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DISSECTION OF HIV-1 ENV-SPECIFIC B CELL RESPONSES IN NONHUMAN PRIMATES

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The cover picture shows a schematic representation of the HIV-1 Envelope glycoprotein trimer. Shown in light blue is a crystallized gp120 core fitted inside a cryo-EM generated native spike shown as the dark blue sheen.

The picture was kindly provided by Christian Poulsen and Christina Corbaci, Scripps research institute, San Diego, CA

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ABSTRACT

Vaccine-induced protection is generally mediated by long-lived antigen-specific B cell responses. Most licensed vaccines target pathogens that display relatively low variability, but for highly variable pathogens, such as HIV-1, vaccine development is more challenging. This thesis is focused on understanding vaccine-induced B cell responses against the HIV-1 envelope glycoproteins (Env), a critical vaccine target. Information about the immunogenic properties of candidate Env immunogens remains limited and so far the elicitation of broadly neutralizing antibodies (bNAbs) were not reported for any vaccine regimen tested in primates. Thus, there is a need to investigate vaccine-induced B cell responses against Env in more detail and to identify means to improve upon current Env-based vaccine strategies. Here, I investigate B cell responses in nonhuman primates immunized with soluble HIV-1 Env trimers to address these questions, as well as to gain an enhanced understanding about B cell responses to complex protein antigens in general.

In **paper I** we established several assays for the evaluation of B cell responses in macaques. Following immunization with soluble trimeric Env, we comprehensively analyzed the B cell responses in the periphery, bone marrow, and mucosal compartments and further evaluated the elicited Abs for neutralization activity and protection in a SHIV challenge model. We observed high levels of Env-specific B cell responses following immunizations, improved breadth of neutralization compared to responses elicited by a monomeric Env vaccine tested in humans and delayed acquisition of SHIV infection compared to in control immunized animals. In **paper II** we evaluated longitudinal B cell responses following immunization with soluble trimeric Env and influenza HA protein, the latter included for comparative purposes. We found that peripheral B cell responses declined rapidly following boost, while antigen-specific long-lived plasma cells were stable for >6 months following immunization, for both antigens. In **paper III** we established a system for high-resolution evaluation of B cell responses in nonhuman primates. We first characterized the rhesus immunoglobulin loci to allow analyses of Ab gene usage and somatic hypermutation. We next isolated monoclonal antibodies (MAbs) targeting the HIV-1 primary receptor binding site (CD4bs) on Env and we examined the binding specificities of these Abs compared to infection-induced MAbs to unravel limitations of current vaccine-induced responses. In **paper IV** we optimized the RT-PCR method used in **paper III** for isolation of Ab V(D)J sequences from rhesus macaque B cells to facilitate future use of the macaque model for B cell studies.

In conclusion, this thesis establishes several methods for the evaluation of B cell responses in nonhuman primates and it demonstrates that the soluble HIV-1 Env trimers induce potent, but relatively short-lived peripheral B cell responses. Additionally, we describe, for the first time, a set of vaccine-induced CD4bs-directed MAbs and we characterize their binding and neutralizing properties and discuss the implications of these results for improved Env vaccine design.

LIST OF PUBLICATIONS

I. **Christopher Sundling**, Mattias N. E. Forsell, Sijy O'Dell, Yu Feng, Bimal Chakrabarti, Srinivas S. Rao, Karin Loré, John R. Mascola, Richard T. Wyatt, Iyadh Douagi, Gunilla B. Karlsson Hedestam. *Soluble HIV-1 Env trimers in adjuvant elicit potent and diverse functional B cell responses in primates*. Journal of Experimental Medicine. 2010. 207;9. 2003-2017.

II. **Christopher Sundling**, Paola Martinez Murillo, Martina Soldemo, Mats Spångberg, Karin Lövgren Bengtsson, Linda Stertman, Mattias N. E. Forsell, Gunilla B. Karlsson Hedestam. *Immunization of macaques with soluble HIV-1 and Influenza virus envelope glycoproteins results in a similarly rapid contraction of peripheral B cell responses after boosting.* Accepted for publication in Journal of Infectious Diseases. Online publication in mid December 2012 and in print February 15, 2013.

III. **Christopher Sundling***, Yuxing Li*, Nick Huynh, Christian Poulsen, Richard Wilson, Sijy O'Dell, Yu Feng, John R. Mascola, Richard T. Wyatt, Gunilla B. Karlsson Hedestam. *High-resolution definition of vaccine-elicited B cell responses against the HIV primary receptor binding site.* Science Translational Medicine. 2012. 4, 142ra96. *Equal contribution

IV. **Christopher Sundling**, Ganesh Phad, Iyadh Douagi, Marjon Navis, Gunilla B. Karlsson Hedestam. *Isolation of antibody V(D)J sequences from single cell sorted rhesus macaque B cells.* Journal of Immunological Methods. 2012. 386:1-2. 85-93.

PUBLICATIONS NOT INCLUDED IN THIS THESIS

Gujer, C. **Sundling, C.** Seder, R. A. Karlsson Hedestam, G. B. Loré, K. *Human and rhesus plasmacytoid dendritic cell and B-cell responses to Toll-like receptor stimulation*. Immunology. 2011. 134:257-69.

Gujer, C. Sandgren, K. J. Douagi, I. Adams, W. C. **Sundling, C.** Smed-Sörensen, A. Seder, R. A. Karlsson Hedestam G. B. Loré, K. *IFN-α produced by human plasmacytoid dendritic cells enhances T cell-dependent naïve B cell differentiation*. Journal of Leukocyte Biology. 2011. 89:811-21.

Sundling, C. O'Dell, S. Douagi, I. Forsell, M. N. Mörner, A. Loré, K. Mascola, J. R. Wyatt, R. T. Karlsson Hedestam, G. B. *Immunization with wild-type or CD4-binding defective HIV-1 Env trimers reduces viremia equivalently following heterologous challenge with simian-human immunodeficiency virus*. Journal of Virology. 2010. 84:9086-96.

Douagi, I.* Forsell,* M. N. E. **Sundling, C**. O'dell, S. Feng, Y. Dosenovic, P. Li, Y. Seder, R. Loré, K. Mascola, J. R. Wyatt, R. T. Karlsson Hedestam, G. B. *Influence of novel CD4 binding-defective HIV-1 Envelope glycoprotein immunogens on neutralizing antibody and T-cell responses in nonhuman primates*. Journal of Virology. 2010. 84:1683-1695 *Equal contribution

Douagi, I. Gujer, C. **Sundling, C**. Adams, W. C. Smed-Sörensen, A. Seder, R. A. Karlsson Hedestam, G. B. Loré, K. *Human B cell responses to TLR ligands are differentially modulated by myeloid and plasmacytoid dendritic cells*. Journal of Immunology. 2009. 182:1991-2001.

Mörner, A. Douagi, I. Forsell, M. N. **Sundling, C**. Dosenovic, P. O'dell, S. Dey, B. Kwong, P. D. Voss, G. Thorstensson, R. Mascola, J. R. Wyatt, R. T. Karlsson Hedestam G. B. *Human immunodeficiency virus type 1 Env trimer immunization of macaques and impact of priming with viral vector or stabilized core protein*. Journal of Virology. 2009. 83:541-551.

Sundling, C. Schön, K. Mörner, A. Forsell, M. N. Wyatt, R. T. Thorstensson, R. Karlsson Hedestam, G. B. Lycke, N. Y. *CTA1-DD adjuvant promotes strong immunity against human immunodeficiency virus type 1 envelope glycoproteins following mucosal immunization*. Journal of General Virology. 2008. 89:2954-2964.

Forsell, M. N. McInerney, G. M. Dosenovic, P. Hidmark, Å. S. **Eriksson, C**. Liljeström, P. Grundner, C. Karlsson Hedestam, G. B. *Increased human immunodeficiency virus type 1 Env expression and antibody induction using an enhanced alphavirus vector*. Journal of General Virology. 2007. 88:2774-2779.

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PREFACE

This thesis will address how antigen-specific B cell responses develop and are maintained following immunization with complex viral glycoproteins, with a focus on the HIV-1 envelope glycoproteins (Env). Immunizations were performed with well-characterized recombinant Env trimers in nonhuman primates from the *Macaca* species, as they are highly relevant biological models due to their similarity to humans. To enable readers outside of this field to obtain a thorough understanding of the work presented in this thesis the introduction will address key areas necessary to understand the problems associated with mounting an effective and long-lasting B cell response following Env immunization. The main areas that will be addressed are:

- The development and maintenance of antigen-specific B cell responses.
- A general introduction to vaccines, how they work, and novel technologies.
- B cell responses to HIV-1 and how the virus evades host immunity.
- Animal models to study infectious disease, with a focus on nonhuman primates.

Following the introduction I will briefly present the major methods used in the papers presented in this thesis and then discuss the results of the papers.

LIST OF ABBREVIATIONS

Ab	Antibody
AID	Activation induced cytidine deaminase
AIDS	Acquired immunodeficiency syndrome
ASC	Antibody-secreting cell
BCR	B cell receptor
bNAbs	Broadly neutralizing antibodies
bp	Base pair (referring to the number of nucleotides)
CD4bs	CD4 receptor binding-site
CDR	Complementary determining region
Con	Constant region (of immunoglobulin)
CoRbs	Co-receptor binding-site
cryo-EM	Cryo-electron tomography
CTL	Cytotoxic T lymphocyte
D	Diversity (region in immunoglobulin)
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
Env	HIV-1 Envelope glycoproteins
FACS	Fluorescence-activated cell sorting
FR	Framework
Gag	Group-specific antigen
GC	Germinal center
HA	Influenza hemagglutinin
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
Igκ	Immunoglobulin kappa chain
Igλ	Immunoglobulin lambda chain
IgL	Immunoglobulin light chain
J	Joining (region in immunoglobulin)
LLPC	Long-lived plasma cell
MAb	Monoclonal antibody
MBC	Memory B cell

MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
Nef	Negative factor
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
Pol	Polymerase gene
RAG	Recombination activating genes
RT	Reverse transcriptase
SHIV	Simian/Human immunodeficiency virus
SHM	Somatic hypermutation
SIV	Simian immunodeficiency virus
TLR	Toll-like receptor
TRIM5α	Tripartite motif protein 5α
V	Variable (region in immunoglobulin)
Vif	Viral infectivity factor
VLP	Virus-like particle

1 AIMS

The specific aims of the individual papers were:

Paper I: To evaluate B cell responses in the periphery and bone marrow following immunization with soluble trimeric HIV-1 Env and to assess the protective effect against mucosal heterologous SHIV challenge.

Paper II: To determine the persistence of B cell responses to soluble HIV-1 Env and influenza virus HA following boost to evaluate if HIV-1 Env displays non-conventional antigenic properties compared to the HA control protein.

Paper III: To characterize the rhesus macaque immunoglobulin loci and to compare it with the human counterpart; and to isolate a panel of monoclonal antibodies (MAbs) directed toward the CD4bs from immunized rhesus macaques and evaluate their functional properties compared to CD4bs-directed MAbs isolated from HIV-1 infected persons.

Paper IV: To improve the efficiency of antibody sequence isolation from bulk, or single sorted, rhesus macaques B cells by adapting an RT-PCR based method described for the human system.

2 B CELL RESPONSES

2.1 A BRIEF INTRODUCTION TO B CELL DEVELOPMENT

The ability of the humoral immune system to respond to and neutralize almost any pathogen lies in the diversity and functional properties of the B cell receptor (BCR). The functional BCR is constructed via a complex series of gene segment recombination events during B cell development in the bone marrow (reviewed in [1]). The BCR is then tested for successful rearrangement and for reactivity to self-antigens, a process called central tolerance, so that mainly B cells expressing functional BCRs that are not self-reactive are released into the circulation. The B cells leaving the bone marrow are immature naïve cells and express surface IgM, but upon reaching secondary lymphoid organs, such as lymph nodes, gut-associated lymphoid tissues, or the spleen, they will develop into mature naïve B cells expressing both IgM and IgD. During the maturation a second step of BCR evaluation will occur, where residual B cells expressing self-reactive BCRs are deleted [2] in a process referred to as peripheral tolerance (for a review on tolerance mechanisms, see [3]). Breakdown of tolerance confers a high risk of developing autoimmune disorders (reviewed in [4] and [5]) illustrating the importance of these mechanisms.

2.2 ANTIBODY STRUCTURE AND GENETICS

It is estimated that rearrangement of the antibody (Ab) gene segments can yield as much as 10^{11}-10^{15} unique combinations [6, 7], enabling Abs to interact with any potential pathogen. This immense diversity originates from the recombination events of variable (V), diversity (D), and joining (J) gene segments localized in the immunoglobulin heavy (IgH) chain locus and the lambda (Igλ), or kappa (Igκ) light chain loci (reviewed in [7, 8]). The numbers of human and rhesus macaque V(D)J and constant region genes as well as chromosome locations of said genes are shown in table I. The overall homology between humans and rhesus genomes is estimated to ~93% [9]. This also applies to the immunoglobulin genes, which are similar both in sequence and organization in the chromosomes [10]. Current knowledge suggests that there are more V-segment open reading frames (ORFs) in the rhesus macaque genome compared to in humans, although a contribution of all V-segments to the functional Ab pool has yet to be confirmed.

Table I. Ig gene numbers in humans and rhesus

	IgH					Igκ				Igλ			
	Ch[c]	V	D	J	Con[d]	Ch	V	J	Con	Ch	V	J	Con
Humans[a]	14	47	23	6	9	2	46	5	1	22	39	7	7
Rhesus[b]	7	63	30	6	8	13	62	5	1	10	50	6	6

[a]Refers to number of functional sequences as determined by IMGT

[b]Refers to number of open reading frames as determined by [10-14].

[c]Ch, chromosome. [d]Constant domain

Flanking the V, D, and J gene segments are recombination signal sequences (RSS). They are composed of highly conserved heptamers and nonamers separated by 12 or 23 base pair (bp) spacers, corresponding to one or two turns of the DNA helix. The recombination of a one-turn spacer with a two-turn spacer is highly favored. In the heavy chain locus the V region is flanked by a two-turn spacer, the D region with one-turn spacers, and the J region with a two-turn spacer. This allows efficient recombination between first the D and J segments and then between the V and DJ segment (Figure 1). Due to the one-turn spacers on both sides of the D segment it can rearrange with the J segment from both the 5' and 3' direction via inversion and deletion respectively allowing translation in all six reading frames [15]. Following transcription of the V(D)J segments they pair with the downstream constant (Con) region, which for naïve B cells is the μ-domain, leading to the production of IgM BCRs.

The recombination events are critically dependent on recombination activating genes (RAG) 1 and 2, which bind the RSS of the donor and acceptor gene segment and catalyze double strand DNA breaks, which then form closed hairpin ends [16, 17]. The hairpins are digested via exonuclease activity and joined by non-homologous end-joining (NHEJ) (reviewed in [18]). In the process of NHEJ palindromic sequences can be added (called P-nucleotides) [19], additionally the enzyme terminal deoxynucleotidyl transferase (TdT) will be recruited and catalyze the incorporation of random non-germline encoded nucleotides (called N-nucleotides) in the heavy chain V-D, D-J, and light chain V-J junctions contributing greatly to the diversity of the complementary determining region 3 (CDR3; see further description of the CDRs below)) [20]. The combined effects of imprecise hairpin digestion and the insertion of P- and N-nucleotides gives rise to the junctional diversity accounting for a large portion of the total variation estimated in the Ab repertoire.

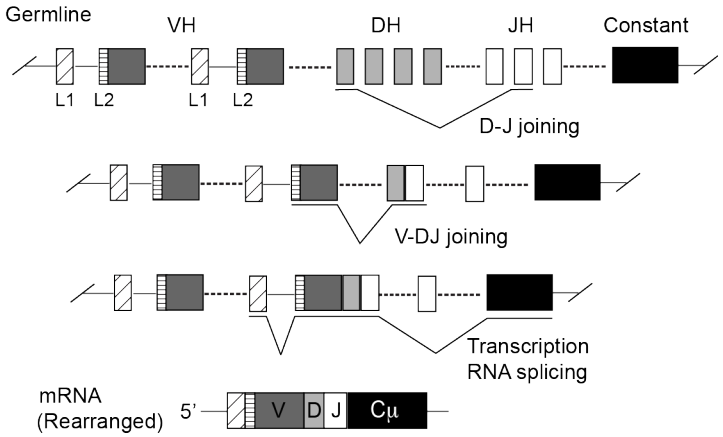

Figure 1. Heavy chain VDJ gene rearrangement. First the diversity (D) and joining (J) segments recombine. This is followed by recombination of a variable (V) and the DJ segments, forming a VDJ gene. After transcription the RNA is spliced to remove an intron between leader 1 (L1) and leader 2 (L2) and between the J segment and the first downstream constant region. The spliced L1 and L2 correspond a signal peptide that directs the antibody mRNA to the rough endoplasmic reticulum and is removed in the translation process. In un-switched cells the constant region is the μ-chain giving rise to IgM antibodies.

Following the formation of a rearranged IgH VDJ and Ig light (IgL) VJ chain they will be produced as heterodimeric proteins that can either be expressed in the form of membrane-bound BCRs or secreted in the form of soluble antibodies. The antibody is divided into a constant and variable domain, where the IgH VDJ and IgL VJ make up the variable domain, while the constant domain is made up from germline encoded constant regions (Figure 2A). It is the constant regions that mediate Fc functions, via binding to complement and Fc receptors (reviewed in [21, 22]), while the variable regions bind the antigen. In humans there are nine IgH constant regions: μ, δ, γ3, γ1, α1, γ2, γ4, ε, and α2 (in order of appearance in the genome), and in rhesus macaques there are eight, as only encode a single α region is encoded [14]. Instead, the rhesus α region displays considerable allelic heterogeneity [12]. The different constant domains are associated with optimal effect against different types of pathogens, and anatomical locations. IgG1 and IgG3 are associated with responses to viruses, IgG2 with encapsulated bacteria, IgG4 and IgE with large extracellular parasites and allergic responses, and IgA with mucosal pathogens. The variable VDJ and VJ domains are further divided into framework (FR) regions 1-4 and CDR1-3. (Figure 2B) [15, 23]. During Ab maturation (covered in section 2.3.1) nucleotide alterations are mainly introduced in the CDR while the FR is kept conserved, possibly due to constraints in the variable domain folding, which is dependent on β-sheets formed by the FR. Additionally, FR2 and 4 form hydrophobic cores that interacts between the heavy and light chains. This folding exposes the highly variable heavy and light chain CDR3 region on the apex of the Ab molecule, increasing the likelihood of antigen interaction [24, 25].

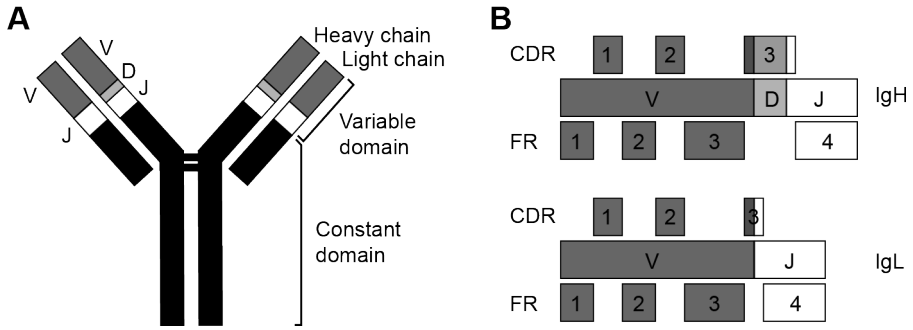

Figure 2. Schematic representation of an antibody. (A) Structural regions of an antibody including heavy and light chain variable and constant regions. The variable region is composed of rearranged V(D)J-segments and the constant regions of germline encoded constant domains. (B) Schematic of heavy (IgH) and light (IgL) chain frameworks (FR) 1-4 and complementary determining regions (CDR) 1-3 within the rearranged VDJ and VJ segments. The same color scheme is used throughout the figure with V (dark gray), D (light gray), J (white), and the constant region (black).

2.3 ANTIBODY DIVERSIFICATION

Following V(D)J recombination the Ab genes can further diversify via two mechanisms; Somatic hypermutation (SHM) and class-switch recombination (CSR). Both mechanisms are critically dependent on the action of the protein activation-induced (cytidine) deaminase (AID), which is upregulated in B cells participating in the

germinal center (GC) reaction following antigen-BCR interaction [26-28]. AID mediates deamination of cytosine (C) to uracil (U), which is mutagenic when paired with guanosine (G) in DNA. Uracil mimics thymidine (T) and during replication the U:G mismatch triggers error-prone DNA repair, which leads to mutations at the site of deamination (reviewed in [29, 30]). Deaminations are mainly introduced in WR**C** and WG**C**W (W=A or T, R=A or G) hotspot motifs [31, 32] and are dependent on ongoing transcription [33, 34].

2.3.1 Somatic hypermutation

Mutations in the V(D)J genes start to appear ~100 bp after the transcription initiation site (promoter) and drops off after ~1 kbp, limiting the variability to the Ig genes [35]. Following AID induced C to U deamination, there are at least three mechanisms for repair that can introduce mutations (Figure 3) [30, 36]. (1) During cell division and DNA replication, the U is read as a T introducing an adenine (A) in the corresponding strand. (2) The U is excised via uracil DNA glycosylase (UNG) resulting in a noninstructive abasic site. Upon replication or DNA repair, any of the nucleotides A, T, G, or C can be incorporated. (3) The U:G mismatch triggers the recruitment of the mismatch repair heterodimers MSH2 and MSH6. MSH2 associates with exonuclease 1 that creates single-stranded sequence gaps. These gaps are then repaired by error-prone DNA polymerases. This mechanisms seems important for mutations in germline encoded A:T nucleotides [37].

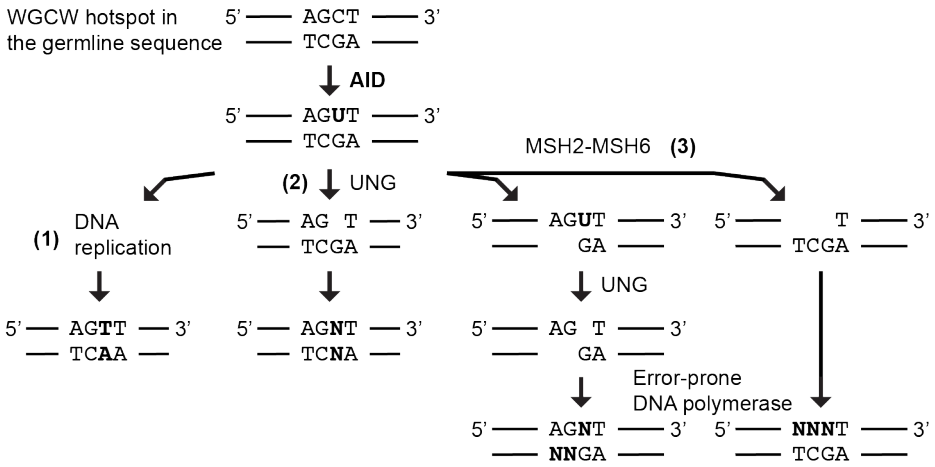

Figure 3. Mechanisms for AID-induced mutations in the V(D)J region. AID catalyzes the deamination of cytosine (C) to uracil (U), which is mutagenic in combination with guanine (G). The deamination can be repaired in at least three ways that leads to changes in the base pair sequence. (1) Upon replication the U can be recognized as a thymidine (T) leading to the formation of a T:A pair at the site of deamination. (2) Uracil DNA glycosylase (UNG) can excise the U leading to an abasic site. This can be repaired by error-prone polymerases or act as a noninstructive base in DNA replication leading to the insertion of any of the nucleotides (A, T, C, or G). (3) The mismatch repair dimers MSH2 and 6 oversee the generation of single-strand gaps spanning several nearby nucleotides. The gaps are then repaired by error-prone DNA polymerases. N indicates either of the nucleotides A, T, C, or G. W indicates nucleotides A or T.

2.3.2 Class-switch recombination

CSR results in the exchange of one Ab constant domain for another while retaining the rearranged Ab variable domain allowing for the B cells to respond to different types of pathogens. In activated mature naïve B cells, the exchange is by default IgM and IgD to a downstream constant domain, determined by the type of innate stimuli and the cytokine milieu associated with the antigenic challenge [38-40]. CSR starts with the recruitment of AID to 5'-AGCT-3' repeats, which are highly concentrated in the switch regions preceding the constant domains and are accessible due to ongoing transcription. AID catalyses the deamination of cytosine on both strands and the resulting UNG and MSH2 base excision leads to DNA double strand breaks [41]. This leads to juxtapositioning of the two switch regions and following repair and ligation, the deletion of the region in between in the form of an extrachromosomal circle (Figure 4) [30, 37].

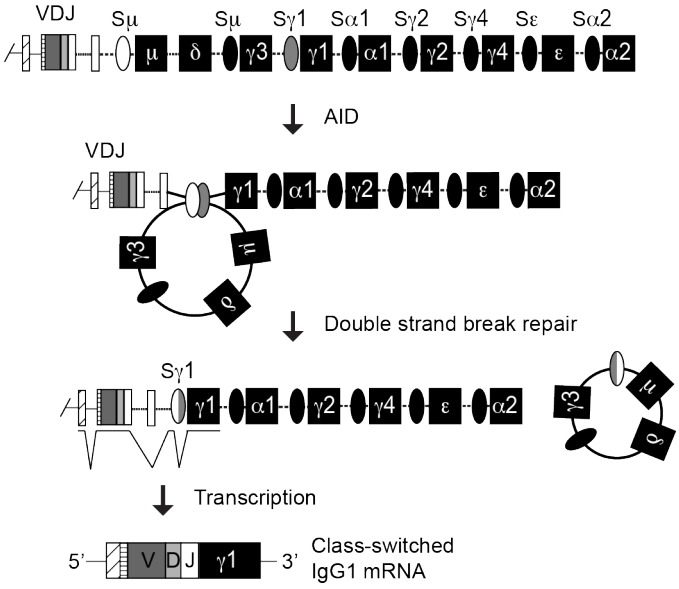

Figure 4. Class-switch recombination (CSR) from the constant μ/δ to γ1 region. AID deaminates cytosine on both DNA strands. UNG and MSH2 mediate base excision forming double strand breaks. The switch regions are juxtaposed and following DNA break repair form an extrachromosomal circle. Since the γ1 constant domain is now most proximal to the VDJ region, IgG1 antibodies will be produced. The cell still retains the capacity to change to an isotype further downstream.

2.4 B CELL RESPONSES TO ANTIGEN STIMULATION

When a mature naïve B cell encounters its cognate antigen it will be internalized, processed and presented on MHC class II molecules. The required affinity to active the naïve BCR is ~1 μM [42], however, this threshold can be reduced to 50 mM if the antigen can support BCR cross-linking to increase the avidity effects [43]. Following BCR ligation, the B cell will be activated leading to upregulation of CCR7 and EBI2, which are important for B cell homing to the interface between the T cell and B cell zones of secondary lymphoid organs, where B cell can receive T cell help [44, 45]. After 1-2 days at the B/T interface, surviving B cells will either enter the germinal center (GC) reaction or become extrafollicular plasma cells [46]. The decision to become an extrafollicular plasma cell or GC B cell is in part dependent on BCR affinity, with lower affinity B cells entering the GC program [47]. B cells with higher affinity BCRs were shown to display increased presentation of MHC class II-restricted peptides making them more competitive for $CD4^+$ T cell help at the initial B/T interface, which stimulates rapid plasma cell differentiation [48]. In the GC reaction,

CD4+ T cell help is mediated by follicular T helper cells characterized as $CXCR5^+$ $CCR7^-$ $CD4^+$ cells that express Bcl-6 and produce IL-21 [49].

B cells designated for the GC reaction also upregulate Bcl-6, a transcription factor that promotes their survival and repress premature differentiation into memory and plasma cells [50, 51]. Furthermore, expression of CXCR5 allows the cells to move toward follicular dendritic cells (FDC) in the light zone where they sample cognate antigen and receive T cell help [52], after which they move to the dark zone where they proliferate extensively and undergo SHM [53, 54]. Evaluation of improved or reduced BCR affinity takes place in the light zone by sampling antigens presented by FDCs, where higher affinity clones outcompete lower affinity clones [55]. However, there appears to be an affinity roof at ~0.1 nM as affinities higher than this do not lead to a higher peptide-MHC II load, and therefore no further competitive advantage [42, 56]. At these affinities there is therefore no further need for Ab SHM.

2.5 B CELL MEMORY

B cell memory alludes to B cell derived responses that persist long after clearance of the antigen that initially generated the response. It is mainly composed of two cell types; Memory B cells (MBC), which are quiescent circulating cells expressing surface bound BCR, but do not produce Abs (reviewed in [57]) and long-lived plasma cells (LLPC), which mainly reside in the bone marrow and continuously produce large amounts of Abs without the need for re-stimulation by antigen (reviewed in [58, 59]). The majority of both cell-types originates from the GC, have undergone SHM, display high affinity and are often class-switched, all of which are desired properties of successful vaccines. These are the cells the current PhD thesis focuses on.

MBCs were shown to persist at low levels without the presence of cognate antigen or T cell help for long periods of time [60-62]. They are mainly localized in proximity to secondary lymphoid organs and especially the spleen to increase the chance of antigen encounter [63-65]. MBCs express a reduced activation threshold coupled with an increased expression of co-stimulatory molecules and activation markers compared to naïve B cells and they can therefore quickly react to antigen challenge [66-69]. Upon re-encounter with the cognate antigen MBCs respond by proliferating and differentiating into short-lived antibody-secreting cells (ASC) that produce large amounts of Abs [70, 71]. Peak IgG responses observed in the periphery following immunization or infections are typically reached after 14 days for a primary encounter and 7 days following boost, and thereafter wane quickly [72-75] and [paper II].

Although circulating Ab titers derived from LLPCs can be detectable for >100 years following immunization [76], LLPCs are not intrinsically long-lived as proposed for the MBCs, although they have been suggested to be imprinted with a maximum lifespan determined by the magnitude of B cell signaling received at the initiation of the immune response [77]. The survival of LLPCs is dependent on the localization to a survival niche [59, 78]. The homing to such a niche is mediated through the surface

expression of chemokine receptors, where CXCR4 will allow homing to the bone marrow (CXCL12 production) [79, 80], CCR5 and CCR28 to the mucosa [81], and CXCR3 to sites of inflammation [81]. In the bone marrow the plasma cells will reside in close proximity to stromal cells that produce high levels of CXCL12, the ligand for CXCR4, and provide interaction between ICAM-1 on the stromal cell and LFA-1 on the LLPC [82]. Further, key cytokines implicated in LLPC survival are IL6, APRIL, and BAFF [83-85]. Stromal cells are not necessarily the main producer of these cytokines, as neutrophils [86], eosinophils [87], basophils [88], and megakaryocytes [89] have been implicated as important contributors.

It is not entirely clear where the LLPCs originate from. It is known that the mutation level and affinities of Abs encoded by LLPCs is slightly higher than those encoded by MBCs [90, 91] allowing Shlomchik and Weisel to hypothesized that there is a temporal switch in the GC reaction where MBCs will be produced first, followed by LLPCs [92]. Following boost Radbruch *et al*. proposed that PCs generated by differentiating MBCs compete with previously resident LLPCs and displace them for access to the survival niche [59]. This hypothesis is supported by observations of both antigen-specific and non-specific plasma cells in the circulation after immunization [93]. However, it is not clear how such competition would occur. Another recently described mechanism for clearing space in the LLPC niche is through selected apoptosis of antigen-specific LLPCs via binding of immune complexes to FcRγIIB expressed on LLPCs [94]. As immune complexes would be highly prevalent in the circulation following boost this would selectively open niche space at a time where new Ab reactivities with potentially higher affinity are generated [95].

3 VACCINES TODAY AND TOMORROW

3.1 A BRIEF HISTORY ON VACCINE DEVELOPMENT

The concept of vaccination started with Edward Jenner and his discovery in 1796, that people previously infected with cowpox were resistant to, or only received mild symptoms from infection with the highly pathogenic smallpox virus. He further learnt, that if he took material from scabs of a cowpox-infected person and gave to a previously unexposed individual, that person would later be protected from smallpox infection. The next big discovery to advance the field of vaccinology was the concept of attenuation, discovered by Louis Pasteur, where less virulent variants of the infectious agent are used for inoculation, inducing protection against challenge but not causing disease.

In the beginning of modern vaccinology, vaccines were mainly developed through chemical inactivation of whole bacteria or viruses, such as for anthrax and rabies. This was followed by attenuation via passaging of viruses *in vivo* (e.g. yellow fever virus and Japanese encephalitis virus) or of bacteria *in vitro* (e.g. Bacille Calmette Guérin). The discovery of cell-culture methods to grow viruses in the mid 20th century, enabled attenuation and production of a wider range of live vaccines (e.g. measles, mumps, varicella, rubella, and the oral polio virus vaccines). Following the discovery and production of whole-particle-based vaccines was the development of subcomponent vaccines, where only parts of the infectious agent are used in the vaccine preparation. These vaccines were considered safer due to the lack of a replicating pathogen and could effectively be given to immune-compromised people. Successful subcomponent vaccines include the diphtheria and tetanus toxoid vaccines as well as the vaccines against flu, anthrax, and rabies, which are based on crude preparation extracts. Even more defined are the recombinant protein vaccines developed for hepatitis B (HBV) [96] and human papilloma virus (HPV) [97], where only the actual immunizing antigen is produced using recombinant DNA technology and expression in defined production cell lines. However, increasing antigen purity often leads to decreased immunogenicity. As a consequence, co-administration of immune-stimulatory components, referred to as adjuvants, which activates innate immune responses and promote adaptive immunity are needed.

3.2 ADJUVANTS

As several new vaccine candidates currently undergoing clinical trials are based on recombinant proteins [98-100], there is an urgent need for improved understanding and licensing of improved adjuvants. To date only three adjuvants are approved for clinical use in humans, although several others currently undergo clinical trials (reviewed in [101] and [102]). Currently approved adjuvants include Alum, which is based on aluminum salts and has been in clinical use for almost a century. Alum was recently found to stimulate the immune response via activation of the inflammasome [103, 104], although redundant mechanisms have been suggested [105]. Other adjuvants approved

for clinical use are MF59, a water-in-oil emulsion [106], and AS04, a combination of Alum and monophosphoryl lipid A. Monophosphoryl lipid A is a ligand for toll-like receptor 4, suggested to enhance local cytokine production, improving the activation of antigen-presenting cells (APCs) [107].

