Cross-reactive neutralizing humoral immunity in HIV-1 disease: dynamics of host-pathogen interactions
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Correlations between HIV-1 subtypes and HIV-1 antibody neutralization sensitivity: significant for vaccine development?
**Abstract**

The correlates of protection against HIV-1 infection or disease progression are still unknown which causes an immense challenge for HIV-1 vaccine design. Existing effective vaccines against other viruses generate antibodies that either block the initial infection or contribute to the eradication of the virus before it can cause disease. For HIV-1, a protective vaccine capable of eliciting protective neutralizing antibodies does not exist and the difficulties for the generation of such a vaccine are multiple. Conserved elements on the viral envelope glycoprotein, the target of HIV-specific neutralizing antibodies, seem to be poorly immunogenic and attempts to generate an immunogen that can elicit broadly reactive neutralizing antibodies have remained largely without success. In addition, the envelope of HIV-1 is highly variable with respect to amino acid sequence, length of the variable loops, and glycosylation pattern. To cope with the high sequence variation, vaccine-elicited subtype-specific neutralizing antibodies have been suggested as an attractive alternative and recent studies have revealed some evidence for the existence of HIV-1 subtype-specific humoral immune responses. Here, we will review these recent findings and hypothesize on the nature of subtype-specific humoral immunity also in light of their relevance for HIV-1 vaccine development.
INTRODUCTION

More than 25 years after the identification of HIV-1 as the causative agent of AIDS, the correlates of protection against HIV-1 infection are still unknown and researchers continue to debate the most effective vaccine strategies to prevent infection or disease progression. The consensus now is that an effective vaccine should elicit both humoral and cellular immunity. 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 design of an immunogen that is capable of eliciting neutralizing antibodies is complicated as the recombinant Env protein, even in trimeric form, and vector-expressed HIV-1 envelopes do not seem to expose the relevant epitopes. In addition, vaccine-elicited antibodies will have a tough job as HIV-1 seems to be relatively resistant to neutralizing antibodies. This resistance can be explained from the inaccessibility of relevant epitopes due to the trimeric structure of the HIV-1 envelope protein and the density of glycosylation. Moreover, some epitopes for neutralizing antibodies only emerge after the conformational changes that are elicited by the engagement of the viral envelope glycoprotein with the CD4 receptor, when spatial constraints no longer allow binding of the relatively large immunoglobulins.

Another major obstacle in the development of an effective HIV-1 vaccine is the large sequence diversity, especially of the viral envelope glycoprotein. This high sequence diversity of the HIV-1 envelope glycoprotein is considered to make it more challenging or even completely impossible for a single vaccine to be capable of eliciting a humoral immune response that would cover protection against all possible variants that are being transmitted in a population. Based on phylogenetic analysis, HIV-1 has been categorized into subtypes of viruses that are more similar to each other than to the rest of the viruses, which has fueled the discussion on so-called HIV-1 subtype-specific vaccines. Here, based on our own findings and on those of others, we will discuss whether the high sequence diversity of HIV-1 may indeed be covered by multiple HIV-1 subtype-specific vaccines. We will review the possible correlation between HIV-1 subtypes and neutralization serotypes, and discuss whether subtype-specific HIV-1 vaccines are a relevant option to pursue.

HUMORAL IMMUNE RESPONSE AGAINST HIV-1 IN NATURAL INFECTION

In order to better understand the immunogenicity of the HIV-1 envelope glycoprotein and the host immune response against it, humoral immunity in the natural course of infection has been extensively studied. 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. These antibodies rapidly select for escape variants of
HIV-1 that have become resistant to neutralization as a result of amino acid substitutions, insertions and/or deletions in the variable regions, and/or changes in the glycan shield. 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. Irrespective of the mechanism, such viral escape variants will rapidly be selected by humoral immune pressure and will replace the neutralization sensitive virus variants.

Generally later in the course of infection, neutralizing activity against heterologous HIV-1 variants evolves but only in a much smaller proportion of the HIV-1 infected population. Indeed, the majority of HIV-1 infected individuals do not develop strong cross-reactive neutralizing activity that is capable of neutralizing HIV-1 variants from different subtypes.

