Cross-reactive neutralizing humoral immunity in HIV-1 disease: dynamics of host-pathogen interactions
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General discussion
Protection against pathogenic infections

The twentieth century witnessed the introduction of several successful vaccines, including those against diphtheria, measles, tetanus, yellow fever, hepatitis A and B, influenza, smallpox and polio. As vaccines became more common, many people began to take them for granted. However, vaccines remain elusive for many important diseases, including malaria and HIV-1 infection.

For each of the known vaccines, protection has been achieved by mimicking infection with the pathogen and thereby establishing immunologic memory that can rapidly respond should an actual infection occur. This has been achieved with the use of live attenuated viruses, killed viruses or recombinant viral proteins which all do not cause the disease but which do elicit strong and long-lasting immune responses.

The tremendous global success with viral vaccines raises the question as to why HIV-1 vaccine development is so difficult. Many of the difficulties lie in distinct properties of this virus compared with other viruses. Foremost among these is the enormous sequence diversity of HIV-1, which can be as high as 35% between viruses from different subtypes and the relative inaccessibility of the conserved epitopes. Moreover, the lack of understanding of the immune responses that can control HIV-1 replication, for instance in elite controllers and high risk seronegative individuals, makes the development of a protective vaccine even more challenging.

It is assumed that a protective vaccine should elicit cross-reactive neutralizing humoral immunity in combination with a cellular immune response. In combination, these responses ideally can protect against acquisition of infection or second best, against disease progression by reducing viral load which will also have an impact on the spread of HIV-1 in the population.

The studies described in this thesis are focused on cross-reactive neutralizing humoral immunity and the interactions between HIV-1 and its host humoral immune responses. In this chapter the implications of the results described in this thesis will be discussed in view of the possibilities for vaccine immunogen design and to elicit sterilizing immunity against HIV-1.

Humoral immunity in HIV-1 infection

The majority of HIV-1-infected individuals mount an HIV-1-specific neutralizing humoral immune response within weeks to months after primary infection. This response is considered to be strain-specific as neutralizing activity is generally restricted to the autologous virus variant and mainly directed against the variable regions of the envelope glycoprotein. Longitudinal studies have shown that HIV-1 rapidly and repeatedly escapes the neutralizing antibody response mounted during HIV-1 infection. The presence of neutralizing antibodies is a burden to the virus as it drives the continuous evolution of the HIV-1 envelope glycoprotein. As a consequence of this selection, the majority of
the virus population in an infected individual is only weakly, if at all, neutralized by the contemporaneous antibody repertoire. With time, as the virus population diversifies and the immune response matures, neutralization can also be detected against heterologous HIV-1 variants. In chapter 2 we observed that the neutralizing activity in sera of participants from the Amsterdam Cohort Studies who are chronically infected with HIV-1 subtype B was preferentially directed against the subtype B HIV-1 variants in a multi-subtype virus panel that also included clade A, C, and D HIV-1 variants. Subtype-specific humoral immunity may provide new leads on the way to a potent HIV-1 vaccine. However, developing and administering multiple HIV-1 vaccines is far less ideal than having a single vaccine that would cover all circulating HIV-1 variants. During the first three years of infection approximately 30% of HIV-1 infected individuals in the Amsterdam Cohort Studies developed cross-reactive neutralizing activity in serum (chapter 4), with the ability to neutralize viruses from different subtypes. In this same cohort one so called “elite neutralizer” was identified with a HIV-1 specific neutralizing activity in serum with tremendous potency and breadth. The prevalence of individuals with cross-reactive and elite neutralizing activity in the Amsterdam Cohort is in agreement with that observed in several other cohorts in other geographic regions. The relatively high prevalence of cross-reactive neutralizing activity suggests that the epitopes that are capable of eliciting these humoral responses are accessible and immunogenic on the native gp160 spike of HIV-1. Moreover, the fact that truly broadly neutralizing antibodies exist, implicates that a single protective antibody-based vaccine against HIV-1 may be an achievable goal. The sequence variation in the HIV-1 envelope glycoprotein may thus be less problematic for the choice of epitope specificities that a vaccine should cover. Indeed, it may not so much be a matter of whether an epitope is present but rather if it is accessible on HIV-1 variants from different subtypes. The fact that recently identified cross-reactive neutralizing antibodies PG9, PG16 and VRC01 have neutralizing activity against the majority of primary HIV-1 variants, suggests that the epitopes of at least these neutralizing antibodies are indeed accessible predicting the potential success of a vaccine that would be capable of eliciting this type of antibodies.

