The effect of adaptive and innate immune response on HIV-1 entry and replication

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THE EFFECT OF ADAPTIVE AND INNATE IMMUNE RESPONSE ON HIV-1 ENTRY AND REPLICATION

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presented by
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1. Summary

Numerous studies suggest that antibodies affect HIV-1 replication in vivo which is best evidenced by the rapid viral escape to antibodies and by passive immunization studies in both primates and humans. Yet the modes of action of these antibodies in vivo remain unclear. Besides direct neutralization activity, antibodies can also induce effector functions as phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), Fc receptor engagement and activation of the complement system.

Direct lysis of HIV-1 upon antibody-mediated activation of the complement system has been demonstrated in vitro. Complement has further been shown to boost the activity of neutralizing antibodies against HIV-1 both in vivo and in vitro. Opposing these observations, several reports have suggested that complement lysis of HIV-1 is limited due to host cell-derived complement control proteins incorporated into the HIV-1 virions and that opsonized virions bind to complement receptor-expressing cells promoting enhanced viral infectivity and transmission.

We developed a novel sensitive virus lysis assay based on real time PCR quantification of HIV-1 RNA. We found that complement-mediated lysis may be very effective already at an early time point of infection. Activity is generally higher in chronic infection and reaches up to 90% in ex vivo experiments. We found a strong association between complement lysis activity and viral load in acute but not in chronic infection, indicating that virolysis contributes to viremia control during the acute phase of HIV-1 infection. In plasma neutralization assays we showed that active complement contributes to the inhibition of viral infectivity in vitro. The antibodies responsible for complement-mediated virolysis activity are directed against the HIV-1 envelope proteins and are predominantly non-neutralizing. Neutralization and lysis activity may overlap to some extent but are not linked obligatorily.

In a longitudinal analysis of a passive immunization trial we assessed the role of complement lysis in the delay of viral rebound induced by the administered monoclonal antibodies. We found that the activity of the neutralizing antibodies 2G12, 2F5 and 4E10 in mediating complement lysis is relatively low. Therefore, the in vivo
activity of the tested monoclonal antibodies is likely dominated by direct neutralization or Fc dependent mechanisms such as ADCC or phagocytosis.

Further experiments are necessary to determine the relative contribution of direct and indirect antibody responses and to unravel the balance between beneficial and detrimental effects of complement. A detailed characterization and quantification of the relative contribution of direct and effector-mediated antibody functions on virus containment in vivo will be of central importance for the definition of relevant immune responses and future vaccine design.
Zusammenfassung

2. Zusammenfassung


Zusammenfassung


3. Outline

After more than 20 years of intensive research on HIV-1 still no vaccine is available. The currently available antiretroviral therapy is effective but has severe side effects and does not eliminate the latently infected cells. Much effort is made in developing a prophylactic or therapeutic vaccine eliciting an autologous immune response against the virus. In vivo both cellular and humoral immune system are responsible for effective control of HIV-1 Infection. The cellular immune response arises early in infection and is thought to be responsible for the simultaneous decline in viral load after the initial peak upon infection.

During my thesis, I got interested in the role of the humoral branch of the immune system in suppressing HIV-1 infection. The capability of the humoral immune system, its modes of action and induction are still only incompletely defined. To date only five broadly neutralizing antibodies are known. These antibodies inhibit HIV-1 by binding directly to free virions. For long only indirect evidence for the activity of neutralizing antibodies in vivo was available. Most important there was the demonstration that HIV-1 rapidly escapes the antibody pressure both during natural infection and in vitro. The effectiveness of neutralizing antibodies in controlling HIV-1 infection in vivo has only recently been demonstrated, when our group brought direct evidence for the activity of a mixture of three of these antibodies in a passive immunization study in 14 HIV-1 infected individuals (Trkola A, 2005, Nat Med 11, 615). Besides the declared mechanism of direct neutralization by binding of the antibodies to free virions, antibodies are known also to elicit effector functions by binding to Fc-receptors and mediate ADCC and phagocytosis or by activating the complement system. These diverse functions and modes of action of both neutralizing and non-neutralizing antibodies are reviewed in detail in chapter 4.1 "Humoral Immunity to HIV-1: Neutralization and beyond". Chapter 4.2 "Antibodies for HIV Treatment and Prevention: Window of Opportunity?" summarizes the present use of antibodies in HIV-1 therapy and future prospects in this field.