**Subtypes of HIV-1 based on genetic diversity**

One of the characteristics of HIV-1 is its enormous sequence diversity. During infection, each day between $10^8$ and $10^{10}$ viral particles are being produced and eliminated. The error-prone viral reverse-transcriptase enzyme and the lack of proofreading mechanisms during reverse transcription of the viral RNA result in frequent mutations in the viral genome. The large turnover of virus in combination with this high mutation rate results in a mixed population of related but distinct HIV-1 variants, also termed the viral quasispecies. Viral variants within a quasispecies are continuously competing, and the dominant sequence reflects the most fit variant at that time point. After accidental introduction of beneficial mutations in the viral genome or due to changing environmental factors, such as the introduction of antiretroviral agents or effective HIV-1 specific immune responses, a previously minor population may become dominant, after which a new, so-called population equilibrium is established.

All viral genes are prone to mutation and the proteins they encode are subject to variation. However, large sequence variation is not allowed in each viral genomic region as this may interfere with viral fitness. For example, the gag and pol regions are relatively conserved as viruses with mutations in those regions, which generally come at a fitness cost, are outcompeted by coexisting viruses that lack this mutation. Only when the positive selection pressure on such mutations is higher than the fitness cost associated with a mutation, will the mutant virus outcompete the wild type variants.

The envelope glycoprotein of HIV-1 is highly variable, creating an enormous sequence variation which may be as high as 10% within the viral quasispecies in a single individual. Apparently, the regions in which this huge sequence variation occur are not critical to the viral replication process.

Despite the high diversity, some viruses are more closely related to each other which has led to a classification of HIV-1 variants into subtypes (Figure 5.1). The main group (M-group) is
subdivided into subtypes A to K and different circulating recombinant forms (CRFs), which have different geographic distributions. Subtype B for instance predominates in Europe, the Americas, and Australia, whereas subtype C predominates in Sub-Saharan Africa and the Indian subcontinent. The prevalence of intersubtype recombinant strains is increasing and creates even more HIV-1 genetic diversity. The viral envelope glycoprotein currently already differs by up to 35% between subtypes and up to 20% within subtypes, with the variable regions and also the third constant region (C3) being the most diverse between subtypes.

The enormity of the challenge to design a vaccine that can elicit host immunity capable of covering the huge sequence diversity can be put into perspective by comparing it with the influenza virus, where a diversity of less than 2% in amino acid changes can already cause failure in the cross-reactivity of the polyclonal response elicited by the vaccine. Every year a new influenza vaccine has to be developed to protect against the newly emerging serotypes of the influenza virus for the upcoming flu season. In comparison, all different subtypes of HIV-1 are present at the same time in the population, emphasizing the enormous obstacle of genetic variation for HIV-1 vaccine development.

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**Figure 5.1: Representation of HIV diversity**

Neighbor Joining tree generated with all envelope gene sequences of the M-group in the Los Alamos database (n=1870) using the GTR distance model with site rate set at gamma shape set at 0.5. Different subtypes and genetic distance bar are indicated.
Evidence for Subtype-Specific Humoral Immunity

The high sequence diversity between HIV-1 variants is challenging the possibility of complete coverage of protection against all circulating viruses by vaccine-elicited neutralizing antibodies. In that light, vaccine-elicited subtype-specific neutralizing antibodies may be an interesting alternative to pursuit although the existence of HIV-1 neutralization serotypes may be questioned 26,27.

Most research has focused on autologous or cross-subtype neutralizing antibodies, while cross-reactive neutralization of different HIV-1 variants within the same subtype has received only little attention. The majority of early studies did not provide evidence for HIV-1 subtype-specific neutralizing activity in serum 27-30. However, these studies were performed with only a limited number of HIV-1 variants and sometimes with a pool of patient sera in which different neutralizing epitope specificities may have been mixed. At that time, well characterized virus panels were not yet developed, which also made it difficult to characterize neutralization specificities. Moreover, these studies strongly focused on broadly neutralizing antibodies that by definition neutralize HIV-1 variants from different subtypes. We recently observed that the neutralizing activity in sera of participants from the Amsterdam Cohort studies who are 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 subtype A, C, and D HIV-1 variants (Figure 5.2). Although not specifically emphasized by the authors, some other recent reports also include data that show that neutralizing activity in patient sera was stronger against viruses that were from the same subtype as the autologous virus that had elicited the neutralizing antibodies in the sera 14,31-38. Simek et al. screened ~1800 sera from individuals from around the world who were infected with HIV-1 of either subtypes A, B, C, D, or recombinant forms, for neutralizing activity against several multi-subtype virus panels. They found that the neutralizing activity in sera indeed tended to be preferentially directed, and with higher potency, against viruses in the panel that were from the same subtype as the virus that had elicited the neutralizing activity, with the strongest correlations for subtypes B and C. These authors also showed that overall, subtype C viruses were neutralized with higher potency than other viruses by heterologous patient plasma, confirming that subtype C viruses are more neutralization sensitive 33,38,39.