Factors associated with the development of cross-reactive neutralizing humoral immunity

To support HIV-1 vaccine development, more insight is needed into the host and viral factors that are associated with the ability of the host to elicit a cross-reactive neutralizing humoral immune response, and how such a neutralizing serum response evolves over time. The development of a potent cross-reactive neutralizing humoral immune response takes at least 2 to 3 years (chapter 3). It has been shown that the breadth of neutralization is correlated with time since infection. This time may be required for the affinity maturation during which the neutralizing antibodies gain affinity and become highly potent.
We have also observed that the ability of serum to neutralize different viruses is directly related to the neutralization titer in serum (chapter 2). This may imply that sera with highly cross-reactive neutralizing ability in general harbor multiple epitope specificities or that a high quantity of a single antibody specificity is more potent, even against unrelated HIV-1 variants. It may also be that the development of broadly neutralizing antibodies is related to the evolution of HIV-1. As discussed above, as neutralizing antibodies emerge during the course of infection, they will rapidly select for HIV-1 escape variants that have mutations in the epitope that is recognized by these antibodies. In turn, these viral escape variants may contribute to the affinity maturation of the neutralizing antibody response. By continuous cycles of selection for escape variants that subsequently drive affinity maturation, antibodies with higher potency and breadth may emerge. This may imply that high concentrations of cross-reactive neutralizing antibodies will increase the chance that such an antibody can bind, for instance when the epitope is only transiently accessible in the trimeric structure. An alternative hypothesis is that instead of affinity maturation of the original antibody response, the constantly emerging escape variants continuously elicit novel antibody responses during the course of infection which in combination may provide a serum with a cross-reactive neutralizing phenotype. Indeed, recent studies have demonstrated that the individual epitope specificities did not account for the breadth of neutralizing activity in serum whereas the combination of these different antibodies did approach the neutralization phenotype of the patient serum.

In chapter 3 we observed that a high plasma viral RNA load set-point and low CD4+ cell count set-point were both associated with the development of cross-reactive neutralizing activity. Furthermore, we observed that higher cross-reactive neutralizing activity was significantly associated with lower CD4+ T cell counts already before and soon after infection (chapter 4). In a model for Lymphocytic Choriomeningitis Virus (LCMV) infection, a reduction in CD4+ T cell numbers prior to infection reduced polyclonal B cell stimulation and enhanced protective antibody responses in terms of earlier onset and higher titers without impairing protective CD8+ T cell responses. The correlation between the development of cross-reactive neutralizing activity and a high plasma viral RNA load indicates that the development of potently neutralizing humoral immunity apparently requires exposure to a sufficient amount of antigen. Indeed the prevalence of cross-reactive neutralizing activity in serum from elite controllers and viremic controllers was much lower as compared to typical progressors.

A certain level of antigen is apparently required to drive the humoral immune response. However not all infected individuals with high viral loads develop cross-reactive neutralizing antibodies. This might relate to differences in the accessible of certain epitopes in the trimeric HIV-1 envelope structure. It can be hypothesized that a transmitted virus with more exposed conserved epitopes might elicit neutralizing antibodies with a larger breadth.Viruses with longer variable loops 1 and 2 and more potential N-linked glycosylation
sites might not elicit such antibodies as the CD4-binding site may be less accessible on these viruses as we observed in chapter 11.

