' are highly useful as they ignore most of the molecular details and simply describe the capacity of a CTL to react to a specific pMHC dose under specific conditions (see for example ref. [148]).

Even if the molecular details remain unclear it is evident that in order to result in T cell activation, the interactions between TCRs, co-receptors and pMHCs must generate sufficient levels of phosphorylation of the intracellular part of the TCR-complex. This is essential for the priming of naïve T-cells by activated DCs and will ultimately result in the killing of target cells by activated effector CTLs. The main outcomes of the priming of naïve $CD8^+$ T-cells include clonal proliferation of the activated CTLs, up-regulation of CD8 and cell surface adhesion molecules, as well as homing to inflamed tissues. Prolonged interaction of TCRs on activated CTLs with cognate pMHCs on target cells will initially lead to tighter interactions with target cells, accompanied by a significant polarization of the CTLs, resulting in the formation of an immunological synapse between the two cells [149] (Detailed reviews of T cell signaling, priming and effector functions can be found in refs. [33,34]). This in turn assures that the subsequent effector functions, aimed at inducing the death of the recognized cell, are efficient and spare surrounding uninfected cells. The differential expression of intra-, extracellular and secreted proteins

that mediate and accompany the differentiation and the effector functions of CTLs provides important markers that allow the characterization of lymphocyte populations.

1.3.4 Modulation of T-cell activation by altered peptide ligands

Important insights into T-cell activation and TCR-pMHC interactions were derived from the understanding that T-cell activation was not a binary process, and that small changes to the presented peptides could gradually affect both the range and magnitude of priming and effector functions [127,150]. Classical altered peptide ligands (APLs) displayed modifications in TCR-contacting residues thereby affecting the functional avidity of CTLs. Starting from an agonist, a peptide that evokes optimal responses in its cognate CTL, modifications can reduce responses (weak/partial agonist), elicit no responses ('null peptide') or even antagonize CTL responses. Additionally, this terminology can also be useful to describe variants of immunodominant viral epitopes with respect to specific CTLs that generally strongly dominate the immune response [151]. Paper **II** explores how APLs modulate TCR recognition and in particular how a specific TCR interacts and recognizes both its cognate agonist pMHC and an altered semi-agonist variant.

In conclusion, the functional avidity of a CTL defines the pMHC density required to cross a signaling threshold of activation (and *vice versa*). However, this threshold is not binary but, within limits, priming and activation of effector function display a dose-response behavior. It should be expected that sensible ranges of pMHC density and functional avidity are more narrow *in vivo* than *in vitro*, thus introducing qualitative limitations to this quantitative relationship. Distinct influences of quality *vs* quantity on T-cell activation within that window have been suggested for $CD4^+$ T-cells [152] but, to my knowledge, not demonstrated for CTLs.

1.3.5 Principles and examples of APLs with enhanced CTL recognition and obstacles of elicitation *in vivo*

The use of APLs has been extended to enhance the immunogenicity of viral escape variants and particularly of poorly immunogenic TAAs. The underlying assumption is that tumors, while not immunogenic, are in principle antigenic and can be targeted by properly activated T-cells [153].

The immunogenicity of MHC-restricted peptides can be enhanced with the aim to directly increase TCR recognition and the functional avidity of elicited CTLs against native epitopes e.g. [154,155]. Alternatively, the MHC-binding properties of TAAs can be enhanced in order to increase pMHC density and thus lower the threshold of functional avidity required for activation. This seems particularly applicable to TAAs as many promising candidates have weak MHC-binding properties, likely limiting their presentation during negative selection (see 1.2.3). An interesting consequence of the central role of pMHC density, which is important for the use of peptides in

immunizations, is that increased peptide doses can elicit CTLs with decreased functional avidity, probably by lowering the avidity threshold of activation and the exhaustion of strongly activated CTLs [156-158].

The aim of peptide enhancement is to elicit more potent responses against native TAAs or viral epitopes presented on target cells. This focus on elicitation deviates somewhat from the original context of APLs and one could argue that this approach somewhat echoes a central obstacle in the design of APLs. Namely, it is becoming increasingly clear that the enhancement of *in vitro* epitope immunogenicity with respect to a small number of CTLs that are specific to the native epitope (termed here cross-recognition) does not necessarily result in the increased elicitation of CTLs with high functional avidity against the native epitope (termed here cross-elicitation) *in vivo* e.g.[159-163].

Strictly applying the principle that minor anchor residues can significantly contribute to MHC-binding, the results presented by van Stipdonk *et al.* described an unconventional modification at position 3 of the D^b restricted gp100-derived TAA EGS (EGSRNQDWL), which is significantly overexpressed in malignant melanoma [164,165]. EGS has very poor MHC stabilization capacity and is poorly immunogenic even though it contains the optimal main anchor motif for D^b [164,166]. A surprisingly large increase in immunogenicity was measured when the serine residue at position 3 of EGS was exchanged to a proline (EGP). The MHC stabilization capacity of the modified EGP epitope was dramatically enhanced and most crucially, vaccination with EGP cross-elicited potent CTL responses against the native epitope EGS on melanoma cells [164].

Manuscript **I** investigates the molecular mechanisms underlying the strong impact of p3P in D^b-restricted peptides on MHC stabilization capacity; expanding the current description of minor anchor residues for this allele. Importantly, this study indicates that the p3P modification has an unforeseen and direct effect on TCR affinity that is independent of its impact on MHC binding. Additionally, Manuscript **III** describes the application of the same modification to increase the immunogenicity of the *Lymphocytic Choriomeningitis Virus* (LCMV)-derived D^b-restricted epitope gp33 (KAVYNFATM) and of the naturally occuring escape variant Y4F (KAVFNFATM) [151]. Importantly and in contrast to the TAA EGS, both gp33 and Y4F already display strong MHC stabilization capacity. Nevertheless, the p3P substitution increased their immunogenicity both in *in vitro* and *in vivo* resulting in focused TCR recognition of Y4F following vaccination with the p3P-modified peptide PF (KAPNFATM).

In summary, these studies demonstrate that it is fully possible to enhance TCR recognition by using APLs with modified secondary anchor residues.

1.4 Introduction to HIV and SIV with a strong emphasis on antibody neutralization

HIV is characterized by its high prevalence in endemic areas, the absence of universally practical and straightforward prevention and its uncontrollable mortality in the absence of treatment [167]. Furthermore, HIV represents a striking evolutionary adaptation, which prevents clearance from the host despite active viral replication and extreme pathology [168,169]. As a lentivirus belonging to the Retroviridae family, HIV relies on a highly error-prone polymerase for the initiation of replication and therefore produces substantial diversity in each cycle of replication [168]. This allows the virus to continuously evade immune responses and display considerable variation during the course of infection [169].

AIDS (acquired immunodeficiency syndrome) is a direct consequence of HIV/SIVs predominant infection and depletion of $CD4^+$ T helper cells in the host [168,170]. Target cell recognition and fusion are mediated by the multimeric, glycosylated cell surface protein complex (Env) in HIV and its simian counterparts SIV [171] (Figure 3). The Env subunits gp41 and gp120 (glycoprotein 41 and 120, named after their respective molecular masses) are the only cell surface proteins and thus the most exposed targets for antibody-mediated immune responses, which can neutralize the virus by preventing interaction and/or fusion with target cells [171]. The continuous replication of HIV results in constant exposure to immune recognition and the virus is accordingly subjected to strong selection pressure from antibody and T-cell responses until the host's immune system eventually collapses [172]. This selection pressure is reflected in specific viral adaptations and the extreme variability of Env, which together have proven successful in impairing immune responses and vaccine efforts alike [171,173-176]. Furthermore, the variability of HIV has resulted in significant plasticity of Env as the virus meets shifting selection pressures from transmission to peak viremia and persistent infection and ultimately to AIDS [177-182].