I experimentally investigated the role of the complement system in HIV-1 infection. The complement system is an essential component of innate immunity and the main
effector mechanism of the humoral branch of the immune system. It comprises of about 20 plasma proteins, which are activated by a self-enhancing cascade. Binding of antibodies to the surface of HIV-1 can activate a pathway that eventually results in disruption of the virus membrane. The results of my studies are presented in two papers. In chapter 5.1 "Complement Lysis Activity in Autologous Plasma Is Associated with Lower Viral Loads during the Acute Phase of HIV-1 Infection" we describe the effective complement-mediated lysis of HIV-1 both in acute and chronic infection. In chapter 5.2 "Potent HIV Neutralizing and Complement Lysis Activity of Antibodies Are Not Obligatorily Linked" we investigated plasma samples of a passive immunization trial (Trkola A, 2005, Nat Med 11, 615). Here we were interested to determine whether complement contributed to the delay of viral rebound observed during this trial.

I hope the presented studies may contribute to a better understanding of the mechanisms and the potential contribution of direct and indirect antibody action against HIV-1 infection.
4. Background

4.1 Humoral Immunity to HIV-1: Neutralization and beyond


Humoral Immunity to HIV-1: Neutralization and Beyond

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Abstract

Humoral immunity is considered a key component of effective vaccines against HIV-1. Hence, an enormous effort has been put into investigating the neutralizing antibody response to HIV-1 over the past 20 years which generated key information on epitope specificity, potency, breadth and in vivo activity of the neutralizing antibodies. Less clear is still the role of antibody mediated effector functions (ADCC, phagocytosis, complement system) and uncertainty prevails whether Fc mediated mechanisms are largely beneficial or detrimental for the host. The current knowledge on the manifold functions of the humoral immune response in HIV infection, their underlying mechanisms and potential in vaccine induced immunity will be discussed in this review.

Keywords

Human Immunodeficiency Virus Type 1, Humoral Immunity, Neutralizing Antibodies, ADCC, Complement

Introduction

"With what success does the human immune system produce antibodies that neutralize the human T-lymphotropic virus type III (HTLV-III), the virus that is aetiologically linked to the
acquired immunodeficiency syndrome (AIDS)? And can the neutralizing antibodies be exploited for prophylaxis or therapy of AIDS?” were the questions raised by Jerome E. Groopman in his News and Views article on the discovery of neutralizing antibodies against HIV by Robin A. Weiss et al. and Marjorie Robert-Guroff et al. in Nature in 1985 [1-3]. More than 20 years later we still pursue these issues. Whether antibodies are successful or not in limiting viral replication during HIV infection remains as zealously debated as their therapeutic potential. That the neutralizing antibody response developed during natural infection is commonly too low to clear infection was evident already in 1985 when Groopman further wrote: “The relatively low titres of neutralizing antibodies in HTLV-III infected persons should not dissuade investigators from pursuing their clinical relevance.....” Well, it did not.

Most likely, more information on the antibody response to HIV has been gathered than on those to any other virus. A dazzling 1722 papers on neutralizing antibody responses in HIV infection have been published to date (Figure 1). In comparison, other major viral diseases lag significantly behind in this statistic, with only 527 publications on neutralizing antibodies in influenza and 418 in measles infection to be found. The knowledge we gained from this wealth of information is plentiful but nonetheless we lack crucial insights into the modes of action and induction of the humoral immune response to HIV that would allow us to utilize its powers for treatment and prophylaxis. The hopes raised upon the discovery of neutralizing antibodies that humoral immunity can be readily employed for active or passive protection gave way to the sobering realization that this is far more challenging to achieve than initially thought. Nevertheless, recent years have brought advances in the field that notably revived the interest to exploit antibodies for protection (Box 1 and Figure 1).