Iscoms, Iscomatrix™, and Matrix™ are experimental adjuvants that have been evaluated in both preclinical and clinical trials [108-110]. They are cage-like structures that are formed when mixing purified fractions of *Quillaia saponaria* extracts, cholesterol, and phospholipids. For Iscomatrix™ and Matrix™ the adjuvant is mixed with the antigen in solution at the time of inoculation, while for Iscoms the antigen is incorporated into the cage-like structures under denaturing conditions during the preparation, limiting their use as conformational B cell epitopes may be disrupted by the treatment. These adjuvants induce strong innate cytokine responses and efficient priming of B cells and $CD4^+$ T cell responses and even some $CD8^+$ T cell responses through cross-presentation [109, 111, 112]. The humoral immune responses typically show a balanced Th1/Th2 profile (reviewed in [113] and [110]). For papers I and II presented in this thesis, the Abisco-100 adjuvant based on the Matrix™ technology was used in combination with the toll-like receptor (TLR) 9 ligand CpG-ODN. The addition of TLR-ligands to non-TLR based adjuvants was shown to improve immune responses in some settings [114-116].

In addition to being required for the induction of *de novo* immune responses to purified protein antigens, adjuvants may be used to improve responses of vaccines that work poorly in the elderly or in partly immune compromised individuals [117]. The addition of an adjuvant also enables a reduction of the antigen dose necessary for the immunization, an important aspect if large numbers of vaccine doses have to be produced quickly, such as during epidemics or pandemics [118, 119]. For influenza vaccination, the addition of the MF59-adjuvant has also been suggested to increase the breadth and affinity of the antigen-specific Ab repertoire [120, 121], but whether the inclusion of adjuvants allow additional specificities to be recruited into the immune reaction remains to be shown.

3.3 CORRELATES OF VACCINE PROTECTION

To enable the evaluation of vaccine candidates in clinical and preclinical research accurately it is important to determine correlates of vaccine protection. For many of the currently licensed vaccines, correlates or surrogate markers have been established, although most are based on empirical evidence rather than known mechanisms of protection (reviewed in [122, 123]) (Table II). For almost all vaccines, Abs have been shown to correlate with protection from infection [122]. However, as discussed by Plotkin (2010), there are several confounding factors to consider when examining potential correlates. For example, high pathogen challenge dose might overcome vaccine-induced immunity. Furthermore, the mechanisms of protection from infection is not necessarily the same as recovery from infection, illustrated by the need for Abs to protect against infection to occur, but cell-mediated immunity to resolve an ongoing

infection [124, 125]. For antibody responses, both specificities and effector functions are important [126-129], and the capacity of different vaccines to stimulate these features may vary between different age groups [130, 131]. Also, the immune system has developed redundancy, where several different mechanisms can mediate protection against, or resolve, an infection individually if necessary. As shown for the HBV vaccine, protection is not necessarily lost because antibody titers fall below the threshold of detection; vaccine-induced memory can induce swift and potent responses abrogating infection [132, 133]. Furthermore, correlates of protection may vary due to the genetic characteristics of different individuals, in particular their major histocompatibility complex (MHC) expression [134, 135].

Table II. Correlates and surrogates for vaccine protection and estimated longevity.

Licenced vaccines (USA)	Read-out [122]	Antibody half-life Years (CI) [76]
Anthrax	Toxin neutralization	
Diphtheria	Toxin neutralization	19 (14-33)
Hepatitis A	ELISA	
Hepatitis B	ELISA	
Hib polysaccharides	ELISA	
Hib conjugate	ELISA	
Human papillomavirus	ELISA	
Influenza	Hemagglutinin inhibition	
Japanese encephalitis	Neutralization	
Lyme disease	ELISA	
Measles	Microneutralization	3014 (104-∞)
Meningococcal	Bactericidal	
Mumps	Not certain	542 (90-∞)
Pertussis	ELISA (toxin)	
Pneumococcus	ELISA; opsonophagocytosis	
Polio	Neutralization	
Rabies	Neutralization	
Rotavirus	Serum IgA	
Rubella	Immunoprecipitation	114 (48-∞)
Tetanus	Toxin neutralization	11 (10-14)
Smallpox	Neutralization	92 (46-∞)
Tick-borne encephalitis	ELISA	
Tuberculosis	Interferon	
Varicella	FAMA* gp ELISA	50 (30-153)
Yellow fever	Neutralization	
Zoster	CD4+ cell; lymphoproliferation	

*FAMA, Fluorescent antibody to membrane antigen

3.3.1 Specificity of the response

A critical factor for effective vaccine-induced responses is the production of highly specific antibodies. For many of the current vaccines it is still unclear what sub-specificities mediate protection and in some cases total antigen-specific ELISA titers from serum is enough as correlate (Table II). However, for infectious agents that readily escape immune recognition it is important to promote the production of antibodies targeting conserved epitopes, which are not subject to variation. It would therefore be helpful to gain an improved understanding about the specificities successful vaccines elicit and to build on this knowledge when designing vaccines against challenging new vaccine targets.

Much of the knowledge regarding Ab specificities to current vaccines comes from the analysis of plasma or serum responses. Smallpox vaccination has been studied extensively due to the life long immunity provided (reviewed in [136]). Smallpox antigen-arrays have indicated Ab reactivities toward a large portion of the surface proteins [137] and Ab binding to several of these surface proteins was shown to neutralize the virus independently of each other, indicating functional redundancy [138]. These studies are important as they improve our understanding of protective immune responses to viral vaccines.

Recent studies have characterized the antigen-specific Ab repertoire, at the clonal level, following tetanus toxoid vaccination [139, 140]. These studies provide valuable information regarding repertoire breadth, affinity maturation, and clonality following sequential protein immunization in a depth not previously performed. The monoclonal Abs (MAbs) were isolated and cloned from plasma cells six days after boost [141]. Similar approaches were performed following immunization with influenza antigens [73] and smallpox [142] and following HIV-1 infection [143]. For the 150 kDa tetanus toxoid antigen it was estimated that a standard vaccination scheme stimulated a repertoire composed of ~100 clonally different Ab lineages [140]. Further boosting did not expand the amount of distinct clones, nor increase SHM rates, indicating that maximal levels were reached. There was, however, a slower average off-rate, translating to slightly higher affinity [139]. The level of affinity reached was between 10 μM and 10 pM with an average of ~1 nM, approaching the suggested upper limit [42, 56]. This affinity was reached with IgH SHM rates of 10-15% at the amino acid level. Similar SHM levels and affinities were observed for influenza vaccination [73, 144] and following HIV-1 Env immunization of rhesus macaques [10]. These studies show that immunization can induce high levels of SHM, translating into affinities close to the suggested maximum roof. However, for HIV-1 many of the broadly neutralizing Abs isolated from infected individuals have mutation rates significantly higher than 20% (amino acid level) [145], indicating that special circumstances might be needed to drive the elicitation of such Abs.

3.3.2 Durability of the response

In addition to the elicitation of appropriate specificities, a successful vaccine needs to induce a durable immune response. One measurement of durability is the antibody half-life, which can be measured in circulation, starting six months to 3 years after vaccination or infection when peak responses have subsided [60, 75, 76] and [paper II].

The Ab half-life of several vaccines and infections were determined [76, 146] and found to vary between 11 years for tetanus to >3000 years for Measles virus (Table II). While antibody titers do not always correlate with vaccine protection, as discussed for the HBV vaccine above, it is interesting to note that different types of antigens vary greatly in the longevity of the response they elicit. Attenuated pathogens, such as measles and rubella, induce antibody half-lives of >100 years, while non-replicating subunit vaccines, such as the tetanus and diphtheria vaccines induce Ab responses that display half-lives of 11 and 19 years respectively [76]. It has been suggested that repetitive structures of antigens (e.g. antigen bound to a virus surface) enabling extensive BCR cross-linking together with T cell help is important for induction of long-lived responses [77]. An additional important factor could be the persistence of antigen which is likely different between a replicating vector and a subunit vaccine. Unfortunately, it is challenging to perform studies to evaluate the longevity of vaccine-induced immune responses due to the time and cost required, especially as responses in small animal models may not be indicative of responses in humans.

3.4 NEXT GENERATION VACCINE DESIGN

Despite the success of current vaccines in limiting- and in some cases eradicating disease, there are still many infectious agents responsible for high morbidity and mortality around the world. For many of these agents the development of new vaccines is of high priority (reviewed in [147]). So far, the most potent and effective vaccines are based on live attenuated strains that replicate with reduced efficiency in the host. These vaccines, however, carry the risk of reverting to pathogenic forms or produce disease in immune-compromised people [148, 149]. Therefore, alternative approaches are explored to reduce or abrogate virulence factors associated with replicating pathogens. For example, target antigens from one pathogen may be express by a non-pathogenic vector, such as the expression of respiratory syncytial virus fusion protein in a parainfluenza virus, instead of the native surface hemagglutinin [150]. Another approach to attenuate live vaccines is the deletion of key virulence factors, as performed for dengue virus and polio [151, 152].

For pathogens, such as HIV-1 and HCV that have a high degree of genetic and structural plasticity, live viral vectors containing substantial parts of the genome will most likely always be considered hazardous due to the risk of reverting to pathogenic forms [153, 154]. Therefore the design of vaccines against these agents is mainly focused on the expression of selected antigens, either via recombinant production, vector expression systems, or plasmid DNA with the aim of inducing immunity targeting conserved regions [155-159]. Furthermore, the approach of using

combinations of vaccine modalities, termed prime-boost, has shown to improve vaccine efficacy in a HIV-1 phase III clinical study [160] and an experimental smallpox vaccine [161], indicating potential positive effects of mixing e.g. viral vectors or DNA to induce strong T cell responses followed by boosting with protein to achieve high Ab titers.

One method of finding potential antigens is via *reverse vaccinology*, where the genomics of the pathogen is used to screen for potential target antigens, followed by expression and functional assays [162]. This approach has been successful in identifying targets for group B meningococcus [163] and several other bacterial species [164, 165], but not yet for viral pathogens. However, viruses display very few proteins on the surface, limiting the need of *reverse vaccinology* approaches. For viruses where the surface proteins contain substantial diversity, an understanding of conserved naturalization-sensitive regions will be more important [166]. For several highly variable viruses, Influenza [167, 168], HCV [169], and HIV-1 [145], potently neutralizing MAbs have been isolated. By crystallizing Ab-antigen complexes as well as native antigens an improved understanding of how neutralization is achieved can be reached [155]. The knowledge can then be applied in *structure-based vaccine design*, where recombinant vaccine-candidates can be generated and tested through rational design [170, 171]. A more focused approach is to graft the epitope of interest onto *scaffold proteins*, unrelated to the pathogen [172]. By consecutively immunizing with different scaffolds expressing the same epitope of interest the immune response should focus on the grafted epitope [173, 174].

4 HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1)

4.1 INTRODUCTION

Since its discovery, HIV-1 has received substantial attention and a large portion of the literature published on infectious diseases every year is directed toward understanding HIV-1 pathogenesis and improving HIV-1 vaccine design. These studies suggest that it is exceptionally difficult to create a vaccine that protects against HIV-1. It is therefore becoming increasingly clear that a thorough understanding of the immunogenicity of individual antigens, in particular the surface-exposed Env antigens, and knowledge about how HIV-1 evades immune recognition is necessary. These issues are discussed below.

4.2 HIV-1 STRUCTURE AND REPLICATION

HIV-1 is a positive stranded RNA virus, possessing a genome of approximately 9.2 kbp and belongs to the Lentivirus genus of the *Retroviridae* family. Viruses in the *Retroviridae* are enveloped by a lipid membrane, which is derived from the infected host cell upon budding. All viruses in the *Retroviridae* family encode three common genes; *gag*, *pol*, and *env* (Figure 5). The HIV-1 *gag* gene encodes a polyprotein, which upon proteolytic cleavage yield the: matrix, capsid, nucleocapsid and p6 proteins. The *pol* gene encodes three enzymes necessary for the viral life cycle; protease, reverse transcriptase and integrase, while *env* encodes the envelope glycoproteins gp41 and gp120. HIV-1 also encodes three accessory proteins; Vif, Vpr, Vpu, and three regulatory proteins; Tat, Rev, and Nef (reviewed in [175, 176]).

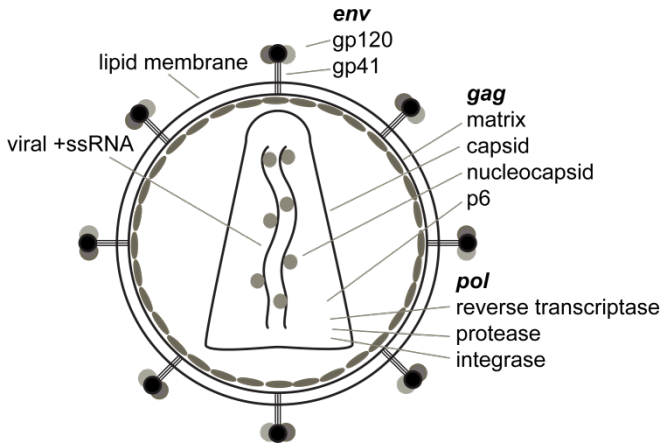

Figure 5. The structure of HIV-1. Shown are the gene products of *env*, *gag*, and *pol* in the context of the mature virion.

HIV-1 binding to the host cell occurs in a two-step process [177]. It is initiated through binding of gp120 to the primary host cell receptor, CD4 [178]. This induces conformational changes in Env, forming the highly conserved co-receptor binding site (CoRbs) [179-182]. The CoRbs then interacts with CCR5 or CXCR4 depending on the tropism of the virus [183]. This interaction initiates extensive conformational changes in gp41, leading to the formation of the six-helix bundle and subsequent membrane fusion [184-187]. Upon entering the cytoplasm the capsid uncoats and the viral RNA is released. The RNA is reverse transcribed into double stranded DNA by the error prone

reverse transcriptase. The cDNA interacts with the HIV-1 integrase and additional viral and cellular components to form the pre-integration complex [188], which is transported into the nucleus where the viral DNA is integrated with the host cell genome [175, 189]. After integration the virus can become latent and persist for the lifetime of the infected cell, making eradication of infection very difficult [190-192].

Starting from the 5' long terminal repeat (LTR), host cell RNA polymerase II performs transcription from the integrated provirus. The initial RNA splice variants encode Tat, Rev, and Nef. Tat binds a secondary RNA structure, the transactivation response region (TAR) in the LTR greatly enhancing RNA synthesis through the phosphorylation of RNA polymerase II [193]. Nef appear to have several effects on host cell molecules, and is responsible for downregulation of host cell CD4, CD28, and MHC class I [175]. Nef has also been implicated in binding to p53, potentially affecting the protein half-life, making the infected cells more resistant to apoptosis [194]. Rev is responsible for the shift to expression of late-phase structural proteins through the interaction with Rev responsive elements (RRE) in single-spliced and non-spliced transcribed mRNA. Rev acts as a transport molecule that is shuttling between the nucleus and the cytosol, mediating the transport of RRE-containing mRNA transcripts to the cytosol to allow their translation [195, 196]. Late stage transcripts include the polyprotein Gag p55 or Gag-Pol p160 and Env together with Vif, Vpu, and Vpr.

Matrix, capsid, and nucleocapsid encoded by the Gag p55 polyprotein are responsible for virus particle assembly at the host cell membrane, while p6 is important in virion budding [197]. The budded virions contain un-processed Gag-Pol p160 and are immature and non-infectious until cleaved by the viral protease to indicated subcomponents (Figure 5). The accessory proteins Vif, Vpu, and Vpr were shown to have a wide array of effects [198-200]. Among the most studied is the mechanisms by which Vif counteract the host cell enzyme apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3 (APOBEC3) [201, 202]. The APOBEC3 proteins belong to the same family as AID, responsible for Ab SHM (described in section 2.3.1). APOBEC3 proteins incorporate in virions upon budding and follow the virus to the next host cell. When the virus infects a new cell, APOBEC3 catalyzes C to U deamination on the negative strand transcripts in the reverse transcription process, leading to G-to-A transitions in the resulting positive strand DNA, with the potential of disrupting downstream gene products [203]. However, it has also been suggested that APOBEC3 deamination can contribute to viral diversity and insertion of drug-resistance mutations [204]. Vif counteracts APOBEC3 by two mechanisms: interfering with its incorporation into virions by targeting it for ubiquitinylation and degradation, and reducing the translation of APOBEC3 mRNA [205, 206]. The effect of Vif is species specific [207], similar to another host innate restriction factor, Tripartite motif protein 5α (TRIM5α), which is also counteracted by HIV-1 in humans [208]. It is not entirely clear how TRIM5α exert its effects, but it contains a PRY/SPRY domain that can recognize incoming capsid structures and has been proposed to interfere in the uncoating of the virus and sequestration into cytoplasmic bodies targeted for

proteasomal degradation [209-211]. For more comprehensive reviews about APOBEC3 and TRIM5α see [202] and [203].

4.3 THE ENVELOPE GLYCOPROTEINS

The HIV-1 envelope glycoproteins (Env) are produced as a singly spliced mRNA from a larger RNA also containing Vif, Vpu, and Vpr. The Env mRNA contains an RRE (described above) enabling its transport from the nucleus to the rough endoplasmic reticulum (rER) where it is translated to an 88-kDa precursor protein, which is co-translationally modified by the addition of N-linked glycans almost doubling the molecular weight to a 160-kDa glycoprotein referred to as gp160. In the ER the precursor protein forms multimers [212], which are proteolytically processed into gp41 and gp120 in the post-ER/Golgi compartment by host cell furin [213]. Transport through the Golgi apparatus allows further modifications of the glycans into complex type N-linked sugars [214]. On the surface, the gp41-gp120 complex is expressed as non-covalently linked heterodimeric trimers [215, 216], the only virally encoded surface exposed proteins. It has been shown, biochemically and via cryo-electron tomography (cryo-EM), that there are only ~10 trimeric Env spikes per virion [217-219]. Several host cell derived proteins are also found in the membrane of the budding virion and have been implicated in viral attachment to target cells [220, 221].

HIV-1 gp41 is responsible for anchoring Env in the cell/virus membrane. It contains a long cytoplasmic tail interacting with the matrix protein, a transmembrane domain, and a glycosylated ectodomain that is mostly shielded by gp120. HIV-1 gp120 is composed of five constant regions (C1-5) and five variable regions (V1-5) [222]. V1-4 contains conserved cysteines flanking the variable regions, enabling the formation of loop structures via disulphide linkage [223], which are exposed on the surface of gp120 and are highly immunogenic [224-226].

Extensive efforts have been made to obtain a crystal structure of the unliganded Env, but due to the gp41-gp120 instability, the inherent conformational flexibility and high density of glycans on gp120, such efforts have been unsuccessful. However, several structures were solved for the individual gp41 and gp120 subdomains. Structures of gp41 have enabled the identification of Env as a likely trimeric complex [216] while the six-helix bundle [227] shed light on the mechanism of virus-host cell membrane fusion. Structures of gp120 were obtained from trimmed and deglycosylated gp120 core molecules unliganded and bound to soluble CD4 and/or MAbs [228-232]. These structures enabled the identification of an inner and outer domain of gp120 connected by a bridging sheet (Figure 6A). The inner domain faces the trimer axis and gp41, while the outer domain is more exposed on the surface of gp120. It is mainly the outer domain that is heavily glycosylated to create a “silent face” not easily recognized by the humoral immune system (Figure 6B) [185, 233]. Furthermore, with the identification of the gp120 “neutralizing face” the core structures have been instrumental in immunogen design efforts, reviewed in [234].

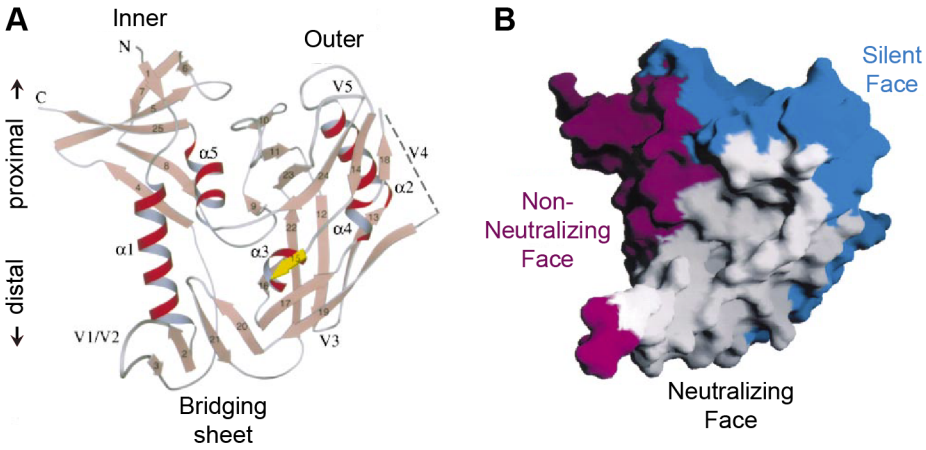

Figure 6. Crystal structure of gp120 core shown in ribbon diagram (A) and surface rendering (B). (A) The division of gp120 core into the inner and outer domain separated by four beta strands forming the bridging sheet are indicated. Beta strands are shown in salmon and alpha helixes in red. The proximal arrow indicate direction toward the virus membrane and the distal arrow toward the host cell membrane, the same orientation is followed in (B), where the gp120 core is divided into three regions based on recognition of the immune system. The non-neutralizing face, directed toward the trimer axis, is shown in magenta. The heavily glycosylated silent face is shown in blue, and the CD4-binding neutralizing face is shown in grey. (A) was adapted from [229] and (B) from [233]. Both figures were reproduced with permission from Nature Publishing Group.

The unliganded structure obtained for HIV-1 gp120 core display the CD4-bound conformation [228], similarly observed in previous gp120 core structures crystallized in combination with ligands [229-232], indicating that the variable loops and gp41 deleted in the gp120 core stabilize Env in a native conformation, while their removal favors a CD4-bound conformation. However, as the CD4-bound conformation is not readily exposed on native spikes it will be necessary to obtain high resolution images of Env in its native state to inform immunogen design efforts. Attempts at using cryo-electron tomography to visualize unliganded surface bound HIV-1 Env have generated images with a resolution of ~10-30 Å. This is not enough to trace individual atoms and protein secondary structures, as with crystallization, but substantial shifts or subdomains in the Env structure are visualized [219, 235-239].

A recent effort to investigate the composition of the native HIV-1 Env was presented by Mao *et al*., where they used single particle cryo-EM to obtain a model of the native Env at high resolution [239]. They expressed cleavage-defective primary HIV-1 Env of the JR-FL strain, with a truncated cytoplasmic tail to increase surface expression [240]. The Env was solubilized from the cell membrane and flash frozen. More than 90,000 images were acquired and merged to obtain a resolution of 10.8 Å. At this resolution Env has a tetrahedral appearance, with a large central cavity separating the protomers. Individual subdomains of Env were visible and five different domains could be identified (Figure 7). Interestingly a novel domain was observed that suggest interactions between the gp120 protomers. The domain was named the "gp120 trimer-association domain" and encompasses a six-way junction between the protomers at the apex of the trimer. The contacts are most likely composed of the V1/2 and V3 loops,

which extend inward toward the trimer axis in a highly structured manner. Further support for the importance of the V1/2 loop for trimer stability is shown when a single N-linked glycan deletion in the V/2 stem or V1/2 loop deletions enables CD4-independent binding to CCR5 and infection of cells lacking CD4 but expressing CCR5 [237, 238, 241].

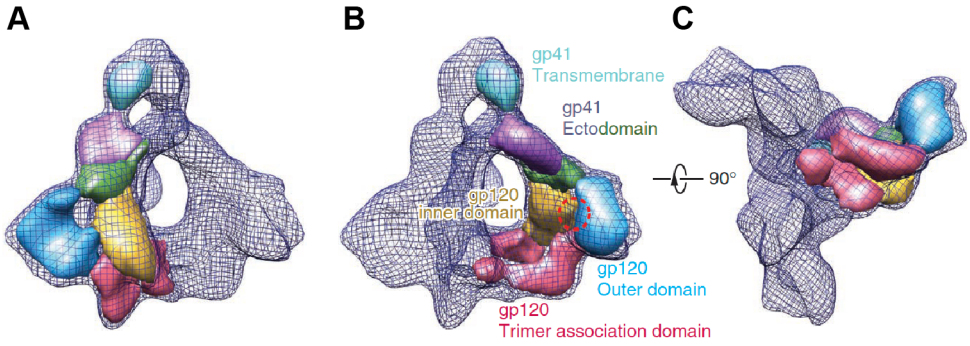

Figure 7. Subdomains included in one HIV-1 Env protomer as determined by Mao *et al.* Shown are the gp41 transmembrane domain (light blue), the g41 ectodomain (purple/green), the gp120 inner domain (gold), the outer domain (turquoise), and the gp120 trimer association domain (pink). The general area of the CD4 binding-site is circled in red. (A) Shows the trimer from the side/back. (B) Shows the trimer from the side/front. (C) Shows the trimer from the apex (from the view of the host membrane). The figure was adapted from [239] and reproduced with permission from Nature Publishing Group.

The Mao *et al*., cryo-EM structure display high homology to a cryo-EM structure described by Liu *et al.,* where native HIV-1 Env was evaluated in conjunction with CD4 and/or MAb-binding, similarly to the first crystallized gp120 core [229]. By fitting the gp120 core into native and post CD4 binding cryo-EM pictures, molecular models could be generated explaining the conformational changes observed. Liu *et al.* showed that CD4 binding causes an outward rotation of the individual gp120 subunits, exposing the CoRbs and extending the V3 loop, as previously proposed [230]. As the gp120 subunits move away from each other, the bound CD4 and V1/V2 loop move away from the center of the spike, creating an open conformation, exposing the gp41 ectodomain, which can then interact with the host cell membrane to mediate fusion (Figure 8).

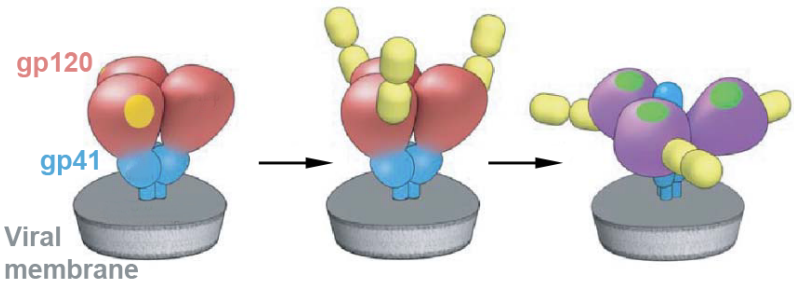

Figure 8. Model describing conformational changes occurring in HIV-1 Env following CD4 binding. The viral membrane is indicated in grey, gp41 in blue, and gp120 in red. Following CD4 binding (yellow) the gp120 monomers rotate outwards, extending the V3 loop (green), and creating a more open conformation exposing the gp41 stalk. The figure was adapted from [235] and reproduced with permission from Nature Publishing Group.

4.4 HIV-1 ENV IMMUNE EVASION STRATEGIES

HIV-1 uses several mechanisms to escape Ab-mediated neutralization and to induce Abs toward regions not exposed on the functional Env spike (reviewed in [234]). These mechanisms are mainly centered on variations in the envelope glycoproteins, which are made possible due to the high mutation rate of the HIV-1 genome and the structural plasticity of Env. Here are four major mechanisms described.

4.4.1 Genetic variability

The major challenge with controlling the HIV-1 epidemic is the genetic variability of the virus. Not only does it complicate vaccine design, but it is also puts severe demands on the human immune system to keep up with ongoing infections, as the antigens change faster than new immune responses can be elicited. The basis for the high genetic diversity of HIV-1 is the error-prone reverse transcriptase, which incorporates ~0.2 mutations per genome and replication cycle [242, 243]. Coupled with a very high replication rate of ~10^{10} new virions per day in an infected individual [244], a large number of different viral variants are generated. A large proportion of the humoral immune responses elicited during infection is directed against the variable regions resulting in strain-specific neutralization. The variable regions can, however, readily change both in sequence and length to escape these Abs (Figure 9) [245-249].

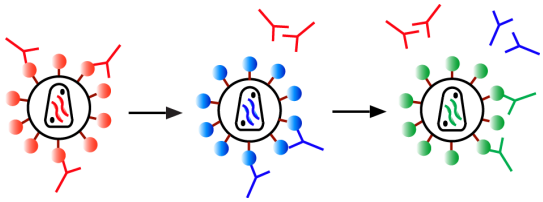

Figure 9. HIV-1 escape from autologous neutralizing antibodies (Abs). When neutralizing Abs are generated escape variants are selected for. This is followed by new autologous neutralizing Abs recognizing the new HIV-1 variant, from which escape will occur again. This cycle will be repeated during the course of the infection. The figure was adapted from [250].

4.4.2 Exposure of non-native Env

As described previously, gp160 is cleaved by the host enzyme furin to gp120 and gp41 that are held together by non-covalent interactions to yield the native Env complex. However, non-native Env structures are also present on the surface of virions, as shown by isolation of virus particles with non-neutralizing Abs [251-253]. Furthermore, the ratio of non-infectious to infectious virus particles is high, potentially due to low expression of viable surface Env [254, 255]. The presence of different Env forms on infectious virus-like particles (VLPs) was studied by Moore et al. Using several complementary methods they observed both gp41 stumps, generated from gp41/gp120 dissociation as indicated in figure 10, and gp41/gp120 monomers [256]. These non-native Env structures are highly immunogenic, but they mainly generate non-neutralizing Abs that do not cross-react with the native Env spike. These decoy immunogens have been proposed to divert the immune system, making the overall Ab response directed against irrelevant target epitopes [257-259].

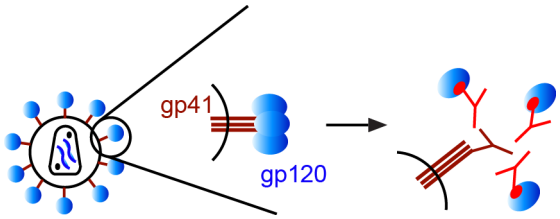

Figure 10. Elicitation of non-neutralizing antibodies toward free gp41 stumps and domains of gp120 not exposed on the functional spike. The figure was adapted from [250].

4.4.3 Conformational masking of Env surfaces

Following CD4 binding, Env undergoes major conformational changes to form the co-receptor binding site (CoRbs), which mediates the interaction with CCR5 or CXCR4 [181, 183]. The co-receptor usage is highly conserved between HIV-1, HIV-2, and SIV [260, 261], suggesting an attractive target for Ab-mediated neutralization. However, despite abundant CoRbs-directed Abs elicited following HIV-1 infection and immunization with Env proteins, no broad neutralization was observed [262-264]. The CoRbs-directed Abs are generated in a CD4-restricted manner, where they are only elicited if there is a high-affinity functional interaction between Env and host cell CD4, as observed in humans and nonhuman primates, but not in e.g. rabbits and mice [112, 264]. The lack of neutralization by this subset of Abs is potentially explained by steric restriction, where Abs fail to gain access to the CoRbs, which is formed after CD4 binding when the Env spike is in close proximity to the host cell membrane [265] (Figure 11A).

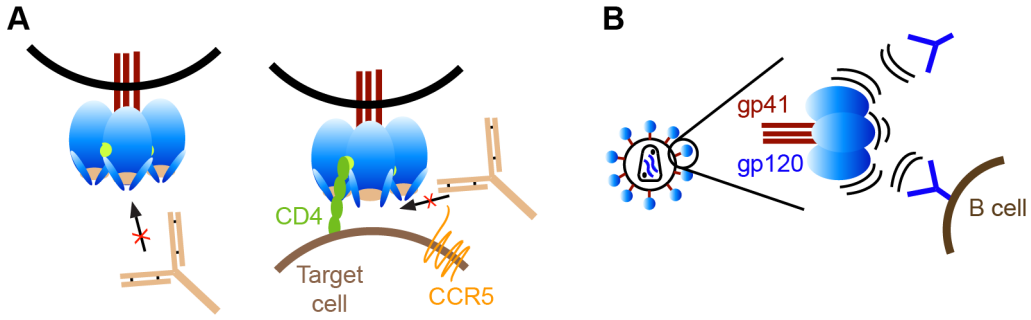

Figure 11. Conformational masking of the CoRbs (A) and via entropic masking (B). (A) The CoRbs of Env only forms after CD4 binding and is therefore inaccessible for circulating Abs. Following CD4 binding, however, the antibodies have restricted access due to steric hindrance. (B) Env possesses a high degree of conformational flexibility impeding strong interaction with a large proportion of the Abs directed toward conserved regions. The figure was adapted from [250].

Another aspect of conformational masking has been proposed by Kwong *et al.* suggesting the existence of an entropic barrier the Abs have to overcome to bind Env stably [266], especially at the CD4-binding site (CD4bs) and CoRbs, which have the capacity to undergo substantial conformational changes [267] (Figure 11B).

More recently it was shown that many of the non-neutralizing Abs elicited toward the CD4bs bind hydrophobic patches in the bridging sheet (described in section 4.3). This has been suggested to elicit substantial conformational changes that are not well tolerated in the context of the functional trimer, with structural clashes between the

binding Abs and protomers or between adjacent protomers, with escape from Ab binding as a result [268]. The moderately broadly neutralizing Ab IgGb12 binds the outer domain of Env (described in section 4.3), which only induce minor entropic changes, and can therefore bind efficiently to functional trimeric Env spikes and neutralize the virus. Another way around the entropic barrier, as suggested by Kwong *et al.* is Ab binding to more than one Env structure, and thereby increasing the binding avidity. This is however complicated by the low density of functional spikes present on the HIV-1 virion [217-219] and will not necessarily overcome the problem with structural clashes occurring from elicited conformational changes.

4.4.4 Glycan masking and quaternary packing

Due to the extremely high density of glycans (approximately half the molecular weight) on the outer surface of Env, large portions of the proteins are more or less inert to the immune system. This protection has been referred to as the "glycan shield" [248] and the area covered, the "silent face" [233]. It was early on, found that immune recognition of glycoproteins was affected by high density of glycans [269]. Since then it was shown that the sites for N-glycosylation (NXS/T) in Env shift readily to allow viral adaption to the host immune response [248, 270] (Figure 12). Addition or removal of glycans, together with insertions and deletions in the V1/2 region can affect the quaternary packing of the Env spike to further shield neutralizing determinants [271].