To be more conclusive on the factors that determine the development of cross-reactive neutralizing humoral immunity, the neutralizing component in serum needs to be identified. This will show whether the breadth of the neutralizing activity in serum is determined by a single high affinity antibody directed against a highly conserved epitope in the envelope glycoprotein, or if it is the combined effect of multiple co-existing neutralizing antibodies directed at multiple distinct regions of the envelope. It cannot be excluded that both scenarios exist and that the number of antibody specificities in cross-reactive neutralizing sera may vary between individuals.

**Effect of Cross-Reacting Neutralizing Humoral Immunity on HIV-1 Disease**

It remains to be established how HIV-1 neutralizing activity in vitro relates to protection from infection in vivo. In non-human primate studies, passive transfer of broadly neutralizing antibodies completely blocked infection by a chimeric simian-human immodeficiency virus, while in humans, passive transfer of broadly neutralizing antibodies delayed HIV-1 rebound after cessation of antiretroviral therapy.

Previous studies have shown that autologous strain specific neutralizing activity does not contribute significantly to the control of HIV-1 infection. In chapter 4 we analyzed the AIDS free survival time of individuals with strong, moderate or absent cross-reactive neutralizing activity, and showed that cross-reactive neutralizing activity in serum did not have an impact on the clinical course of HIV-1 infection. Moreover a similar prevalence of cross-reactive neutralizing serum activity in long-term non-progressors (LTNP) and progressors at 2 and 4 years post-SC was observed. The absent correlation between the presence of cross-reactive neutralizing immunity and disease progression could point towards a fading humoral immunity in the progressive course of infection. Previous studies have shown that the autologous neutralizing antibody response decreases over time, probably as a result of the depletion of CD4+ T-cell help during chronic infection. In addition, vaccination of HIV-1 infected individuals against other pathogens showed reduced immune responses. In the longitudinal analysis that is described in chapter 7, cross-reactive neutralizing humoral immunity was preserved in both LTNP and progressors, even after the moment of AIDS diagnosis. In contrast, autologous neutralizing activity was only observed against viruses that were isolated early in infection. Moreover, the limited autologous neutralizing activity against early viruses was lost after AIDS diagnosis.

The absent association between cross-reactive neutralizing immunity and the clinical course of HIV-1 infection together with the limited autologous neutralizing activity might also be suggestive of rapid viral escape from cross-reactive neutralizing humoral immune pressure, despite the fact that cross-reactive neutralizing antibodies are considered to be
directed against conserved epitopes. We indeed observed that HIV-1 can rapidly escape from autologous humoral immunity with cross-reactive neutralizing activity together with the inability of the infected host to generate novel neutralizing antibody specificities against these escape variants (chapter 7). Furthermore, these escape mutations do not come at a fitness cost to the virus, as has been described for certain escape mutations in conserved epitopes for cytotoxic T lymphocytes. Overall, the similar potency of humoral immunity, the similar dynamics of viral escape, and the absent impact of escape on the replication kinetics of viruses from both LTNP and progressors argue against a role for neutralizing antibodies in the clinical course of infection. Possibly HIV-1 cellular immunity and host genetic background, rather than neutralizing antibodies may contribute to the control of already established infections while neutralizing antibodies may be essential for protection from infection.

**The adaptation of HIV-1 to humoral immunity**

As previously mentioned, neutralizing antibodies rapidly select for escape variants of HIV-1 that have become resistant to neutralization. Escape from neutralizing antibodies may be mediated by mutations in the epitope as a consequence of which the antibody is no longer able to bind, or by changes in other regions of the envelope that prevent access of the antibody to the neutralizing epitope.