The antibody response to HIV is generally vigorous and predominantly directed to the structural proteins of the virus. Within few weeks of infection antibodies against the envelope proteins gp120 and gp41, the core (p24) and matrix (p17) become detectable in plasma of HIV positive individuals [4-10]. However, only a fraction of the elicited antibodies has been found to have neutralizing (infection inhibiting) activity [11,12]. While neutralization is often considered as the most efficient mode of antibody mediated defence against viral infections, we have learned from other viral diseases that effector functions mediated by virus-specific, non-neutralizing antibodies can have substantial impact through clearing virus particles and infected cells by actions of the complement system, phagocytes or killer cells (Figure 2) [13-22]. However, in HIV infection the impact of these immune functions is not yet clear and may be contradicted by infection enhancing effects of antibodies which have been observed in
vitro. Here we summarize the current knowledge on the various functional properties of antibodies in HIV infection with a particular emphasis on areas that need further investigation to allow future exploitation of humoral immunity for vaccination and therapeutic purposes.

Box 1: Summing up two decades of research on neutralizing antibodies to HIV-1

The 1980's
- HIV-specific antibody test developed for blood screening
- Detection of neutralizing antibodies against HIV [2,3,23,24]

The 1990s
- Primary HIV isolates are highly resistant to neutralization [25]
- Viruses rapidly escape neutralizing antibodies in vivo [8,26-34]
- Most V3 loop antibodies are strain-specific, with few exceptions [11,35,36]
- CD4 binding site antibodies are abundant but with few exceptions do not neutralize primary HIV-1 isolates [36-38]
- Majority of envelope-specific antibodies are non-neutralizing and do not recognize native envelope trimer [39]
- Broadly neutralizing antibodies: IgG1b12 [38], 2F5 [40-42], 2G12 [43] identified
- Interference with CD4 and coreceptor binding is main mechanism of neutralizing antibodies against gp120 [43-45]
- Structure-function analysis of gp120 confirms modes of antibody interaction [46,47]

Since 2000
- Importance of MPER in gp41 underlined through identification of broadly neutralizing MAbs 4E10 and Z13 [48-50]
- Broadly neutralizing MAbs Ig1B12, 2G12, 2F5, 4E10 have characteristic structural features [51-54]
- Passive immunization studies in animal models and humans provide in vivo evidence of neutralization activity of broadly neutralizing MAbs [11,55-58]
- Virus escape to antibody neutralization occurs already in acute infection. Neutralization response during acute infection is higher than previously thought [8,27].
- Neutralizing antibodies lead to a selection in viral transmission [59-61] and are suggested to limit superinfection [62,63]
- Immune system of host shapes response (twin studies) [64,65]
- HIV envelope evades immune recognition through masking of epitopes, oligomeric exclusion, steric interference and the heavy glycosylation [27,66]
- Continuous failure of vaccines to elicit broadly effective neutralizing antibody response [67]
- Cryo-electron tomographic structures give insight on spike organization on virion [68,69]
Neutralizing antibodies in HIV infection

Across diverse virus infections, neutralizing antibodies have been found to employ multiple mechanisms to interfere with viral replication. Receptor binding and fusion mediated by the envelope proteins are essential steps in the life cycle of all viruses and therefore a primary target for neutralizing antibodies. The majority of antibodies neutralize free virions by preventing receptor engagement or by interfering with the fusion process [70]. Later in the life cycle, antibodies that block replication by preventing virus uncoating or budding have been described in several viral diseases [71-78].