The development of highly active antiretroviral therapy (HAART) against HIV efficiently prevented disease progression and dramatically changed the life of those infected [183]. Despite this huge success for drug design and the recent improvements in drug availability in poorer countries, HIV still has a dramatic prevalence in certain parts of the world and the associated mortality and morbidity cause extreme socio-economic damages to affected societies [167]. A significant number of effective prevention strategies and factors decreasing the spread of HIV have been described, including HAART, condom use, male circumcision, treatment of other sexually transmitted disease, pre- and post exposure prophylaxis, education, women's empowerment, stable family structures as well as social stability [167,184]. However, the failure and inherent difficulties of their implementation as well as the financial costs, the occurrence of drug resistance and the side effects of HAART, together with the precedence of the global vaccination campaigns against Polio and Smallpox, provide continuous motivation for the development of a vaccine against HIV. Essentially, these efforts have encountered the same challenges as did our immune

system and success is likely to require a combination of 'simply' trying, characterizing the most relevant targets and improving our understanding of the immunological principles underlying B-cell activation [173,185-187].

Although the HIV-2 variant shares its genome structure and main proteins with HIV-1, significant differences nevertheless exist between the two [188]. HIV-2, which is believed to have crossed independently to humans, is endemic mainly in West Africa [188]. Overall, HIV-2 causes a relatively less aggressive infection, mainly characterized by decreased virema, slower and less certain progression to AIDS associated with reduced $CD4^+$ T-cell loss and decreased infectivity [188-190]. Notably, HIV-2 is more susceptible to NAbs *in vitro* and more commonly neutralized by concurring autologous NAbs isolated from patients [191,192].

1.5 Antibody responses against HIV/SIV Env and viral evolution in the host

1.5.1 Env structure and function

The Env complex is a hexamer consisting of trimers of gp41/gp120 heterodimers, with gp41 spanning the viral membrane and gp120 facing target cells. Each Env trimer has a mushroom-shaped form with a three-fold symmetry perpendicular to the cell membrane [175,193,194] (Figure 3a). The prominently exposed gp120 is the main target for antibody responses and contains several crucial structural elements. It consists of a conserved site for interaction with gp41 and trimerisation, a variable outer face, a number of variable regions and two highly conserved binding sites [171,175,194] (Figure 3d). Furthermore, gp120 is highly glycosylated, flexible and a large proportion of its secondary structure is undefined [175]. Out of the five variable regions V1-V5, V4 and V5 are real loops that make up a significant part of the variable outer face of gp120 (Figure 3c,d) and play an important role in NAb escape (see 1.5.2). The V1/V2 region is highly variable, glycosylated and seems to display considerable flexibility with respect to the core domain of gp120 [195-197]. Although it has been called the 'V1/V2 loop', its size of roughly 70 amino acids and its three-dimensional crystal structure [197] strongly indicate that 'domain' or 'region' are more appropriate terms. The main function of the V1/V2 region is to shield the rest of the gp120 molecule, including the V3 region, from antibody recognition (Figure 3d). Furthermore, the V1/V2 region has also been implicated in direct interactions with integrins on target cell membranes [198]. As for V1/V2, the V3 region has also been referred to as 'loop' but it has been convincingly argued that 'region' is more appropriate based on similar arguments [199]. The V3 region is believed to extend towards target cells following initial interaction with CD4 (Figure 3f) [200,201 {Liu, 2008 #287]. It plays an important role in viral tropism and while parts of its 35 residues are highly variable, it contains significantly conserved elements, including the signature motifs GPGR or GPGQ at the tip of its extended structure [202]. Binding of gp120 to CD4 and to the chemokine receptors CXCR4/CCR5 is mediated by two distinct and highly

conserved binding sites on gp120 and is a prerequisite for large structural rearrangements that lead to membrane fusion driven by coiled-coil formation by gp41 [203].

1.5.2 Immune evasion from antibody responses against Env

HIV Env features an impressive array of antibody evasion mechanisms including heavy glycosylation and the dominance of highly variable regions on the exposed faces of gp120 [171,175]. Furthermore, the conserved receptor interaction sites are hidden and inherently flexible, requiring CD4 binding to stabilize gp120 at a significant entropic cost [204]. This stabilization exposes the CXCR4/CCR5 binding site, which in turn is therefore only available for binding in great proximity to the target cell membrane, sterically preventing antibody neutralization (Figure 3f) [174,193]. Additionally, only a few copies of the Env trimer are expressed on the cell surface, potentially hindering bivalent antibody binding [205]. Finally, the functional Env trimer is highly unstable and the released gp120 monomers can cause antibody responses against sites that are buried within the functional Env complex [206].

As a consequence of this adaptation, NAb responses against HIV, which are generally observed with a significant delay and fail to suppress the infection [207]. The majority of the elicited antibodies are directed against monomeric gp120, non-neutralizing sites or highly variable parts of Env [207,208]. Nevertheless, it is important to note that a number of well-described epitopes targeted by very broad NAbs (the term 'broad' generally describes the ability to cross-neutralize heterologous viruses, including isolates that are less sensitive to neutralization) have been identified [174,209-211]. Examples of such conserved epitopes comprise quaternary epitopes on the V1/V2 and the V3 regions, specifically on a site that has been implicated in binding to integrins on the target cells [198]. Additionally, the CD4 binding site (CD4bs), the MPER (membrane proximal external region) and even conserved glycans are targeted by very broad NAbs. Interestingly, while it is believed that a significant fraction of patients eventually develops broad NAb specificities, these are generally observed late and fail to significantly impact disease progression [207,212]. These broad NAbs all display extensive affinity maturation and other features such as above-average CDRH3 lengths and high hydrophobicity that together explain their rare and late occurrence [213-216]; reflecting the potent evasive properties of the Env proteins.

Crystal structure analysis combined with mutational and biophysical studies of broad NAbs have provided a deep understanding of their mechanism of action as well as of their specific features (see or example refs. [185,204,217-219]). NAbs that efficiently target the V3 region are of particular interest to the work presented in this thesis (Papers **IV** and **V**; Manuscript **VI**). Recognized very early for its immunodominance, the V3 region elicits high titers of antibodies, including NAbs [195,220]. Generally however, these NAbs are highly specific and bind mostly to variable regions of the V3 region. Nevertheless, NAbs with some degree of breadth and potency, particularly against the conserved

GPGR/GPGQ motif, have been identified [220-223]. X-ray structure determination of the human anti-V3 antibody 447-52D in complex with peptide from the V3 region [218] demonstrated that this antibody makes an unusually high proportion of direct contacts with the backbone of the gp120 molecule around the GPGR/GRGQ motif, which allows it to defy some degree of sequence variation. Additionally, a number of other NAbs with considerable breadth and potency have been recently isolated and demonstrated to bind a quaternary epitope formed by V3 and V1/V2 [220,223]. Another interesting example is represented by the group of NAbs that bind to the conserved CD4bs and display highly potent and broad neutralizing activity, [185,217,224]. The extreme sterical hindrance from glycans and the V1/V2 region that antibodies have to overcome upon binding to the Env trimer (Figure 3d) was revealed by the crystal structure of b12, the first member of this group, in complex with monomeric gp120 [225] and subsequently based on Env structures determined with cryo-electron microscopy (cryo-EM) [226]. Using thermodynamic measurements it was demonstrated that a decisive feature of b12 and similar NAbs was the capacity to recognize only a specific fraction of the CD4bs. This allowed the antibody to bind to gp120 without the entropic penalty associated with the binding of CD4 and non-neutralizing antibodies to the CD4bs [217,224,227,228].