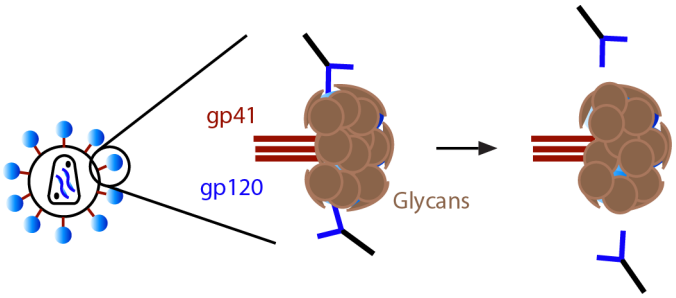

Figure 12. Following the appearance of autologous neutralizing antibodies, N-linked glycosylation sites in Env can be added or deleted to enable antibody escape by occlusion of the target epitope. Adapted from [250].

4.5 HIV-1 TRANSMISSION AND ESTABLISHMENT OF INFECTION

The most prevalent form of HIV-1 transmission, accounting for >95% of all cases, is via sexual contact, with the majority being from heterosexual transmissions. There are several factors influencing the transmission efficiency, such as viral load in the infected partner, presence of other sexually transmitted diseases, lesions in the mucosa, male circumcision, and type of sexual act [272-276]. The majority of the remaining 5% of transmission events is due to inoculations using contaminated needles or other minor routes of transmission, such as mother-to-child and contaminated blood products. Productive infection is thought to arise from as few as a single founder virus in many cases [277, 278] and it is almost exclusively CCR5-using viruses that establish infection [278]. The dependence on CCR5 as the co-receptor for the majority of transmission events is illustrated by the "resistant" phenotype expressed by people homozygous for the CCR5 Δ32 mutant [279, 280]. The mutant consists of a 32 bp deletion, which introduces a premature stop codon in the CCR5 gene, abolishing functional expression on the cell surface. People heterozygous for the Δ32 mutant display partial protection to infection and have a slower disease progression [281, 282].

The mutation is mainly observed in Caucasians and is prevalent in ~10 % of the population.

Upon entering the mucosa- (or gut-) associated lymphoid tissue (MALT) the virus infects and replicates to high levels in activated $CCR5^+$ memory CD4 T cells [283-287], which are the main MALT-resident T cells [283-285]. At peak viremia, as much as 80% of the MALT memory T cells can be infected or killed. A large proportion of the human memory T cells reside in the MALT and even after initiation of antiretroviral therapy and suppression of viremia the reconstitution of the memory T cell compartment is poor [288, 289]. Even more profoundly depleted following infection are $CD4^+$ IL17-producing T helper (Th17) cells [290]. These cells have been implicated in gut homeostasis and production of microbial defensins, which are important for the maintenance of the mucosal barrier [291, 292], thus their removal has been linked to chronic immune activation observed following HIV-1 infection [293]. Chronic immune activation shows a strong correlation with HIV-1 disease progression and has been implicated as a major mechanism behind the pathogenicity of HIV-1 infection [293] (Figure 13). SIV infection of its natural host (e.g. sooty mangabeys) cause a similar rapid and profound depletion of $CD4^+$ T cells; however, infected monkeys do not develop chronic immune activation and do not progress to AIDS [294, 295].

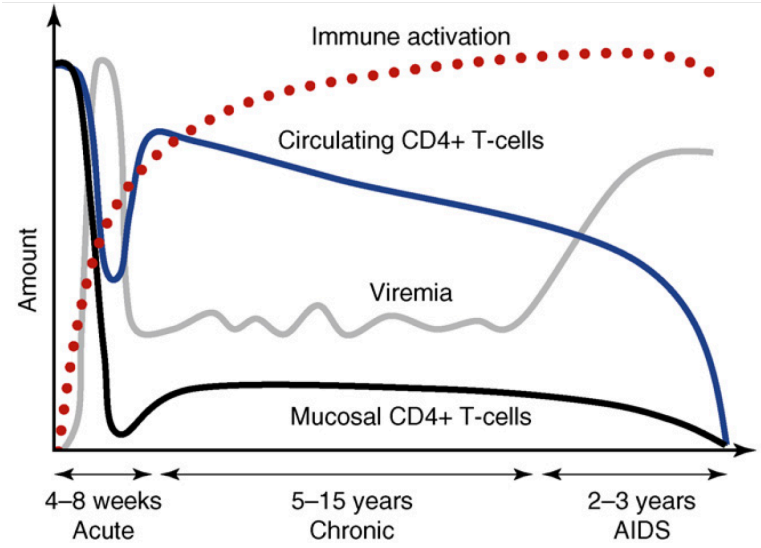

Figure 13. HIV-1 replication (gray), CD4+ T cell counts in the periphery (blue) and mucosa (black), and immune activation (red) following HIV-1 infection. Originally printed in [296]. Reproduced with permission from Elsevier

The earliest T cell-mediated response detected in infected individuals appear around the time of peak viremia [297] (reviewed in [298]). Up until this time the virus replicates in the absence of an adaptive immune responses, but escape mutants will be selected for rapidly as viremia decreases to set-point levels [299]. The magnitude of the viral load set-point, which is largely attributed to the effectiveness of the cytotoxic T lymphocytes (CTLs), is predictive of the time for disease progression to AIDS, with lower level indicating slower progression [300]. Disease progression is further affected by MHC class I, called human leukocyte antigen (HLA) for humans and (Mamu) for rhesus monkeys, expressed by the infected individual [301]. Some HLA types, such as HLA-B*57, B*58, and B*27 are associated with improved control of viral replication in humans [302] and Mamu-B08, B13, and B17 in rhesus monkeys [303], due to their

capacity to present peptides from conserved epitope regions [304, 305]. After the decline to set-point the infected individual is often clinically asymptomatic, and so called clinical latency ensues. However, even during this period there is a high level of ongoing viral replication, resulting in the production of escape mutants. The control of viral replication during this period is attributed to both cellular and humoral responses [306-308]. The latency can last for several years, however, gradually the $CD4^+$ T cell count drops and eventually upon reaching below 200 cells per µl blood, the infected individual usually reaches the clinical status of AIDS and becomes sensitive to opportunistic pathogens [309].

4.6 B CELL MEDIATED RESPONSES TO HIV-1 ENV

4.6.1 The role of B cell responses in HIV-1 infection

The initial B cell response to HIV-1 is observed in the form of immune complexes, as early as eight days after detectable viremia. These Abs are directed toward the gp41 ectodomain of the surface envelope glycoproteins. It takes an additional two weeks before the appearance of Abs directed toward gp120 [310]. Autologous neutralizing Abs to HIV-1 are not detectable until months after infection [247, 248, 311, 312] and are generally strain-specific with limited breadth [313, 314]. However, upon elicitation they exert a significant selective pressure on the circulating virus [315] resulting in rapid generation of escape variants, as discussed in section 4.4, enabling continued high viral replication [316, 317].

Abs that neutralizes a broader range of virus isolates are usually not detected until after several years of chronic infection [318-321]. However, up to 25% of infected individuals have been shown to produce moderate to broadly neutralizing antibodies (bNAbs) after more than two years of infection [318, 320, 322]. Among these a subset are exceptionally broad and can neutralize a large portion of the known HIV-1 strains [320, 323, 324]. Mikell *et al.* analyzed longitudinal samples following HIV-1 infection and observed that the cross-reactive neutralizing Abs mainly developed in individuals where the early Env-specific B cell response targeted epitopes on the functional spike, whereas individuals that did not develop cross-reactive Abs targeted epitopes on monomeric gp120. These findings have implications for vaccine-design where it might be beneficial to mimic the early response observed in individuals developing bNAbs [325]. A similar study by Lynch *et al.* analyzed longitudinal serum specificity toward the CD4bs (more specifically the RSC3 protein, indicative of broadly neutralizing reactivity), and showed that such Abs arose quite early after infection although the time of appearance was variable between donors (10-152 weeks). The titers were also low and up to three years were needed to expand enough to make a significant impact on plasma neutralization [326].

Selective adsorption followed by mapping of broadly neutralizing sera has shown that both the membrane-proximal region of gp41 and several regions on gp120 can be targeted, with frequent observation of glycan-dependent and CD4bs-directed neutralization [319, 321, 327-329]. It has further been shown that the neutralization can

be mediated by relatively few specificities [329]. These findings provide proof-of-concept that the immune system can elicit broad and potent Abs and give hope to the vaccine field, that such Abs can be elicited via immunization. Furthermore, if elicited in sufficient concentrations they are likely to protect the individual from infection or attenuate replication, as indicated by passive transfer studies of bNAbs in macaques followed by subsequent chimeric SIV/HIV (SHIV) challenge [330-334].

In addition to direct neutralization, anti-Env Abs mediate effector functions via the Fc portion of the Abs. Fc-mediated effects have been implicated both for neutralizing [330] and non-neutralizing Abs [335] in protecting monkeys from mucosal viral challenge. However, of non-neutralizing Abs, only those targeting gp41 were shown to have an effect, possibly due to the presence of gp41 stumps on viral membranes, while the non-neutralizing gp120-directed Abs recognize epitopes that are not exposed on infectious viruses [335]. Other gp41-directed Abs have been implicated in delayed HIV-1 disease progression by effectively activating NK cells [336].

4.6.2 Broadly neutralizing antibodies and their implication for vaccine design

For more than 10 years only four broadly neutralizing Abs isolated from HIV-1 infected individuals were known. IgGb12 targeting the CD4bs [337], 2F5 and 4E10 targeting the membrane-proximal region of gp41 [338, 339], and 2G12 targeting high-mannose glycans on gp120 [340]. Thanks to the application of several new technologies additional bNAbs have now been isolated, some of which identify new target epitopes on Env (reviewed in [145]). The techniques used for isolating the MAbs were either from hybridoma immortalization of memory B cells [341, 342], flow cytometric sort of antigen-specific B cells followed by single-cell RT-PCR and cloning [343-346], or screening of supernatants from stimulated memory B cells plated at low cell numbers per well (2-3 cells) for neutralizing activity followed by antibody cloning [347-350]. The flow cytometric sorting strategy uses well-defined baits to fish for specific reactivities, such as B cells directed toward the CD4bs [345, 346]. This approach, however, limits the type of broadly neutralizing reactivities that can be isolated, to previously known determinants. Unbiased screening of stimulated memory B cell cultures circumvents this problem, as shown by the discovery of novel broadly neutralizing reactivities toward trimer and glycan specific regions on Env [347, 348]. However, this method has the caveat that a large number of cells must be screened to find interesting targets. Another recently published method utilizes an approach where flow cytometric sorting is performed with transfected cells expressing Env on the surface. This method enables isolation of memory B cells reactive with the native Env spike [351]. This approach is based on previous findings showing that bNAb but not non-neutralizing Env-directed Abs bind cell surface-expressed Env, while both sets of Abs bind soluble Env antigen [352-354]. Although the method is less efficient than recombinant protein baits it has a higher likelihood of finding Abs neutralizing Env via novel epitopes [351].

The new generation bNAbs display a vastly improved breadth and potency in comparison to the first generation MAbs and several groups have mapped how the Abs bind and neutralize Env to get an improved understanding of how they can circumvent the immune evasion strategies exhibited by Env [355-360].

To investigate how Abs evolve to become broadly neutralizing Wu *et al.* performed 454 sequencing on PBMCs from the donor that the bNAbs VRC01 and VRC03 were isolated from [346] By using Ig family-specific primers for PCR amplification coupled with bioinformatic analysis of obtained sequences, thousands of related clones were identified allowing the Ab linage to be traced in the infected individual [357]. A similar approach was taken for the bNAbs PGT135-137 described by Walker *et al*. [348, 361]. The identification of different evolutionary variants of the bNAbs, together with an improved understanding of potential vaccine targets on the Env trimer, have implications for vaccine design as it may be possible to design antigens that selectively drive clones in the evolutionary chain in a desired direction (reviewed in [362, 363]). Additionally, the design of different antigens for sequential boosting may be a necessary to promote SHM as many of the infection-induced bNAbs display very high mutation rates (>15% of VH nucleotides), something that might be difficult to achieve with a single antigen [10, 139].

4.6.3 Immunization-elicited antibodies

Since the identification of HIV-1 as the causative agent for AIDS numerous attempts were made to design an effective vaccine. Four candidates have made it to phase IIb/III clinical trials (described in section 4.6.3.1) and the results indicate that both T cell and B cell responses are necessary for a successful vaccine [364]. The Env antigens used were recombinant gp120 monomers, which in standardized pseudovirus assays [365, 366] display limited neutralization breadth [72, 367]. Current preclinical vaccine candidates based on recombinant stabilized trimeric Env elicit improved breadth although at low levels and limited to few primary viruses [72, 368-371]. As a proof-of-concept, however, Barnett *et al*. showed that immunization with recombinant trimeric Env could protect monkeys from challenge with homologous SIV-HIV chimeric virus (SHIV), providing evidence that parenteral immunizations can induce sufficient humoral responses to protect against mucosal challenge if the correct Ab specificities are generated [372].

The majority of Abs elicited following trimeric Env immunization are directed toward the variable loops [368, 373-375], limiting pseudovirus neutralization to mainly autologous or T cell lab-adapted virus strains. However, reactivities toward more conserved determinants, such as the CD4bs are also elicited [10, 112]. Following isolation and epitope mapping, a panel of CD4bs-directed MAbs were shown to resemble non-broadly neutralizing CD4bs-directed MAbs elicited during chronic HIV-1 infection [10]. Fine mapping of the Ab binding footprint show that they bind Env in close proximity to the trimer axis (see section 4.3) often interacting with residues in the bridging sheet, possibly inducing conformational changes resulting in steric clashes as

described in section 4.4.3. In contrast, the broadly neutralizing MAb, VRC01, binds an overlapping footprint, but more distally on the gp120 outer domain, perhaps allowing better access of this type of Ab on the functional Env spike. As mentioned previously, attempts to focus vaccine-elicited immune responses on conserved and neutralization sensitive determinants [271, 376, 377] have met with limited success. However, there are some indications that stabilization of specific epitopes can generate enhanced antibody responses to these determinants [378], suggesting that such engineering efforts are promising for the future.

A promising finding was recently published by McCoy et al., where they isolated a broadly neutralizing Ab, called J3, from a llama immunized with trimeric gp140 Env [379]. Llamas display both single heavy chain Abs (VHH) and heavy and light chain paired Abs (similar to humans). The expression of VHH Abs allows efficient evaluation of phagemid libraries where only the VHH repertoire is screened (no artificial IgH-IgL chain pairs has to be made) [380]. J3 was isolated by screening a phagemid library directly for neutralization without previous panning steps to recombinant protein. Interestingly the serum neutralization of the llama did not reflect the breadth and potency of J3 and based on the screening protocol the authors estimated that <0.012% of the total VHH repertoire are J3-like Abs, indicating that J3-like clones did not have a selective advantage following antigen boost, consistent with the observation that J3-binding to recombinant gp140 and gp120 protein in ELISA was similar or reduced to that observed for non-neutralizing VHH clones [380, 381]. This shows the importance of using a screening method where bNAbs can be separated from non-bNAbs.

4.6.3.1 HIV-1 phase III clinical trials

To date four phase IIb/III clinical efficacy trials were conducted. The first two trials, performed by the company Vaxgen were based on recombinant monomeric gp120 Env (AIDSVAX) given with alum adjuvant in seven consecutive doses in North America and the Netherlands (clade B) [382] or Thailand (clade B/E) [367]. The primary target groups were men who have sex with men (MSM) and women with high risk of HIV-1 exposure or injection drug users. More than 7500 persons participated in the two studies and the HIV-1 incidence was 7% and 8.4% respectively in the placebo groups. No effect on HIV-1 acquisition [367, 382] or other immune parameters [383] was observed between the placebo and vaccine arms although all vaccine recipients developed high, but transient, Env-specific Ab responses, with limited breadth [384].

Due to the challenge of eliciting broadly neutralizing antibody responses, focus turned toward T cells as the main targets for a vaccine. Promising data showed that adenovirus vectors could protect monkeys from SHIV infection [385] and it was known that CTL responses were important for suppression of viremia in natural HIV-1 infection [301, 386, 387]. A clinical study was initiated by Merck with a replication-defective adenovirus type 5 vector expressing the HIV-1 genes *gag, pol*, and *nef* of clade B [388]. The vaccine was given three times to 3000 participants. At a planned interim analysis it was found that the vaccine had no effect upon acquisition of infection or viral loads and

when evaluating vaccine-induced immunity it was also found that participants presenting high levels of adenovirus type 5-directed Abs upon study initiation and were un-circumcised exhibited an increased risk of HIV-1 infection in comparison to the placebo group [389]. The STEP trial findings were recently recapitulated in a nonhuman primate study where the monkeys had been made seropositive to Ad5 before immunization and challenge with SIVmac251. There was a trend toward enhanced infection in the Ad5 seropositive group compared with controls but the study was too low-powered to definitively determine if pre-existing adenovirus immunity could enhance acquisition of infection [390]. In another recent study, enhanced acquisition of infection to low dose challenge with SIVmac251 was correlated with high levels of SIV-specific interferon gamma producing cells in rhesus macaques immunized with DNA and VLPs [391]. These findings indicate a potential risk with the presence of large numbers of non-protective activated T cells at the time of infection.

The fourth late phase clinical trial, called RV144, was based on a prime-boost regimen with the canary-pox vector (ALVAC) encoding a modified clade B/E Env and clade B *gag/protease* together with clade B/E gp120 protein monomers (AIDSVAX) [160, 392]. The vaccine was given to more than 16 000 participants in Thailand and upon completion; vaccine efficacy was calculated to 31% in the modified intention-to-treat analysis, with the largest effect in low to medium risk groups [160]. In follow-up analyses, it was suggested that the presence of IgG Abs to the V2 loop correlated with reduced risk of infection [393] and that this effect was ablated if high levels of Env-specific IgA was present [394]. Considering that the RV144 and Vaxgen trials used the same recombinant protein for immunizations, but had different outcomes (31% protection in RV144 and no protection in the AIDSVAX trial) it will be important to further define potential correlates of protection in RV144, as well as to repeat this vaccine concept in independent trials.

5 ANIMAL MODELS IN VACCINE RESEARCH

Animal models are imperative for understanding human immunology and host response to infectious agents. They are frequently used as preclinical gatekeepers for vaccine candidates and they are needed for toxicity and pathogenesis studies. The immune system is a complex biological system that would be impossible to accurately recapitulate by *in vitro* models or *in silico*. However, when using animal models it is important to define confounding factors in the translation to human immunology. It is therefore of great interest to identify the model most suited to the question addressed. In this section I will mainly focus on the nonhuman primate models, the focus of this thesis. I will further briefly discuss additional models important for infectious disease research.

5.1 NONHUMAN PRIMATE MODELS

Nonhuman primates are divided into two groups: old-world monkeys, including baboons, macaques, gibbons, and great apes; and new-world monkeys, including capuchin, howler, and squirrel monkeys. It is mainly old-world monkeys that are used in HIV-1 research due to their similarity to humans and the existence of model viruses inducing disease resembling HIV-1 infection in humans. HIV-1 can only productively infect chimpanzees due to the host restriction factors APOBEC3 and TRIM5α (described in section 4.3), which limit infection if not countered by HIV-1-encoded proteins. Early in the field of HIV-1 research, chimpanzees were used for pathogenesis and vaccine studies [395-397]. However, due to ethical considerations, cost and the fact that they seldom develop AIDS after infection, the field has turned toward other models [398, 399].

Currently, the most prominent models for HIV-1 translational studies are Asian macaques (rhesus, cynomolgus, and pig-tail macaques), and especially rhesus monkeys (reviewed in [400]). They share a high level of homology to humans both in sequence (average 93% [9]), gene expression, [401] immune cell composition [402, 403], and responsiveness to stimulation with pathogen-associated danger signals [404, 405]. They can be efficiently infected with SIV and chimeric SIV/HIV viruses (SHIV) to evaluate pathogenicity and T cell-based vaccines as well as Ab-mediated vaccine protection, respectively. SIV infection recapitulates HIV-1 infection in humans well, and has provided much of the current knowledge regarding mucosal transmission [406], although disease progression to AIDS tends to be faster than observed for humans (reviewed in [400, 407]). Depending on the SIV isolate and the route of infection used for challenge, it is also possible to mimic conditions resembling natural infection in humans [408]. SHIVs were constructed specifically to enable the evaluation of Ab-based vaccines in monkeys. The classical SHIVs contain the *env*, *tat, vpu* and *rev* genes from HIV-1 and the remaining genes, important to counter the host cell restriction factors from SIV (Figure 14). Additional SHIVs have been constructed, e.g. SIV containing only RT from HIV-1, to enable evaluation of RT inhibitor drugs [409] or

HIV-1 containing only SIV-*vif*, closely resembling native HIV-1 but with the caveat that it can only infect pigtail macaques efficiently due to their lack of TRIM5α (Vif counteracts APOBEC3) [410].

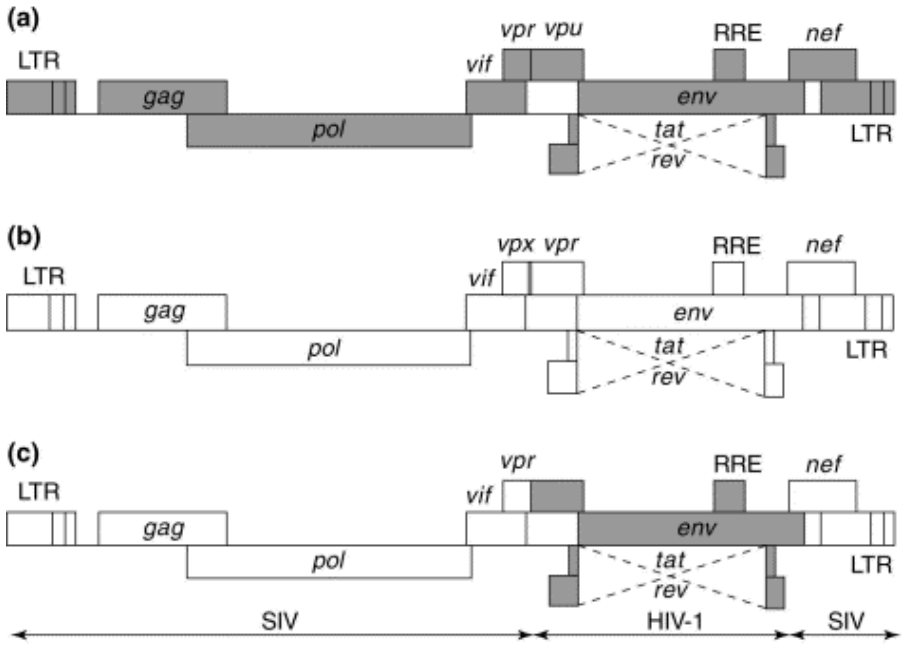

Figure 14. Genetic organization of (a) HIV-1 (gray areas), (b) SIV (white areas), and (c) SHIV, where the SIV Env, tat, rev, and vpu has been exchanged for corresponding HIV-1 variants. Adapted from [411] and reproduced with permission from Elsevier.

Initially, SHIVs using the CXCR4 co-receptor were constructed [412-418]. These viruses were after passage often very pathogenic and replicated well in macaques depleting peripheral but not gut $CD4^+$ T cells [419]. However upon vaccination they were relatively easy to protect against [385, 420-422], raising concerns about whether CXCR4-using SHIVs are appropriate model viruses for HIV-1 challenge studies [423]. CCR5 using SHIVs were subsequently developed, initially only with HIV-1 clade B Env [424-426], but also more recently with Env from clade C [427-429]. The CCR5 using SHIVs are considered to mimic natural HIV-1 infection better and different stocks that are more or less resistant to neutralizing Abs were generated [419, 430, 431].

As with all models, there are caveats. This is also true for the rhesus macaque model and there are some differences that are important to consider (reviewed in [432]). For example rhesus macaques express a larger number of MHC class I and II alleles than humans and were shown to mount stronger T cell responses to experimental vaccines [432]. However, the result from the human STEP trial was recapitulated in macaques [390], indicating that the model is predictive under specific circumstances (here using an SIV challenge instead of a CXCR4 SHIV that initially indicated protective effect in immunized monkeys [385]). It was also recently shown that rhesus macaques likely express a larger number of Ab Ig genes compared to humans (Table I) [9, 10], although the effect of this is unknown. So far immunization of nonhuman primates with

recombinant proteins closely resembles responses in humans to similar antigens [367, 433, 434]. Furthermore, MAbs isolated from HIV-1 Env immunized rhesus macaques display reactivities closely resembling those elicited during infection in humans, indicating that both species mount similar Ab responses [10]. This is further supported by the homologous V(D)J family usage profiles in human and rhesus B cell populations [10, 11, 13, 90, 435-439].

Another important difference, especially when evaluating Ab Fc-mediated effects, is the variability in Fcγ-receptors (FcγR). In a study by Warncke *et al.,* they evaluated the FcγR in cynomolgus macaques and compared effector function and IgG subtype binding to the human counterparts [440]. They show that IgG1 (which is the most abundant circulating Fc subtype) have identical binding and effector function in both species (likely being highly predictive between the species), while IgG2 and IgG4 display fundamental differences. In humans IgG2 and IgG4 display weak binding to FcγRs, while cynomolgus macaques show the opposite with strong binding. To balance the increased signaling the macaques instead express higher affinity between IgG2 and the inhibitory FcγRIIB, displaying an overall more reactive profile.

5.2 OTHER MODELS

Although several nonhuman primate species are used as models for HIV-1 pathogenesis and vaccine evaluation, their usage is limited by cost, infrastructure, and availability. Also according to the three R's (Refine, Reduce, Replace), higher order organisms should be replaced with lower order organisms or preferentially *in vitro* methods if possible.

Due to the possibility to genetically and physiologically modify mice they have been adapted to become possible models for human disease. Several types of humanized mice have been generated and evaluated for how well they model the human immune system and how they respond to pathogen or vaccine challenge (reviewed in [441, 442]). The models are generally based on three different approaches: (1) Mice are transgenic for one or more human genes, only allowing evaluation of very specific interactions, with the caveat that the system is highly artificial. (2) Immunodeficient mice grafted with human $CD34^+$ progenitor cells. These mice produce human cells but due to the cells maturing in mouse tissues they display significant difference to the human counterparts. (3) Immunodeficient mice transplanted with human cells and tissues. One example is the BLT mouse that has transplanted human bone marrow, liver, and thymus to allow maturation of humanized cells in a humanized environment [443] Of these three types of humanized mice models, the BLT mice produce the most homologous immune system, with similar tissue distribution, receptor expression, and response to antigen or pathogen challenge, warranting further evaluation as a translational model [442].

6 MATERIALS AND METHODS

In this section the major materials and methods used throughout the papers will be presented. For more detailed information, see papers I-IV.

6.1 RECOMBINANT ENVELOPE GLYCOPROTEINS

All proteins were produced via transient transfection of 293-F cell in serum free suspension cultures as previously described [264]. Briefly, cells were transfected at 1-1.2×10^6 ml^{-1} using 293-Fectin and incubated for four days. Supernatants were harvested and cleared via centrifugation and filtration before the proteins were separated by affinity chromatography. The first separation was via surface glycan binding to lentil-lectin coated beads. After extensive washing the proteins were eluted and captured via their His-tag in a second column containing nickel-chelated beads. Following further washing the proteins were eluted and the buffer was exchanged to PBS. Protein integrity and purity was assessed by SDS-PAGE. Protein probes for use in ELISpot analysis were biotinylated enzymatically on the C-terminal avitag [225]. All antigens used for immunizations were purified from LPS using Triton X-114 extraction prior to inoculation.

The soluble, wildtype gp140-F constructs composed of gp120 and the ectodomain of gp41 were first described by Yang *et al.* [444]. They contain a C-terminal trimerization motif from the T4 bacteriophage fibritin stabilizing Env in a trimeric context. Furthermore, to eliminate gp120 dissociation from the soluble gp140-F trimers, a substitution mutation was introduced in the furin cleavage site. A number of variant Env proteins were also made as shown in figure 15 and described in [225]. All proteins contain a His-tag and the protein used as probes contain a sequence encoding the Avi-tag at the C-terminus of the protein.

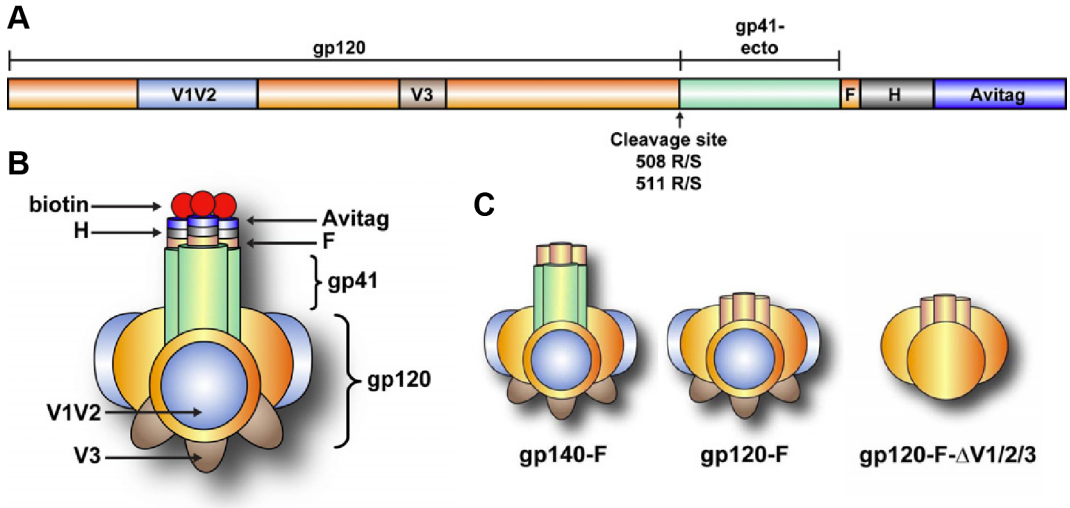

Figure 15. Graphical representation of the Env glycoproteins used for immunization and as probes. (A) The gp140-F protein composed of gp120, the gp41 ectodomain, the fibritin domain (F), the His-tag (H),

and the Avitag. (B) Representation of a biotinylated, trimeric Env (gp140-F-bio) probe. (C) Trimeric Env probes lacking selected structural determinants for use in the subtractive B cell ELIspot analysis. The figure was modified from [225].

6.2 ANIMALS

Macaques were immunized and sampled for papers I and II. No new animals were used in paper III and IV as samples from the animals described in paper I were used. In paper I, 12 female rhesus macaques and 4 cynomolgus macaques were included and in paper II, 6 female cynomolgus macaques were included. The macaques were kept in pairs in 4 m^3 cages with enrichment according to general guidelines from the Swedish board of agriculture. All experiments were approved by the local ethical committee on animal experiments. Before initiation of the experiments all animals were habituated to the housing conditions for >6 weeks and confirmed negative for SIV, simian T lymphotropic virus, and simian retrovirus type D.

6.3 IMMUNIZATIONS AND SAMPLING

All immunizations were given with Abisco-100 and CpG ODN 2395 adjuvants. The total volume was divided in two halves, which were given in the quadriceps muscle, one half in each leg. In paper I, immunizations were given monthly while in paper II they were given at 0, 4, and 33 weeks.

Immunizations and blood samplings were performed under sedation with 10 mg/kg ketamine (given i.m.). When sampled for mucosal lavages and bone marrow, animals were given an additional 0.5 mg/kg Xylazine (given i.m.) to induce muscle relaxation and analgesia. Blood was collected in 6-9 ml vacutainer tubes containing EDTA. Cells were isolated from blood and bone marrow through density gradient centrifugation with Ficoll-Hypaque. After red blood cell lysis and extensive washing with PBS the cells were frozen in fetal calf serum supplemented with 10% DMSO. Following sampling of mucosal surfaces, the lavages were added to a concentrated protease inhibitor cocktail, spun and the supernatant frozen at -80 °C.

6.4 MEMORY B CELL STIMULATION AND ELISPOT ANALYSIS

Memory B cells were differentiated to antibody-secreting cells (ASCs) by stimulating 1×10^6 PBMCs for four days in 48-well plates with a cocktail composed of pokeweed mitogen (PWM), staphylococcus aureus cowan stain lysate (SAC), and CpG ODN 10103. Following culture the cells were washed and transferred to ELISpot plates coated with anti-IgG and incubated over night. If bone marrow or peripheral plasma cells were enumerated they were plated directly on anti-IgG coated ELISpot plates without previous stimulation. After washing away the cells, antigen-specific IgG was detected by the addition of biotinylated Env probes (see section 6.1). Following the addition of streptavidin-ALP and BCIP/NBT, antigen-specific spots were formed where ASCs had produced Ab during the overnight incubation. Differential B cell

analysis was performed as previously described to determine the frequency of memory B cells directed toward specific sub-determinants of Env [225].

6.5 FLOW CYTOMETRY

In this thesis flow cytometry was used for two purposes: evaluation of the memory B cell stimulation protocol on B- and T cells in paper I (performed on a FACS-Calibur) and sorting single B cells in paper III and IV (performed on a FACS-Aria).

To evaluate the stimulatory capacity of the cocktail on B- and T cells in paper I, PBMCs were labeled with CFSE before culture initiation. After six days of culture the cells were washed and additionally stained with MAbs for CD20 (B cells), CD27 (activation/memory B cell marker), CD4 and CD8 (T cell markers). Proliferation was measured in B- and T cells as decrease in CFSE content. In another set of experiments PBMCs were stimulated for four days before staining with MAbs for CD20, CD4 and CD8, as well as for intracellular IgG. Intracellular staining requires permeabilization of the cells, and was therefore performed after the cell surface staining. All Ab staining incubations were performed in the dark for 20 min followed by washing with PBS supplemented with 2% FCS.