The existence of subtype-specific neutralizing antibodies may also have been underestimated in studies that focused on the number of neutralized viruses from different subtypes, while not taking into account the neutralizing titers. With that approach, cross-reactivity will be seen as evidence against subtype-specific neutralizing antibodies while neutralizing titers against viruses from the same subtype as the infecting variant that had elicited these antibodies may have been significantly higher. This would then have pointed to the existence of subtype-specific neutralizing antibodies, or at least neutralizing antibodies with a higher affinity for epitopes that are only present or accessible on viruses from a specific subtype 32,38.

Another indication for the existence of subtype-specific humoral immunity is the specificity
Figure 5.2: Heatmap and clustering analysis of neutralizing activity in serum

Sera of 35 patients from the Amsterdam Cohort Studies were tested for neutralization activity against a panel of 23 tier 1 viruses (5 HIV-1 variants from subtype A, 6 from subtype B, 7 from subtype C, and 5 from subtype D). IC_{50} values are shown in gray shade, with the lowest values in white and the highest values in dark gray. Each column shows the IC_{50} values of a single serum, each row shows the neutralization data per virus isolate. The viruses are clustered according to their neutralization sensitivity and the sera are clustered according to their neutralization capacity. The subtype to which the virus belongs is indicated at the end of each cluster branch. In the clustering procedure, the Euclidean distance between the log_{10} IC_{50} values of a set of neutralization values in one row or column was calculated and repeated 10,000 times to find the best fit. Raw data are taken from van Gils et al. 38.
of the neutralizing activity of some of the known broadly neutralizing antibodies. Most broadly neutralizing antibodies have been isolated from subtype B-infected individuals and some of these monoclonal antibodies (mAbs) do not efficiently neutralize non-subtype B HIV-1 variants, either reflected in a lower number of non-subtype B viruses that could be neutralized and/or in lower neutralizing titers against these viruses. For example mAb b12 neutralized up to 75% of subtype B viruses, but less than 50% of non-subtype B viruses. Also mAb 2G12 was shown to have a rather limited neutralizing activity against non-subtype B viruses. Although the MPER is relatively conserved, the 2F5 epitope is absent in a large proportion of subtype C viruses, which explains the limited neutralizing activity of the 2F5 antibody against HIV-1 variants of this subtype. MAb 447-52D may be the best example of subtype-specific neutralizing humoral immunity as it completely lacks the ability to neutralize non-subtype B HIV-1 variants but has neutralizing activity against the vast majority of unrelated subtype B viruses, albeit it mainly against highly neutralization sensitive tier 1 isolates. Using a heat-map, neutralization data can be organized into meaningful patterns, making it easier to interpret the data. With this tool, Binley et al. have shown a clustering of the different subtypes that was based on the neutralization profiles of the broadly neutralizing mAbs mentioned above.

**Variations in the humoral response elicited by HIV-1 variants from different subtypes**

Because of the high sequence diversity between subtypes and the existence of subtype-specific neutralizing antibodies, it cannot be excluded that the potency and quality of the elicited envelope-specific humoral immune response is associated with the subtype of the HIV-1 variant by which an individual is infected. Epitopes in the first and second variable regions (V1V2) appear to be common immunogens between HIV-1 variants from different subtypes in early infection. However, this region is highly variable and antibodies that target this region are mostly type-specific. The V1V2 region is also highly involved in shielding neutralizing epitopes in other parts of the envelope protein and is frequently associated with the escape from neutralizing antibodies via amino acid substitutions, insertions and deletions, and/or changes in glycosylation. An additional region that is targeted by the early humoral immune response elicited by different subtypes is the third variable region (V3). Anti-V3 antibodies are among the first antibodies to be elicited in HIV-1 infection and can have cross-subtype neutralizing capacity. This cross-reactivity appears to be more common for V3-specific antibodies elicited by HIV-1 subtype A infection than for antibodies elicited by subtype B HIV-1 variants, indicating that the epitopes in the V3 domain are indeed different, at least between some subtypes. These differences in V3 epitopes may be used for diagnostic purposes as it turned out to be possible to determine the specific subtype of an infecting strain using a V3 loop peptide immunoassay. However, anti-V3 antibodies play a minimal role in neutralizing humoral immunity, due to the occlusion of the
V3 loop within the trimeric envelope glycoprotein of primary viruses \(^{44,46,48}\).