As previously mentioned, HIV-2 is generally less resistant to NAbs *in vitro* and more successfully controlled *in vivo* compared to HIV-1. This feature has been associated to a less potent shielding by the variable regions [188,229]. Unfortunately, still relatively few comparative studies have been performed to adequately address the mechanistic differences and evolutionary adaptations that underlay the observed differences in NAbs resistance. Particularly, the three-dimensional structure of the HIV-2 gp120 (HIV-2_{gp120}) molecule has so far not been determined and comparative thermodynamic studies that would address entropic masking have not yet been performed.

1.5.3 Antibody pressure and the evolution of HIV/SIV in the host

While this introduction focuses on the direct evolution of Env in response to antibody pressure, it should be noted that HIV and SIV also adapt to CTL responses and possess several other immune evasion mechanisms unrelated to Env [187,230]. The extreme variability of HIV and SIV as well as the specific adaptations of Env allow the viruses to continuously escape from NAbs produced by the host [172,174]. This 'evolvability' is concentrated to the large proportion of loops and regions on Env (Figure 3c) that can vary significantly in both sequence and length [177,231,232]. Additionally, Env displays considerable variation in both amount and localization of glycans, with an increase generally associated with escape from antibody responses [181,231-233]. Finally, the degree to which conserved functional regions, such as the CD4 and coreceptor binding sites, are sterically masked in the functional trimer can vary.

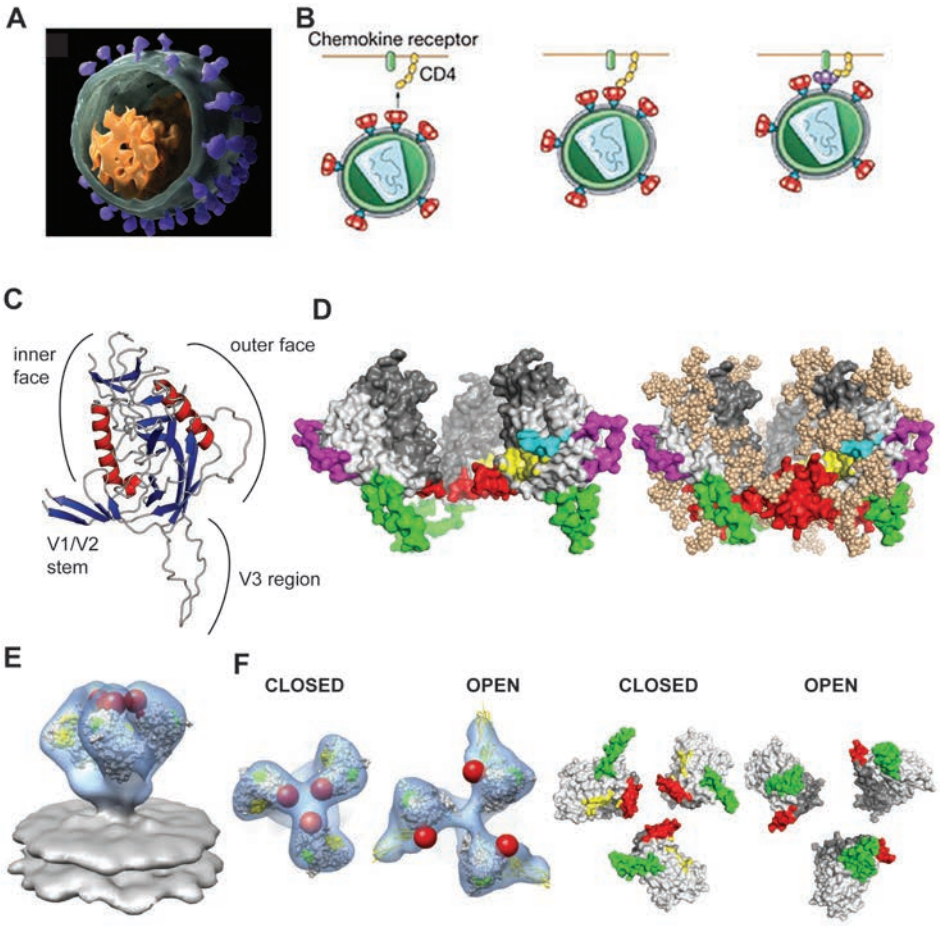

Figure 3: Key properties of HIV Env

A) Electron microscopy reconstruction of HIV particles with the trimeric 'Env (blue) on the viral membrane. B) Schematic representation of the events preceding target cell fusion. Interaction of Env (red) with CD4 (yellow) results in a conformation change in Env (purple), leading to interaction with the chemokine receptor (green). C) Molecular model of the core domain of gp120 including the V3. The molecule is colored according to secondary structure highlighting the high proportion of unstructured regions D) Left panel: Hypothetical model of gp120 in trimeric Env prior to CD4 binding. The variable inner and more conserved outer faces are colored in light and dark grey, respectively. The V1/V2 stem, the V3 region, V4 and V5 as well as the CD4bs are colored in red, green, purple, cyan and yellow, respectively. Right panel: A hypothetical model of the V1/V2 region (red) and predicted glycans (sand) have been added to visualize the extreme shielding of gp160. E) 3D cryo-EM reconstruction of Env (blue) to which crystal structures of gp120 (colored as in D) have been fitted. F) Cryo-EM with fitted X-ray structures before (closed) and after (open) CD4 engagement. CD4 binding leads to a rotation of the monomers resulting in exposure of the V3 and sites associated to chemokine receptor binding. Figure A, E and the left panel of F have been reproduced from [2] under the creative commons attribution license; Figure B has been reproduced with permission from [3].

While there is conflicting evidence on whether the virus that is transmitted and establishes the infection displays inherently increased neutralization sensitivity [208,234,235], it has become evident that transmission is mediated by a single or very few viral species [208,236]. Additionally, it has also been demonstrated that the rapid onset of variation in Env is at least partially driven by antibody responses and but appeared to be constrained by the need to maintain replicative capacity [178,180] A potential trade-off between replicative capacity and antibody resistance has also been suggested as laboratory-adapted HIV strains, in contrast to primary HIV isolates, display strongly increased neutralization sensitivity and seem overall more adapted for *in vitro* replicative capacity [237]. Interestingly, these features were reflected to some degree in changes observed upon comparing HIV isolates from patients suffering from AIDS and those that are chronically infected. HIV isolates from AIDS patients display reduced glycosylation, increased neutralization sensitivity and increased *in vitro* replicative capacity correlated with changes in electrostatic surface potential and reduced glycosylation [229,238] and **Paper VIII**.

A fascinating and somewhat worrying recent addition to the described plasticity of Env was provided on the basis of a comparison between the neutralization sensitivity of primary isolates that were obtained at similar times after infection but 15 years apart [176,239]. These studies demonstrated that over the course of 15 years HIV-1 has evolved significantly decreased neutralization sensitivity. The decreased neutralization sensitivity was correlated to increases in the length of both V1/V2 and V4 loops as well as to the amount of predicted glycosylation sites. Interestingly, the increased neutralization resistance was accompanied by an overall decrease in the potency and breadth of NAbs produced by the respective patients [176]. Finally, very similar changes to gp120 were found when comparing the sequences deposited in the global HIV sequence database from corresponding years.

In conclusion, HIV/SIV successfully evades humoral immune responses against the highly conserved epitopes on Env through the extreme variability and specific adaptations of the envelope proteins. This ability generally results in the failure of the host to mount effective NAb responses, while also providing tremendous challenges to vaccine development. Finally, recent evidence suggests that HIV has over the years of the epidemic evolved its resistance to human antibody responses without evident loss of infectivity.