In paper III and IV specific B cell subsets were sorted at single cell density into 96-well PCR plates based on their expression of CD27 (differentiate memory and naïve cells), surface IgG (indicate switched memory B cells), surface IgM (in conjunction with low CD27 indicate naïve cells) and Env probes (used only for the antigen-specific B cell sort performed in paper III). The cells were further stained to exclude T cells (CD3 and CD8), monocytes (CD14), and dead cells (Aqua blue or Grivid). The single cell lymphocyte population was identified based on (SSC-A vs FSC-A) and further removal of cell doublets based on (FSC-H vs FSC-A in paper III, and SSC-W vs SSC-H followed by FSC-W vs FSC-H in paper IV). Final populations sorted were: $CD20^{+}CD27^{+}IgG^{+}$ memory B cells (paper III and IV), $CD20^{+}CD27^{-}IgG^{-}IgM^{+}$ naïve B cells (paper IV), $CD20^{+}CD27^{+}IgG^{+}gp140\text{-}F^{+}$ total Env-specific memory B cells (paper III), and $CD20^{+}CD27^{+}IgG^{+}gp140\text{-}F^{+}gp140\text{-}F\text{-}D368R^{-}$ CD4bs-specfic memory B cells (paper III).

6.6 SINGLE-CELL ANTIBODY CLONING AND EXPRESSION

Single-cell RT-PCR was used in papers III and IV with the only difference that the primer sets were different. In paper III we used previously published primers designed for the isolation of human Ig genes [343] while in paper IV we constructed our own primers adapted to the rhesus genome. We only cloned the RT-PCR generated sequences for Ab expression in paper III.

RT-PCR was performed on single B cells directly sorted into 96-well PCR plates containing lysis buffer as described in section 6.5. In paper IV we show that the addition of carrier RNA in the lysis buffer helps recovery. Reverse transcription was performed with superscript III using random hexamers. Subsequent PCR was

performed in different plates for IgH, Igκ and Igλ, using 50 cycles for the outer primers followed by nested PCR with inner primers for another 50 cycles. Positive amplification was determined with 96-well gels prestained with EtBr or Sybr Safe. The 5' primers consist of mixes covering the V-segment families while the 3' primes are located in the Ab constant regions. A major difference between the primer sets used for paper III and IV is that the new rhesus 5' primers are relocalized further upstream into the leader sequence (see section 2.2), which is less exposed to SHM and should stay relatively constant even in very mutated B cells.

Positive wells from the nested PCR were sent for sequencing to determine V(D)J usage. Cloning primers containing restriction enzyme motifs were then matched to the obtained sequences and were used in a third high fidelity PCR starting from material from the nested PCR. PCR products were enzymatically digested and ligated into expression vectors containing Ab constant regions. Following transformation of bacteria, colonies were screened for positive inserts, which were sent for sequencing to verify the original sequence obtained after the nested PCR. If the sequence was consistent, the matching IgH and IgL chain vectors were co-transfected into 293-F cells using Freestyle MAX reagent. On day four of transfection the supernatants were evaluated for Ab production and Env-binding by ELISA. Successful Ab production was verified through coating with anti-IgG and antigen-specific Abs through coating with different forms of Env. CD4bs reactivity was determined as reduced/abrogated binding to the gp140-F-D368R mutant in comparison to wildtype gp140-F protein. If the cultures were positive for IgG and Env binding they were kept an additional 1-3 days (5-7 days total) after which the Abs were purified.

Ab purification was performed by incubating the cleared supernatant (centrifuged and passed through a 0.22 μm nylon mesh) with protein-G coated beads for 2h. The beads were then extensively washed before the Abs were eluted with low pH buffer. Following neutralization the buffer was exchanged for PBS by centrifugation using 30 kDa cut-off centricons. Finally, the Ab concentration was measured by nanodrop and purity was assessed by SDS-PAGE. The specificity and function of the isolated Abs were evaluated by multiple assays as described in paper III.

6.7 PSEUDOVIRUS NEUTRALIZATION ASSAY

Pseudovirus neutralization assays were used in papers I-III to evaluate neutralizing properties of vaccine-induced plasma or isolated MAbs [365]. In this assay, the capacity of Abs to block infection of the TZM-bl reporter cell line by HIV-1 Env pseudotyped virus particles is assessed. The TZM-bl cells contain a luciferase reporter gene under transcriptional control of the HIV-1 LTR (see section 4.2). They are also stably transfected with the CD4, CCR5 and CXCR4 surface receptors allowing infection by the vast majority of HIV-1 viruses. Following infection and integration the pseudovirus drives the expression of the luciferase gene via production of Tat. The pseudoviruses are generated by co-transfection of 293T cells with a plasmid encoding the HIV-1 Env of interest, and another plasmid encoding the HIV-1 gag-pol backbone.

The resulting pseudovirus is replication-deficient as it lacks the *env* and *rev* in the genome. By pre-incubating the pseudovirus with plasma or MAbs in a dilution series a curve is generated indicating concentration Ab needed to inhibit virus infection. Commonly the titer/concentration necessary to inhibit 50% (ID_{50} or IC_{50} respectively) of infections is shown.

To standardize and categorize the pseudoviruses they were divided into Tiers depending on how difficult they are to neutralize, as determined by well characterized, MAbs and the IgG fractions of plasma or serum from HIV-1 infected individuals [365, 366]. Tier 1 viruses are divided into A and B, where both represent easy-to-neutralize viruses with 1A more so than 1B. Tier 1 viruses generally have more open conformation associated with lab-adapted strains. Tier 2 viruses are more difficult to neutralize and represent primary circulating HIV-1 strains. Neutralization against tier 2 viruses is occasionally observed in vaccine studies, but then usually at high plasma concentrations. Tier 3 viruses represent very-difficult-to-neutralize primary viruses and are not commonly included in neutralization panels to evaluate vaccine-induced responses.

7 RESULTS AND DISCUSSION

Much of the knowledge about B cell responses to HIV-1 Env is derived from serological studies, in particular from small animal models such as mice, rabbits and guinea pigs. However, macaques are also used extensively as preclinical models in HIV-1 vaccine research, but up until this thesis was initiated they were primarily used to evaluate different vaccine modalities in virus challenge experiments. In some cases circulating Ab responses were also evaluated, but there was little focus on the Env-specific B cells themselves, the cells that are the bases for humoral immune responses. This was in part because reliable methods to study macaque B cell responses were lacking. In this thesis several new methods that facilitate studies of humoral immune responses in rhesus macaques were developed. This thesis also provides new knowledge about the rhesus macaque Ig locus enabling the evaluation of B cell responses at the genetic level. Here, these methods have been used to characterize LLPC and MBC responses following immunization with recombinant HIV-1 Env trimers and to characterize the properties of vaccine-induced monoclonal Abs directed against the conserved CD4 binding-site of Env. In this section, the results from **papers I-IV** are presented and discussed

7.1 GENETIC EVALUATION OF RHESUS MACAQUES AS MODELS FOR B CELL RESEPONSES

With the overall high sequence homology between rhesus macaques and humans, as well as phenotypic conservation of many immune cell subsets, macaques represent an excellent preclinical model for vaccine studies (see section 5.1). In an effort to refine the model and gain an improved understanding of Ab-mediated B cell responses, we characterized the immunoglobulin (Ig) loci of a previously published and assembled female Indian rhesus macaque genome [9] in **paper III**. We used previously published rhesus germline Ig genes [11, 445-447] in addition to bioinformatics tools [448] and sequences generated in our own lab to search the rhesus genome for Ig genes. We localized the IgH locus to chromosome 7, the Igκ locus to chromosome 13 and the Igλ locus to chromosome 10. Additionally we extracted V(D)J sequences likely corresponding to Ig gene open-reading frames (ORFs) (Table I). An intact ORF was defined based on the presence of an intact leader sequence and the absence of stop codons in the coding regions. Although the number of proposed Ig gene segments was larger in the rhesus macaque genome compared to in humans, the chromosomal organization was similar and the sequence homology was high (close to the average 93% genome homology between human and rhesus). Additionally when performing joint sequence analysis between the human and rhesus V-segments they group according to gene family, rather than species, indicating a high conservation of the Ig loci (Figure 16).

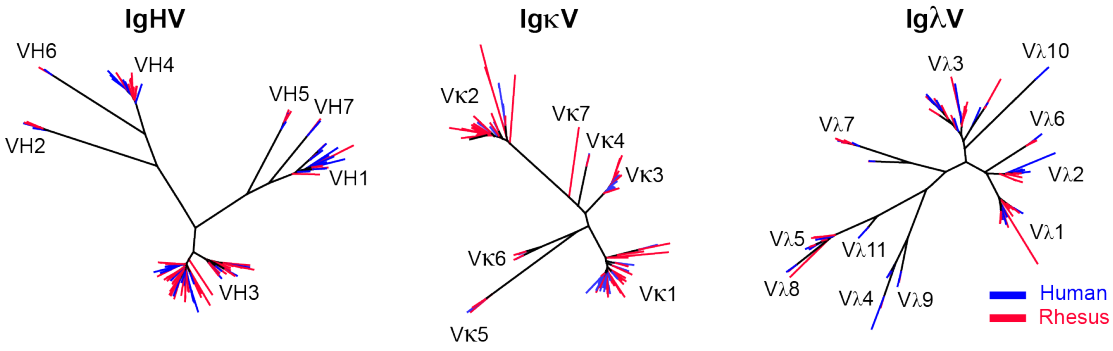

Figure 16. Joined align between rhesus (red) and human (blue) V-segments. Numbers indicates gene family. The figure is adapted from paper IV and reproduced with permission from Elsevier.

In addition to high Ig sequence homology and genetic structure of the heavy and light chain loci, the expression of Ab V(D)J gene families is also similar in sorted IgG^+ MBCs between rhesus [10, 13] and humans [90] indicating that both species utilize their Ab repertoires similarly. Collectively, these results support the use of rhesus macaques as a model for human B cell biology.

7.2 ASSAYS FOR THE EVALUATION OF B CELL RESPONSES IN MACAQUES

7.2.1 Memory B cell differentiation

Due to the quiescent nature of memory B cells (MBCs) it is difficult to quantitatively and qualitatively evaluate the compartment in their native state, however, an inherent effect of MBCs is that they readily differentiate to Ab-secreting cells (ASCs) upon stimulation. This property can be used to quantify antigen-specific memory B cells as ASCs are readily enumerated by ELISpot analysis. In **paper I** we established a protocol for macaque MBCs differentiation into ASCs, which we then used to analyze Env-specific responses following immunizations in **paper I** and **II** (Figure 17).

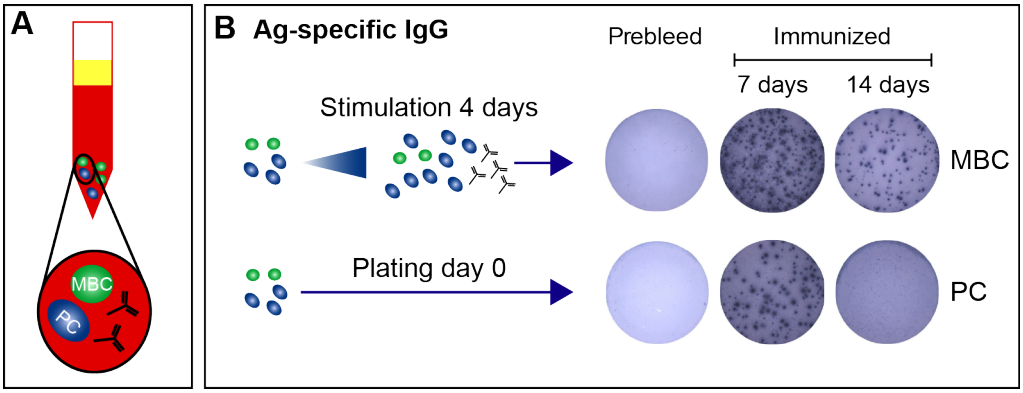

Figure 17. Memory B cells (MBCs) and plasma cells (PC) were enumerated from peripheral blood via ELISpot analysis (A). (B) PBMCs were stimulated for 4 days to allow proliferation and differentiation to Ab-secreting cells (ASC), which were detected via ELISpot. PCs were detected through direct addition of the cells to ELISpot plates without previous stimulation. Representative ELISpot wells are shown before immunization (prebleed), and 1 and 2 weeks after immunization. No antigen-specific PCs were detected in prebleeds and at 2 weeks following immunization.

We evaluated several cytokines and mitogens that had been implicated in MBC differentiation based on previous publications for the human system. The combinations included Pokeweed mitogen (PWM) with the TLR9-ligand CpG and *Staphylococcus aureus* cowan strain lysate (SAC) [449], IL-21 with CD40-ligand [450], and IL-2 with IL-10 and CD40-ligand, or in combinations with IL-6 [451, 452]. We added CpG B or C to all stimulations, as TLR9-ligands were shown to drive human B cell differentiation efficiently, especially for MBCs ([453, 454] and unpublished observations). CpG was slightly less effective at driving rhesus B cell differentiation, although there is still a marked positive effect [405]. The stimulations were evaluated on cynomolgus and/or rhesus macaque cells depending on availability. Of the different combinations tested, two stood out as very potent: the PMW+SAC+CpG combination and the IL-21+CD40-ligand+CpG combination, both which promoted MBC differentiation into ASCs similarly with peak numbers of IgG-producing ASCs obtained after 3-4 days of culture (Figure 18).

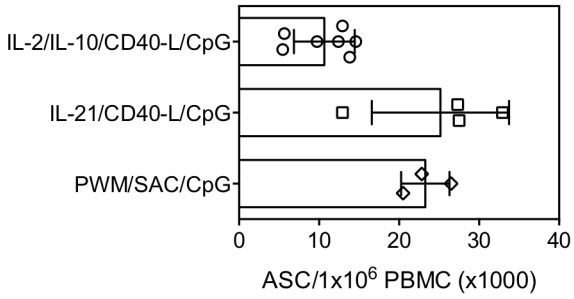

Figure 18. PBMCs were stimulation with indicated combination for 3-4 days followed by enumeration of IgG Ab-secreting cells (ASC) via ELISpot.

While comparable results were obtained with these two combinations, we decided to use PWM+SAC+CpG for several reasons; first, because we had previously observed variability in the CD40-L stimulations when reagents were purchased from different vendors; second, because the IL-21+CD40-L+CpG mix was more expensive; and third, because of reports suggesting that IL-21 with CD40-ligand also drive naïve B cell differentiation, which could potentially act as a confounding factor in the assays [450].

To further understand how the PWM+SAC+CpG stimulation exerted its effect we stained PBMCs with CFSE and cultured them for six days. We observed a high level of proliferation in B cells, but also in T cells. To determine if the T cells had a role in the stimulation we sorted both rhesus and human B cells and T cells and stimulated B cells only or B cell/T cell co-cultures. IgG production from the PWM+SAC+CpG stimulation was almost exclusively detected in the B cell/T cell co-cultures, clearly indicating the dependence of the stimulation on the presence of T cells. CpG alone promote some Ab production even in the absence of T cells, although at a much lower level compared to the T cell dependent stimulations including PWM.

7.2.2 Bone marrow culture

As described in section 2.5 a large proportion of long-lived plasma cells (LLPC) reside in survival niches in the bone marrow, where they are dependent on close contact with

stromal cells. The LLPCs are responsible for producing high-affinity Abs that provide a first line of defense to invading pathogens by blocking infection or reducing the infectious dose, attenuating the infection [455]. The LLPC-dependent Ab production can be sustained for the life of an individual, although different antigens seem to induce different half-lives of the response by mechanisms not yet understood [77]. These qualities show how important it is to obtain an improved understanding of the B cell responses elicited through immunizations. Following Env inoculations we collected bone marrow biopsies at different time points to enumerate LLPCs by ELISpot analysis (Figure 19A). As observed in figure 19B top panel, the antigen-specific LLPC counts are low even when plating large numbers of cells (plated 1×10^6 cells). To see if we could increase the sensitivity of the assay, we cultured bone marrow in complete media for 1-3 weeks (Figure 19B lower panel) followed by antigen-specific ELISA. In the culture wells we observed the formation of stromal networks, likely supporting the survival of the LLPCs. Addition of exogenous IL-6 or APRIL, which were implicated for *in vitro* survival of LLPCs, did not increase the amount of Ab produced. The major limiting factor in observing a positive ELISA signal appears to be the amount of cells cultured. Bone marrow ELISpot was used in both **paper I** and **II**, while the bone marrow culture method was used in **paper II**.

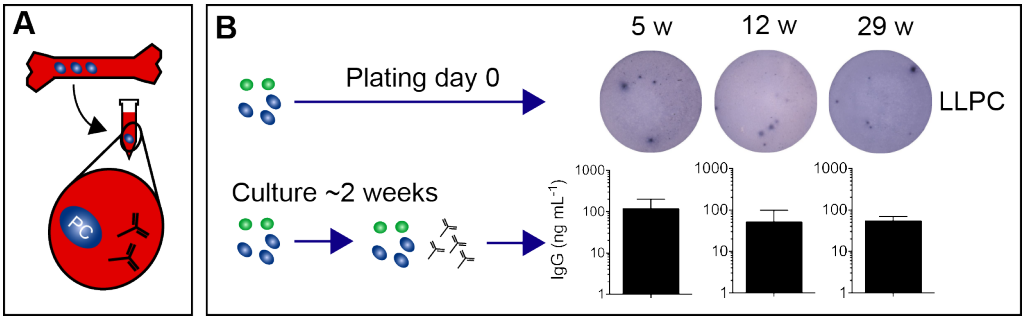

Figure 19. Schematic of quantification of bone marrow PCs. (A) Bone marrow was aspirated and the mononuclear cell fraction purified and frozen. (B) Top panel; long-lived PCs (LLPC) were enumerated via ELISpot by plating without previous culture. Shown are representative wells for Env-specific IgG-producing PCs. Bottom panel; Bone marrow was culture for 1-3 weeks in complete media and thereafter the supernatant was measured for Ab secretion. Shown is Env-specific IgG at indicated time points (n=2-4 donors). (w) indicates weeks following immunization.

7.2.3 Flow cytometric single memory B cell sort and Ab cloning

To gain an improved insight in the type of Abs that are elicited following immunization, in **paper III** we adapted previously published protocols for flow cytometric staining of antigen-specific MBCs followed by subsequent Ab cloning [343, 344, 346]. IgG^+ MBCs were sorted at single-cell density into PCR plates. The RNA was reversed transcribed and the V(D)J families amplified by nested PCR using mixes of primers covering the different Ab gene families. Following sequence verification the products were cloned into Ab expression vectors via restriction sites introduced by an additional PCR with cloning primers [2]. The primers used in **paper III** were based on human Ab sequences and had low efficiency for amplification of especially the kappa

and lambda light chains. In **paper IV** we therefore designed new primers based on the rhesus macaque germline sequences described in **paper III,** an additional two IgHV sequences we found during the work with **paper IV** and previously described rhesus Ig sequences [11, 14, 456, 457] (Figure 20).

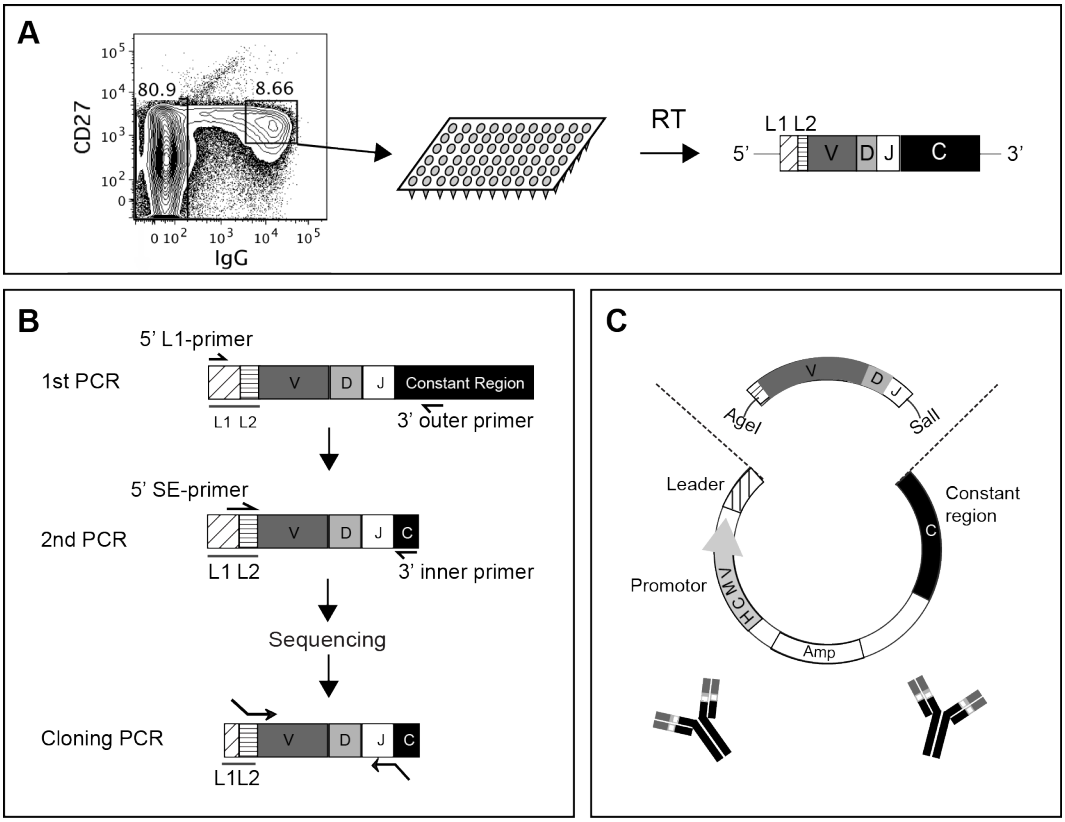

Figure 20. Workflow for the isolation of MAbs from sorted single cells. (A) The cells of interest were sorted at single cell density via flow cytometry into 96-well PCR plates containing lysis buffer. Reverse transcription was performed with random hexamers. Shown in the FACS plot is a gate for ($CD20^{+}$)$CD27^{+}IgG^{+}$ memory B cells. (B) In paper IV the 5' primers used in the 1st PCR were named the L1-primers since they anneal to the beginning of leader 1 (L1). In the 2nd PCR the 5' primes were designed to anneal to the L1/L2 junction, to specifically amplify rearranged transcribed sequences. The primers were named the SE-primers. Following successful nested PCR the products were sequenced. Cloning primers were chosen depending on the V- and J-segment and run in a third PCR introducing restriction sites. (C) Products from the cloning PCR were digested with restriction enzymes and cloned into CMV-driven Ab expression vectors already containing a leader sequence and a constant region. After verification of vector insert, the Abs were produced via transient transfection of 293-F cells.

The 3' primes were located in the Ab constant regions, which are relatively conserved, making the design fairly easy. For the human 5' primers, however, they were located mainly in the V-region FR1 [343]. In addition to imprinting the PCR fragment with the primer sequence and thereby affecting SHM calculations the V-region is exposed to SHM. This makes the primer set vulnerable to extensive SHM, commonly observed in the broadly neutralizing Abs isolated to date [145]. A problem that is solved when the primers are moved to the Ab leader sequence as shown by Scheid *et al*. [345].

The Ab leader sequence constitutes two exons (L1 and L2) separated by a short intron (Figure 1) [24]. In the mRNA the intron is excised and the joined L1/L2 leader sequence functions as a signal peptide directing the mRNA to the rER where it is removed upon translocation of the nascent polypeptide into the ER. Each V-segment (IgH, Igκ, and Igλ) has a leader sequence upstream in the DNA and although relatively conserved there is some variation (usually <20%) within each gene family. Therefore the larger families commonly need more than one primer to enable amplification of all variants.

Due to the reasons described above we decided to design the rhesus-specific 5' primers so that they align to the leader sequence. A first nested primer set was generated to the most conserved regions within the Ig leaders. Designing primers toward conserved regions enabled a smaller number of primers to cover all variants, which usually improves the PCR, however, when testing the primer mixes on cDNA and single-cells the amplification was very poor. Further design and evaluation showed that it was important to place the primers in the beginning of L1 and also in the junction between the L1 and L2 exons (Figure 20). Since the RT-PCR is performed on single cells, both DNA and RNA is present, therefore using primers toward the L1 and L2 junction will allow specific amplification of mRNA with recombined V(D)J sequences. Additionally placing the primers in the beginning of the leader sequence indicates that the downstream sequence potentially forms secondary structures inhibiting efficient primer binding.

The final 5' primer sets consisted of 25 IgHV primers (11 outer and 14 inner), 18 IgκV primers (9 outer and 9 inner), and 21 IgλV primers (11 outer and 10 inner). The IgH 5' primers can be paired with 3' primers specific for IgG, IgA, IgD, or IgM. Additionally cloning primers containing restriction sites were designed for all rhesus germline V and J genes described in **paper III** and **IV**. However, since the Ab sequences are exposed to SHM additional cloning primers have to be generated to accommodate specific mutations.

7.3 MAGNITUDE AND DURABILITY OF B CELL RESPONSES TO ENVELOPE GLYCOPROTEINS

As discussed in section 3.1 and 3.4 an increasing number of licensed vaccines and vaccine candidates are based on recombinant proteins. However, the successful HBV and HPV vaccines are based on mutimeric VLPs while HIV-1 Env is based on soluble trimeric protein that additionally is heavily glycosylated. The high amount of glycans was shown to enable interaction with lectin receptors on APCs and exert downstream signaling effects [458]. Additionally Env binding to CD4 and or the co-receptors CCR5 and CXCR4 may induce immunomodulatory functions in T cells, as suggested by *in vitro* studies [459-461]. All these properties potentially affect immune responses to Env following immunization [462], and several studies have indicated that the responses induced are relatively short-lived [146, 463, 464], suggesting that perhaps HIV-1 Env is

an intrinsically poor antigen [462]. In **papers I** and **II** we evaluated humoral and cellular B cell responses following immunization with trimeric HIV-1 Env (paper I and II) and Influenza HA (paper II) in Abisco-100 and CpG adjuvant. In **paper I** we immunized the macaques monthly five times while in **paper II** the macaques were immunized three times, with a long interval between the second and third immunization to allow evaluation of longitudinal responses (Figure 21).

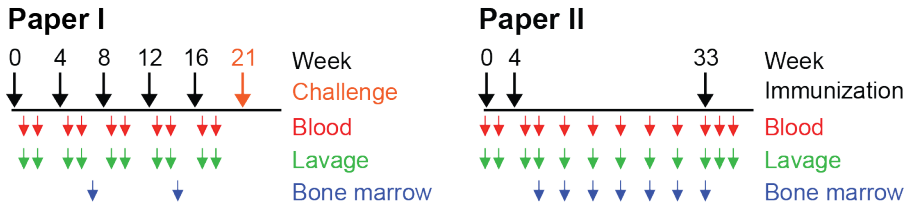

Figure 21. Immunization schedules and samplings for **paper I** and **II**. In paper I, rhesus macaqeus (n=12, 6 receiving trimeric Env and 6 adjuvant controls) were immunized monthly five times. In paper II, cynomolgus macaques (n=6) were immunized three times, at 0, 4, and 33 weeks. Blood samplings are indicated by red arrows, bone marrow samplings by blue arrows, mucosal samplings by green arrows, and immunizations by black arrows. Numbers above the arrows indicate week of immunization or for the orange arrow, SHIV challenge.

For both studies, peak Ab titers were reached after two immunizations and subsequent boosting did not elevate the titers further. A similar profile was observed in the MBC compartment, with peak frequencies of 10-20% of the total IgG^+ MBCs being specific for Env. The circulating PC compartment reached similar frequencies as the MBCs, although they only produced very transient peaks of ASCs at 1 week following boost and were undetectable at 2 weeks. This is consistent with rapid differentiation of MBCs to ASCs upon antigen challenge. These results are also consistent with observations following immunization with influenza [73], and rabies vaccines [74], and following dengue virus infection [465] suggesting that peak Env responses elicited from immunizations appear to be within a normal range of those elicited by protective vaccines or following infection. Additionally, we detected Env-specific LLPCs in the bone marrow of immunized macaques, indicating that long-lived responses were likely induced, although similarly to the other B cell compartments, maximum frequencies were also reached following the second immunization and were not boosted further.

In **paper II** we evaluated the longevity of B cell responses following immunization. We compared Env responses to those of recombinant influenza HA, a soluble protein antigen that is not as glycosylated and does not interact with T cells. We produced HA in the same expression system as Env to allow a direct comparison of the responses against these antigens. Longitudinal responses were evaluated for 29 weeks following the second immunization, when peak B cell responses were reached. HA responses were slightly lower than Env responses, possibly because HA is a smaller antigen. However, there were similar frequencies of antigen-specific LLPCs in the bone marrow for both antigens, perhaps indicating differential requirements for entering the bone marrow niche. Following peak responses, plasma Ab titers and MBC levels rapidly

contracted with a half-life of ~2 weeks, while the LLPC frequencies remained constant. The peripheral Ab responses were highly correlated with the mucosal Ab levels, indicating that the Abs detected at the mucosal surfaces result from transcytosis of circulating Abs [466], a mechanism that was shown to afford protection in HPV vaccinated women [467]. After 5-6 months, the Env and HA Ab titers reached a plateau ~1.5 log lower than peak Ab titers, while the MBC frequencies declined further indicating that the Ab levels at the plateau levels were likely disconnected from the MBC compartment. Instead the frequency of antigen-specific Abs and LLPCs were at a similar level (~1% of total IgG responses) indicating that the LLPCs were responsible for maintaining the circulating Ab titers at this time as also suggested for other vaccines [76].

In comparison to the responses elicited by the soluble Env trimers in macaques, a previous study in humans using monomeric HIV-1 Env in the MF59 adjuvant required three inoculations to reach peak titers [468]. This was followed by a similarly rapid decline of Ab responses before reaching a less steep curve where the Ab waning was considerably slower for up to 200 days. In contrast, following immunization with the VLP-based HPV vaccine, there is a ~1 log decline in Ab titers over 30 months, after which the titers stabilize above baseline and are protective for >5 years [469]. Therefore, it is possible that soluble proteins in general elicit responses that decline rapidly, while this is different for particle-based vaccines. Collectively **papers I** and **II** indicate that soluble HIV-1 Env trimers induce B cell responses of similar magnitude and durability as other viral antigens suggesting that it is not an intrinsically poor antigen, at least not in the context of a strong adjuvant, as used in these studies.

7.4 QUALITY OF B CELL RESPONSES FOLLOWING HIV-1 ENV IMMUNIZATION

To gain an improved understanding of why current HIV-1 vaccine candidates are not eliciting protective responses to HIV-1 it is necessary to dissect the quality of the elicited B cell responses at higher resolution. Thanks to the development of several new methods this can be achieved at different levels for both cellular and humoral B cell responses. [225, 327] and [**paper I, III,** and **IV**].

In **paper I** we used a differential ELISpot [225] together with the MBC stimulation protocol to determine how Env region-specific MBC responses evolved following sequential immunizations. We observed a shift from early gp41-specific MBCs to more variable region-specific responses during the course of the study although a substantial portion of the response was directed toward non-gp41 and non-V1-3 regions. The observation that gp41-directed responses were prominent in early immunizations is similar to kinetics following infection, where gp41-specific responses are commonly detected before gp120-specific responses [310], although the reason for this is not clear. We also tried to use the differential probes to determine Env-specificities in the plasma and MBC culture supernatants of the immunized macaques. However, the major

reactivities observed were to the V1-3 loops, likely explained by a selective advantage of these Abs in the ELISA format competing out the other reactivities.

To evaluate the functional properties of the Ab responses, neutralization of a panel of tier 1 and 2 pseudoviruses was performed. There was a clear improvement in neutralization to the panel between the second and fifth immunization, which was interesting considering that the overall Env-specific Ab titers were the same at these two time points, as described in section 7.3. As an indicator of Ab maturation we measured the avidity index of the plasma Abs. The avidity index is determined by comparing Ab bound to antigen before and after incubating with a chaotropic agent [470]. The more Ab bound, the higher the index, with 100% indicating no displacement of Abs. In **paper I** there was a significant increase in the avidity index during the immunization schedule potentially signifying an increased affinity, translating into improved neutralization in the pseudovirus assay. However, to formally show that this occurs for individual B cells, antigen-specific cells would have to be sorted and MAbs isolated for analysis of SHM and neutralization. Interestingly, when performing the avidity assay on the plasma in **paper II**, a similar increase in avidity index was observed during the extended interval between the second and third immunization, suggesting ongoing Ab maturation in the absence of booster immunizations, potentially due to persisting antigen and ongoing GC reactions (Figure 22) [471, 472]. In future studies it would be valuable to determine if the week 29 Abs (just before immunization three) in **paper II** are of similar quality as those elicited from five immunizations in **paper I**, as that would be informative for immunization schedules in future clinical studies using recombinant Env protein. Additionally, immunization schedules including long intervals improved the magnitude of longitudinal responses in other studies [468, 473].

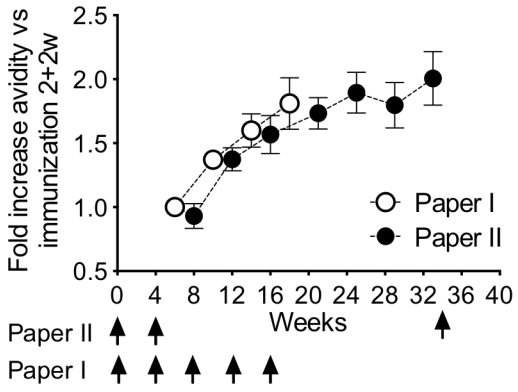

Figure 22. Ab maturation in paper I (open circles) and II (filled circles) as measured by avidity index. Arrows indicate immunizations. The Y-axis show folds increase in avidity index to following immunization 2 + 2 weeks. The X-axis shows weeks since study initiation.

Although neutralization assays and Ab mapping provide valuable information regarding the immunogenicity of candidate vaccines, fine details regarding how elicited Abs react with the antigen requires studies of MAbs. The identification of broadly reactive Abs in the plasma of individuals chronically infected with HIV-1 [474] and the subsequent isolation of multiple bNAbs from such individuals [145] has clearly shown that the human immune system is capable of eliciting Abs to HIV-1 that can target highly conserved epitopes and mediate neutralization against diverse HIV-1 variants. Several of the isolated bNAbs target the Env CD4bs [337, 345, 346], a region that only

tolerates limited variability to retain the capacity to bind human CD4. However, all CD4bs-directed Abs are not broadly neutralizing and even MAbs with largely overlapping epitopes, such as IgGb12 and IgGb13 can display very different breadth and potency in neutralization assays [268].