As mentioned above, HIV-1 subtype C viruses seem to be more sensitive to neutralization than HIV-1 variants from other subtypes \(^{31-33,38}\). Moreover, individuals infected with subtype C HIV-1 variants seem to have a better and stronger neutralizing antibody response than individuals infected with a non-subtype C virus \(^{33,37,49,50}\). Indeed, subtype C HIV-1 infected individuals were most prevalent among elite neutralizers \(^{32}\), confirming the higher immunogenicity of this subtype. It is tempting to speculate that vulnerable epitopes in subtype C viruses are less well occluded by the variable loops and/or the glycan shield of the envelope glycoprotein, making it easier for neutralizing antibodies to access their epitopes and neutralize these viruses. In addition, this enhanced epitope exposure will improve immunogenicity, making it easier to elicit neutralizing antibodies.

One of the major differences between subtype C HIV-1 and viruses from other subtypes is the C3 region in the viral envelope gp120 which for subtype C viruses is a target for neutralizing antibodies \(^{51}\). There are structural differences between subtype B and subtype C HIV-1 variants in the alpha 2-helix of C3 and it has been shown that this region is a target for antibodies \(^{8,48}\). In viruses from other subtypes this region may be much more occluded, interfering with the possibility to elicit neutralizing antibody responses.

Other regions of the envelope glycoprotein, such as the CD4 and co-receptor binding sites and the membrane-proximal (MPER) region of gp41, are much more conserved and very similar between different subtypes. In line with this is the enormous neutralization breadth of antibody 4E10, albeit not very potent, against the MPER region of gp41 and the breadth and very strong potency of the recently described antibody VRC01 that targets the CD4 binding site. The limited breadth of mAb b12, which is also directed against the conserved CD4 binding site, can be explained by the fact that mAb b12 is directed at subtype-specific residues while VRC01 is directed against much more cross-subtype conserved residues in the CD4 binding site \(^{40,52,53}\).

**Characteristics of cross-reactive HIV-1 specific neutralizing activity**

Factors that determine whether broadly neutralizing antibodies develop are largely unknown. A positive correlation between the viral load setpoint in plasma and the breadth of humoral immunity \(^{13,54}\) implicates that at least sufficient antigen exposure is required to elicit potently neutralizing antibodies. Indeed, the prevalence of cross-reactive neutralizing activity is low among elite controllers and long-term non-progressors with low viral load \(^{13,55-57}\). It has also been shown that the breadth of neutralization is correlated with the time since infection \(^{13,15}\). It generally takes almost 2 to 3 years for broadly neutralizing antibodies to develop. This time may be required for the affinity maturation during which the neutralizing antibodies gain affinity and become highly potent, which corresponds with the finding that the breadth of neutralization is also correlated with antibody avidity \(^{13}\). It has been hypothesized that
the development of broadly neutralizing antibodies may also be related to the evolution of HIV-1. As neutralizing antibodies emerge during the course of infection, they will rapidly select 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. There indeed is a strong correlation between the serum titer and breadth of the neutralizing response and both seem to increase simultaneously during the course of infection (Figure 5.3). 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.

To be more conclusive on the nature 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 protein, or whether cross-reactive neutralizing activity in serum can be attributed to a combination of multiple co-existing neutralizing antibodies directed against a number of distinct regions of the envelope that

![Figure 5.3: Correlation between serum titer and neutralizing breadth](image)

On the x-axis an increasing heterologous neutralization titer is suggested, on the y-axis an increasing breadth of the response in three categories. Line represents the association between neutralizing titer and breadth, increasing gray tone in the background shows increasing potency of neutralizing activity. Figure is reproduced from van Gils et al.
together give the phenotype of a cross-reactive serum neutralization. It cannot be excluded that both scenarios exist and that the number of antibody specificities in cross-reactive neutralizing sera may vary between individuals.