1.6 HIV vaccine efforts

1.6.1 HIV Env immunogen design and the main obstacles to HIV vaccine efforts

The following section will attempt to describe previous and ongoing efforts to generate prophylactic antibody-based protection against HIV-1. It should however, be noted that

other prophylactic measures such as pre- and post exposure prophylaxis as well as microbicides are also being investigated and have shown some promises [184,240]. Although not addressed in the frame of this thesis, it is furthermore conceivable that T-cell responses will contribute to a prophylactic vaccine and T-cell based immunogens thus seem strong candidates for therapeutic applications [208].

The use of attenuated viruses as immunogens is being explored and although they promise some effectivity, this approach is generally considered unsafe due to high risk for reversion to pathogenicity [241]. Therefore, most efforts have been focused on the use of recombinant forms of gp41 and gp120 as immunogens and particularly on the design of stable gp140 trimers. These recombinant trimers, which consist of gp120 and the extracellular part of gp41, are intended to mimic functional Env with respect to overall structure as well as disposition and accessibility of NAb epitopes. The resulting efforts have led to the elicitation of increasingly potent and broad NAb responses in small animal models and non-human primates [242-245]. Additionally, the development of increasingly sophisticated DNA-prime protein-boost vaccination strategies has improved the outcome of immunizations [246,247]. Nevertheless, despite these achievements none of the immunogens and vaccination strategies tested so far has resulted in the elicitation of truly broad and potent NAbs [245]. This seems entirely reasonable (admittedly in retrospect) considering the ability of the virus to successfully evade immune responses, the long time it requires for patients to mount broad NAbs, and the potential bias resulting from different CDR repertoires in animal models and humans [207]. More specifically, one could argue that the design of immunogens is constrained by an underlying conundrum in that those with facilitated access to highly conserved epitopes should elicit neutralizing antibodies that are unable to recognize the epitopes in their native restricted context [173,187]. Conversely, the use of immunogens that fully mimic the restrictions of native Env is most likely to replicate natural exposure and -at least initially- simply not yield NAbs against conserved epitopes.

Accordingly, current efforts are increasingly focused on the design of immunogens and vaccination strategies that take into account the unusual properties of broad NAbs that have been isolated from patients. Proposed strategies aim for example at the initial elicitation of antibodies with common precursors to the highly affinity-maturated CDRs generally observed in broad NAbs [185,207,213,215]. Additionally, it might be necessary to repeatedly boost the elicited antibody responses in order to maximize their neutralization capacity [248]. In parallel, increasingly intricate epitope-based immunogens are being designed with the aim to directly elicit potent NAbs [219,223,249,250]. Finally, novel ideas including the work presented in Manuscript **VI** as well as increasing understanding of optimal priming, adjuvants, delivery, vaccination strategies and combination with CTL responses will most probably provide crucial contributions to successful vaccines.

The study presented in Manuscript **VI** is unusual as DNA priming was boosted with a recombinant gp140 trimer that was derived from SIV_{mac239} instead of HIV. This strategy attempted to benefit from the comparatively higher stability of the SIV trimer and test if highly heterologous protein boosts would preferentially increase responses against highly conserved epitopes. The strategy resulted in the elicitation of comparably high-titer and potent NAbs in rabbits and thus provides a novel and promising platform for further optimization.

Another important aspect of vaccine design against HIV is that the exact or minimal requirements for sterilizing immunity (the correlates of protection) are only beginning to be established. This has been highlighted by the results from the RV144 phase 3 efficacy trial, which was based on the use of a monomeric gp120 protein boost and reported some indications of efficacy [251,252]. The indicated protection is notable given that monomeric gp120 had been abandoned in immunogen design and is generally considered to not elicit broad NAbs in small animal or non-human primate studies. Accordingly, while relatively high but transient titers of NAbs were demonstrated in vaccinees, no broad NAbs were observed [253]. It seems certain that DNA priming was an important part of the protective responses, as earlier trials with similar recombinant proteins did not result in similar protection [245]. Additionally, the results from the RV144 study provide support for the argument that antibodies might be protective in mucosal transmission without being necessarily truly broad and potent as long as they are present in sufficient concentrations in the mucosa before viral transmission [187,236,254,255] The latter is based on the assumption that the virus is particularly sensitive to neutralization in the mucosa but also to antibody-based retention, antibody-dependent cellular cytotoxicity (ADCC) and other innate factors [187,236,255]. It has to be kept in mind however, that the RV144 trial was conducted with comparably low-risk volunteers and that the observed protection might be not representative for other parts of the world. Nevertheless, and while the induction of highly potent and very broad NAbs would undoubtedly be of huge advantage and should remain a goal, the need to induce truly broad and potent NAbs potentially sets the bar for recombinant gp140 too high given their superiority over the gp120 used in RV144 [244,245,256]. Additionally, the importance of protection at the mucosa gives additional momentum to studies addressing how to optimize mucosal Ig(immunoglobulin)G and IgA induction. Finally, additional clues for vaccine design are likely to come from the understanding of how to manipulate B-cell activation and affinity maturation in the course of vaccination[185].

In conclusion, the efforts underlying immunogen design demonstrated that the strategy to 're-elicit' [173] known broad NAb specificities is highly challenging and has largely failed so far. Nevertheless, the notable advances in immunogen design, the increasing description of vulnerable sites on Env, the improving understanding of mucosal transmission combined with the detailed characterization of the responses elicited in the

RV144 trial, increasing immunological understanding as well as the optimization of vaccination strategies offer hope and provide a number of specific paths forward.

2 AIMS OF THE THESIS

- To provide a detailed characterization of the molecular mechanisms underlying the enhanced immunogenicity observed upon introduction of p3P into D^b-restricted epitopes, including the generation of soluble TCRs for structural and biophysical analysis.

- To achieve a structural understanding of the observed functional hierarchy of gp33 APLs and to apply the p3P modification on escape variants of the immunodominant *LCMV* epitope gp33.

- To apply the p3P modification or the principles underlying its enhancement of immunogenicity to MHC-restricted TAAs.

- To increase our molecular understanding of HIV neutralization and the interplay between the elicitation of neutralizing antibody responses and SIV evolution *in vivo*.

- To test a novel vaccination strategy for its ability to induce potent neutralizing antibody responses against HIV.

3 RESULTS AND DISCUSSION

3.1 Enhancement of class I restricted viral and tumor-associates epitopes

3.1.1 Recognition of gp33 variants by the P14 TCR

LCMV infection elicits a potent immune response that is particularly directed against the immunodominant D^b and H-$2K^b$ (K^b) restricted epitope gp33 [257]. Crystal structure determination of gp33 in complex with D^b revealed that the side chain of the tyrosine at position 4 of the peptide (Y4) protrude towards the TCR [258]. The highly efficient naturally occurring escape variant Y4F has been identified in mice that were transgenic for the murine D^b/gp33-restricted TCR P14 [151]. Subsequently, Y4F as well as a number of designed APLs, including Y4A (KAVANFATM) and Y4S (KAVSNFATM), have been used extensively to characterize how peptide modifications affect CTL activation and TCR affinity [126,151,259,260] (Paper **II** Figure 1, Table 1). In summary, the results presented in Paper **II**, in agreement with previous studies have revealed the existence of an immunological hierarchy in which gp33 is a full agonist, Y4A a semi agonist and Y4F and Y4S are not recognized [151,259,261]. Furthermore, differences in the affinities of soluble P14 to the different D^b/APL complexes followed a similar pattern [125,126,262].