It was previously shown that CD4bs-directed Abs are elicited by trimeric Env immunization of rhesus macaques [112]; however, the plasma neutralization breadth was limited indicating that the Abs were mostly strain-specific or targeting regions not exposed on the functional Env spike. In **paper III** we sorted MBCs targeting the CD4bs elicited using samples described in **paper I**. The aim was to gain an improved understanding of the types of CD4bs-directed Abs elicited by immunization and to investigate how they compare to CD4bs-elicited MAbs from chronically HIV-1 infected individuals to inform future Env immunogen design efforts.

CD4bs-directed MBCs were sorted using two differential probes, gp140-F (wildtype) and gp140-F-D368R (CD4bs-defective) conjugated to different fluorochromes. The D368R mutant abrogates binding by most known CD4bs-directed MAbs while retaining binding to MAbs targeting epitopes outside of the CD4bs. IgG^+ MBCs binding to gp140-F but not gp140-F-D368R were defined as CD4bs-specific and were sorted at single cell density into 96-well PCR plates. The Ab IgH and IgL V(D)J gene segments were amplified by nested RT-PCR using a primer set designed for human V(D)J amplification [343]. PCR reactions giving products of the correct size were sequenced and products encoding IgH and IgL chains derived from the same single cell were independently cloned into expression vectors containing a leader sequence and constant region for the heavy and light chains, respectively. Functional Abs were produced via transient transfection of 293-F cells and tested for binding and specificity by ELISA. A panel of eight MAbs Abs specific for the CD4bs was isolated. One MAb (GE147) bound recombinant Env strongly in ELISA format, but displayed low affinity in an Octet system, which is similar to biacore and measure both on-rate and off-rate, indicating a high dependence on avidity for efficient binding. This is consistent with the low SHM rate displayed by GE147, with 1.4% divergence from germline on the nucleotide level. The other seven MAbs displayed a level of SHM more similar to the level observed in the total IgG^+ MBC population in the same macaque, which is also similar to that observed following tetanus [140] or influenza vaccination [73]. In addition to gp120, to which all MAbs except GE147 displayed nM affinity, we also determined the affinity for the 2CC core. 2CC is an Env gp120 core stabilized in the CD4 bound conformation [378]. When measuring MAb binding it shows selective interaction with bNAbs in comparison to non-bNAbs and it was used successfully as a probe in flow cytometric single-cell sorts to isolate bNAbs [345]. Interestingly we detected binding, although at low affinity, to 2CC by MAb GE148 indicating the possibility that with the correct antigenic drive and additional SHM, this MAb lineage might be driven to elicit improved neutralizing activity.

To evaluate the vaccine-induced MAbs functionally they were tested for neutralization of a panel of clade B viruses as well as one T cell lab adapted virus from clade A and

one from clade C. All MAbs except GE147 neutralized one or more viruses, with several MAbs neutralizing those of clade A and C indicating targeting of conserved regions within the CD4bs. These neutralization profiles were similar to non-bNAbs isolated from HIV-1 infected individuals. To further define the binding site of the individual MAbs, we performed a selected alanine (Ala) scan consisting of 27 gp120 Env mutants each with an alanine substitution at a single residue [475]. Mutants were chosen that had previously been implicated in CD4 binding or interaction with CD4bs-directed MAbs [229, 268, 358]. The Ala-scan revealed that the binding region of the vaccine-induced MAbs largely overlapped with that of the infection-induced non-bNAbs. Additionally, when modeled in the context of the functional spike [235], the binding region of the vaccine-induced MAbs was shown to be more proximal to the trimer axis compared to that of the infection-induced, broadly neutralizing MAb, VRC01. We speculate that this is an angle of approach that potentially is occluded on primary virus Env spikes, explaining the limited neutralization breadth displayed by the vaccine-induced CD4bs-directed MAbs isolated in this thesis (Figure 23).

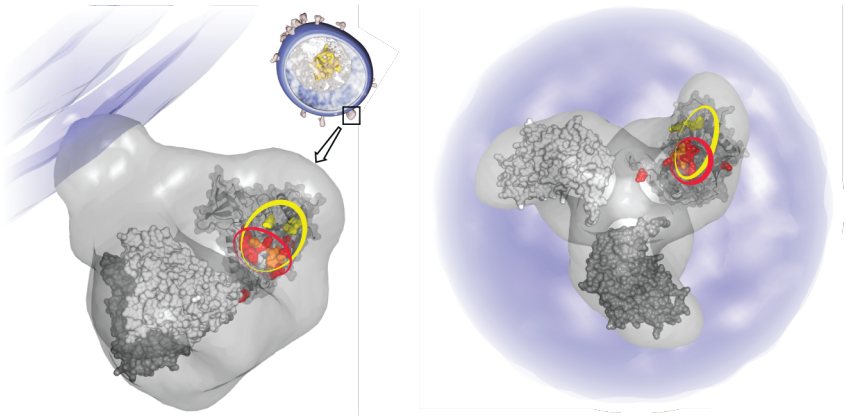

Figure 23. gp120 core [229] modeled in a trimeric context in the cryo-EM derived native spike [235]. The spike is shown in side (left panel) and top (right panel) view. Circles indicate binding regions and highlighted amino acids indicate residues important for MAb binding. Red indicate vaccine-induced NHP MAbs and yellow the bNAb VRC01. Orange indicates overlapping residues.

A further piece of information was obtained by testing neutralization to JRFL, a neutralization resistant virus, in which an N-linked glycosylation site was removed to disrupt the tight quaternary packaging of the Env spike (JRFLΔ301) [271]. While no neutralization was detected against the wildtype JRFL virus, effective neutralization was achieved against the JRFLΔ301 variant. Importantly, however, to get a complete understanding of the interactions between the MAbs and Env it will be necessary to crystallize the Abs in complex with antigen, experiments that are currently ongoing for GE148 and GE136. Although a panel of eight MAbs is only a small fraction of the Abs elicited toward the CD4bs following immunization, they provide a first step towards understanding why Env vaccine-elicited responses are not more broadly neutralizing. By defining the limitations with the current antigens, efforts can be made to attempt to restrict immune responses to broadly neutralizing surfaces on Env.

7.4.1 Ab breadth and potency elicited by trimeric Env versus monomeric Env

Studies in small animal models have shown that soluble trimeric Env is superior to monomers in terms of eliciting Abs exhibiting neutralization breadth and potency [368, 370, 476]. In **paper I** we therefore evaluated the trimeric Env-elicited Ab responses from three nonhuman primate studies [72, 112, 477] to gp120 monomeric responses elicited in twenty randomly selected human subjects from the VAX04 human clinical trial [367]. To do the comparison we evaluated the neutralizing capacity in plasma or serum against several tier 1 and 2 viruses from clade B and selected viruses from clade A and C using standardized methods [365] (Table III).

Table III. Neutralization following immunization with monomeric or trimeric Env.

			VAX04[b]	NHP[c]
	Virus	Clade	monomers	trimers
Tier 1	MN	B	100[a]	100
	HxB2	B	15	100
	SF162	B	5	100
	BaL0.1	B	0	37.5
Tier 2	YU2	B	0	75
	89.6	B	0	27
	6536	B	0	38
	ADA	B	95	55
Tier 1	DJ263	A	0	75
Tier 1	MW965	C	95	100

[a]values indicate percent responders (ID_{50}>10) vs non-responders (ID_{50}<10)

[b]n=20 randomly selected human donors from the VAX04 clinical trial.

[c]n=16 macaques pooled from paper I, [477], and [112].

The plasma obtained from trimeric Env-immunized macaques expressed a broader neutralization profile with activities against all the viral isolates tested. The monomer-induced human responses, on the other hand, showed a more narrow neutralizing profile, although when reactivity was detected it was generally potent. These responses indicate that the Env trimers used here elicit superior Abs breadth in the highly relevant translational macaque models in addition to small animals. However, it is also important to address the potential role of the different adjuvants for their capacity to influence the response. In this regard, Alum was used in the human study while AS01B or Abisco-100 together with CpG was used in the nonhuman primate studies, being a potential caveat to the comparison performed in **paper I**.

7.5 ASSESSMENT OF PROTECTION FROM HETEROLOGOUS SHIV CHALLENGE

Following immunization with Env a large proportion of the vaccine elicited antibody response is directed toward the variable regions [368, 373], which can protect against SHIV challenge with homologous virus [372, 478]. However, so far only partial protection against heterologous strains was observed [470, 479]. To evaluate the protective capacity of the Env-specific Abs elicited in **paper I** we first determined the neutralizing antibody titers *in vitro* to the CCR5 tropic SHIV-SF162P4. We observed a

moderate ID_{50} neutralization titer of ~200 against both the challenge virus and a cloned pseudovirus generated from the SHIV stock [479]. When performing repeated low/medium dose rectal SHIV-SF162P4 challenge a trend toward delayed infection and reduced viremia following infection was observed in Env-immunized animals compared to control animals. To investigate the basis for the limited protection, we determined the levels of Env-specific Abs at the virus portal of entry. We quantified the levels of Env-specific IgG and IgA in rectal and vaginal washes and analyzed these levels in relation to the circulating Abs in blood in each animal (Figure 24).

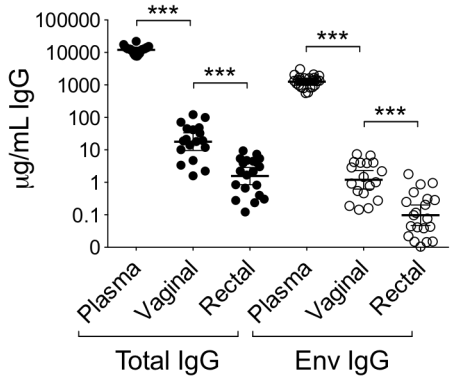

Figure 24. Total IgG and Env-specific IgG antibody responses in plasma and in mucosal washes (n=21-24 depending on successful sampling). Statistics was evaluated with ANOVA on log transformed values.

Although there was a correlation between the plasma and mucosal compartments, the levels of Abs in the vaginal wash were 1000-fold lower and in the rectal wash 10 000-fold lower than measured in circulation. Additionally, Env-specific IgA levels were below detection. This raises the possibility that the neutralizing antibody titers, although high in blood, were too low at the mucosal surfaces for efficient protection. These results highlight the importance of inducing Abs possessing specificities with enhanced neutralizing activity so that lower effective titers are needed.

8 CONCLUDING REMARKS

With this thesis my aim was to provide an improved understanding of antigen-specific B cell responses following immunizations with protein-based vaccines. In particular, I used a recombinant trimeric HIV-1 Env glycoprotein as a model antigen as it is highly characterized both functionally and structurally in addition to being a clinically relevant vaccine candidate. In the studies included in this thesis, we immunized rhesus or cynomolgus macaques as a highly relevant model for human biology. By characterizing the Ab Ig loci from a rhesus macaque in **paper III** we further refined the model to facilitate analysis of B cell responses. We show that the human and rhesus Ig loci are highly homologous. Additionally following analysis of Ab V(D)J-segments from sorted cells in **paper III** and **IV** we confirmed that the gene usage was highly similar between different macaques as well as to that used in humans, further supporting the relevance of the macaque model for studies of human B cell biology.

In **papers I** and **II** we developed several assays to enable the evaluation of cellular B cell responses and sought to determine the magnitude, durability, and quality of trimeric Env-specific B cell responses. We showed that recombinant trimeric Env elicit potent B cell responses with similar magnitude and kinetics as those of influenza and rabies vaccination. When comparing longitudinal samples from HIV-1 Env and influenza HA immunized macaques there was a similarly rapid contraction of antigen-specific peripheral B cell responses following immunization, suggesting that soluble HIV-1 Env is not an unusually weak immunogen when administered in adjuvant, at least compared to influenza HA. Furthermore, in both **papers I** and **II** bone marrow resident plasma cells specific for HIV-1 Env were detectable, indicating that long-lived plasma cells were elicited through our immunizations.

In addition to evaluating the magnitude and durability of B cell responses to trimeric Env, we also used several assays to gain insight in the quality of the elicited responses. In **paper I** we used a differential ELISpot [225] to show how the Env-specific memory B cell compartment developed from early gp41-specific responses to variable region 1-3 specific responses during the immunization schedule. We further showed that despite no difference in magnitude of the antigen-specific B cell compartment there was a significant increase in neutralization titers to a panel of tier 1 and 2 pseudoviruses between the second and fifth immunization. This increase was likely due to Ab affinity maturation as indicated by an avidity assay. Furthermore, in **paper II** we show that the increase in Ab maturation was not necessarily dependent on repeated boosting as a similar increase was observed during a 29-week interval following only two immunizations, supporting the use of immunization schedules with long intervals between the second and third immunization [468].

We further compared the Env trimer-elicited neutralizing Ab responses from our immunized macaques with the responses elicited in a large clinical trial using monomeric Env and observed a improvement in the responses induced by the trimers.

These findings warrant further investigation in more controlled studies where the same adjuvant and HIV-1 Env strain is used in both groups. Such results would have direct implications for future clinical HIV-1 vaccine trials.

Recently several bNAbs were isolated from chronically infected individuals. These Abs provide valuable information about vulnerable regions on the HIV-1 spike that can be targeted in vaccine design. We believe that in addition to isolating new bNAbs it is important to understand the responses elicited by current vaccine-candidates to inform immunogen-design efforts. In **paper III** we established a system for high-resolution evaluation of vaccine-induced B cell responses. We sorted Env-specific memory B cells recognizing a highly conserved Ab target, the primary receptor binding site (CD4bs), from the Env trimer-immunized animals described in **paper I**. We cloned and expressed a panel of Abs to determine qualitative differences to CD4bs-directed infection-induced bNAbs and non-bNAbs. Although all MAbs displayed similarly high affinity to recombinant trimeric Env they differed in neutralizing capacity. When mapping important residues for MAb binding to the CD4bs the vaccine-induced MAbs were shown to be dependent on residues located closer toward the center of the spike, likely being occluded in the tightly packed Env spike on primary virus strains, while a well-studied bNAb, VRC01, bound more distally on the outer domain of gp120, which likely is more accessible. These finding have implications for vaccine design as it may be possible to generate Env trimers that are more restricted in terms of which epitopes they expose. Finally, we adapted an RT-PCR-based protocol for the amplification and cloning of MAbs from sorted single cells to the rhesus macaque system in **paper IV,** to facilitate future efforts to characterize B cell responses at high resolution in rhesus macaques, an approach that has broad applications for studies of humoral immune responses.

9 FUTURE DIRECTIONS

Nonhuman primates are invaluable as translational models for the evaluation of preclinical vaccine-candidates and for an improved understanding of human biology. Although recent improvement in the characterization of macaque B cell biology there is still a need for further studies to refine the model. Important aspects that need to be resolved are the flow cytometric definition of circulating plasma cells and long-lived plasma cells as well as improved insight in memory B cell subsets and distribution. These factors are highly important when evaluating B cell responses elicited by vaccine candidates. In addition to the definition of likely Ab germline repertoires as presented in **paper III** it will be important to characterize V(D)J-segment recombination and expression as functional Abs.

Many of the CD4bs-directed bNAbs display specific characteristics, such as restricted gene family usage, commonly the IgHV1-2 segment (in humans). It will be important to determine if such restricted segments are also used in CD4bs-directed Abs following immunizations and if so, determine how they bind Env and if further affinity maturation of such Abs drives them in the right direction. As discussed in this thesis, the Env vaccine-induced B cell responses studied here appear to reach an affinity roof, corresponding to an average level of SHM, while bNAbs isolated from infected individuals display a considerably higher level of SHM. One important question for the future will be to identify strategies to promote SHM of vaccine-induced B cell responses.

Although immunization with trimeric Env elicits Ab responses with improved neutralization breadth compared to monomeric Env, the breadth is still limited compared to broadly reactive responses in HIV-1 infected individuals. It will be important to continuously evaluate current and novel vaccine-candidates for iterative improvements in immunogen design to more selectively drive Ab responses to conserved and neutralization-sensitive regions on the Env spike. The methods described in **paper III** and **IV** will be valuable to meet this goal. An additional important future task is to crystallize vaccine-induced, CD4bs-directed MAbs with Env to obtain more definitive insights into the Ab-antigen interaction, knowledge that may lead to the design of improved vaccine approaches.

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11 REFERENCES

1. Billips, L.G., K. Lassoued, C. Nunez, J. Wang, H. Kubagawa, G.L. Gartland, . . . M.D. Cooper, *Human B-cell development.* Ann N Y Acad Sci, 1995. 764: p. 1-8.
2. Wardemann, H., S. Yurasov, A. Schaefer, J.W. Young, E. Meffre, and M.C. Nussenzweig, *Predominant autoantibody production by early human B cell precursors.* Science, 2003. 301(5638): p. 1374-7.
3. Nemazee, D., D. Russell, B. Arnold, G. Haemmerling, J. Allison, J.F. Miller, . . . K. Buerki, *Clonal deletion of autospecific B lymphocytes.* Immunol Rev, 1991. 122: p. 117-32.
4. Wardemann, H. and M.C. Nussenzweig, *B-cell self-tolerance in humans.* Adv Immunol, 2007. 95: p. 83-110.
5. von Boehmer, H. and F. Melchers, *Checkpoints in lymphocyte development and autoimmune disease.* Nat Immunol, 2010. 11(1): p. 14-20.
6. Glanville, J., W. Zhai, J. Berka, D. Telman, G. Huerta, G.R. Mehta, . . . J. Pons, *Precise determination of the diversity of a combinatorial antibody library gives insight into the human immunoglobulin repertoire.* Proc Natl Acad Sci U S A, 2009. 106(48): p. 20216-21.
7. Schroeder, H.W., Jr., *Similarity and divergence in the development and expression of the mouse and human antibody repertoires.* Dev Comp Immunol, 2006. 30(1-2): p. 119-35.
8. Yancopoulos, G.D. and F.W. Alt, *Regulation of the assembly and expression of variable-region genes.* Annu Rev Immunol, 1986. 4: p. 339-68.
9. Gibbs, R.A., J. Rogers, M.G. Katze, R. Bumgarner, G.M. Weinstock, E.R. Mardis, . . . A.S. Zwieg, *Evolutionary and biomedical insights from the rhesus macaque genome.* Science, 2007. 316(5822): p. 222-34.
10. Sundling, C., Y. Li, N. Huynh, C. Poulsen, R. Wilson, S. O'Dell, . . . G.B. Karlsson Hedestam, *High-Resolution Definition of Vaccine-Elicited B Cell Responses Against the HIV Primary Receptor Binding Site.* Sci Transl Med, 2012. 4(142): p. 142ra96.
11. Link, J.M., M.A. Hellinger, and H.W. Schroeder, Jr., *The Rhesus monkey immunoglobulin IGHD and IGHJ germline repertoire.* Immunogenetics, 2002. 54(4): p. 240-50.
12. Rogers, K.A., L. Jayashankar, F. Scinicariello, and R. Attanasio, *Nonhuman primate IgA: genetic heterogeneity and interactions with CD89.* J Immunol, 2008. 180(7): p. 4816-24.
13. Sundling, C., G. Phad, I. Douagi, M. Navis, and G.B. Hedestam, *Isolation of antibody V(D)J sequences from single cell sorted rhesus macaque B cells.* J Immunol Methods, 2012. 386(1-2): p. 85-93.
14. Scinicariello, F., C.N. Engleman, L. Jayashankar, H.M. McClure, and R. Attanasio, *Rhesus macaque antibody molecules: sequences and heterogeneity of alpha and gamma constant regions.* Immunology, 2004. 111(1): p. 66-74.
15. Kabat, E.A. and T.T. Wu, *Identical V region amino acid sequences and segments of sequences in antibodies of different specificities. Relative contributions of VH and VL genes, minigenes, and complementarity-determining regions to binding of antibody-combining sites.* J Immunol, 1991. 147(5): p. 1709-19.
16. Jung, D. and F.W. Alt, *Unraveling V(D)J recombination; insights into gene regulation.* Cell, 2004. 116(2): p. 299-311.
17. Schatz, D.G. and P.C. Swanson, *V(D)J recombination: mechanisms of initiation.* Annu Rev Genet, 2011. 45: p. 167-202.
18. van Gent, D.C. and M. van der Burg, *Non-homologous end-joining, a sticky affair.* Oncogene, 2007. 26(56): p. 7731-40.
19. Meier, J.T. and S.M. Lewis, *P nucleotides in V(D)J recombination: a fine-structure analysis.* Mol Cell Biol, 1993. 13(2): p. 1078-92.
20. Tonegawa, S., *Somatic generation of antibody diversity.* Nature, 1983. 302(5909): p. 575-81.
21. Nguyen, D.C., F. Scinicariello, and R. Attanasio, *Characterization and allelic polymorphisms of rhesus macaque (Macaca mulatta) IgG Fc receptor genes.* Immunogenetics, 2011. 63(6): p. 351-62.
22. Nimmerjahn, F. and J.V. Ravetch, *FcgammaRs in health and disease.* Curr Top Microbiol Immunol, 2011. 350: p. 105-25.
23. Padlan, E.A., *Anatomy of the antibody molecule.* Mol Immunol, 1994. 31(3): p. 169-217.
24. Schroeder, H.W., Jr. and L. Cavacini, *Structure and function of immunoglobulins.* J Allergy Clin Immunol, 2010. 125(2 Suppl 2): p. S41-52.

25. Al-Lazikani, B., A.M. Lesk, and C. Chothia, *Standard conformations for the canonical structures of immunoglobulins.* J Mol Biol, 1997. 273(4): p. 927-48.
26. Niiro, H. and E.A. Clark, *Regulation of B-cell fate by antigen-receptor signals.* Nat Rev Immunol, 2002. 2(12): p. 945-56.
27. Muramatsu, M., V.S. Sankaranand, S. Anant, M. Sugai, K. Kinoshita, N.O. Davidson, and T. Honjo, *Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells.* J Biol Chem, 1999. 274(26): p. 18470-6.
28. Arakawa, H., J. Hauschild, and J.M. Buerstedde, *Requirement of the activation-induced deaminase (AID) gene for immunoglobulin gene conversion.* Science, 2002. 295(5558): p. 1301-6.
29. Maul, R.W. and P.J. Gearhart, *AID and somatic hypermutation.* Adv Immunol, 2010. 105: p. 159-91.
30. Longerich, S., U. Basu, F. Alt, and U. Storb, *AID in somatic hypermutation and class switch recombination.* Curr Opin Immunol, 2006. 18(2): p. 164-74.
31. Rogozin, I.B. and N.A. Kolchanov, *Somatic hypermutagenesis in immunoglobulin genes. II. Influence of neighbouring base sequences on mutagenesis.* Biochim Biophys Acta, 1992. 1171(1): p. 11-8.
32. Rada, C., M.R. Ehrenstein, M.S. Neuberger, and C. Milstein, *Hot spot focusing of somatic hypermutation in MSH2-deficient mice suggests two stages of mutational targeting.* Immunity, 1998. 9(1): p. 135-41.
33. Bachl, J., C. Carlson, V. Gray-Schopfer, M. Dessing, and C. Olsson, *Increased transcription levels induce higher mutation rates in a hypermutating cell line.* J Immunol, 2001. 166(8): p. 5051-7.
34. Fukita, Y., H. Jacobs, and K. Rajewsky, *Somatic hypermutation in the heavy chain locus correlates with transcription.* Immunity, 1998. 9(1): p. 105-14.
35. Lebecque, S.G. and P.J. Gearhart, *Boundaries of somatic mutation in rearranged immunoglobulin genes: 5' boundary is near the promoter, and 3' boundary is approximately 1 kb from V(D)J gene.* J Exp Med, 1990. 172(6): p. 1717-27.
36. Teng, G. and F.N. Papavasiliou, *Immunoglobulin somatic hypermutation.* Annu Rev Genet, 2007. 41: p. 107-20.
37. Xu, Z., H. Zan, E.J. Pone, T. Mai, and P. Casali, *Immunoglobulin class-switch DNA recombination: induction, targeting and beyond.* Nat Rev Immunol, 2012. 12(7): p. 517-31.
38. Pone, E.J., J. Zhang, T. Mai, C.A. White, G. Li, J.K. Sakakura, . . . P. Casali, *BCR-signalling synergizes with TLR-signalling for induction of AID and immunoglobulin class-switching through the non-canonical NF-kappaB pathway.* Nat Commun, 2012. 3: p. 767.
39. Rawlings, D.J., M.A. Schwartz, S.W. Jackson, and A. Meyer-Bahlburg, *Integration of B cell responses through Toll-like receptors and antigen receptors.* Nat Rev Immunol, 2012. 12(4): p. 282-94.
40. Lundgren, M., U. Persson, P. Larsson, C. Magnusson, C.I. Smith, L. Hammarstrom, and E. Severinson, *Interleukin 4 induces synthesis of IgE and IgG4 in human B cells.* Eur J Immunol, 1989. 19(7): p. 1311-5.
41. Han, L., S. Masani, and K. Yu, *Overlapping activation-induced cytidine deaminase hotspot motifs in Ig class-switch recombination.* Proc Natl Acad Sci U S A, 2011. 108(28): p. 11584-9.
42. Batista, F.D. and M.S. Neuberger, *Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate.* Immunity, 1998. 8(6): p. 751-9.
43. Dal Porto, J.M., A.M. Haberman, M.J. Shlomchik, and G. Kelsoe, *Antigen drives very low affinity B cells to become plasmacytes and enter germinal centers.* J Immunol, 1998. 161(10): p. 5373-81.
44. Kelly, L.M., J.P. Pereira, T. Yi, Y. Xu, and J.G. Cyster, *EBI2 guides serial movements of activated B cells and ligand activity is detectable in lymphoid and nonlymphoid tissues.* J Immunol, 2011. 187(6): p. 3026-32.
45. Gatto, D., K. Wood, and R. Brink, *EBI2 operates independently of but in cooperation with CXCR5 and CCR7 to direct B cell migration and organization in follicles and the germinal center.* J Immunol, 2011. 187(9): p. 4621-8.
46. Pereira, J.P., L.M. Kelly, and J.G. Cyster, *Finding the right niche: B-cell migration in the early phases of T-dependent antibody responses.* Int Immunol, 2010. 22(6): p. 413-9.
47. Paus, D., T.G. Phan, T.D. Chan, S. Gardam, A. Basten, and R. Brink, *Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation.* J Exp Med, 2006. 203(4): p. 1081-91.

48. Schwickert, T.A., G.D. Victora, D.R. Fooksman, A.O. Kamphorst, M.R. Mugnier, A.D. Gitlin, . . . M.C. Nussenzweig, *A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center.* J Exp Med, 2011. 208(6): p. 1243-52.
49. Crotty, S., *Follicular helper CD4 T cells (TFH).* Annu Rev Immunol, 2011. 29: p. 621-63.
50. Tunyaplin, C., A.L. Shaffer, C.D. Angelin-Duclos, X. Yu, L.M. Staudt, and K.L. Calame, *Direct repression of prdm1 by Bcl-6 inhibits plasmacytic differentiation.* J Immunol, 2004. 173(2): p. 1158-65.
51. Kuo, T.C., A.L. Shaffer, J. Haddad, Jr., Y.S. Choi, L.M. Staudt, and K. Calame, *Repression of BCL-6 is required for the formation of human memory B cells in vitro.* J Exp Med, 2007. 204(4): p. 819-30.
52. Suzuki, K., I. Grigorova, T.G. Phan, L.M. Kelly, and J.G. Cyster, *Visualizing B cell capture of cognate antigen from follicular dendritic cells.* J Exp Med, 2009. 206(7): p. 1485-93.
53. Okada, T., M.J. Miller, I. Parker, M.F. Krummel, M. Neighbors, S.B. Hartley, . . . J.G. Cyster, *Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells.* PLoS Biol, 2005. 3(6): p. e150.
54. Victora, G.D., T.A. Schwickert, D.R. Fooksman, A.O. Kamphorst, M. Meyer-Hermann, M.L. Dustin, and M.C. Nussenzweig, *Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter.* Cell, 2010. 143(4): p. 592-605.
55. Shih, T.A., E. Meffre, M. Roederer, and M.C. Nussenzweig, *Role of BCR affinity in T cell dependent antibody responses in vivo.* Nat Immunol, 2002. 3(6): p. 570-5.
56. Foote, J. and H.N. Eisen, *Kinetic and affinity limits on antibodies produced during immune responses.* Proc Natl Acad Sci U S A, 1995. 92(5): p. 1254-6.
57. Tangye, S.G. and D.M. Tarlinton, *Memory B cells: effectors of long-lived immune responses.* Eur J Immunol, 2009. 39(8): p. 2065-75.
58. Tangye, S.G., *Staying alive: regulation of plasma cell survival.* Trends Immunol, 2011. 32(12): p. 595-602.
59. Radbruch, A., G. Muehlinghaus, E.O. Luger, A. Inamine, K.G. Smith, T. Dorner, and F. Hiepe, *Competence and competition: the challenge of becoming a long-lived plasma cell.* Nat Rev Immunol, 2006. 6(10): p. 741-50.
60. Crotty, S., P. Felgner, H. Davies, J. Glidewell, L. Villarreal, and R. Ahmed, *Cutting edge: long-term B cell memory in humans after smallpox vaccination.* J Immunol, 2003. 171(10): p. 4969-73.
61. Lee, F.E., J.L. Halliley, E.E. Walsh, A.P. Moscatiello, B.L. Kmush, A.R. Falsey, . . . I. Sanz, *Circulating human antibody-secreting cells during vaccinations and respiratory viral infections are characterized by high specificity and lack of bystander effect.* J Immunol, 2011. 186(9): p. 5514-21.
62. Benson, M.J., R. Elgueta, W. Schpero, M. Molloy, W. Zhang, E. Usherwood, and R.J. Noelle, *Distinction of the memory B cell response to cognate antigen versus bystander inflammatory signals.* J Exp Med, 2009. 206(9): p. 2013-25.
63. Liu, Y.J., C. Barthelemy, O. de Bouteiller, C. Arpin, I. Durand, and J. Banchereau, *Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2.* Immunity, 1995. 2(3): p. 239-48.
64. Dunn-Walters, D.K., P.G. Isaacson, and J. Spencer, *Analysis of mutations in immunoglobulin heavy chain variable region genes of microdissected marginal zone (MGZ) B cells suggests that the MGZ of human spleen is a reservoir of memory B cells.* J Exp Med, 1995. 182(2): p. 559-66.
65. Mamani-Matsuda, M., A. Cosma, S. Weller, A. Faili, C. Staib, L. Garcon, . . . J.C. Weill, *The human spleen is a major reservoir for long-lived vaccinia virus-specific memory B cells.* Blood, 2008. 111(9): p. 4653-9.
66. Tangye, S.G., D.T. Avery, E.K. Deenick, and P.D. Hodgkin, *Intrinsic differences in the proliferation of naive and memory human B cells as a mechanism for enhanced secondary immune responses.* J Immunol, 2003. 170(2): p. 686-94.
67. Good, K.L., D.T. Avery, and S.G. Tangye, *Resting human memory B cells are intrinsically programmed for enhanced survival and responsiveness to diverse stimuli compared to naive B cells.* J Immunol, 2009. 182(2): p. 890-901.
68. Good, K.L. and S.G. Tangye, *Decreased expression of Kruppel-like factors in memory B cells induces the rapid response typical of secondary antibody responses.* Proc Natl Acad Sci U S A, 2007. 104(33): p. 13420-5.
69. Martin, S.W. and C.C. Goodnow, *Burst-enhancing role of the IgG membrane tail as a molecular determinant of memory.* Nat Immunol, 2002. 3(2): p. 182-8.

70. Ahmed, R. and D. Gray, *Immunological memory and protective immunity: understanding their relation.* Science, 1996. 272(5258): p. 54-60.
71. Rajewsky, K., *Clonal selection and learning in the antibody system.* Nature, 1996. 381(6585): p. 751-8.
72. Sundling, C., M.N. Forsell, S. O'Dell, Y. Feng, B. Chakrabarti, S.S. Rao, . . . G.B. Karlsson Hedestam, *Soluble HIV-1 Env trimers in adjuvant elicit potent and diverse functional B cell responses in primates.* J Exp Med, 2010. 207(9): p. 2003-17.
73. Wrammert, J., K. Smith, J. Miller, W.A. Langley, K. Kokko, C. Larsen, . . . P.C. Wilson, *Rapid cloning of high-affinity human monoclonal antibodies against influenza virus.* Nature, 2008. 453(7195): p. 667-71.
74. Blanchard-Rohner, G., A.S. Pulickal, C.M. Jol-van der Zijde, M.D. Snape, and A.J. Pollard, *Appearance of peripheral blood plasma cells and memory B cells in a primary and secondary immune response in humans.* Blood, 2009. 114(24): p. 4998-5002.
75. Hammarlund, E., M.W. Lewis, S.G. Hansen, L.I. Strelow, J.A. Nelson, G.J. Sexton, . . . M.K. Slifka, *Duration of antiviral immunity after smallpox vaccination.* Nat Med, 2003. 9(9): p. 1131-7.
76. Amanna, I.J., N.E. Carlson, and M.K. Slifka, *Duration of humoral immunity to common viral and vaccine antigens.* N Engl J Med, 2007. 357(19): p. 1903-15.
77. Amanna, I.J. and M.K. Slifka, *Mechanisms that determine plasma cell lifespan and the duration of humoral immunity.* Immunol Rev, 2010. 236: p. 125-38.
78. Minges Wols, H.A., G.H. Underhill, G.S. Kansas, and P.L. Witte, *The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity.* J Immunol, 2002. 169(8): p. 4213-21.
79. Tokoyoda, K., T. Egawa, T. Sugiyama, B.I. Choi, and T. Nagasawa, *Cellular niches controlling B lymphocyte behavior within bone marrow during development.* Immunity, 2004. 20(6): p. 707-18.
80. Nakayama, T., K. Hieshima, D. Izawa, Y. Tatsumi, A. Kanamaru, and O. Yoshie, *Cutting edge: profile of chemokine receptor expression on human plasma cells accounts for their efficient recruitment to target tissues.* J Immunol, 2003. 170(3): p. 1136-40.
81. Kunkel, E.J. and E.C. Butcher, *Plasma-cell homing.* Nat Rev Immunol, 2003. 3(10): p. 822-9.
82. Ellyard, J.I., D.T. Avery, C.R. Mackay, and S.G. Tangye, *Contribution of stromal cells to the migration, function and retention of plasma cells in human spleen: potential roles of CXCL12, IL-6 and CD54.* Eur J Immunol, 2005. 35(3): p. 699-708.
83. Ellyard, J.I., D.T. Avery, T.G. Phan, N.J. Hare, P.D. Hodgkin, and S.G. Tangye, *Antigen-selected, immunoglobulin-secreting cells persist in human spleen and bone marrow.* Blood, 2004. 103(10): p. 3805-12.
84. Roldan, E. and J.A. Brieva, *Terminal differentiation of human bone marrow cells capable of spontaneous and high-rate immunoglobulin secretion: role of bone marrow stromal cells and interleukin 6.* Eur J Immunol, 1991. 21(11): p. 2671-7.
85. Benson, M.J., S.R. Dillon, E. Castigli, R.S. Geha, S. Xu, K.P. Lam, and R.J. Noelle, *Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL.* J Immunol, 2008. 180(6): p. 3655-9.
86. Huard, B., T. McKee, C. Bosshard, S. Durual, T. Matthes, S. Myit, . . . E. Roosnek, *APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa.* J Clin Invest, 2008. 118(8): p. 2887-95.
87. Chu, V.T., A. Frohlich, G. Steinhauser, T. Scheel, T. Roch, S. Fillatreau, . . . C. Berek, *Eosinophils are required for the maintenance of plasma cells in the bone marrow.* Nat Immunol, 2011. 12(2): p. 151-9.
88. Rodriguez Gomez, M., Y. Talke, N. Goebel, F. Hermann, B. Reich, and M. Mack, *Basophils support the survival of plasma cells in mice.* J Immunol, 2010. 185(12): p. 7180-5.
89. Winter, O., K. Moser, E. Mohr, D. Zotos, H. Kaminski, M. Szyska, . . . R.A. Manz, *Megakaryocytes constitute a functional component of a plasma cell niche in the bone marrow.* Blood, 2010. 116(11): p. 1867-75.
90. Scheid, J.F., H. Mouquet, J. Kofer, S. Yurasov, M.C. Nussenzweig, and H. Wardemann, *Differential regulation of self-reactivity discriminates between IgG+ human circulating memory B cells and bone marrow plasma cells.* Proc Natl Acad Sci U S A, 2011. 108(44): p. 18044-8.
91. Smith, K.G., A. Light, G.J. Nossal, and D.M. Tarlinton, *The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response.* EMBO J, 1997. 16(11): p. 2996-3006.