In chapter 7 we observed that the escape of HIV-1 to cross-reactive neutralizing humoral immunity was correlated with an increase in length of the viral envelope glycoprotein (Env) and the number of potential N-linked glycosylation sites (PNGS) in Env. Positive selection pressure was observed in the variable regions in Env, suggesting that escape is not mediated by mutations in the conserved epitopes but rather by changes in the variable regions that then prevent access of the neutralizing antibodies to their target epitopes. This also explains why escape from cross-reactive neutralizing humoral immunity does not coincide with a reduced replication fitness of the virus (chapter 7). Interestingly, the exchange of the different variable regions from a neutralization sensitive into a neutralization resistant HIV-1 variant that were obtained respectively early and late in infection from a single individual, revealed that the V1V2 region is indeed a strong determinant for neutralization sensitivity (chapter 11). This was confirmed by the observation that increasing neutralization sensitivity coincided with shorter V1V2 loops and fewer PNGS in tier categorized neutralization sensitive and resistant HIV-1 variants (chapter 11).

The adaptation of HIV-1 at a population level to neutralizing humoral immunity also coincided with an increased length of Env and number of PNGS in Env, mainly concentrated in the V1 region. Moreover, exchange of the V1V2 regions from neutralization sensitive HIV-1 variants from historical seroconverters with V1V2 regions from neutralization resistant HIV-1 variants from contemporary seroconverters could decrease the neutralization sensitivity (chapter 11). These findings, together with studies from others, demonstrate...
that the increase in length and number of PNGS of the Env V1V2 region of the HIV-1 envelope glycoprotein is directly responsible for the protection of HIV-1 against CD4-binding site directed neutralizing antibodies, possibly by shielding underlying epitopes in the envelope glycoprotein from antibody recognition.

In addition to the changes in the Env V1V2 region also other changes in the envelope glycoprotein may influence neutralization resistance. It has been shown that mutations outside an epitope may influence the conformational structure of the envelope and thereby the exposure of an epitope. In chapters 8 and 9 we observed that over the course of infection in a substantial proportion of HIV-1-infected individuals viruses emerged that were resistant to one or more broadly neutralizing antibodies while the patients from whom these viruses were isolated lacked HIV-1 specific humoral and cellular immunity. For vaccine design, it will be important to understand which mechanisms may drive the selection of neutralization resistant virus variants.

In chapter 6 we studied in detail the HIV-1 evolution in several patients using different viral sources to better understand the selective pressure of humoral immunity on HIV-1 evolution. We observed that clonal HIV-1 variants isolated from PBMC may equally represent the viral quasispecies in blood as sequences obtained from serum and PBMC proviral DNA. However, certain selective forces, such as neutralizing humoral immunity, may drive differential evolution of the cell-free and cell-associated virus pool, reflected in separate clusters of HIV-1 sequences that were obtained from serum RNA in some patients at certain time points. In chapter 10 it was shown that the serum HIV-1 variants that were unable to persist in peripheral blood were more sensitive to autologous serum neutralization and had shorter Env with fewer potential N-linked glycosylation sites than successfully evolving HIV-1 variants, suggestive of a role for neutralizing antibody pressure on HIV-1 evolution.

DIRECTIONS FOR HIV-1 VACCINE DEVELOPMENT

The nature of neutralizing antibody responses in natural HIV-1 infection may offer new clues for vaccine design. Recently, the extremely potent and broadly neutralizing antibodies VRC01, and PG9 and PG16 were identified, which all seem to target conserved regions of the envelope glycoprotein. One of the current approaches is to use the epitopes of very potent broadly neutralizing antibodies as immunogens to elicit HIV-1 specific neutralizing antibodies with similar potency and breadth. The epitopes targeted by the currently known broadly neutralizing antibodies are the conserved domains on the envelope trimer, located at the CD4-binding site (VRC01 and b12), glycan shield (2G12), conserved regions of the V1,V2 and V3 region (PG9 and PG16), and the membrane proximal external region (MPER) of gp41 (2F5 and 4E10). The fact that the majority of primary HIV-1 variants are neutralized by one or more of the currently known broadly neutralizing antibodies, implies that the epitopes for these
broadly neutralizing antibodies are accessible on primary viruses. While it is not precisely known what level of Abs are required for protection against HIV-1 infection, recent work examining the efficacy of low antibody titers against low dose repeated pathogenic simian-human immunodeficiency virus challenge in macaques indicates that high concentrations of antibodies may not be needed to provide protective benefit \(^{52,53}\).