Surface stabilization assays and circular dichroism (CD) analysis confirmed that the observed functional differences could not be explained by the different MHC stabilization capacity of the APLs (Paper **II** Figure 2, Suppl. Figure 1). These results highlight the specificity of TCR binding and recognition described in the introduction where removal of a hydroxyl at the tip of Y4F is sufficient to abolish functional responses. Even more impressive is the fact that gp33 and Y4A display significant differences in immunogenicity *in vitro* while their binding by P14 differs only by 1 kcal/mol in free binding energy and/or roughly eight-fold in association rate. Although these values were not determined at 37°C they seem a fair approximation) (Paper **II** Table 1). Furthermore, the observed hierarchy of gp33 APLs is highly relevant with respect to the discussion regarding TCR specificity *vs* cross-reactivity given that P14 is able to at least partially recognize Y4A but neither Y4S nor Y4F. This means that P14 is able to partially tolerate the almost complete loss of the exposed side chain at position 4, whereas the relatively more conservative modification of Y4 of to a serine or a phenylalanine could not be tolerated (Paper **II** Figure 1a). While it seemed reasonable to assume that the hydroxyl group of tyrosine at position 4 rather than its aromatic ring is crucial for binding by P14, this would not explain why Y4A but not Y4S can compensate for the loss of this hydroxyl group.

X-ray structure determination of the four different pMHCs did not reveal any conformational changes that could explain the observed differences in TCR recognition (Paper **II** Figure 4a, Suppl. Figure 3, Table 2). Nevertheless, inspection of the amount and

localization of the water molecules that surrounded the four peptide-binding clefts suggested a higher degree of coordination of waters only in the complex of D^b/Y4S (Paper **II** Suppl. Figure 4) (see below). Furthermore, a thermodynamic analysis of the interactions of P14 with D^b/gp33 and D^b/Y4A revealed significantly distinct thermodynamic signatures (Paper **II** Figure 3; Table 1). While, binding of D^b/Y4A by P14 was associated with less favorable binding enthalpy, it did not result in the entropic penalty observed upon binding of P14 to D^b/gp33. Instead, the P14 - D^b/Y4A interaction derived its favorable free binding energy from a combination of both favorable binding enthalpy and binding entropy.

Different thermodynamic strategies are not uncommon in TCR-pMHC interactions [263-265], but it was nevertheless very interesting to observe that the small structural changes between Y4A and gp33 resulted in a reversal of the binding entropy despite being recognized by the same TCR. It could be possible that increased water expulsion from hydrophobic interactions and/or CDR rearrangements and flexibility drive the observed increase in binding entropy and association rate of the P14-D^b/Y4A. One could further speculate that the increased water coordination in D^b/Y4S when compared to D^b/Y4A hinder this path of recognition by P14.

In conclusion, this study highlights the yet highly unpredictable nature of the TCR-pMHC interaction with respect to even small changes in the presented peptides. Furthermore, it also emphasizes how the very small energetic and/or kinetic differences, that often characterize distinct TCR/pMHC interactions, make it entirely possible that the causative molecular changes are missed or simply not apparent in X-ray structures.

3.1.2 Enhanced stability, immunogenicity and TCR-recognition of proline-substituted LCMV epitopes and a melanoma-associated TAA

The p3P modification of D^b-restricted epitopes described by van Stipdonk *et al.* [164] was both intriguing and promising for the design of APLs with enhanced stabilization capacity and immunogenicity. Position 3 has also been previously described as a secondary anchor residue for D^b [266]. Furthermore, proline at this position had been noted as favorable but not enough to predict the striking increase in immunogenicity observed when EGS was modified to EGP. Targeting such minor anchor residues meant that the p3P modification could be applicable to a wide range of peptides that display poor immunogenicity despite containing favorable main anchor residues. Ultimately, the promise of the p3P modification was highlighted by the fact that it did not disturb the molecular mimicry between EGS and EGP as revealed by comparative structural analysis. Finally and most importantly, immunization of C57BL/6 mice with EGP cross-elicited CTL responses with sufficient avidity to lyse target cells that presented the native EGS epitope and thus delayed tumor growth [164].

The study presented in Manuscript **III** was based on these promising results and applied the p3P modification to possibly increase the immunogenicity of the LCMV peptides gp33, Y4A and Y4F. Furthermore, both studies summarized in Manuscript **I** and Manuscript **III** evaluated the direct impact of the p3P modification on TCR binding and pMHC stability. In each of the complexes tested, p3P increased the thermostability of the respective soluble pMHCs. However, the immunodominant gp33 as well as Y4F and Y4A displayed very strong binding to D^b and the thermostability of the respective pMHCs was measured to around 53°C (Manuscript **I** Figure 1a) thus exceeding by far the 37°C measured for the TAA EGS (Manuscript **III** Figure 1, Table 1). In line with their already potent MHC stabilization capacity, the thermostability of the LCMV D^b complexes was enhanced by only about 3-4°C upon p3P modification, while the thermostability of D^b/EGS jumped to almost 50°C for D^b/EGP in agreement with cellular stabilization assays [164].

SPR measurements with soluble recombinant P14 demonstrated that each of the p3P modified LCMV epitopes was recognized with small but significantly increased TCR affinity when presented by D^b and compared to their native counterparts (Manuscript **III** Figure 1). The production, refolding and purification of a soluble recombinant form of Pmel, a murine TCR that recognizes D^b/EGS, was established and the TCR used for SPR measurements comparing D^b/EGS to D^b/EGP (Manuscript **I** Figure 3a). These biophysical measurements confirmed that the p3P modification directly leads to a small but significant increase in TCR affinity. Furthermore, measuring the activation of $P14^+$ CTLs with the enhanced epitopes PA (KAPANFATM) and PF (KAPFNFATM) demonstrated that the p3P modification resulted in significantly increased immunogenicity and converted the unrecognized Y4F into a semi-agonist (Manuscript **III** Figures 1+3, Suppl. Figures 2+3). Finally, vaccination of C57BL/6 with PF but not Y4F elicited a more focused CTL response against the native escape variant Y4F (Manuscript **III** Figure 4).

In conclusion the results presented in Manuscripts **I** and **III,** combined with the previously published study [164], clearly demonstrated that p3P enhances MHC binding affinity, stabilization capacity, direct TCR affinity and overall immunogenicity of TAAs and immunodominant viral epitopes. p3P does not alter the conformation of the modified epitope, which leads to enhanced elicitation of CTL responses that cross-react with the native epitope.

Additionally, the SPR measurements with the D^b/EGS-specific Pmel TCR demonstrated that the native gp100 epitope EGS was recognized with an affinity generally characteristic for agonist epitopes, while it had been established that this peptide is not immunogenic *in vivo* [164,166]. This however, seems fitting with the hypothesis that promising TAAs are defined by the existence of cognate CTLs with sufficient avidity, which is made possible by their weak MHC stabilization capacity and limited negative selection [95-99].

On the other hand this summary does not address the molecular mechanism responsible for the increases in MHC stabilization capacity upon p3P modification. While the observation that the p3P modification results in direct increases of TCR affinity was interesting, it should be noted that they were generally small and that the strongly increased stabilization capacity observed for EGP could sufficiently explain its increased immunogenicity. Nevertheless, the fact that PF and PA also displayed enhanced immunogenicity *in vitro* when compared to their native counterparts supports the notion that the increases in TCR affinity are relevant given that the associated differences in MHC binding appear too small to have such an impact in these assays.