92. Shlomchik, M.J. and F. Weisel, *Germinal center selection and the development of memory B and plasma cells.* Immunol Rev, 2012. 247(1): p. 52-63.
93. Odendahl, M., H. Mei, B.F. Hoyer, A.M. Jacobi, A. Hansen, G. Muehlinghaus, . . . T. Dorner, *Generation of migratory antigen-specific plasma blasts and mobilization of resident plasma cells in a secondary immune response.* Blood, 2005. 105(4): p. 1614-21.
94. Xiang, Z., A.J. Cutler, R.J. Brownlie, K. Fairfax, K.E. Lawlor, E. Severinson, . . . K.G. Smith, *FcgammaRIIb controls bone marrow plasma cell persistence and apoptosis.* Nat Immunol, 2007. 8(4): p. 419-29.
95. Nimmerjahn, F. and J.V. Ravetch, *Antibody-mediated modulation of immune responses.* Immunol Rev, 2010. 236: p. 265-75.
96. McAleer, W.J., E.B. Buynak, R.Z. Maigetter, D.E. Wampler, W.J. Miller, and M.R. Hilleman, *Human hepatitis B vaccine from recombinant yeast.* Nature, 1984. 307(5947): p. 178-80.
97. Harper, D.M., E.L. Franco, C. Wheeler, D.G. Ferris, D. Jenkins, A. Schuind, . . . G. Dubin, *Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial.* Lancet, 2004. 364(9447): p. 1757-65.
98. Bejon, P., J. Lusingu, A. Olotu, A. Leach, M. Lievens, J. Vekemans, . . . L. von Seidlein, *Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age.* N Engl J Med, 2008. 359(24): p. 2521-32.
99. Zhu, F.C., J. Zhang, X.F. Zhang, C. Zhou, Z.Z. Wang, S.J. Huang, . . . N.S. Xia, *Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial.* Lancet, 2010. 376(9744): p. 895-902.
100. Agnandji, S.T., B. Lell, S.S. Soulanoudjingar, J.F. Fernandes, B.P. Abossolo, C. Conzelmann, . . . P. Vansadia, *First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children.* N Engl J Med, 2011. 365(20): p. 1863-75.
101. Lore, K. and G.B. Karlsson Hedestam, *Novel adjuvants for B cell immune responses.* Curr Opin HIV AIDS, 2009. 4(5): p. 441-6.
102. Kenney, R.T. and R. Edelman, *Survey of human-use adjuvants.* Expert Rev Vaccines, 2003. 2(2): p. 167-88.
103. Eisenbarth, S.C., O.R. Colegio, W. O'Connor, F.S. Sutterwala, and R.A. Flavell, *Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants.* Nature, 2008. 453(7198): p. 1122-6.
104. Kool, M., T. Soullie, M. van Nimwegen, M.A. Willart, F. Muskens, S. Jung, . . . B.N. Lambrecht, *Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells.* J Exp Med, 2008. 205(4): p. 869-82.
105. McKee, A.S., M.W. Munks, M.K. MacLeod, C.J. Fleenor, N. Van Rooijen, J.W. Kappler, and P. Marrack, *Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity.* J Immunol, 2009. 183(7): p. 4403-14.
106. Seubert, A., E. Monaci, M. Pizza, D.T. O'Hagan, and A. Wack, *The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells.* J Immunol, 2008. 180(8): p. 5402-12.
107. Didierlaurent, A.M., S. Morel, L. Lockman, S.L. Giannini, M. Bisteau, H. Carlsen, . . . N. Garcon, *AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity.* J Immunol, 2009. 183(10): p. 6186-97.
108. Clements, D.E., B.A. Coller, M.M. Lieberman, S. Ogata, G. Wang, K.E. Harada, . . . T. Humphreys, *Development of a recombinant tetravalent dengue virus vaccine: immunogenicity and efficacy studies in mice and monkeys.* Vaccine, 2010. 28(15): p. 2705-15.
109. Drane, D., E. Maraskovsky, R. Gibson, S. Mitchell, M. Barnden, A. Moskwa, . . . R. Basser, *Priming of CD4+ and CD8+ T cell responses using a HCV core ISCOMATRIX vaccine: a phase I study in healthy volunteers.* Hum Vaccin, 2009. 5(3): p. 151-7.
110. Sjolander, A., D. Drane, E. Maraskovsky, J.P. Scheerlinck, A. Suhrbier, J. Tennent, and M. Pearse, *Immune responses to ISCOM formulations in animal and primate models.* Vaccine, 2001. 19(17-19): p. 2661-5.
111. Davis, I.D., W. Chen, H. Jackson, P. Parente, M. Shackleton, W. Hopkins, . . . J.S. Cebon, *Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4(+) and CD8(+) T cell responses in humans.* Proc Natl Acad Sci U S A, 2004. 101(29): p. 10697-702.

112. Douagi, I., M.N. Forsell, C. Sundling, S. O'Dell, Y. Feng, P. Dosenovic, . . . G.B. Karlsson Hedestam, *Influence of novel CD4 binding-defective HIV-1 envelope glycoprotein immunogens on neutralizing antibody and T-cell responses in nonhuman primates.* J Virol, 2010. 84(4): p. 1683-95.
113. Morelli, A.B., D. Becher, S. Koernig, A. Silva, D. Drane, and E. Maraskovsky, *ISCOMATRIX: a novel adjuvant for use in prophylactic and therapeutic vaccines against infectious diseases.* J Med Microbiol, 2012. 61(Pt 7): p. 935-43.
114. Wiley, S.R., V.S. Raman, A. Desbien, H.R. Bailor, R. Bhardwaj, A.R. Shakri, . . . D. Carter, *Targeting TLRs expands the antibody repertoire in response to a malaria vaccine.* Sci Transl Med, 2011. 3(93): p. 93ra69.
115. Klinman, D.M., H. Xie, S.F. Little, D. Currie, and B.E. Ivins, *CpG oligonucleotides improve the protective immune response induced by the anthrax vaccination of rhesus macaques.* Vaccine, 2004. 22(21-22): p. 2881-6.
116. Wille-Reece, U., B.J. Flynn, K. Lore, R.A. Koup, A.P. Miles, A. Saul, . . . R.A. Seder, *Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates.* J Exp Med, 2006. 203(5): p. 1249-58.
117. Puig Barbera, J. and D. Gonzalez Vidal, *MF59-adjuvanted subunit influenza vaccine: an improved interpandemic influenza vaccine for vulnerable populations.* Expert Rev Vaccines, 2007. 6(5): p. 659-65.
118. Leroux-Roels, I., A. Borkowski, T. Vanwolleghem, M. Drame, F. Clement, E. Hons, . . . G. Leroux-Roels, *Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial.* Lancet, 2007. 370(9587): p. 580-9.
119. Boyle, J., D. Eastman, C. Millar, S. Camuglia, J. Cox, M. Pearse, . . . D. Drane, *The utility of ISCOMATRIX adjuvant for dose reduction of antigen for vaccines requiring antibody responses.* Vaccine, 2007. 25(14): p. 2541-4.
120. Khurana, S., N. Verma, J.W. Yewdell, A.K. Hilbert, F. Castellino, M. Lattanzi, . . . H. Golding, *MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines.* Sci Transl Med, 2011. 3(85): p. 85ra48.
121. Khurana, S., W. Chearwae, F. Castellino, J. Manischewitz, L.R. King, A. Honorkiewicz, . . . H. Golding, *Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus.* Sci Transl Med, 2010. 2(15): p. 15ra5.
122. Plotkin, S.A., *Correlates of protection induced by vaccination.* Clin Vaccine Immunol, 2010. 17(7): p. 1055-65.
123. Plotkin, S.A., *Vaccines: correlates of vaccine-induced immunity.* Clin Infect Dis, 2008. 47(3): p. 401-9.
124. Permar, S.R., S.A. Klumpp, K.G. Mansfield, A.A. Carville, D.A. Gorgone, M.A. Lifton, . . . N.L. Letvin, *Limited contribution of humoral immunity to the clearance of measles viremia in rhesus monkeys.* J Infect Dis, 2004. 190(5): p. 998-1005.
125. Chen, R.T., L.E. Markowitz, P. Albrecht, J.A. Stewart, L.M. Mofenson, S.R. Preblud, and W.A. Orenstein, *Measles antibody: reevaluation of protective titers.* J Infect Dis, 1990. 162(5): p. 1036-42.
126. Onorato, I.M., J.F. Modlin, A.M. McBean, M.L. Thoms, G.A. Losonsky, and R.H. Bernier, *Mucosal immunity induced by enhance-potency inactivated and oral polio vaccines.* J Infect Dis, 1991. 163(1): p. 1-6.
127. Huber, V.C., R.M. McKeon, M.N. Brackin, L.A. Miller, R. Keating, S.A. Brown, . . . J.A. McCullers, *Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza.* Clin Vaccine Immunol, 2006. 13(9): p. 981-90.
128. Terajima, M., J. Cruz, M.D. Co, J.H. Lee, K. Kaur, J. Wrammert, . . . F.A. Ennis, *Complement-dependent lysis of influenza a virus-infected cells by broadly cross-reactive human monoclonal antibodies.* J Virol, 2011. 85(24): p. 13463-7.
129. Lee, L.H., C.E. Frasch, L.A. Falk, D.L. Klein, and C.D. Deal, *Correlates of immunity for pneumococcal conjugate vaccines.* Vaccine, 2003. 21(17-18): p. 2190-6.
130. Romero-Steiner, S., D.M. Musher, M.S. Cetron, L.B. Pais, J.E. Groover, A.E. Fiore, . . . G.M. Carlone, *Reduction in functional antibody activity against Streptococcus pneumoniae in vaccinated elderly individuals highly correlates with decreased IgG antibody avidity.* Clin Infect Dis, 1999. 29(2): p. 281-8.
131. Maslanka, S.E., J.W. Tappero, B.D. Plikaytis, R.S. Brumberg, J.K. Dykes, L.L. Gheesling, . . . G.M. Carlone, *Age-dependent Neisseria meningitidis serogroup C class-specific antibody*

concentrations and bactericidal titers in sera from young children from Montana immunized with a licensed polysaccharide vaccine. Infect Immun, 1998. 66(6): p. 2453-9.
132. Tuaillon, E., Y.A. Tabaa, G. Petitjean, M.F. Huguet, G. Pajeaux, J.M. Fondere, . . . J.P. Vendrell, *Detection of memory B lymphocytes specific to hepatitis B virus (HBV) surface antigen (HBsAg) from HBsAg-vaccinated or HBV-immunized subjects by ELISPOT assay.* J Immunol Methods, 2006. 315(1-2): p. 144-52.
133. Liao, S.S., R.C. Li, H. Li, J.Y. Yang, X.J. Zeng, J. Gong, . . . K.L. Zhang, *Long-term efficacy of plasma-derived hepatitis B vaccine: a 15-year follow-up study among Chinese children.* Vaccine, 1999. 17(20-21): p. 2661-6.
134. Ovsyannikova, I.G., R.M. Jacobson, R.A. Vierkant, S.J. Jacobsen, V.S. Pankratz, and G.A. Poland, *The contribution of HLA class I antigens in immune status following two doses of rubella vaccination.* Hum Immunol, 2004. 65(12): p. 1506-15.
135. Hohler, T., E. Reuss, N. Evers, E. Dietrich, C. Rittner, C.M. Freitag, . . . R. Fimmers, *Differential genetic determination of immune responsiveness to hepatitis B surface antigen and to hepatitis A virus: a vaccination study in twins.* Lancet, 2002. 360(9338): p. 991-5.
136. Amanna, I.J., M.K. Slifka, and S. Crotty, *Immunity and immunological memory following smallpox vaccination.* Immunol Rev, 2006. 211: p. 320-37.
137. Davies, D.H., D.M. Molina, J. Wrammert, J. Miller, S. Hirst, Y. Mu, . . . P.L. Felgner, *Proteome-wide analysis of the serological response to vaccinia and smallpox.* Proteomics, 2007. 7(10): p. 1678-86.
138. Benhnia, M.R., M.M. McCausland, H.P. Su, K. Singh, J. Hoffmann, D.H. Davies, . . . S. Crotty, *Redundancy and plasticity of neutralizing antibody responses are cornerstone attributes of the human immune response to the smallpox vaccine.* J Virol, 2008. 82(7): p. 3751-68.
139. Poulsen, T.R., A. Jensen, J.S. Haurum, and P.S. Andersen, *Limits for antibody affinity maturation and repertoire diversification in hypervaccinated humans.* J Immunol, 2011. 187(8): p. 4229-35.
140. Poulsen, T.R., P.J. Meijer, A. Jensen, L.S. Nielsen, and P.S. Andersen, *Kinetic, affinity, and diversity limits of human polyclonal antibody responses against tetanus toxoid.* J Immunol, 2007. 179(6): p. 3841-50.
141. Meijer, P.J., P.S. Andersen, M. Haahr Hansen, L. Steinaa, A. Jensen, J. Lantto, . . . L.S. Nielsen, *Isolation of human antibody repertoires with preservation of the natural heavy and light chain pairing.* J Mol Biol, 2006. 358(3): p. 764-72.
142. Lantto, J., M. Haahr Hansen, S.K. Rasmussen, L. Steinaa, T.R. Poulsen, J. Duggan, . . . A. Jensen, *Capturing the natural diversity of the human antibody response against vaccinia virus.* J Virol, 2011. 85(4): p. 1820-33.
143. Scheid, J.F., H. Mouquet, N. Feldhahn, M.S. Seaman, K. Velinzon, J. Pietzsch, . . . M.C. Nussenzweig, *Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals.* Nature, 2009. 458(7238): p. 636-40.
144. Yu, X., T. Tsibane, P.A. McGraw, F.S. House, C.J. Keefer, M.D. Hicar, . . . J.E. Crowe, Jr., *Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors.* Nature, 2008. 455(7212): p. 532-6.
145. Kwong, P.D. and J.R. Mascola, *Human Antibodies that Neutralize HIV-1: Identification, Structures, and B Cell Ontogenies.* Immunity, 2012. 37(3): p. 412-25.
146. Bonsignori, M., M.A. Moody, R.J. Parks, T.M. Holl, G. Kelsoe, C.B. Hicks, . . . B.F. Haynes, *HIV-1 envelope induces memory B cell responses that correlate with plasma antibody levels after envelope gp120 protein vaccination or HIV-1 infection.* J Immunol, 2009. 183(4): p. 2708-17.
147. Plotkin, S.A., *Vaccines: the fourth century.* Clin Vaccine Immunol, 2009. 16(12): p. 1709-19.
148. Piyasirisilp, S. and T. Hemachudha, *Neurological adverse events associated with vaccination.* Curr Opin Neurol, 2002. 15(3): p. 333-8.
149. Lindsey, N.P., B.A. Schroeder, E.R. Miller, M.M. Braun, A.F. Hinckley, N. Marano, . . . E.B. Hayes, *Adverse event reports following yellow fever vaccination.* Vaccine, 2008. 26(48): p. 6077-82.
150. Schmidt, A.C., D.R. Wenzke, J.M. McAuliffe, M. St Claire, W.R. Elkins, B.R. Murphy, and P.L. Collins, *Mucosal immunization of rhesus monkeys against respiratory syncytial virus subgroups A and B and human parainfluenza virus type 3 by using a live cDNA-derived vaccine based on a host range-attenuated bovine parainfluenza virus type 3 vector backbone.* J Virol, 2002. 76(3): p. 1089-99.

151. Macadam, A.J., G. Ferguson, D.M. Stone, J. Meredith, S. Knowlson, G. Auda, . . . P.D. Minor, *Rational design of genetically stable, live-attenuated poliovirus vaccines of all three serotypes: relevance to poliomyelitis eradication.* J Virol, 2006. 80(17): p. 8653-63.
152. Men, R., M. Bray, D. Clark, R.M. Chanock, and C.J. Lai, *Dengue type 4 virus mutants containing deletions in the 3' noncoding region of the RNA genome: analysis of growth restriction in cell culture and altered viremia pattern and immunogenicity in rhesus monkeys.* J Virol, 1996. 70(6): p. 3930-7.
153. Whitney, J.B. and R.M. Ruprecht, *Live attenuated HIV vaccines: pitfalls and prospects.* Curr Opin Infect Dis, 2004. 17(1): p. 17-26.
154. Roohvand, F. and N. Kossari, *Advances in hepatitis C virus vaccines, part two: advances in hepatitis C virus vaccine formulations and modalities.* Expert Opin Ther Pat, 2012. 22(4): p. 391-415.
155. Burton, D.R., P. Poignard, R.L. Stanfield, and I.A. Wilson, *Broadly neutralizing antibodies present new prospects to counter highly antigenically diverse viruses.* Science, 2012. 337(6091): p. 183-6.
156. Yu, C.I. and B.L. Chiang, *A new insight into hepatitis C vaccine development.* J Biomed Biotechnol, 2010. 2010: p. 548280.
157. Stoll-Keller, F., H. Barth, S. Fafi-Kremer, M.B. Zeisel, and T.F. Baumert, *Development of hepatitis C virus vaccines: challenges and progress.* Expert Rev Vaccines, 2009. 8(3): p. 333-45.
158. Saunders, K.O., R.S. Rudicell, and G.J. Nabel, *The design and evaluation of HIV-1 vaccines.* AIDS, 2012. 26(10): p. 1293-302.
159. Liu, M.A., *Immunologic basis of vaccine vectors.* Immunity, 2010. 33(4): p. 504-15.
160. Rerks-Ngarm, S., P. Pitisuttithum, S. Nitayaphan, J. Kaewkungwal, J. Chiu, R. Paris, . . . J.H. Kim, *Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand.* N Engl J Med, 2009. 361(23): p. 2209-20.
161. Heraud, J.M., Y. Edghill-Smith, V. Ayala, I. Kalisz, J. Parrino, V.S. Kalyanaraman, . . . G. Franchini, *Subunit recombinant vaccine protects against monkeypox.* J Immunol, 2006. 177(4): p. 2552-64.
162. Rappuoli, R., *Reverse vaccinology, a genome-based approach to vaccine development.* Vaccine, 2001. 19(17-19): p. 2688-91.
163. Pizza, M., V. Scarlato, V. Masignani, M.M. Giuliani, B. Arico, M. Comanducci, . . . R. Rappuoli, *Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing.* Science, 2000. 287(5459): p. 1816-20.
164. Schroeder, J. and T. Aebischer, *Vaccines for leishmaniasis: from proteome to vaccine candidates.* Hum Vaccin, 2011. 7 Suppl: p. 10-5.
165. Sette, A. and R. Rappuoli, *Reverse vaccinology: developing vaccines in the era of genomics.* Immunity, 2010. 33(4): p. 530-41.
166. Karlsson Hedestam, G.B., R.A. Fouchier, S. Phogat, D.R. Burton, J. Sodroski, and R.T. Wyatt, *The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus.* Nat Rev Microbiol, 2008. 6(2): p. 143-55.
167. Dreyfus, C., N.S. Laursen, T. Kwaks, D. Zuijdgeest, R. Khayat, D.C. Ekiert, . . . R.H. Friesen, *Highly conserved protective epitopes on influenza B viruses.* Science, 2012. 337(6100): p. 1343-8.
168. Corti, D., J. Voss, S.J. Gamblin, G. Codoni, A. Macagno, D. Jarrossay, . . . A. Lanzavecchia, *A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins.* Science, 2011. 333(6044): p. 850-6.
169. Johansson, D.X., C. Voisset, A.W. Tarr, M. Aung, J.K. Ball, J. Dubuisson, and M.A. Persson, *Human combinatorial libraries yield rare antibodies that broadly neutralize hepatitis C virus.* Proc Natl Acad Sci U S A, 2007. 104(41): p. 16269-74.
170. Walker, L.M. and D.R. Burton, *Rational antibody-based HIV-1 vaccine design: current approaches and future directions.* Curr Opin Immunol, 2010. 22(3): p. 358-66.
171. Lingwood, D., P.M. McTamney, H.M. Yassine, J.R. Whittle, X. Guo, J.C. Boyington, . . . G.J. Nabel, *Structural and genetic basis for development of broadly neutralizing influenza antibodies.* Nature, 2012.
172. Azoitei, M.L., B.E. Correia, Y.E. Ban, C. Carrico, O. Kalyuzhniy, L. Chen, . . . W.R. Schief, *Computation-guided backbone grafting of a discontinuous motif onto a protein scaffold.* Science, 2011. 334(6054): p. 373-6.

173. Correia, B.E., Y.E. Ban, M.A. Holmes, H. Xu, K. Ellingson, Z. Kraft, . . . W.R. Schief, *Computational design of epitope-scaffolds allows induction of antibodies specific for a poorly immunogenic HIV vaccine epitope.* Structure, 2010. 18(9): p. 1116-26.
174. Guenaga, J., P. Dosenovic, G. Ofek, D. Baker, W.R. Schief, P.D. Kwong, . . . R.T. Wyatt, *Heterologous epitope-scaffold prime:boosting immuno-focuses B cell responses to the HIV-1 gp41 2F5 neutralization determinant.* PLoS One, 2011. 6(1): p. e16074.
175. Frankel, A.D. and J.A. Young, *HIV-1: fifteen proteins and an RNA.* Annu Rev Biochem, 1998. 67: p. 1-25.
176. Vaishnav, Y.N. and F. Wong-Staal, *The biochemistry of AIDS.* Annu Rev Biochem, 1991. 60: p. 577-630.
177. Wilen, C.B., J.C. Tilton, and R.W. Doms, *HIV: Cell Binding and Entry.* Cold Spring Harb Perspect Med, 2012. 2(8).
178. Dalgleish, A.G., P.C. Beverley, P.R. Clapham, D.H. Crawford, M.F. Greaves, and R.A. Weiss, *The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus.* Nature, 1984. 312(5996): p. 763-7.
179. Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, . . . N.R. Landau, *Identification of a major co-receptor for primary isolates of HIV-1.* Nature, 1996. 381(6584): p. 661-6.
180. Dragic, T., V. Litwin, G.P. Allaway, S.R. Martin, Y. Huang, K.A. Nagashima, . . . W.A. Paxton, *HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5.* Nature, 1996. 381(6584): p. 667-73.
181. Wu, L., N.P. Gerard, R. Wyatt, H. Choe, C. Parolin, N. Ruffing, . . . J. Sodroski, *CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5.* Nature, 1996. 384(6605): p. 179-83.
182. Rizzuto, C.D., R. Wyatt, N. Hernandez-Ramos, Y. Sun, P.D. Kwong, W.A. Hendrickson, and J. Sodroski, *A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding.* Science, 1998. 280(5371): p. 1949-53.
183. Mosier, D.E., *How HIV changes its tropism: evolution and adaptation?* Curr Opin HIV AIDS, 2009. 4(2): p. 125-30.
184. Markosyan, R.M., F.S. Cohen, and G.B. Melikyan, *HIV-1 envelope proteins complete their folding into six-helix bundles immediately after fusion pore formation.* Mol Biol Cell, 2003. 14(3): p. 926-38.
185. Wyatt, R. and J. Sodroski, *The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens.* Science, 1998. 280(5371): p. 1884-8.
186. Chan, D.C. and P.S. Kim, *HIV entry and its inhibition.* Cell, 1998. 93(5): p. 681-4.
187. Melikyan, G.B., R.M. Markosyan, H. Hemmati, M.K. Delmedico, D.M. Lambert, and F.S. Cohen, *Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion.* J Cell Biol, 2000. 151(2): p. 413-23.
188. Hare, S., S.S. Gupta, E. Valkov, A. Engelman, and P. Cherepanov, *Retroviral intasome assembly and inhibition of DNA strand transfer.* Nature, 2010. 464(7286): p. 232-6.
189. Tang, H., K.L. Kuhen, and F. Wong-Staal, *Lentivirus replication and regulation.* Annu Rev Genet, 1999. 33: p. 133-70.
190. Chomont, N., M. El-Far, P. Ancuta, L. Trautmann, F.A. Procopio, B. Yassine-Diab, . . . R.P. Sekaly, *HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation.* Nat Med, 2009. 15(8): p. 893-900.
191. North, T.W., J. Higgins, J.D. Deere, T.L. Hayes, A. Villalobos, L.A. Adamson, . . . P.A. Luciw, *Viral Sanctuaries during Highly Active Antiretroviral Therapy in a Nonhuman Primate Model for Aids.* J Virol, 2009.
192. Dinoso, J.B., S.Y. Kim, A.M. Wiegand, S.E. Palmer, S.J. Gange, L. Cranmer, . . . F. Maldarelli, *Treatment intensification does not reduce residual HIV-1 viremia in patients on highly active antiretroviral therapy.* Proc Natl Acad Sci U S A, 2009. 106(23): p. 9403-8.
193. Brigati, C., M. Giacca, D.M. Noonan, and A. Albini, *HIV Tat, its TARgets and the control of viral gene expression.* FEMS Microbiol Lett, 2003. 220(1): p. 57-65.
194. Greenway, A.L., D.A. McPhee, K. Allen, R. Johnstone, G. Holloway, J. Mills, . . . P. Lambert, *Human immunodeficiency virus type 1 Nef binds to tumor suppressor p53 and protects cells against p53-mediated apoptosis.* J Virol, 2002. 76(6): p. 2692-702.
195. Wu, Y. and J.W. Marsh, *Gene transcription in HIV infection.* Microbes Infect, 2003. 5(11): p. 1023-7.
196. Freed, E.O., *HIV-1 replication.* Somat Cell Mol Genet, 2001. 26(1-6): p. 13-33.

197. Scarlata, S. and C. Carter, *Role of HIV-1 Gag domains in viral assembly.* Biochim Biophys Acta, 2003. 1614(1): p. 62-72.
198. Emerman, M. and M.H. Malim, *HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology.* Science, 1998. 280(5371): p. 1880-4.
199. Majumder, B., N.J. Venkatachari, A. Srinivasan, and V. Ayyavoo, *HIV-1 mediated immune pathogenesis: spotlight on the role of viral protein R (Vpr).* Curr HIV Res, 2009. 7(2): p. 169-77.
200. Kremer, M. and B.S. Schnierle, *HIV-1 Vif: HIV's weapon against the cellular defense factor APOBEC3G.* Curr HIV Res, 2005. 3(4): p. 339-44.
201. Sheehy, A.M., N.C. Gaddis, J.D. Choi, and M.H. Malim, *Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein.* Nature, 2002. 418(6898): p. 646-50.
202. Niewiadomska, A.M. and X.F. Yu, *Host restriction of HIV-1 by APOBEC3 and viral evasion through Vif.* Curr Top Microbiol Immunol, 2009. 339: p. 1-25.
203. Huthoff, H. and G.J. Towers, *Restriction of retroviral replication by APOBEC3G/F and TRIM5alpha.* Trends Microbiol, 2008. 16(12): p. 612-9.
204. Mulder, L.C., A. Harari, and V. Simon, *Cytidine deamination induced HIV-1 drug resistance.* Proc Natl Acad Sci U S A, 2008. 105(14): p. 5501-6.
205. Sheehy, A.M., N.C. Gaddis, and M.H. Malim, *The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif.* Nat Med, 2003. 9(11): p. 1404-7.
206. Navarro, F. and N.R. Landau, *Recent insights into HIV-1 Vif.* Curr Opin Immunol, 2004. 16(4): p. 477-82.
207. Mariani, R., D. Chen, B. Schrofelbauer, F. Navarro, R. Konig, B. Bollman, . . . N.R. Landau, *Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif.* Cell, 2003. 114(1): p. 21-31.
208. Stremlau, M., C.M. Owens, M.J. Perron, M. Kiessling, P. Autissier, and J. Sodroski, *The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys.* Nature, 2004. 427(6977): p. 848-53.
209. Yap, M.W., S. Nisole, and J.P. Stoye, *A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction.* Curr Biol, 2005. 15(1): p. 73-8.
210. Stremlau, M., M. Perron, M. Lee, Y. Li, B. Song, H. Javanbakht, . . . J. Sodroski, *Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor.* Proc Natl Acad Sci U S A, 2006. 103(14): p. 5514-9.
211. Campbell, E.M., O. Perez, J.L. Anderson, and T.J. Hope, *Visualization of a proteasome-independent intermediate during restriction of HIV-1 by rhesus TRIM5alpha.* J Cell Biol, 2008. 180(3): p. 549-61.
212. Earl, P.L., B. Moss, and R.W. Doms, *Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein.* J Virol, 1991. 65(4): p. 2047-55.
213. Moulard, M., S. Hallenberger, W. Garten, and H.D. Klenk, *Processing and routage of HIV glycoproteins by furin to the cell surface.* Virus Res, 1999. 60(1): p. 55-65.
214. Mizuochi, T., T.J. Matthews, M. Kato, J. Hamako, K. Titani, J. Solomon, and T. Feizi, *Diversity of oligosaccharide structures on the envelope glycoprotein gp 120 of human immunodeficiency virus 1 from the lymphoblastoid cell line H9. Presence of complex-type oligosaccharides with bisecting N-acetylglucosamine residues.* J Biol Chem, 1990. 265(15): p. 8519-24.
215. Center, R.J., R.D. Leapman, J. Lebowitz, L.O. Arthur, P.L. Earl, and B. Moss, *Oligomeric structure of the human immunodeficiency virus type 1 envelope protein on the virion surface.* J Virol, 2002. 76(15): p. 7863-7.
216. Lu, M., S.C. Blacklow, and P.S. Kim, *A trimeric structural domain of the HIV-1 transmembrane glycoprotein.* Nat Struct Biol, 1995. 2(12): p. 1075-82.
217. Zhu, P., E. Chertova, J. Bess, Jr., J.D. Lifson, L.O. Arthur, J. Liu, . . . K.H. Roux, *Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions.* Proc Natl Acad Sci U S A, 2003. 100(26): p. 15812-7.
218. Chertova, E., J.W. Bess, Jr., B.J. Crise, I.R. Sowder, T.M. Schaden, J.M. Hilburn, . . . L.O. Arthur, *Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), Is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus.* J Virol, 2002. 76(11): p. 5315-25.
219. Zhu, P., J. Liu, J. Bess, Jr., E. Chertova, J.D. Lifson, H. Grise, . . . K.H. Roux, *Distribution and three-dimensional structure of AIDS virus envelope spikes.* Nature, 2006. 441(7095): p. 847-52.