The individual identified as elite neutralizer in chapter 4 represents a new resource for the identification of novel monoclonal antibodies that are both broad and potent against HIV-1. Another new development is the use of a B-cell mosaic vaccine \(^{102-104}\) to optimize the immunogenicity in an attempt to elicit subtype-specific or even cross-clade neutralizing antibodies as described in chapter 5. A different direction of immunogen design is the use of only the epitope itself, such as the CD4-binding site \(^{45,105}\). By using glycans to cover other immunogenic targets of the protein except for the desired epitope, the chance may be increased that antibodies against that particular epitope are elicited. However it should be taken into account that the immunogen or epitope that will be used to elicit an antibody response is also accessible on currently circulating primary HIV-1 variants. As illustrated in chapter 11, HIV-1 has the ability to protect the conserved epitopes by increasing length and glycosylation of the envelope glycoprotein. It is therefore important to use an immunogen that has the natural characteristics of the envelope glycoprotein, but will direct the response to conserved epitopes to get a broad and potent response.

To date, no immunogen has been able to elicit protective neutralizing immunity in animal models \(^{84}\). In most studies on immunogenicity, animals are primed and boosted only a few times and total follow-up times are often restricted to several weeks \(^{101}\). It is intriguing that while we know that the development of a cross-reactive potently neutralizing antibody response in HIV-1 infected humans may take several years, we still expect this same process to happen within weeks in animal models. Although in the ideal situation one would like to achieve at least some level of protection already after priming, it cannot be excluded that the affinity maturation of HIV-1 neutralizing antibodies that is probably essential to get cross-reactive neutralizing antibodies, requires a longer period of time and multiple antigen exposures also in animal models. Therefore, in addition to the development of novel immunogens, novel designs of immunization schedules may be required.

It also remains to be established in what formulation the immunogen should be delivered. Many possibilities are being developed, from soluble proteins to DNA plasmids and viral vectors, which can all be used in multiple prime-boost combinations \(^{101,106}\). The type of response that needs to be elicited also depends on the delivery system. Soluble proteins will elicit only humoral immune responses, while DNA plasmids and viral vectors can elicit both humoral and cellular responses. These gene delivery systems can deliver any type of gene into a cell and get expression of the protein. Depending on the protein, the immune response will be directed into Th1 or Th2 depending on the HLA type by which presentation of the epitope is restricted \(^{101,106}\).
A first modest success was obtained with a pox virus prime, gp120 protein boost vaccine regimen in the so-called Thai trial (RV144). This vaccine included gag, nef, and pol and in addition monomeric envelope glycoproteins from clades B and E, which are the major circulating clades in the region where the vaccine trial was performed. The vaccine-induced protective effect was however only modest and the identification of the immune correlates of protection and the relative contribution of each vaccine component need to be elucidated. First analyses have shown that vaccinated individuals developed HIV-1 binding antibodies in serum, however no neutralizing antibodies could be detected. It cannot be excluded that other antibody functions, such as ADCC or ADVCI may play a role in the achieved protection.

Our studies only emphasized on neutralizing antibody responses with a focus on cross-reactive activity, however other antibody functions, such as ADCC or ADVCI, and cellular immunity may also play a role in HIV-1 evolution and disease course and may be worth studying in our patients in the future as well.

**Concluding Remarks**

Although neutralizing antibodies may not be able to influence HIV-1 disease course, neutralizing antibodies do have an impact on HIV-1 evolution. New insights in these interactions have revealed the importance of the accessibility of the vulnerable epitopes on the HIV-1 envelope glycoprotein in a vaccine immunogen. The fact that HIV-1 rapidly escapes from even the most potent and cross-reactive neutralizing antibodies implicates that by all means, viral replication in a new host should be prevented. A vaccine therefore should elicit protection against acquisition of HIV-1.

**References**


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