3.1.3 Molecular basis underlying the enhanced MHC stabilization capacity and TCR recognition of D^b/EGP

Previous crystal structure analysis of D^b/EGP indicated that the pyrrolidine ring of p3P closely interacted with the side chain of the MHC-I heavy chain tyrosine residue Y159, which is highly conserved among all known MHC alleles [164]. Accordingly, the authors proposed that specific interactions between proline and the aromatic ring of Y159 were responsible for the enhanced MHC binding and immunogenicity. Interactions between aliphatic CH donors, particularly prolines, and aromatic rings, so-called CH-π interactions, are commonly found in enzymatic systems and protein-protein interactions [267-275]. However, the role of this interaction has to date not been explicitly analyzed for peptide binding by MHCs. Additional analysis of crystal structures of D^b/peptide complexes deposited in the Protein Data Bank (pdb, www.rcsb.org) revealed that the juxtaposition between Y159 and the side chain of position 3 (p3) of the bound peptides was highly conserved. These interactions were mediated in part by an absolutely conserved hydrogen bond interaction between the hydroxyl group of Y159 and the backbone carbonyl of peptide residue 1 [50] (Manuscript **I** Suppl. Figure 1). The conserved juxtaposition suggested that CH-π interactions at p3 may play an important and underappreciated role for the binding of D^b restricted epitopes.

The prominence of Y159 made it possible to use side-directed mutagenesis to address the contribution of the p3P-Y159 interaction to the enhanced Pmel affinity and stabilization capacity observed for EGP. Residue Y159 was mutated to phenylalanine (Y159F), leucine (Y159L) and to alanine (Y159A) in order to successively remove the chemical properties of Y159. Essentially, the measurements demonstrated that the striking difference in thermostability between D^b/EGS and D^b/EGP was entirely dependent on the aromatic rings of Y159 or F159 (Manuscript **I** Figure 1), as expected for CH-π interactions [271]. The data presented here therefore suggest that CH-π interactions between Y159 and p3P lead to enhanced MHC binding and stabilization capacity of D^b-restricted epitopes.
Furthermore, analysis of published epitopes eluted from D^{b+} cells demonstrated an overrepresentation of proline, as well as other aliphatic residues at position 3 that should be favorable for CH-π interactions [55,64,276]. However, to our knowledge, no

rationalization of such preferences has been published and it was interesting to note that while the prediction servers of the immunoepitope database (IEDB, http://www.immuneepitope.org) [277-280] and netMHC (http://www.cbs.dtu.dk/services/NetMHC/ [278]) did correctly predict higher binding affinity of EGP over EGS, proline was not listed as a favorable residue at position 3 in the binding motif of D^b. In conclusion we therefore believe that the results of these studies combined with the published epitope elution and the conserved juxtaposition of p3 and Y159, indicate that CH-π interactions play an important role in shaping the amino acid preferences for position 3 of D^b-restricted epitopes. Additionally, it should be noted that proline is described as main anchor at position 3 of H-$2D^d$ epitopes [52,59,281], is observed in several K^b epitopes [55] and particularly in 9-mers as well as other unconventional K^b epitopes [5,282]. It is therefore tempting to speculate that these are aspects of the same mechanism, which is highly favorable for the binding of peptides containing p3P, while being constrained by the specific geometry of the MHC allele.

Having established that the stability advantage conferred by EGP was lost in D^b-Y159A made it possible to address if the differences in Pmel affinity were connected to the enhanced stabilization and dependent on interactions with Y159. Accordingly, the binding affinities of Pmel to D^b-Y159F and D^b-Y159A were determined in complex with EGS and EGP (Manuscript **I** Figure 3). Surprisingly, our results clearly demonstrated that the enhanced Pmel recognition of D^b/EGP was conserved if not increased in D^b-Y159F/EGP and D^b-Y159A/EGP. This clearly indicates that p3P leads to an increase in TCR recognition that is independent of direct interactions with the MHC peptide-binding cleft and therefore likely to be an effect that is mediated through the modified peptide.

Subsequently, tetrameric complexes of D^b/EGP, D^b/EGS and D^b-Y159F/EGP were generated and used to stain $CD8^+$ Pmel T-cells (Manuscript **I** Figure 4). The use of pMHC tetramers at known concentrations made it possible to probe the functional significance of the small affinity differences observed in SPR, while avoiding the confounding effects from distinct MHC stabilization capacities and ensuing pMHC densities associated with cellular assays. In accordance with the SPR-measurements, a significant difference in staining intensity was observed between D^b/EGP and D^b/EGS. Furthermore, $CD8^+$ Pmel T cells were stained with higher intensity by D^b-Y159F/EGP tetramers compared to D^b/EGS as expected from the thermostability and SPR measurements. Together with the increased immunogenicity observed for gp33-PF, whose stability advantage was too small to result in distinct cellular stabilization (data not shown), this suggests that the TCR affinity increases induced by p3P are correlated with functional differences in TCR recognition.

Since the comparative analysis of the crystal structures of the various pMHCs provided no insights into the enhancement conferred by p3P [164], MD simulations were conducted to gain additional insights into differences between D^b/EGP and D^b/EGS. These results

(Manuscript **I** Figure 5) suggest that p3P stabilizes the D^b/EGP complex by reducing the flexibility of the peptide-binding cleft, particularly at elevated temperatures (Manuscript **III** Figure 2). Additionally, the MD simulations indicated that the flexibility of the p3P-modified EGP peptide was decreased when comparing D^b/EGP and D^b/EGS. While detailed analysis of the interactions between the peptide and the binding cleft as well as of TCR contact residues are still ongoing, these results are well in line with the striking differences in thermostability. Furthermore, other recent studies that investigated the influence of pMHC dynamics on TCR binding have suggested that increased pMHC and peptide flexibility is associated with decreased TCR affinity [113-115,283].

In conclusion, the presented results demonstrate that interactions between Y159 and p3P are essential for the measured increase in MHC-I binding stability and point towards a fundamental role of CH-π interactions in D^b-restriction. Nevertheless, p3P enhanced the TCR affinity of modified peptides independently of the p3P-Y159, suggesting a novel and unexpected role of p3P in the modulation of TCR recognition of D^b-restricted epitopes.

3.2 Elicitation and structural characterization of anti-HIV antibodies

3.2.1 Crystal structure of the HIV-2 neutralizing anti-V3 Fab fragment 7C8

Fab fragments of the murine monoclonal antibody 7C8 have been demonstrated to neutralize HIV-2 and bind to a stretch of V3-residues, which contain the highly conserved immunodominant motif FHSQ [284,285]. Papers **IV** and **V** summarize the crystallization and structure determination of the Fab fragment of 7C8 providing the first structural analysis of an HIV-2-neutralizing immunoglobulin. With the exception of the generally more variable CDRH3, all of the CDRs of 7C8 display one of the canonical classes defined for murine antibodies [286]. In line with most of the described potent anti-HIV antibodies, 7C8 features an elongated CDRH3 loop comprising 13 amino acid residues (Paper **V** Figure 1B). Fitting to its binding of the extended V3 region, the canonical classes found in 7C8 are most commonly observed in so-called peptide-binding antibodies and form, generally with shorter CDRH3 loops, a deep cleft that runs through the antigen-binding site [287,288]. On the other hand, the elongated CDRH3 of 7C8 is localized at one end of its deep, narrow and highly hydrophobic cleft (Paper **V** Figure 2), similarly to the murine anti-HIV-1 V3 antibody 58.2 [289]. Although the need for these elongated CDRs in anti-HIV antibodies is still not entirely understood, it is likely related to the extensive shielding of epitopes on gp120 and might allow for more extensive contacts with the V3 epitope as exemplified in a number of human anti-V3 antibodies [218,290,291]. Finally, an analysis of the electrostatic surface potential of the Fab fragment of 7C8 demonstrated that the two sides of the deep antigen-binding cleft had distinct positive and negative surface charges (Paper **V** Figure 2), a property that is likely to play an important role in epitope binding and specificity.