220. Berman, P.W. and G.R. Nakamura, *Adhesion mediated by intercellular adhesion molecule 1 attenuates the potency of antibodies that block HIV-1 gp160-dependent syncytium formation.* AIDS Res Hum Retroviruses, 1994. 10(5): p. 585-93.
221. Cantin, R., S. Methot, and M.J. Tremblay, *Plunder and stowaways: incorporation of cellular proteins by enveloped viruses.* J Virol, 2005. 79(11): p. 6577-87.
222. Starcich, B.R., B.H. Hahn, G.M. Shaw, P.D. McNeely, S. Modrow, H. Wolf, . . . et al., *Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS.* Cell, 1986. 45(5): p. 637-48.
223. Leonard, C.K., M.W. Spellman, L. Riddle, R.J. Harris, J.N. Thomas, and T.J. Gregory, *Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells.* J Biol Chem, 1990. 265(18): p. 10373-82.
224. Moore, J.P., Q.J. Sattentau, R. Wyatt, and J. Sodroski, *Probing the structure of the human immunodeficiency virus surface glycoprotein gp120 with a panel of monoclonal antibodies.* J Virol, 1994. 68(1): p. 469-84.
225. Dosenovic, P., B. Chakrabarti, M. Soldemo, I. Douagi, M.N. Forsell, Y. Li, . . . G.B. Karlsson Hedestam, *Selective expansion of HIV-1 envelope glycoprotein-specific B cell subsets recognizing distinct structural elements following immunization.* J Immunol, 2009. 183(5): p. 3373-82.
226. Goudsmit, J., C. Debouck, R.H. Meloen, L. Smit, M. Bakker, D.M. Asher, . . . D.C. Gajdusek, *Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees.* Proc Natl Acad Sci U S A, 1988. 85(12): p. 4478-82.
227. Tan, K., J. Liu, J. Wang, S. Shen, and M. Lu, *Atomic structure of a thermostable subdomain of HIV-1 gp41.* Proc Natl Acad Sci U S A, 1997. 94(23): p. 12303-8.
228. Kwon, Y.D., A. Finzi, X. Wu, C. Dogo-Isonagie, L.K. Lee, L.R. Moore, . . . P.D. Kwong, *Unliganded HIV-1 gp120 core structures assume the CD4-bound conformation with regulation by quaternary interactions and variable loops.* Proc Natl Acad Sci U S A, 2012. 109(15): p. 5663-8.
229. Kwong, P.D., R. Wyatt, J. Robinson, R.W. Sweet, J. Sodroski, and W.A. Hendrickson, *Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody.* Nature, 1998. 393(6686): p. 648-59.
230. Huang, C.C., M. Tang, M.Y. Zhang, S. Majeed, E. Montabana, R.L. Stanfield, . . . P.D. Kwong, *Structure of a V3-containing HIV-1 gp120 core.* Science, 2005. 310(5750): p. 1025-8.
231. Kwong, P.D., R. Wyatt, S. Majeed, J. Robinson, R.W. Sweet, J. Sodroski, and W.A. Hendrickson, *Structures of HIV-1 gp120 envelope glycoproteins from laboratory-adapted and primary isolates.* Structure, 2000. 8(12): p. 1329-39.
232. Pancera, M., S. Majeed, Y.E. Ban, L. Chen, C.C. Huang, L. Kong, . . . P.D. Kwong, *Structure of HIV-1 gp120 with gp41-interactive region reveals layered envelope architecture and basis of conformational mobility.* Proc Natl Acad Sci U S A, 2010. 107(3): p. 1166-71.
233. Wyatt, R., P.D. Kwong, E. Desjardins, R.W. Sweet, J. Robinson, W.A. Hendrickson, and J.G. Sodroski, *The antigenic structure of the HIV gp120 envelope glycoprotein.* Nature, 1998. 393(6686): p. 705-11.
234. Pantophlet, R. and D.R. Burton, *GP120: target for neutralizing HIV-1 antibodies.* Annu Rev Immunol, 2006. 24: p. 739-69.
235. Liu, J., A. Bartesaghi, M.J. Borgnia, G. Sapiro, and S. Subramaniam, *Molecular architecture of native HIV-1 gp120 trimers.* Nature, 2008. 455(7209): p. 109-13.
236. Zanetti, G., J.A. Briggs, K. Grunewald, Q.J. Sattentau, and S.D. Fuller, *Cryo-electron tomographic structure of an immunodeficiency virus envelope complex in situ.* PLoS Pathog, 2006. 2(8): p. e83.
237. White, T.A., A. Bartesaghi, M.J. Borgnia, J.R. Meyerson, M.J. de la Cruz, J.W. Bess, . . . S. Subramaniam, *Molecular architectures of trimeric SIV and HIV-1 envelope glycoproteins on intact viruses: strain-dependent variation in quaternary structure.* PLoS Pathog, 2010. 6(12): p. e1001249.
238. Hu, G., J. Liu, K.A. Taylor, and K.H. Roux, *Structural comparison of HIV-1 envelope spikes with and without the V1/V2 loop.* J Virol, 2011. 85(6): p. 2741-50.
239. Mao, Y., L. Wang, C. Gu, A. Herschhorn, S.H. Xiang, H. Haim, . . . J. Sodroski, *Subunit organization of the membrane-bound HIV-1 envelope glycoprotein trimer.* Nat Struct Mol Biol, 2012.

240. Wang, B.Z., W. Liu, S.M. Kang, M. Alam, C. Huang, L. Ye, . . . R.W. Compans, *Incorporation of high levels of chimeric human immunodeficiency virus envelope glycoproteins into virus-like particles.* J Virol, 2007. 81(20): p. 10869-78.
241. Kolchinsky, P., E. Kiprilov, P. Bartley, R. Rubinstein, and J. Sodroski, *Loss of a single N-linked glycan allows CD4-independent human immunodeficiency virus type 1 infection by altering the position of the gp120 V1/V2 variable loops.* J Virol, 2001. 75(7): p. 3435-43.
242. Abram, M.E., A.L. Ferris, W. Shao, W.G. Alvord, and S.H. Hughes, *Nature, position, and frequency of mutations made in a single cycle of HIV-1 replication.* J Virol, 2010. 84(19): p. 9864-78.
243. Drake, J.W., B. Charlesworth, D. Charlesworth, and J.F. Crow, *Rates of spontaneous mutation.* Genetics, 1998. 148(4): p. 1667-86.
244. Perelson, A.S., A.U. Neumann, M. Markowitz, J.M. Leonard, and D.D. Ho, *HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time.* Science, 1996. 271(5255): p. 1582-6.
245. Tremblay, M. and M.A. Wainberg, *Neutralization of multiple HIV-1 isolates from a single subject by autologous sequential sera.* J Infect Dis, 1990. 162(3): p. 735-7.
246. Arendrup, M., C. Nielsen, J.E. Hansen, C. Pedersen, L. Mathiesen, and J.O. Nielsen, *Autologous HIV-1 neutralizing antibodies: emergence of neutralization-resistant escape virus and subsequent development of escape virus neutralizing antibodies.* J Acquir Immune Defic Syndr, 1992. 5(3): p. 303-7.
247. Richman, D.D., T. Wrin, S.J. Little, and C.J. Petropoulos, *Rapid evolution of the neutralizing antibody response to HIV type 1 infection.* Proc Natl Acad Sci U S A, 2003. 100(7): p. 4144-9.
248. Wei, X., J.M. Decker, S. Wang, H. Hui, J.C. Kappes, X. Wu, . . . G.M. Shaw, *Antibody neutralization and escape by HIV-1.* Nature, 2003. 422(6929): p. 307-12.
249. Sagar, M., X. Wu, S. Lee, and J. Overbaugh, *Human immunodeficiency virus type 1 V1-V2 envelope loop sequences expand and add glycosylation sites over the course of infection, and these modifications affect antibody neutralization sensitivity.* J Virol, 2006. 80(19): p. 9586-98.
250. Eriksson, C., M.N. Forsell, and G.B. Karlsson Hedestam, *Hur HIV-1 värjer sig från neutraliserande antikroppar: Implikationer för vaccinutveckling*, in *Incitament*2007. p. 535-539.
251. Poignard, P., M. Moulard, E. Golez, V. Vivona, M. Franti, S. Venturini, . . . D.R. Burton, *Heterogeneity of envelope molecules expressed on primary human immunodeficiency virus type 1 particles as probed by the binding of neutralizing and nonneutralizing antibodies.* J Virol, 2003. 77(1): p. 353-65.
252. Cavacini, L. and M. Posner, *Native HIV type 1 virion surface structures: relationships between antibody binding and neutralization or lessons from the viral capture assay.* AIDS Res Hum Retroviruses, 2004. 20(4): p. 435-41.
253. Herrera, C., C. Spenlehauer, M.S. Fung, D.R. Burton, S. Beddows, and J.P. Moore, *Nonneutralizing antibodies to the CD4-binding site on the gp120 subunit of human immunodeficiency virus type 1 do not interfere with the activity of a neutralizing antibody against the same site.* J Virol, 2003. 77(2): p. 1084-91.
254. Layne, S.P., M.J. Merges, M. Dembo, J.L. Spouge, S.R. Conley, J.P. Moore, . . . P.L. Nara, *Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus.* Virology, 1992. 189(2): p. 695-714.
255. Dimitrov, D.S., R.L. Willey, H. Sato, L.J. Chang, R. Blumenthal, and M.A. Martin, *Quantitation of human immunodeficiency virus type 1 infection kinetics.* J Virol, 1993. 67(4): p. 2182-90.
256. Moore, P.L., E.T. Crooks, L. Porter, P. Zhu, C.S. Cayanan, H. Grise, . . . J.M. Binley, *Nature of nonfunctional envelope proteins on the surface of human immunodeficiency virus type 1.* J Virol, 2006. 80(5): p. 2515-28.
257. Parren, P.W., D.R. Burton, and Q.J. Sattentau, *HIV-1 antibody--debris or virion?* Nat Med, 1997. 3(4): p. 366-7.
258. Wyatt, R., E. Desjardin, U. Olshevsky, C. Nixon, J. Binley, V. Olshevsky, and J. Sodroski, *Analysis of the interaction of the human immunodeficiency virus type 1 gp120 envelope glycoprotein with the gp41 transmembrane glycoprotein.* J Virol, 1997. 71(12): p. 9722-31.
259. Moore, J.P. and J. Sodroski, *Antibody cross-competition analysis of the human immunodeficiency virus type 1 gp120 exterior envelope glycoprotein.* J Virol, 1996. 70(3): p. 1863-72.
260. Decker, J.M., F. Bibollet-Ruche, X. Wei, S. Wang, D.N. Levy, W. Wang, . . . G.M. Shaw, *Antigenic conservation and immunogenicity of the HIV coreceptor binding site.* J Exp Med, 2005. 201(9): p. 1407-19.

261. Marcon, L., H. Choe, K.A. Martin, M. Farzan, P.D. Ponath, L. Wu, . . . J. Sodroski, *Utilization of C-C chemokine receptor 5 by the envelope glycoproteins of a pathogenic simian immunodeficiency virus, SIVmac239.* J Virol, 1997. 71(3): p. 2522-7.
262. Chen, W., Z. Zhu, Y. Feng, and D.S. Dimitrov, *Human domain antibodies to conserved sterically restricted regions on gp120 as exceptionally potent cross-reactive HIV-1 neutralizers.* Proc Natl Acad Sci U S A, 2008. 105(44): p. 17121-6.
263. Robinson, J.E., D.H. Elliott, E.A. Martin, K. Micken, and E.S. Rosenberg, *High frequencies of antibody responses to CD4 induced epitopes in HIV infected patients started on HAART during acute infection.* Hum Antibodies, 2005. 14(3-4): p. 115-21.
264. Forsell, M.N., B. Dey, A. Morner, K. Svehla, S. O'Dell, C.M. Hogerkorp, . . . R.T. Wyatt, *B cell recognition of the conserved HIV-1 co-receptor binding site is altered by endogenous primate CD4.* PLoS Pathog, 2008. 4(10): p. e1000171.
265. Labrijn, A.F., P. Poignard, A. Raja, M.B. Zwick, K. Delgado, M. Franti, . . . D.R. Burton, *Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1.* J Virol, 2003. 77(19): p. 10557-65.
266. Kwong, P.D., M.L. Doyle, D.J. Casper, C. Cicala, S.A. Leavitt, S. Majeed, . . . J. Arthos, *HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites.* Nature, 2002. 420(6916): p. 678-82.
267. Myszka, D.G., R.W. Sweet, P. Hensley, M. Brigham-Burke, P.D. Kwong, W.A. Hendrickson, . . . M.L. Doyle, *Energetics of the HIV gp120-CD4 binding reaction.* Proc Natl Acad Sci U S A, 2000. 97(16): p. 9026-31.
268. Chen, L., Y.D. Kwon, T. Zhou, X. Wu, S. O'Dell, L. Cavacini, . . . P.D. Kwong, *Structural basis of immune evasion at the site of CD4 attachment on HIV-1 gp120.* Science, 2009. 326(5956): p. 1123-7.
269. Alexander, S. and J.H. Elder, *Carbohydrate dramatically influences immune reactivity of antisera to viral glycoprotein antigens.* Science, 1984. 226(4680): p. 1328-30.
270. Cheng-Mayer, C., A. Brown, J. Harouse, P.A. Luciw, and A.J. Mayer, *Selection for neutralization resistance of the simian/human immunodeficiency virus SHIVSF33A variant in vivo by virtue of sequence changes in the extracellular envelope glycoprotein that modify N-linked glycosylation.* J Virol, 1999. 73(7): p. 5294-300.
271. Koch, M., M. Pancera, P.D. Kwong, P. Kolchinsky, C. Grundner, L. Wang, . . . R. Wyatt, *Structure-based, targeted deglycosylation of HIV-1 gp120 and effects on neutralization sensitivity and antibody recognition.* Virology, 2003. 313(2): p. 387-400.
272. Boily, M.C., R.F. Baggaley, L. Wang, B. Masse, R.G. White, R.J. Hayes, and M. Alary, *Heterosexual risk of HIV-1 infection per sexual act: systematic review and meta-analysis of observational studies.* Lancet Infect Dis, 2009. 9(2): p. 118-29.
273. Gupta, R., T. Warren, and A. Wald, *Genital herpes.* Lancet, 2007. 370(9605): p. 2127-37.
274. Russell, D.B., *Herpes and HIV infection--has the time come to act?* Sex Health, 2006. 3(2): p. 67-71.
275. Hladik, F. and T.J. Hope, *HIV infection of the genital mucosa in women.* Curr HIV/AIDS Rep, 2009. 6(1): p. 20-8.
276. Ganor, Y. and M. Bomsel, *HIV-1 transmission in the male genital tract.* Am J Reprod Immunol, 2011. 65(3): p. 284-91.
277. Salazar-Gonzalez, J.F., E. Bailes, K.T. Pham, M.G. Salazar, M.B. Guffey, B.F. Keele, . . . B.H. Hahn, *Deciphering human immunodeficiency virus type 1 transmission and early envelope diversification by single-genome amplification and sequencing.* J Virol, 2008. 82(8): p. 3952-70.
278. Keele, B.F., E.E. Giorgi, J.F. Salazar-Gonzalez, J.M. Decker, K.T. Pham, M.G. Salazar, . . . G.M. Shaw, *Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection.* Proc Natl Acad Sci U S A, 2008. 105(21): p. 7552-7.
279. Samson, M., F. Libert, B.J. Doranz, J. Rucker, C. Liesnard, C.M. Farber, . . . M. Parmentier, *Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene.* Nature, 1996. 382(6593): p. 722-5.
280. Liu, R., W.A. Paxton, S. Choe, D. Ceradini, S.R. Martin, R. Horuk, . . . N.R. Landau, *Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection.* Cell, 1996. 86(3): p. 367-77.
281. Dean, M., M. Carrington, C. Winkler, G.A. Huttley, M.W. Smith, R. Allikmets, . . . S.J. O'Brien, *Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS*

Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. Science, 1996. 273(5283): p. 1856-62.
282. Huang, Y., W.A. Paxton, S.M. Wolinsky, A.U. Neumann, L. Zhang, T. He, . . . R.A. Koup, *The role of a mutant CCR5 allele in HIV-1 transmission and disease progression.* Nat Med, 1996. 2(11): p. 1240-3.
283. Mattapallil, J.J., D.C. Douek, B. Hill, Y. Nishimura, M. Martin, and M. Roederer, *Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection.* Nature, 2005. 434(7037): p. 1093-7.
284. Brenchley, J.M., T.W. Schacker, L.E. Ruff, D.A. Price, J.H. Taylor, G.J. Beilman, . . . D.C. Douek, *CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract.* J Exp Med, 2004. 200(6): p. 749-59.
285. Veazey, R.S., P.A. Marx, and A.A. Lackner, *Vaginal CD4+ T cells express high levels of CCR5 and are rapidly depleted in simian immunodeficiency virus infection.* J Infect Dis, 2003. 187(5): p. 769-76.
286. Veazey, R.S., M. DeMaria, L.V. Chalifoux, D.E. Shvetz, D.R. Pauley, H.L. Knight, . . . A.A. Lackner, *Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection.* Science, 1998. 280(5362): p. 427-31.
287. Li, Q., L. Duan, J.D. Estes, Z.M. Ma, T. Rourke, Y. Wang, . . . A.T. Haase, *Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells.* Nature, 2005. 434(7037): p. 1148-52.
288. Guadalupe, M., E. Reay, S. Sankaran, T. Prindiville, J. Flamm, A. McNeil, and S. Dandekar, *Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy.* J Virol, 2003. 77(21): p. 11708-17.
289. Mehandru, S., M.A. Poles, K. Tenner-Racz, P. Jean-Pierre, V. Manuelli, P. Lopez, . . . M. Markowitz, *Lack of mucosal immune reconstitution during prolonged treatment of acute and early HIV-1 infection.* PLoS Med, 2006. 3(12): p. e484.
290. Brenchley, J.M., M. Paiardini, K.S. Knox, A.I. Asher, B. Cervasi, T.E. Asher, . . . D.C. Douek, *Differential Th17 CD4 T-cell depletion in pathogenic and nonpathogenic lentiviral infections.* Blood, 2008. 112(7): p. 2826-35.
291. Kolls, J.K. and A. Linden, *Interleukin-17 family members and inflammation.* Immunity, 2004. 21(4): p. 467-76.
292. Liang, S.C., X.Y. Tan, D.P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L.A. Fouser, *Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides.* J Exp Med, 2006. 203(10): p. 2271-9.
293. Brenchley, J.M., D.A. Price, and D.C. Douek, *HIV disease: fallout from a mucosal catastrophe?* Nat Immunol, 2006. 7(3): p. 235-9.
294. Silvestri, G., D.L. Sodora, R.A. Koup, M. Paiardini, S.P. O'Neil, H.M. McClure, . . . M.B. Feinberg, *Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia.* Immunity, 2003. 18(3): p. 441-52.
295. Kaur, A., M. Di Mascio, A. Barabasz, M. Rosenzweig, H.M. McClure, A.S. Perelson, . . . R.P. Johnson, *Dynamics of T- and B-lymphocyte turnover in a natural host of simian immunodeficiency virus.* J Virol, 2008. 82(3): p. 1084-93.
296. Forsman, A. and R.A. Weiss, *Why is HIV a pathogen?* Trends Microbiol, 2008. 16(12): p. 555-60.
297. Koup, R.A., J.T. Safrit, Y. Cao, C.A. Andrews, G. McLeod, W. Borkowsky, . . . D.D. Ho, *Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.* J Virol, 1994. 68(7): p. 4650-5.
298. Boutwell, C.L., M.M. Rolland, J.T. Herbeck, J.I. Mullins, and T.M. Allen, *Viral evolution and escape during acute HIV-1 infection.* J Infect Dis, 2010. 202 Suppl 2: p. S309-14.
299. Bernardin, F., D. Kong, L. Peddada, L.A. Baxter-Lowe, and E. Delwart, *Human immunodeficiency virus mutations during the first month of infection are preferentially found in known cytotoxic T-lymphocyte epitopes.* J Virol, 2005. 79(17): p. 11523-8.
300. Mellors, J.W., C.R. Rinaldo, Jr., P. Gupta, R.M. White, J.A. Todd, and L.A. Kingsley, *Prognosis in HIV-1 infection predicted by the quantity of virus in plasma.* Science, 1996. 272(5265): p. 1167-70.
301. Kaslow, R.A., M. Carrington, R. Apple, L. Park, A. Munoz, A.J. Saah, . . . D.L. Mann, *Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection.* Nat Med, 1996. 2(4): p. 405-11.

302. Goulder, P.J., M. Bunce, P. Krausa, K. McIntyre, S. Crowley, B. Morgan, . . . A.J. McMichael, *Novel, cross-restricted, conserved, and immunodominant cytotoxic T lymphocyte epitopes in slow progressors in HIV type 1 infection.* AIDS Res Hum Retroviruses, 1996. 12(18): p. 1691-8.
303. Hendel, H., S. Caillat-Zucman, H. Lebuanec, M. Carrington, S. O'Brien, J.M. Andrieu, . . . J.F. Zagury, *New class I and II HLA alleles strongly associated with opposite patterns of progression to AIDS.* J Immunol, 1999. 162(11): p. 6942-6.
304. Emu, B., E. Sinclair, H. Hatano, A. Ferre, B. Shacklett, J.N. Martin, . . . S.G. Deeks, *HLA class I-restricted T-cell responses may contribute to the control of human immunodeficiency virus infection, but such responses are not always necessary for long-term virus control.* J Virol, 2008. 82(11): p. 5398-407.
305. O'Connell, K.A., J.R. Bailey, and J.N. Blankson, *Elucidating the elite: mechanisms of control in HIV-1 infection.* Trends Pharmacol Sci, 2009. 30(12): p. 631-7.
306. Yamamoto, H. and T. Matano, *Anti-HIV adaptive immunity: determinants for viral persistence.* Rev Med Virol, 2008. 18(5): p. 293-303.
307. Huang, K.H., D. Bonsall, A. Katzourakis, E.C. Thomson, S.J. Fidler, J. Main, . . . P. Klenerman, *B-cell depletion reveals a role for antibodies in the control of chronic HIV-1 infection.* Nat Commun, 2010. 1: p. 102.
308. Miller, C.J., M. Genesca, K. Abel, D. Montefiori, D. Forthal, K. Bost, . . . J.M. McCune, *Antiviral antibodies are necessary for control of simian immunodeficiency virus replication.* J Virol, 2007. 81(10): p. 5024-35.
309. Johnson, R.P., R.F. Siliciano, and M.J. McElrath, *Cellular immune responses to HIV-1.* AIDS, 1998. 12 Suppl A: p. S113-20.
310. Tomaras, G.D., N.L. Yates, P. Liu, L. Qin, G.G. Fouda, L.L. Chavez, . . . B.F. Haynes, *Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia.* J Virol, 2008. 82(24): p. 12449-63.
311. Aasa-Chapman, M.M., A. Hayman, P. Newton, D. Cornforth, I. Williams, P. Borrow, . . . A. McKnight, *Development of the antibody response in acute HIV-1 infection.* AIDS, 2004. 18(3): p. 371-81.
312. Gray, E.S., P.L. Moore, I.A. Choge, J.M. Decker, F. Bibollet-Ruche, H. Li, . . . L. Morris, *Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection.* J Virol, 2007. 81(12): p. 6187-96.
313. Cheng-Mayer, C., J. Homsy, L.A. Evans, and J.A. Levy, *Identification of human immunodeficiency virus subtypes with distinct patterns of sensitivity to serum neutralization.* Proc Natl Acad Sci U S A, 1988. 85(8): p. 2815-9.
314. Profy, A.T., P.A. Salinas, L.I. Eckler, N.M. Dunlop, P.L. Nara, and S.D. Putney, *Epitopes recognized by the neutralizing antibodies of an HIV-1-infected individual.* J Immunol, 1990. 144(12): p. 4641-7.
315. Frost, S.D., T. Wrin, D.M. Smith, S.L. Kosakovsky Pond, Y. Liu, E. Paxinos, . . . D.D. Richman, *Neutralizing antibody responses drive the evolution of human immunodeficiency virus type 1 envelope during recent HIV infection.* Proc Natl Acad Sci U S A, 2005. 102(51): p. 18514-9.
316. Albert, J., B. Abrahamsson, K. Nagy, E. Aurelius, H. Gaines, G. Nystrom, and E.M. Fenyo, *Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera.* AIDS, 1990. 4(2): p. 107-12.
317. Montefiori, D.C., I.Y. Zhou, B. Barnes, D. Lake, E.M. Hersh, Y. Masuho, and L.B. Lefkowitz, Jr., *Homotypic antibody responses to fresh clinical isolates of human immunodeficiency virus.* Virology, 1991. 182(2): p. 635-43.
318. Sather, D.N., J. Armann, L.K. Ching, A. Mavrantoni, G. Sellhorn, Z. Caldwell, . . . L. Stamatatos, *Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection.* J Virol, 2009. 83(2): p. 757-69.
319. Li, Y., K. Svehla, M.K. Louder, D. Wycuff, S. Phogat, M. Tang, . . . R. Wyatt, *Analysis of neutralization specificities in polyclonal sera derived from human immunodeficiency virus type 1-infected individuals.* J Virol, 2009. 83(2): p. 1045-59.
320. Doria-Rose, N.A., R.M. Klein, M.M. Manion, S. O'Dell, A. Phogat, B. Chakrabarti, . . . M. Connors, *Frequency and phenotype of human immunodeficiency virus envelope-specific B cells from patients with broadly cross-neutralizing antibodies.* J Virol, 2009. 83(1): p. 188-99.
321. Binley, J.M., E.A. Lybarger, E.T. Crooks, M.S. Seaman, E. Gray, K.L. Davis, . . . J.R. Mascola, *Profiling the specificity of neutralizing antibodies in a large panel of plasmas from patients*

chronically infected with human immunodeficiency virus type 1 subtypes B and C. J Virol, 2008. 82(23): p. 11651-68.
322. Moore, P.L., E.S. Gray, D. Sheward, M. Madiga, N. Ranchobe, Z. Lai, . . . L. Morris, *Potent and broad neutralization of HIV-1 subtype C by plasma antibodies targeting a quaternary epitope including residues in the V2 loop.* J Virol, 2011. 85(7): p. 3128-41.
323. Simek, M.D., W. Rida, F.H. Priddy, P. Pung, E. Carrow, D.S. Laufer, . . . W.C. Koff, *Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm.* J Virol, 2009. 83(14): p. 7337-48.
324. Gray, E.S., N. Taylor, D. Wycuff, P.L. Moore, G.D. Tomaras, C.K. Wibmer, . . . L. Morris, *Antibody specificities associated with neutralization breadth in plasma from human immunodeficiency virus type 1 subtype C-infected blood donors.* J Virol, 2009. 83(17): p. 8925-37.
325. Mikell, I., D.N. Sather, S.A. Kalams, M. Altfeld, G. Alter, and L. Stamatatos, *Characteristics of the earliest cross-neutralizing antibody response to HIV-1.* PLoS Pathog, 2011. 7(1): p. e1001251.
326. Lynch, R.M., L. Tran, M.K. Louder, S.D. Schmidt, M. Cohen, R. Dersimonian, . . . J.R. Mascola, *The development of CD4 binding site antibodies during HIV-1 infection.* J Virol, 2012. 86(14): p. 7588-95.
327. Li, Y., S.A. Migueles, B. Welcher, K. Svehla, A. Phogat, M.K. Louder, . . . J.R. Mascola, *Broad HIV-1 neutralization mediated by CD4-binding site antibodies.* Nat Med, 2007. 13(9): p. 1032-4.
328. Tomaras, G.D., J.M. Binley, E.S. Gray, E.T. Crooks, K. Osawa, P.L. Moore, . . . L. Morris, *Polyclonal B cell responses to conserved neutralization epitopes in a subset of HIV-1-infected individuals.* J Virol, 2011. 85(21): p. 11502-19.
329. Walker, L.M., M.D. Simek, F. Priddy, J.S. Gach, D. Wagner, M.B. Zwick, . . . D.R. Burton, *A limited number of antibody specificities mediate broad and potent serum neutralization in selected HIV-1 infected individuals.* PLoS Pathog, 2010. 6(8): p. e1001028.
330. Hessell, A.J., P. Poignard, M. Hunter, L. Hangartner, D.M. Tehrani, W.K. Bleeker, . . . D.R. Burton, *Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques.* Nat Med, 2009. 15(8): p. 951-4.
331. Hessell, A.J., E.G. Rakasz, P. Poignard, L. Hangartner, G. Landucci, D.N. Forthal, . . . D.R. Burton, *Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers.* PLoS Pathog, 2009. 5(5): p. e1000433.
332. Hessell, A.J., E.G. Rakasz, D.M. Tehrani, M. Huber, K.L. Weisgrau, G. Landucci, . . . D.R. Burton, *Broadly neutralizing monoclonal antibodies 2F5 and 4E10 directed against the human immunodeficiency virus type 1 gp41 membrane-proximal external region protect against mucosal challenge by simian-human immunodeficiency virus SHIVBa-L.* J Virol, 2010. 84(3): p. 1302-13.
333. Ferrantelli, F., R.A. Rasmussen, K.A. Buckley, P.L. Li, T. Wang, D.C. Montefiori, . . . R.M. Ruprecht, *Complete protection of neonatal rhesus macaques against oral exposure to pathogenic simian-human immunodeficiency virus by human anti-HIV monoclonal antibodies.* J Infect Dis, 2004. 189(12): p. 2167-73.
334. Ng, C.T., J.P. Jaworski, P. Jayaraman, W.F. Sutton, P. Delio, L. Kuller, . . . N.L. Haigwood, *Passive neutralizing antibody controls SHIV viremia and enhances B cell responses in infant macaques.* Nat Med, 2010. 16(10): p. 1117-9.
335. Burton, D.R., A.J. Hessell, B.F. Keele, P.J. Klasse, T.A. Ketas, B. Moldt, . . . J.P. Moore, *Limited or no protection by weakly or nonneutralizing antibodies against vaginal SHIV challenge of macaques compared with a strongly neutralizing antibody.* Proc Natl Acad Sci U S A, 2011. 108(27): p. 11181-6.
336. Vieillard, V., J. Crouzet, F. Boufassa, A. Sennepin, R.H. Fang, P. Debre, and L. Meyer, *Specific Anti-gp41 Antibodies Predict HIV-1 Disease Progression.* J Acquir Immune Defic Syndr, 2012.
337. Burton, D.R., J. Pyati, R. Koduri, S.J. Sharp, G.B. Thornton, P.W. Parren, . . . et al., *Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody.* Science, 1994. 266(5187): p. 1024-7.
338. Muster, T., F. Steindl, M. Purtscher, A. Trkola, A. Klima, G. Himmler, . . . H. Katinger, *A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1.* J Virol, 1993. 67(11): p. 6642-7.

339. Stiegler, G., R. Kunert, M. Purtscher, S. Wolbank, R. Voglauer, F. Steindl, and H. Katinger, *A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1.* AIDS Res Hum Retroviruses, 2001. 17(18): p. 1757-65.
340. Trkola, A., M. Purtscher, T. Muster, C. Ballaun, A. Buchacher, N. Sullivan, . . . H. Katinger, *Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1.* J Virol, 1996. 70(2): p. 1100-8.
341. Traggiai, E., S. Becker, K. Subbarao, L. Kolesnikova, Y. Uematsu, M.R. Gismondo, . . . A. Lanzavecchia, *An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus.* Nat Med, 2004. 10(8): p. 871-5.
342. Corti, D., J.P. Langedijk, A. Hinz, M.S. Seaman, F. Vanzetta, B.M. Fernandez-Rodriguez, . . . A. Lanzavecchia, *Analysis of memory B cell responses and isolation of novel monoclonal antibodies with neutralizing breadth from HIV-1-infected individuals.* PLoS One, 2010. 5(1): p. e8805.
343. Tiller, T., E. Meffre, S. Yurasov, M. Tsuiji, M.C. Nussenzweig, and H. Wardemann, *Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning.* J Immunol Methods, 2008. 329(1-2): p. 112-24.
344. Scheid, J.F., H. Mouquet, N. Feldhahn, B.D. Walker, F. Pereyra, E. Cutrell, . . . M.C. Nussenzweig, *A method for identification of HIV gp140 binding memory B cells in human blood.* J Immunol Methods, 2009. 343(2): p. 65-7.
345. Scheid, J.F., H. Mouquet, B. Ueberheide, R. Diskin, F. Klein, T.Y. Oliveira, . . . M.C. Nussenzweig, *Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding.* Science, 2011. 333(6049): p. 1633-7.
346. Wu, X., Z.Y. Yang, Y. Li, C.M. Hogerkorp, W.R. Schief, M.S. Seaman, . . . J.R. Mascola, *Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1.* Science, 2010. 329(5993): p. 856-61.
347. Walker, L.M., S.K. Phogat, P.Y. Chan-Hui, D. Wagner, P. Phung, J.L. Goss, . . . D.R. Burton, *Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target.* Science, 2009. 326(5950): p. 285-9.
348. Walker, L.M., M. Huber, K.J. Doores, E. Falkowska, R. Pejchal, J.P. Julien, . . . P. Poignard, *Broad neutralization coverage of HIV by multiple highly potent antibodies.* Nature, 2011. 477(7365): p. 466-70.
349. Huang, J., G. Ofek, L. Leub, M.K. Louder, N.A. Doria-Rose, N.S. Longo, . . . M. Connors, *Broad and potent neutralization of HIV-1 by a gp41-specific human antibody.* Nature, 2012.
350. Bonsignori, M., K.K. Hwang, X. Chen, C.Y. Tsao, L. Morris, E. Gray, . . . B.F. Haynes, *Analysis of a clonal lineage of HIV-1 envelope V2/V3 conformational epitope-specific broadly neutralizing antibodies and their inferred unmutated common ancestors.* J Virol, 2011. 85(19): p. 9998-10009.
351. Klein, F., C. Gaebler, H. Mouquet, D.N. Sather, C. Lehmann, J.F. Scheid, . . . M.C. Nussenzweig, *Broad neutralization by a combination of antibodies recognizing the CD4 binding site and a new conformational epitope on the HIV-1 envelope protein.* J Exp Med, 2012.
352. Fouts, T.R., A. Trkola, M.S. Fung, and J.P. Moore, *Interactions of polyclonal and monoclonal anti-glycoprotein 120 antibodies with oligomeric glycoprotein 120-glycoprotein 41 complexes of a primary HIV type 1 isolate: relationship to neutralization.* AIDS Res Hum Retroviruses, 1998. 14(7): p. 591-7.
353. Fouts, T.R., J.M. Binley, A. Trkola, J.E. Robinson, and J.P. Moore, *Neutralization of the human immunodeficiency virus type 1 primary isolate JR-FL by human monoclonal antibodies correlates with antibody binding to the oligomeric form of the envelope glycoprotein complex.* J Virol, 1997. 71(4): p. 2779-85.
354. Moore, J.P., Y. Cao, L. Qing, Q.J. Sattentau, J. Pyati, R. Koduri, . . . D.D. Ho, *Primary isolates of human immunodeficiency virus type 1 are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120.* J Virol, 1995. 69(1): p. 101-9.
355. Falkowska, E., A. Ramos, Y. Feng, T. Zhou, S. Moquin, L.M. Walker, . . . D.R. Burton, *PGV04, an HIV-1 gp120 CD4 binding site antibody, is broad and potent in neutralization but does not induce conformational changes characteristic of CD4.* J Virol, 2012. 86(8): p. 4394-403.
356. Li, Y., S. O'Dell, R. Wilson, X. Wu, S.D. Schmidt, C.M. Hogerkorp, . . . R.T. Wyatt, *HIV-1 Neutralizing Antibodies Display Dual Recognition of the Primary and Coreceptor Binding Sites and Preferential Binding to Fully Cleaved Envelope Glycoproteins.* J Virol, 2012. 86(20): p. 11231-41.