It is likely that epitopes that are less well conserved between subtypes but conserved within a subtype are capable of eliciting subtype-specific, rather than cross-reactive neutralizing antibodies. The epitope of the already mentioned 446-52D in the V3 loop is an example of this. This epitope is quite different between subtypes but highly conserved in subtype B HIV-1 variants, underscoring that subtype-specific neutralizing antibodies indeed exist.

The potential relevance of subtype-specific neutralizing antibodies as a target in vaccine development remains to be established. The idea to use this type of antibodies stems from the notion that cross-subtype neutralizing antibodies are extremely rare. However, several recent studies have demonstrated that a relatively high proportion (~30%) of individuals has cross-reactive neutralizing humoral immunity, which suggests that the epitopes that are capable of eliciting these humoral responses are accessible and immunogenic on the native gp160 spike of HIV-1 and that the B cell repertoire in humans is indeed capable of producing these potently neutralizing antibodies, at least in a significant proportion of HIV-1 infected individuals. Interestingly, although cross-reactive neutralizing activity is considered to be directed against epitopes that are conserved in HIV-1 variants from different subtypes, also here a rapid selection of escape variants is observed without a major impact on viral fitness. This suggests that escape from cross-reactive neutralizing activity 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.

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 protective immunity that protects against acquisition of HIV-1.

**Directions for vaccine development**

It is generally assumed that an HIV-1 vaccine should elicit humoral immunity, ideally in combination with a cellular immune response. In line with this assumption is the observation that all vaccine formulations that have been tested in phase 2b trials, all lacked an immunogen that was capable of eliciting strongly neutralizing antibodies, which had little or no impact on HIV-1 transmission. In initial trials, subtype B monomeric envelope glycoproteins elicited high titer type-specific antibody responses against the vaccine strain, but did not provide protection against acquisition of HIV-1 in the population. A polyvalent envelope glycoprotein vaccine-elicited neutralizing activity against tier 1 viruses, but still no protection from infection by a heterologous SHIV in macaques was achieved. 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 subtypes B en E, which are the major circulating subtypes 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 but data on neutralizing activity of these antibodies has not yet been reported. It cannot be excluded that other antibody functions, such as ADCC or ADVCI play a role in the achieved protection. Another interesting question to address is whether the HIV-1 variants that established infection in the vaccine recipients are genetically different from the vaccine strains or from the viruses in the infected placebo recipients. 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. Similar to mAb b12, VRC01 targets the CD4 binding site, but with a much higher potency. PG9 and PG16 recognize a conformational epitope in the V2 region that for its conformation is dependent on glycosylation of the V2 region. In the retrovaccinology approach, these epitopes will serve the design of novel immunogens.

In addition to the approach described above, progress has been made on the characterization of the consensus and/or most recent common ancestor sequences that are considered to resemble HIV-1 variants that have not yet escaped from immunity and that may harbor important epitopes. A new development is the use of a mosaic vaccine to optimize the immunogenicity in an attempt to elicit subtype-specific or even cross-subtype neutralizing antibodies. Mosaic sequences closely resemble natural strains and by design are assembled from common mutational solutions that the virus itself favors to balance fitness constraints with immune escape. B-cell mosaics are being developed to optimize epitope coverage. A cocktail of mosaics in a vaccine might elicit potently neutralizing antibody responses.

**Conclusion**

Subtype-specific humoral immunity has been suggested as an interesting alternative to deal with the huge sequence variation that is challenging HIV-1 vaccine development. As reviewed here, at least one neutralizing antibody is more or less subtype-specific as its epitope is mainly present in viruses that belong to subtype B. The fact that truly broadly neutralizing antibodies that neutralize HIV-1 variants from different subtypes exist, implicates that specific epitopes that can elicit these antibody responses are highly conserved across HIV-1 subtypes. The sequence variation in the HIV-1 envelope may thus be less problematic for the choice of epitope specificities 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.

Novel antibodies such as VRC01, PG9 and PG16, with unmet breadth and potency,
should be the starting point for vaccine design as these antibodies, in real life, seem to have overcome the problem of subtype-specificities.

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HIV-1 neutralization serotypes