A molecular model of the core domain of the HIV-2 gp125 (the homolog to HIV-1 gp120) monomer, including the V3 region, was generated and a previously published analysis [194] used to create a putative disposition of the gp125 monomers in functional Env (Paper **V** Figure 3). Interestingly, and somewhat in contrast to previous comparisons [285], the sequence analysis clearly demonstrated that the conserved and immunodominant FHSQ motif does not align with the conserved and immunodominant GPGR motif found at tip of the V3 regions from HIV-1 clade B virus. Accordingly, the generated molecular model suggested that FHSQ is not localized at the tip but rather in the middle section of the V3 region (Paper **V** Figure 3). It should however, be noted that the precise conformation of the V3 region in the model remains speculative given its lack of secondary structure. The generated molecular models of gp125 were used to test if sensible binding modes could be established between the 7C8 Fab fragment and gp120 using molecular docking simulations. Docking simulations are notoriously ambiguous especially if little experimental data is available to constrain them or validate their predictions [292,293]. These limitations prevented a reasonable detailed analysis of the interactions formed between V3 and 7C8 particularly as the two main solutions derived from the computational analysis displayed distinct orientations (Manuscript **V** Figure 4). However, all of the main docking solutions were highly focused on the FHSQ motif as expected from previous preliminary epitope characterizations [294] despite the fact that the docking procedure was not specifically directed towards this epitope (Manuscript **V** Figures 3). The two solutions could be superposed onto the trimeric model of g125 (Manuscript **V** Figure 4) even if the size of potential glycans was taken into account (data not shown). The absence of sterical clashes together with the focus of 7C8 in the docking solutions on FHSQ provided at least an indirect validation of the obtained results and corresponded to the demonstrated immunodominance of this motif [284,285,295,296]. Finally, besides enforcing the importance of the FHSQ epitope for recognition by 7C8, the two models provide a potential mechanism for neutralization by 7C8, as the size of bound Fab fragments would sterically hinder subsequent engagement of the gp125 trimer with the co-receptor on the target cell.

3.2.2 Induction of cross-reactive neutralizing antibodies in rabbits through a highly heterologous prime boost vaccination strategy

As introduced above, our understanding of the binding sites of very broad NAbs has increased dramatically over the last years. Nevertheless, to date it has not been possible to fully translate this into the design of immunogens that elicit focused B-cell responses against these conserved and potentially vulnerable sites within Env. Therefore, a somewhat unusual approach was taken in Manuscript **VI** where a recombinant gp140 trimer from SIV_{mac239} was used to boost antibody responses against HIV-1. The SIV boost followed repeating priming with a plasmid mix of three different clade B *envs*, which were injected intra-dermally together with adjuvant and electroporation. This strategy was based on the observation that recombinant trimers from SIV display increased stability during production, purification and storage when compared to their HIV-1 counterparts

(Manuscript **VI** Figure 1, GBSJ personal communication). While difficult to specifically address *in vivo*, this should be an important advantage for the induction of responses following injection. Additionally, the study was aimed at testing if the highly heterologous nature of the SIV boost with respect to the HIV prime would contribute to the elicitation of NAb responses particularly against the conserved and thus vulnerable epitopes. Although clade B HIV-1 and SIV_{mac239} Env share limited sequence conservation, certain important regions are conserved including the CD4bs (Manuscript **VI** Figure 1).

Analysis of the resulting NAb responses indeed revealed high titer neutralization of clade B HIV-1 as well as persistent neutralization in sera collected up to three month after the last boost (Manuscript **VI** Figure 3). Importantly, significant levels of neutralization were also observed against viral isolates, which are less sensitive to neutralization (so called 'Tier 2' isolates) as well as isolates of clades that were not represented in the HIV-1 DNA prime (Manuscript **VI** Figure 3F-I). A comparison of neutralization before and after the SIV_{mac239} boost (week 16 and week 20, respectively) demonstrated that boosting increased NAb responses against most of the tested virus. Not surprisingly, no neutralization against HIV-1 was found in a control group that only received the recombinant SIV gp140 trimer. Likewise, no neutralization of SIV was observed in serum collected prior to the SIV boost and the induced responses were not increased in primed animals (Manuscript **VI** Figure 3J, K). In conclusion, this study clearly demonstrated that the vaccination strategy resulted in highly potent and notably broad NAb responses. Furthermore, the heterologous boost clearly increased the strength of the elicited antibody response including reactivity against heterologous virus.

Epitope scanning for antibody binding responses revealed that the SIV boost primarily increased the responses that were already elicited by the prime (Manuscript **VI** Figure 4C). However, a site-wise analysis demonstrated that the level of responses prior to the boost did not necessarily determine the strength of the boost (Manuscript **VI** Figure 4A). For example responses against C1, C2 and V5 were most strongly increased. Interestingly, and in contrast to the observed neutralization, strong binding titers against SIV gp140 were detected already after HIV-1 priming and before the SIV protein boost (Manuscript **VI** Figure 2C).

Using peptide-inhibition assays it was further demonstrated that a significant portion of the NAb responses was directed against the tip of the V3 region of gp120 (Manuscript **VI** Figure 5). Interestingly however, the neutralization inhibition by the V3 peptide was not complete, especially in some of the animals tested. Furthermore, a fraction of the neutralization responses could be inhibited with a heterologous V3 peptide from HIV-1 clade A. Finally, further testing of peptide inhibition of neutralization and ELISA binding responses demonstrated that -as far as investigated- the observed parallel reactivity against HIV-1 and SIV was truly parallel and not due to antibody cross-reactivity (data not shown). In conclusion, the characterization of the antibody specificity demonstrated that a

significant fraction of the NAb response was directed against the immunodominant but escape-prone V3 regions, while lower levels of broad V3 neutralization responses as well as unrelated specificities were also observed.

The influence of the adjuvants used during the prime was very limited and while some increase in antibody binding-titers were found (Manuscript **VI** Figures 2,4) no significant effect was observed in the neutralization assays. It should be noted that all neutralization assays were performed with specific target cells and are therefore insensitive to antibody effector functions such as ADCC.

In summary these results demonstrate that the vaccination strategy led to the induction of NAb responses with considerable potency and breadth. The extent to which the observed effects were caused by the highly heterologous nature of the boost remains to be tested in detail. Nevertheless, this study clearly demonstrates that SIV_{mac239} gp140 trimers can be highly useful immunogens for the induction of NAbs against HIV-1. Accordingly, it seems appealing to identify the elements that confer higher stability to the SIV gp140 trimer and transfer those to recombinant HIV trimers. This might lead to the creation of more potent HIV-1 based immunogens and would allow to individually test the contributions of gp140 trimer stability and heterologous nature for the capacity to boost NAb responses.

3.3 Antibody responses and viral evolution in the host

3.3.1 Neutralizing antibody responses and evolution of SIV after early short-term antiretroviral therapy

The study presented in Paper **VII** presents a comprehensive longitudinal analysis of the appearance of NAbs in the context of viral evolution in 12 experimentally-infected macaques that were subjected to early but short-term antiretroviral therapy (ART). During the course of 14 months the viral load, $CD4^+$ T-cell counts, total antibody levels as well as the breadth and strength of NAb responses were measured. While early single drug treatment effectively controlled viremia in nearly all animals and while more than half of the animals demonstrated persistent control, another group only transiently controlled the infection and eventually developed high viremia and declining CD4 counts (Manuscript **VII** Figure 1, Table 1). Furthermore, these two groups were clearly segregated with respect to viral loads, NAb responses, Env diversity and divergence.