357. Wu, X., T. Zhou, J. Zhu, B. Zhang, I. Georgiev, C. Wang, . . . J.R. Mascola, *Focused evolution of HIV-1 neutralizing antibodies revealed by structures and deep sequencing.* Science, 2011. 333(6049): p. 1593-602.
358. Li, Y., S. O'Dell, L.M. Walker, X. Wu, J. Guenaga, Y. Feng, . . . J.R. Mascola, *Mechanism of neutralization by the broadly neutralizing HIV-1 monoclonal antibody VRC01.* J Virol, 2011. 85(17): p. 8954-67.
359. Ringe, R., S. Phogat, and J. Bhattacharya, *Subtle alteration of residues including N-linked glycans in V2 loop modulate HIV-1 neutralization by PG9 and PG16 monoclonal antibodies.* Virology, 2012. 426(1): p. 34-41.
360. Pejchal, R., K.J. Doores, L.M. Walker, R. Khayat, P.S. Huang, S.K. Wang, . . . I.A. Wilson, *A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield.* Science, 2011. 334(6059): p. 1097-103.
361. Zhu, J., S. O'Dell, G. Ofek, M. Pancera, X. Wu, B. Zhang, . . . P.D. Kwong, *Somatic populations of PGT135-137 HIV-1-neutralizing antibodies identified by 454 pyrosequencing and bioinformatics.* Front Microbiol, 2012.
362. Kwong, P.D., J.R. Mascola, and G.J. Nabel, *Rational Design of Vaccines to Elicit Broadly Neutralizing Antibodies to HIV-1.* Cold Spring Harb Perspect Med, 2011. 1(1): p. a007278.
363. Haynes, B.F., G. Kelsoe, S.C. Harrison, and T.B. Kepler, *B-cell-lineage immunogen design in vaccine development with HIV-1 as a case study.* Nat Biotechnol, 2012. 30(5): p. 423-33.
364. McElrath, M.J. and B.F. Haynes, *Induction of immunity to human immunodeficiency virus type-1 by vaccination.* Immunity, 2010. 33(4): p. 542-54.
365. Li, M., F. Gao, J.R. Mascola, L. Stamatatos, V.R. Polonis, M. Koutsoukos, . . . D.C. Montefiori, *Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies.* J Virol, 2005. 79(16): p. 10108-25.
366. Seaman, M.S., H. Janes, N. Hawkins, L.E. Grandpre, C. Devoy, A. Giri, . . . J.R. Mascola, *Tiered categorization of a diverse panel of HIV-1 Env pseudoviruses for assessment of neutralizing antibodies.* J Virol, 2010. 84(3): p. 1439-52.
367. Pitisuttithum, P., P. Gilbert, M. Gurwith, W. Heyward, M. Martin, F. van Griensven, . . . K. Choopanya, *Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand.* J Infect Dis, 2006. 194(12): p. 1661-71.
368. Li, Y., K. Svehla, N.L. Mathy, G. Voss, J.R. Mascola, and R. Wyatt, *Characterization of antibody responses elicited by human immunodeficiency virus type 1 primary isolate trimeric and monomeric envelope glycoproteins in selected adjuvants.* J Virol, 2006. 80(3): p. 1414-26.
369. Beddows, S., M. Franti, A.K. Dey, M. Kirschner, S.P. Iyer, D.C. Fisch, . . . J.P. Moore, *A comparative immunogenicity study in rabbits of disulfide-stabilized, proteolytically cleaved, soluble trimeric human immunodeficiency virus type 1 gp140, trimeric cleavage-defective gp140 and monomeric gp120.* Virology, 2007. 360(2): p. 329-40.
370. Kim, M., Z.S. Qiao, D.C. Montefiori, B.F. Haynes, E.L. Reinherz, and H.X. Liao, *Comparison of HIV Type 1 ADA gp120 monomers versus gp140 trimers as immunogens for the induction of neutralizing antibodies.* AIDS Res Hum Retroviruses, 2005. 21(1): p. 58-67.
371. Forsell, M.N., W.R. Schief, and R.T. Wyatt, *Immunogenicity of HIV-1 envelope glycoprotein oligomers.* Curr Opin HIV AIDS, 2009. 4(5): p. 380-7.
372. Barnett, S.W., I.K. Srivastava, E. Kan, F. Zhou, A. Goodsell, A.D. Cristillo, . . . M. Vajdy, *Protection of macaques against vaginal SHIV challenge by systemic or mucosal and systemic vaccinations with HIV-envelope.* AIDS, 2008. 22(3): p. 339-48.
373. Derby, N.R., Z. Kraft, E. Kan, E.T. Crooks, S.W. Barnett, I.K. Srivastava, . . . L. Stamatatos, *Antibody responses elicited in macaques immunized with human immunodeficiency virus type 1 (HIV-1) SF162-derived gp140 envelope immunogens: comparison with those elicited during homologous simian/human immunodeficiency virus SHIVSF162P4 and heterologous HIV-1 infection.* J Virol, 2006. 80(17): p. 8745-62.
374. Pinter, A., *Roles of HIV-1 Env variable regions in viral neutralization and vaccine development.* Curr HIV Res, 2007. 5(6): p. 542-53.
375. Ching, L.K., G. Vlachogiannis, K.A. Bosch, and L. Stamatatos, *The first hypervariable region of the gp120 Env glycoprotein defines the neutralizing susceptibility of heterologous human immunodeficiency virus type 1 isolates to neutralizing antibodies elicited by the SF162gp140 immunogen.* J Virol, 2008. 82(2): p. 949-56.
376. Wu, L., T. Zhou, Z.Y. Yang, K. Svehla, S. O'Dell, M.K. Louder, . . . G.J. Nabel, *Enhanced exposure of the CD4-binding site to neutralizing antibodies by structural design of a membrane-*

anchored human immunodeficiency virus type 1 gp120 domain. J Virol, 2009. 83(10): p. 5077-86.
377. Humbert, M., R.A. Rasmussen, H. Ong, F.M. Kaiser, S.L. Hu, and R.M. Ruprecht, *Inducing cross-clade neutralizing antibodies against HIV-1 by immunofocusing.* PLoS One, 2008. 3(12): p. e3937.
378. Dey, B., K. Svehla, L. Xu, D. Wycuff, T. Zhou, G. Voss, . . . R.T. Wyatt, *Structure-based stabilization of HIV-1 gp120 enhances humoral immune responses to the induced co-receptor binding site.* PLoS Pathog, 2009. 5(5): p. e1000445.
379. McCoy, L.E., A.F. Quigley, N.M. Strokappe, B. Bulmer-Thomas, M.S. Seaman, D. Mortier, . . . R.A. Weiss, *Potent and broad neutralization of HIV-1 by a llama antibody elicited by immunization.* J Exp Med, 2012. 209(6): p. 1091-103.
380. Forsman, A., E. Beirnaert, M.M. Aasa-Chapman, B. Hoorelbeke, K. Hijazi, W. Koh, . . . R.A. Weiss, *Llama antibody fragments with cross-subtype human immunodeficiency virus type 1 (HIV-1)-neutralizing properties and high affinity for HIV-1 gp120.* J Virol, 2008. 82(24): p. 12069-81.
381. Koh, W.W., S. Steffensen, M. Gonzalez-Pajuelo, B. Hoorelbeke, A. Gorlani, A. Szynol, . . . R.A. Weiss, *Generation of a family-specific phage library of llama single chain antibody fragments that neutralize HIV-1.* J Biol Chem, 2010. 285(25): p. 19116-24.
382. Flynn, N.M., D.N. Forthal, C.D. Harro, F.N. Judson, K.H. Mayer, and M.F. Para, *Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection.* J Infect Dis, 2005. 191(5): p. 654-65.
383. Connor, R.I., B.T. Korber, B.S. Graham, B.H. Hahn, D.D. Ho, B.D. Walker, . . . S.M. Wolinsky, *Immunological and virological analyses of persons infected by human immunodeficiency virus type 1 while participating in trials of recombinant gp120 subunit vaccines.* J Virol, 1998. 72(2): p. 1552-76.
384. Gilbert, P., M. Wang, T. Wrin, C. Petropoulos, M. Gurwith, F. Sinangil, . . . D.C. Montefiori, *Magnitude and breadth of a nonprotective neutralizing antibody response in an efficacy trial of a candidate HIV-1 gp120 vaccine.* J Infect Dis, 2010. 202(4): p. 595-605.
385. Shiver, J.W., T.M. Fu, L. Chen, D.R. Casimiro, M.E. Davies, R.K. Evans, . . . E.A. Emini, *Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity.* Nature, 2002. 415(6869): p. 331-5.
386. Yang, O.O., S.A. Kalams, A. Trocha, H. Cao, A. Luster, R.P. Johnson, and B.D. Walker, *Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms.* J Virol, 1997. 71(4): p. 3120-8.
387. Carrington, M., G.W. Nelson, M.P. Martin, T. Kissner, D. Vlahov, J.J. Goedert, . . . S.J. O'Brien, *HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage.* Science, 1999. 283(5408): p. 1748-52.
388. Priddy, F.H., D. Brown, J. Kublin, K. Monahan, D.P. Wright, J. Lalezari, . . . E. Quirk, *Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults.* Clin Infect Dis, 2008. 46(11): p. 1769-81.
389. Buchbinder, S.P., D.V. Mehrotra, A. Duerr, D.W. Fitzgerald, R. Mogg, D. Li, . . . M.N. Robertson, *Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial.* Lancet, 2008. 372(9653): p. 1881-93.
390. Qureshi, H., Z.M. Ma, Y. Huang, G. Hodge, M.A. Thomas, J. DiPasquale, . . . C.J. Miller, *Low-dose penile SIVmac251 exposure of rhesus macaques infected with adenovirus type 5 (Ad5) and then immunized with a replication-defective Ad5-based SIV gag/pol/nef vaccine recapitulates the results of the phase IIb step trial of a similar HIV-1 vaccine.* J Virol, 2012. 86(4): p. 2239-50.
391. Tenbusch, M., R. Ignatius, V. Temchura, G. Nabi, B. Tippler, G. Stewart-Jones, . . . K. Uberla, *Risk of immunodeficiency virus infection may increase with vaccine-induced immune response.* J Virol, 2012.
392. Thongcharoen, P., V. Suriyanon, R.M. Paris, C. Khamboonruang, M.S. de Souza, S. Ratto-Kim, . . . J.H. Kim, *A phase 1/2 comparative vaccine trial of the safety and immunogenicity of a CRF01_AE (subtype E) candidate vaccine: ALVAC-HIV (vCP1521) prime with oligomeric gp160 (92TH023/LAI-DID) or bivalent gp120 (CM235/SF2) boost.* J Acquir Immune Defic Syndr, 2007. 46(1): p. 48-55.

393. Nakamura, G.R., D.P. Fonseca, S.M. O'Rourke, A.L. Vollrath, and P.W. Berman, *Monoclonal antibodies to the V2 domain of MN-rgp120: fine mapping of epitopes and inhibition of alpha4beta7 binding.* PLoS One, 2012. 7(6): p. e39045.
394. Haynes, B.F., P.B. Gilbert, M.J. McElrath, S. Zolla-Pazner, G.D. Tomaras, S.M. Alam, . . . J.H. Kim, *Immune-correlates analysis of an HIV-1 vaccine efficacy trial.* N Engl J Med, 2012. 366(14): p. 1275-86.
395. Boyer, J.D., K.E. Ugen, B. Wang, M. Agadjanyan, L. Gilbert, M.L. Bagarazzi, . . . D.B. Weiner, *Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination.* Nat Med, 1997. 3(5): p. 526-32.
396. Girard, M., M.P. Kieny, A. Pinter, F. Barre-Sinoussi, P. Nara, H. Kolbe, . . . et al., *Immunization of chimpanzees confers protection against challenge with human immunodeficiency virus.* Proc Natl Acad Sci U S A, 1991. 88(2): p. 542-6.
397. Fultz, P.N., P. Nara, F. Barre-Sinoussi, A. Chaput, M.L. Greenberg, E. Muchmore, . . . M. Girard, *Vaccine protection of chimpanzees against challenge with HIV-1-infected peripheral blood mononuclear cells.* Science, 1992. 256(5064): p. 1687-90.
398. Novembre, F.J., M. Saucier, D.C. Anderson, S.A. Klumpp, S.P. O'Neil, C.R. Brown, 2nd, . . . H.M. McClure, *Development of AIDS in a chimpanzee infected with human immunodeficiency virus type 1.* J Virol, 1997. 71(5): p. 4086-91.
399. Prince, A.M. and L. Andrus, *AIDS vaccine trials in chimpanzees.* Science, 1998. 282(5397): p. 2195-6.
400. Baroncelli, S., D.R. Negri, Z. Michelini, and A. Cara, *Macaca mulatta, fascicularis and nemestrina in AIDS vaccine development.* Expert Rev Vaccines, 2008. 7(9): p. 1419-34.
401. Rotger, M., J. Dalmau, A. Rauch, P. McLaren, S.E. Bosinger, R. Martinez, . . . A. Telenti, *Comparative transcriptomics of extreme phenotypes of human HIV-1 infection and SIV infection in sooty mangabey and rhesus macaque.* J Clin Invest, 2011. 121(6): p. 2391-400.
402. Lore, K., *Isolation and immunophenotyping of human and rhesus macaque dendritic cells.* Methods Cell Biol, 2004. 75: p. 623-42.
403. Pichyangkul, S., P. Saengkrai, K. Yongvanitchit, C. Limsomwong, M. Gettayacamin, D.S. Walsh, . . . D.G. Heppner, *Isolation and characterization of rhesus blood dendritic cells using flow cytometry.* J Immunol Methods, 2001. 252(1-2): p. 15-23.
404. Kwissa, M., H.I. Nakaya, H. Oluoch, and B. Pulendran, *Distinct TLR adjuvants differentially stimulate systemic and local innate immune responses in nonhuman primates.* Blood, 2012. 119(9): p. 2044-55.
405. Gujer, C., C. Sundling, R.A. Seder, G.B. Karlsson Hedestam, and K. Lore, *Human and rhesus plasmacytoid dendritic cell and B-cell responses to Toll-like receptor stimulation.* Immunology, 2011. 134(3): p. 257-69.
406. Lackner, A.A., M.M. Lederman, and B. Rodriguez, *HIV Pathogenesis: The Host.* Cold Spring Harb Perspect Med, 2012. 2(9).
407. Geretti, A.M., *Simian immunodeficiency virus as a model of human HIV disease.* Rev Med Virol, 1999. 9(1): p. 57-67.
408. Keele, B.F., H. Li, G.H. Learn, P. Hraber, E.E. Giorgi, T. Grayson, . . . G.M. Shaw, *Low-dose rectal inoculation of rhesus macaques by SIVsmE660 or SIVmac251 recapitulates human mucosal infection by HIV-1.* J Exp Med, 2009. 206(5): p. 1117-34.
409. Uberla, K., C. Stahl-Hennig, D. Bottiger, K. Matz-Rensing, F.J. Kaup, J. Li, . . . et al., *Animal model for the therapy of acquired immunodeficiency syndrome with reverse transcriptase inhibitors.* Proc Natl Acad Sci U S A, 1995. 92(18): p. 8210-4.
410. Hatziioannou, T., Z. Ambrose, N.P. Chung, M. Piatak, Jr., F. Yuan, C.M. Trubey, . . . P.D. Bieniasz, *A macaque model of HIV-1 infection.* Proc Natl Acad Sci U S A, 2009. 106(11): p. 4425-9.
411. Nath, B.M., K.E. Schumann, and J.D. Boyer, *The chimpanzee and other non-human-primate models in HIV-1 vaccine research.* Trends Microbiol, 2000. 8(9): p. 426-31.
412. Crawford, J.M., P.L. Earl, B. Moss, K.A. Reimann, M.S. Wyand, K.H. Manson, . . . D.C. Montefiori, *Characterization of primary isolate-like variants of simian-human immunodeficiency virus.* J Virol, 1999. 73(12): p. 10199-207.
413. Etemad-Moghadam, B., G.B. Karlsson, M. Halloran, Y. Sun, D. Schenten, M. Fernandes, . . . J. Sodroski, *Characterization of simian-human immunodeficiency virus envelope glycoprotein epitopes recognized by neutralizing antibodies from infected monkeys.* J Virol, 1998. 72(10): p. 8437-45.
414. Reimann, K.A., J.T. Li, R. Veazey, M. Halloran, I.W. Park, G.B. Karlsson, . . . N.L. Letvin, *A chimeric simian/human immunodeficiency virus expressing a primary patient human*

immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. J Virol, 1996. 70(10): p. 6922-8.
415. Shibata, R., F. Maldarelli, C. Siemon, T. Matano, M. Parta, G. Miller, . . . M.A. Martin, *Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing.* J Infect Dis, 1997. 176(2): p. 362-73.
416. Karlsson, G.B., M. Halloran, J. Li, I.W. Park, R. Gomila, K.A. Reimann, . . . J. Sodroski, *Characterization of molecularly cloned simian-human immunodeficiency viruses causing rapid CD4+ lymphocyte depletion in rhesus monkeys.* J Virol, 1997. 71(6): p. 4218-25.
417. Joag, S.V., Z. Li, L. Foresman, E.B. Stephens, L.J. Zhao, I. Adany, . . . O. Narayan, *Chimeric simian/human immunodeficiency virus that causes progressive loss of CD4+ T cells and AIDS in pig-tailed macaques.* J Virol, 1996. 70(5): p. 3189-97.
418. Li, J.T., M. Halloran, C.I. Lord, A. Watson, J. Ranchalis, M. Fung, . . . J.G. Sodroski, *Persistent infection of macaques with simian-human immunodeficiency viruses.* J Virol, 1995. 69(11): p. 7061-7.
419. Harouse, J.M., A. Gettie, R.C. Tan, J. Blanchard, and C. Cheng-Mayer, *Distinct pathogenic sequela in rhesus macaques infected with CCR5 or CXCR4 utilizing SHIVs.* Science, 1999. 284(5415): p. 816-9.
420. Amara, R.R., F. Villinger, J.D. Altman, S.L. Lydy, S.P. O'Neil, S.I. Staprans, . . . H.L. Robinson, *Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine.* Science, 2001. 292(5514): p. 69-74.
421. Barouch, D.H., S. Santra, J.E. Schmitz, M.J. Kuroda, T.M. Fu, W. Wagner, . . . N.L. Letvin, *Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination.* Science, 2000. 290(5491): p. 486-92.
422. Nehete, P.N., S. Chitta, M.M. Hossain, L. Hill, B.J. Bernacky, W. Baze, . . . K.J. Sastry, *Protection against chronic infection and AIDS by an HIV envelope peptide-cocktail vaccine in a pathogenic SHIV-rhesus model.* Vaccine, 2001. 20(5-6): p. 813-25.
423. Feinberg, M.B. and J.P. Moore, *AIDS vaccine models: challenging challenge viruses.* Nat Med, 2002. 8(3): p. 207-10.
424. Nishimura, Y., M. Shingai, R. Willey, R. Sadjadpour, W.R. Lee, C.R. Brown, . . . M.A. Martin, *Generation of the Pathogenic R5-Tropic Shivad8 by Serial Passaging in Rhesus Macaques.* J Virol, 2010.
425. Luciw, P.A., E. Pratt-Lowe, K.E. Shaw, J.A. Levy, and C. Cheng-Mayer, *Persistent infection of rhesus macaques with T-cell-line-tropic and macrophage-tropic clones of simian/human immunodeficiency viruses (SHIV).* Proc Natl Acad Sci U S A, 1995. 92(16): p. 7490-4.
426. Pal, R., B. Taylor, J.S. Foulke, R. Woodward, M. Merges, R. Praschunus, . . . M. Reitz, *Characterization of a simian human immunodeficiency virus encoding the envelope gene from the CCR5-tropic HIV-1 Ba-L.* J Acquir Immune Defic Syndr, 2003. 33(3): p. 300-7.
427. Liu, Q., Y. Li, G. Yang, J. Dai, R.M. Ruprecht, and Y. Shao, *Molecularly cloned SHIV-CN97001: a replication-competent, R5 simian/human immunodeficiency virus containing env of a primary Chinese HIV-1 clade C isolate.* J Med Primatol, 2011.
428. Song, R.J., A.L. Chenine, R.A. Rasmussen, C.R. Ruprecht, S. Mirshahidi, R.D. Grisson, . . . R.M. Ruprecht, *Molecularly cloned SHIV-1157ipd3N4: a highly replication- competent, mucosally transmissible R5 simian-human immunodeficiency virus encoding HIV clade C Env.* J Virol, 2006. 80(17): p. 8729-38.
429. Siddappa, N.B., G. Hemashettar, Y.L. Wong, S. Lakhashe, R.A. Rasmussen, J.D. Watkins, . . . R.M. Ruprecht, *Development of a tier 1 R5 clade C simian-human immunodeficiency virus as a tool to test neutralizing antibody-based immunoprophylaxis.* J Med Primatol, 2011. 40(2): p. 120-8.
430. Tan, R.C., J.M. Harouse, A. Gettie, and C. Cheng-Mayer, *In vivo adaptation of SHIV(SF162): chimeric virus expressing a NSI, CCR5-specific envelope protein.* J Med Primatol, 1999. 28(4-5): p. 164-8.
431. Siddappa, N.B., J.D. Watkins, K.J. Wassermann, R. Song, W. Wang, V.G. Kramer, . . . R.M. Ruprecht, *R5 clade C SHIV strains with tier 1 or 2 neutralization sensitivity: tools to dissect env evolution and to develop AIDS vaccines in primate models.* PLoS One, 2010. 5(7): p. e11689.
432. Shedlock, D.J., G. Silvestri, and D.B. Weiner, *Monkeying around with HIV vaccines: using rhesus macaques to define 'gatekeepers' for clinical trials.* Nat Rev Immunol, 2009. 9(10): p. 717-28.
433. Berman, P.W., T.J. Gregory, L. Riddle, G.R. Nakamura, M.A. Champe, J.P. Porter, . . . J.W. Eichberg, *Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160.* Nature, 1990. 345(6276): p. 622-5.

434. Davis, D., H. Donners, B. Willems, K. Lovgren-Bengtsson, L. Akerblom, G. Vanham, . . . G. van der Groen, *Neutralization of primary HIV-1 SF13 can be detected in extended incubation phase assays with sera from monkeys immunized with recombinant HIV-1 SF2 gp120.* Vaccine, 2004. 22(5-6): p. 747-54.
435. Margolin, D.H., K.A. Reimann, J. Sodroski, G.B. Karlsson, K. Tenner-Racz, P. Racz, and N.L. Letvin, *Immunoglobulin V(H) usage during primary infection of rhesus monkeys with chimeric simian-human immunodeficiency viruses.* J Virol, 1997. 71(11): p. 8582-91.
436. Arnaout, R., W. Lee, P. Cahill, T. Honan, T. Sparrow, M. Weiand, . . . S.B. Koralov, *High-resolution description of antibody heavy-chain repertoires in humans.* PLoS One, 2011. 6(8): p. e22365.
437. Brezinschek, H.P., S.J. Foster, R.I. Brezinschek, T. Dorner, R. Domiati-Saad, and P.E. Lipsky, *Analysis of the human VH gene repertoire. Differential effects of selection and somatic hypermutation on human peripheral CD5(+)/IgM+ and CD5(-)/IgM+ B cells.* J Clin Invest, 1997. 99(10): p. 2488-501.
438. Foster, S.J., H.P. Brezinschek, R.I. Brezinschek, and P.E. Lipsky, *Molecular mechanisms and selective influences that shape the kappa gene repertoire of IgM+ B cells.* J Clin Invest, 1997. 99(7): p. 1614-27.
439. Andersen, P.S., M. Haahr-Hansen, V.W. Coljee, F.R. Hinnerfeldt, K. Varming, S. Bregenholt, and J.S. Haurum, *Extensive restrictions in the VH sequence usage of the human antibody response against the Rhesus D antigen.* Mol Immunol, 2007. 44(4): p. 412-22.
440. Warncke, M., T. Calzascia, M. Coulot, N. Balke, R. Touil, F. Kolbinger, and C. Heusser, *Different adaptations of IgG effector function in human and nonhuman primates and implications for therapeutic antibody treatment.* J Immunol, 2012. 188(9): p. 4405-11.
441. Boberg, A., A. Brave, S. Johansson, B. Wahren, J. Hinkula, and E. Rollman, *Murine models for HIV vaccination and challenge.* Expert Rev Vaccines, 2008. 7(1): p. 117-30.
442. Denton, P.W. and J.V. Garcia, *Humanized mouse models of HIV infection.* AIDS Rev, 2011. 13(3): p. 135-48.
443. Lan, P., N. Tonomura, A. Shimizu, S. Wang, and Y.G. Yang, *Reconstitution of a functional human immune system in immunodeficient mice through combined human fetal thymus/liver and CD34+ cell transplantation.* Blood, 2006. 108(2): p. 487-92.
444. Yang, X., J. Lee, E.M. Mahony, P.D. Kwong, R. Wyatt, and J. Sodroski, *Highly stable trimers formed by human immunodeficiency virus type 1 envelope glycoproteins fused with the trimeric motif of T4 bacteriophage fibritin.* J Virol, 2002. 76(9): p. 4634-42.
445. Howard, W.A., J.M. Bible, E. Finlay-Dijsselbloem, S. Openshaw, and D.K. Dunn-Walters, *Immunoglobulin light-chain genes in the rhesus macaque II: lambda light-chain germline sequences for subgroups IGLV1, IGLV2, IGLV3, IGLV4 and IGLV5.* Immunogenetics, 2005. 57(9): p. 655-64.
446. Howard, W.A., J.M. Bible, E. Finlay-Dijsselbloem, S. Openshaw, and D.K. Dunn-Walters, *Immunoglobulin light-chain genes in the rhesus macaque I: kappa light-chain germline sequences for subgroups IGKV1, IGKV and IGKV3.* Immunogenetics, 2005. 57(3-4): p. 210-8.
447. Bible, J.M., W. Howard, H. Robbins, and D.K. Dunn-Walters, *IGHV1, IGHV5 and IGHV7 subgroup genes in the rhesus macaque.* Immunogenetics, 2003. 54(12): p. 867-73.
448. Lane, J., P. Duroux, and M.P. Lefranc, *From IMGT-ONTOLOGY to IMGT/LIGMotif: the IMGT standardized approach for immunoglobulin and T cell receptor gene identification and description in large genomic sequences.* BMC Bioinformatics, 2010. 11: p. 223.
449. Crotty, S., R.D. Aubert, J. Glidewell, and R. Ahmed, *Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system.* J Immunol Methods, 2004. 286(1-2): p. 111-22.
450. Good, K.L., V.L. Bryant, and S.G. Tangye, *Kinetics of human B cell behavior and amplification of proliferative responses following stimulation with IL-21.* J Immunol, 2006. 177(8): p. 5236-47.
451. Jego, G., R. Bataille, and C. Pellat-Deceunynck, *Interleukin-6 is a growth factor for nonmalignant human plasmablasts.* Blood, 2001. 97(6): p. 1817-22.
452. Tangye, S.G., D.T. Avery, and P.D. Hodgkin, *A division-linked mechanism for the rapid generation of Ig-secreting cells from human memory B cells.* J Immunol, 2003. 170(1): p. 261-9.
453. Bernasconi, N.L., E. Traggiai, and A. Lanzavecchia, *Maintenance of serological memory by polyclonal activation of human memory B cells.* Science, 2002. 298(5601): p. 2199-202.
454. Douagi, I., C. Gujer, C. Sundling, W.C. Adams, A. Smed-Sorensen, R.A. Seder, . . . K. Lore, *Human B cell responses to TLR ligands are differentially modulated by myeloid and plasmacytoid dendritic cells.* J Immunol, 2009. 182(4): p. 1991-2001.

455. Dorner, T. and A. Radbruch, *Antibodies and B cell memory in viral immunity.* Immunity, 2007. 27(3): p. 384-92.
456. Scinicariello, F. and R. Attanasio, *Intraspecies heterogeneity of immunoglobulin alpha-chain constant region genes in rhesus macaques.* Immunology, 2001. 103(4): p. 441-8.
457. Rogers, K.A., J.P. Richardson, F. Scinicariello, and R. Attanasio, *Molecular characterization of immunoglobulin D in mammals: immunoglobulin heavy constant delta genes in dogs, chimpanzees and four old world monkey species.* Immunology, 2006. 118(1): p. 88-100.
458. Shan, M., P.J. Klasse, K. Banerjee, A.K. Dey, S.P. Iyer, R. Dionisio, . . . J.P. Moore, *HIV-1 gp120 mannoses induce immunosuppressive responses from dendritic cells.* PLoS Pathog, 2007. 3(11): p. e169.
459. Fernando, K., H. Hu, H. Ni, J.A. Hoxie, and D. Weissman, *Vaccine-delivered HIV envelope inhibits CD4(+) T-cell activation, a mechanism for poor HIV vaccine responses.* Blood, 2007. 109(6): p. 2538-44.
460. Hu, H., K. Fernando, H. Ni, and D. Weissman, *HIV envelope suppresses CD4+ T cell activation independent of T regulatory cells.* J Immunol, 2008. 180(8): p. 5593-600.
461. Vlahakis, S.R., A. Algeciras-Schimnich, G. Bou, C.J. Heppelmann, A. Villasis-Keever, R.G. Collman, and C.V. Paya, *Chemokine-receptor activation by env determines the mechanism of death in HIV-infected and uninfected T lymphocytes.* J Clin Invest, 2001. 107(2): p. 207-15.
462. Klasse, P.J., R.W. Sanders, A. Cerutti, and J.P. Moore, *How can HIV-type-1-Env immunogenicity be improved to facilitate antibody-based vaccine development?* AIDS Res Hum Retroviruses, 2012. 28(1): p. 1-15.
463. Gupta, K., M. Hudgens, L. Corey, M.J. McElrath, K. Weinhold, D.C. Montefiori, . . . P. Goepfert, *Safety and immunogenicity of a high-titered canarypox vaccine in combination with rgp120 in a diverse population of HIV-1-uninfected adults: AIDS Vaccine Evaluation Group Protocol 022A.* J Acquir Immune Defic Syndr, 2002. 29(3): p. 254-61.
464. Berman, P.W., K.K. Murthy, T. Wrin, J.C. Vennari, E.K. Cobb, D.J. Eastman, . . . J.F. Obijeski, *Protection of MN-rgp120-immunized chimpanzees from heterologous infection with a primary isolate of human immunodeficiency virus type 1.* J Infect Dis, 1996. 173(1): p. 52-9.
465. Wrammert, J., N. Onlamoon, R.S. Akondy, G.C. Perng, K. Polsrila, A. Chandele, . . . R. Ahmed, *Rapid and massive virus-specific plasmablast responses during acute dengue virus infection in humans.* J Virol, 2012. 86(6): p. 2911-8.
466. Rojas, R. and G. Apodaca, *Immunoglobulin transport across polarized epithelial cells.* Nat Rev Mol Cell Biol, 2002. 3(12): p. 944-55.
467. Nardelli-Haefliger, D., D. Wirthner, J.T. Schiller, D.R. Lowy, A. Hildesheim, F. Ponci, and P. De Grandi, *Specific antibody levels at the cervix during the menstrual cycle of women vaccinated with human papillomavirus 16 virus-like particles.* J Natl Cancer Inst, 2003. 95(15): p. 1128-37.
468. Graham, B.S., M.C. Keefer, M.J. McElrath, G.J. Gorse, D.H. Schwartz, K. Weinhold, . . . P.E. Fast, *Safety and immunogenicity of a candidate HIV-1 vaccine in healthy adults: recombinant glycoprotein (rgp) 120. A randomized, double-blind trial. NIAID AIDS Vaccine Evaluation Group.* Ann Intern Med, 1996. 125(4): p. 270-9.
469. Villa, L.L., R.L. Costa, C.A. Petta, R.P. Andrade, J. Paavonen, O.E. Iversen, . . . E. Barr, *High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up.* Br J Cancer, 2006. 95(11): p. 1459-66.
470. Zhao, J., L. Lai, R.R. Amara, D.C. Montefiori, F. Villinger, L. Chennareddi, . . . H.L. Robinson, *Preclinical studies of human immunodeficiency virus/AIDS vaccines: inverse correlation between avidity of anti-Env antibodies and peak postchallenge viremia.* J Virol, 2009. 83(9): p. 4102-11.
471. Bachmann, M.F., B. Odermatt, H. Hengartner, and R.M. Zinkernagel, *Induction of long-lived germinal centers associated with persisting antigen after viral infection.* J Exp Med, 1996. 183(5): p. 2259-69.
472. Allen, C.D. and J.G. Cyster, *Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function.* Semin Immunol, 2008. 20(1): p. 14-25.
473. Anderson, K.P., C. Lucas, C.V. Hanson, H.F. Londe, A. Izu, T. Gregory, . . . J.W. Eichberg, *Effect of dose and immunization schedule on immune response of baboons to recombinant glycoprotein 120 of HIV-1.* J Infect Dis, 1989. 160(6): p. 960-9.
474. Stamatatos, L., L. Morris, D.R. Burton, and J.R. Mascola, *Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine?* Nat Med, 2009. 15(8): p. 866-70.

475. Pantophlet, R., E. Ollmann Saphire, P. Poignard, P.W. Parren, I.A. Wilson, and D.R. Burton, *Fine mapping of the interaction of neutralizing and nonneutralizing monoclonal antibodies with the CD4 binding site of human immunodeficiency virus type 1 gp120.* J Virol, 2003. 77(1): p. 642-58.
476. Earl, P.L., W. Sugiura, D.C. Montefiori, C.C. Broder, S.A. Lee, C. Wild, . . . B. Moss, *Immunogenicity and protective efficacy of oligomeric human immunodeficiency virus type 1 gp140.* J Virol, 2001. 75(2): p. 645-53.
477. Morner, A., I. Douagi, M.N. Forsell, C. Sundling, P. Dosenovic, S. O'Dell, . . . G.B. Karlsson Hedestam, *Human immunodeficiency virus type 1 env trimer immunization of macaques and impact of priming with viral vector or stabilized core protein.* J Virol, 2009. 83(2): p. 540-51.
478. Bogers, W.M., D. Davis, I. Baak, E. Kan, S. Hofman, Y. Sun, . . . J.L. Heeney, *Systemic neutralizing antibodies induced by long interval mucosally primed systemically boosted immunization correlate with protection from mucosal SHIV challenge.* Virology, 2008. 382(2): p. 217-25.
479. Sundling, C., S. O'Dell, I. Douagi, M.N. Forsell, A. Morner, K. Lore, . . . G.B. Karlsson Hedestam, *Immunization with wild-type or CD4-binding-defective HIV-1 Env trimers reduces viremia equivalently following heterologous challenge with simian-human immunodeficiency virus.* J Virol, 2010. 84(18): p. 9086-95.