Animals in which the virus eventually escaped control displayed greater *env* divergence and a larger number of positively-selected amino-acid substitutions including a number of well-characterized antibody escape mutations as well as mutations associated with increased replicative capacity (Manuscript **VII** Figures 2, 7, 8; Table 3; Suppl. Figure 3). The positively selected mutations within gp120 could be mapped to surface-exposed areas

and largely clustered in variable regions (Manuscript **VII** Figure 8; Suppl. Figure 5). Phylogenic analysis suggested that the increases in viral diversity and positively selected mutations occurred early and likely before differences in viremia became apparent (Manuscript **VII** Figures 2, 8; Suppl. Figures 3, 4). Not only did virus isolated from controlling macaques entirely lack notable escape mutations or mutations associated with increased replicative capacity, they also displayed less divergence and even signs of decreased neutralization resistance during the course of the study (Manuscript **VII** Figure 5). Finally, it should be noted that a significant number of positively and negatively selected mutations in *env* have, at the time of the study, not been described in the literature, which highlights our limited understanding of SIV/HIV fitness and immune escape.

Analysis of antibody responses in the different macaques demonstrated that animals controlling the infection displayed earlier signs of breadth in the neutralization response although they generally had markedly decreased NAb potency when compared to animals with higher viremia (Manuscript **VII** Figures 3, 5; Tables 2, 3). While this indicated that the potency of the NAb responses required a certain level of viremia and Env diversity, it is also tempting to speculate that early NAb breadth was responsible for the observed viral control.

In conclusion, it was not possible to determine causalities in the interplay of ART, viral evolution and antibody responses. Nevertheless, this study allowed us to propose several hypothetical non-excluding scenarios, which could be specifically tested in subsequent experiments. Additionally, the different findings have important implications with respect to the application of post-exposure prophylaxis, therapeutic vaccination combined with treatment interruption and the elicitation of potent antibody responses:

- The treatment could have played a crucial role in long-term control of virema by allowing the immune system to expand the HIV-specific $CD4^+$ T cell repertoire without fueling infection.
- Another crucial factor could have been the suppression of early increases in viral diversity (a hallmark of early HIV/SIV infection) as it might have allowed initial maturation of the adaptive immune responses without viral escape.
- A number of crucial mutations might have, nevertheless, allowed the viral populations to increase in diversity eventually escaping immune control. This suggests a stochastic, somewhat 'virus-centric' process and could have re-established the 'normal' infection dynamic to the advantage of the virus.
- A more 'host-centric' view could be proposed in which early development of NAbs of a certain breadth sufficiently crippled viral expansion during and after ART to allow long-term control of the infection.
- Of importance for vaccination is that some NAb breadth was observed without high viral titers, while potent NAb responses were clearly correlated to high viremia.

However, it should be noted, that a large number of non-excluding factors that have not been addressed are probably also important and may thus play a crucial role. Those include CTL responses in combination with MHC polymorphisms and the possible appearance of drug resistance during ART.

3.3.2 Properties of late-stage HIV-1 Env

The results presented in Paper **VIII** are part of a series of studies that investigate how the characteristics of Env change in patients after the onset of AIDS [182,229,238,297]. This period is characterized by a significant depletion of the primary, CCR5-positive target cells as well as decreasing antibody immune pressure. Previous work demonstrated that late virus isolates were generally characterized by decreased CCR5 sensitivity, increased *in vitro* fitness, decreased DC-SIGN usage and increased surface charge. All together these studies suggested that changes in tropism and probably lower selection pressure from immune responses, resulted in significant changes to Env and viral fitness. Here, these characteristics were expanded by demonstrating that HIV-1 isolates, obtained after AIDS onset, were found to be more sensitive to neutralization by a commonly used mix of three broad NAbs (Paper **VIII** Figure 1). Furthermore, the increased neutralization sensitivity was correlated to increased replicative capacity and decreased CD4 counts (Paper **VIII** Table 2). Analysis of *env* revealed that the late-stage Env displayed reduced numbers of predicted glycosylation sites, which were localized in proximity to the 2G12 glycan epitope (Paper **VIII** Figures 4, 6; Table 2).

In conclusion, this study demonstrates that the transition to late-stage disease is accompanied by significant changes in Env, which seem to arise in an opportunistic manner as the immune response begins to collapse break down and allow the virus to maximize replicative capacity over neutralization resistance. The observed decrease in glycosylation sites during the late-stage of the disease is well in line with results from studies that have strongly linked glycosylation to neutralization resistance and specifically demonstrated glycosylation to increase during the early phase of infection [181,229,231,233] as well as during the epidemic [176]. The changes highlight the extreme variability and plasticity of the viral fusion machinery and to some extent mirror the changes in Env observed in laboratory-isolates. However, significant changes might also be forced upon Env as the primary $CCR5^+$ $CD4^+$ T-cell population is increasingly depleted.

FUTURE DIRECTIONS

The following section will suggest one or two key experiments or directions that could be taken to expand the findings for each of the projects comprised in this thesis.

Manuscript I: Molecular basis underlying enhanced immunogenicity of optimized H-2D^b-restricted melanoma-associated epitopes

- Thermodynamic measurements of pMHC dissociation and TCR binding with the pMHC complexes used in this study to provide a more complete basis for the understanding of how p3P enhances TCR binding.
- Transfection of D^b-negative APCs with constructs expressing the different D^b-mutants in order to further dissect the relative contributions of increased stabilization capacity and increased TCR binding to the enhanced immunogenicity of EGP *in vivo*.

Paper II: The unexpected T-cell recognition of an altered peptide ligand is driven by reversed thermodynamics

- Crystal structure determination of P14 in complexes with D^b/gp33 and D^b/Y4A complemented by thermodynamic measurements with P14-D^b/Y4S that aim at modifying water coordination.

Manuscript III: Induction of efficient CTL responses against a viral escape mutant through an unconventional peptide optimization

- Using LCMV infections of C57BL/6 and P14 transgenic mice to establish the effect of prophylactic and therapeutic vaccination with PF.
- Determination of the crystal structures of P14 in complex with D^b/gp33 and D^b/V3P.

Papers IV, V: Crystal structure of the HIV-2 neutralizing Fab fragment 7C8 with high specificity to the V3 region of gp125

- Crystal structure determination of HIV-2 gp125 alone and in complex with neutralizing Fab fragments.

Manuscript VI: SIVmac239 gp140 trimers induce potent cross-reactive HIV-1 neutralization when primed with HIV-1 Envelope DNA mixture

- Attempts to more extensively characterize the specificities of the elicited neutralisation response, potentially through the use of specific antibody-depletion steps.
- Extensive comparisons of how modifications to the HIV DNA prime or SIV protein boost affect the elicited neutralization response including a direct comparison of SIV and HIV protein boost.

Manuscript VII: Generation of neutralizing antibodies and divergence of SIVmac239 in cynomolgus macaques following short-term early antiretroviral therapy

- Testing if the occurrence of mutations that confer resistance to ART could have affected the ability of the virus to expand its diversity and escape immunological control.
- Beginning to address the contributions of CTL pressure and escape on the outcome of this study.

Manuscript VIII: Increased sensitivity to broadly neutralizing antibodies of end-stage disease R5 HIV-1 correlates with evolution in Env glycosylation and charge

- Parallel analysis of neutralization responses, $CD4^+$ T-cell repertoire and preferred target cells to establish if changes in neutralization pressure or tropism primarily drive the in the changes observed in Env.

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