HIV infection is initiated by the interaction of the viral envelope proteins gp120 and gp41 with the cellular receptor CD4 and a coreceptor (most commonly CCR5 or CXCR4) which enables the virus to fuse with the host cell membrane and to enter the cell [12,79]. Neutralizing antibodies against HIV described to date interfere with attachment to CD4, binding to the coreceptor, or post receptor engagement by binding to domains involved in the actual fusion process [12,44,80-84]. Whether HIV specific antibodies that employ other mechanisms to inhibit virus replication do not exist, or if current screening methods are not suited to detect these activities is uncertain. To what extent antibodies that bind to viral envelope proteins and inhibit entry also interfere with viral egress, or if distinct specificities

Figure 1: Number of research papers published on neutralizing antibodies.
Indicated terms were searched in the PubMed library on 29.09.2006: “neutralization AND antibodies AND HIV” (red), “neutralization AND antibodies AND influenza” (blue).
are required for both processes has yet not been systematically investigated. Notably, virus neutralization is generally probed for by assessing the ability of antibodies to bind to free virus particles and consequently inhibit viral infection. What role transmission of free virus particles plays in vivo compared to spread from cell-to-cell needs yet to be determined. Modes of cellular transmission have only begun to be understood in recent years: In close cellular contact HIV particles are spread via formation of so-called virological synapses. This process has been documented for viral transmission between CD4 T cells as also between CD4 T cells and dendritic cells [85-90]. Notably, virus that is transmitted via the synapse could be less accessible for neutralizing antibodies and not all antibodies may remain active in this context. In vitro the capacity of neutralizing antibodies in limiting cell-to-cell spread has been found to be considerably lower than the activity against free virions [91,92].

Defining epitopes on the viral envelope proteins that elicit potent neutralizing activities remains an ongoing challenge for vaccine design. Despite the pronounced antibody response to the viral envelope proteins, only a small fraction of these antibodies have neutralizing activity. Functional glycoprotein spikes on HIV particles consist of trimers of non-covalently linked gp120 (surface subunit) and gp41 (transmembrane subunit) [12]. On these spikes regions on gp120 facing inwards the trimer as well as the greater part of gp41 are considered not to be accessible for antibodies [12,39,47,68] while potentially accessible domains on gp120 are highly glycosylated further impeding antibody recognition [47,93]. Notably, this spatial organization allows the virus to occlude antigenic sites that are exposed on monomeric subunits. Only antibodies that react with the intact trimer are considered to bear neutralizing activity [94,95]. However these responses are minute compared to reactivities to the monomers which are likely presented in greater abundance to the immune system. So are both gp120 and gp41 monomers, as also gp160 precursor proteins released upon disintegration of infected cells [12]. Gp120 monomers can further dissociate from spikes due to the inherent instability of the trimer leaving behind anchored gp120 monomers and imbedded gp41 stumps on the viral surface [12,96].

Following receptor binding and the ensuing conformational changes within both envelope proteins, previously hidden regions within gp120 and gp41 that are vulnerable to neutralization attack could become transiently exposed. However, in general the time window between receptor binding and initiation of fusion as well as space constraints are thought to limit antibody action during this entry stage with notable exceptions [97]. Despite the hurdles the immune system has to overcome, neutralizing activity develops during natural infection and a number of epitopes for neutralizing antibodies have been identified on
Background

the Env glycoproteins over the years (for extensive reviews see [36,84,98,99]). Within gp120, the V3 loop, the V1/V2 loop, the CD4 binding site (CD4BS) and the CD4-induced (CD4i) binding domain, which promotes interaction with the co-receptors, were identified as target sites for neutralizing antibodies. In comparison only few gp41 specific neutralizing antibodies have been identified which likely reflects the low accessibility of the trans-membrane protein on native trimers [100-102]. The best characterized are the antibodies directed to the membrane proximal external region (MPER) within gp41, the monoclonal antibodies 2F5, 4E10 and Z13 [40,41,48,49,103]. Recently, additional neutralization sensitive epitopes within gp41, signified by antibodies to the CBD1 binding domain [104] and fusion intermediates [97], have been described and await verification of their potency and in vivo relevance.

On large, the initial hopes on exploiting antibody based immunity for treatment and prevention were dampened once it became clear that most of the neutralization activity elicited to HIV-1 is strain and subtype specific. The V3-loop specific antibody 447-52D has within subtype B relatively broad reactivity and has therefore been extensively investigated [35,103]. However, only a handful of human monoclonal antibodies with potent and comparably broad cross-neutralizing activity have been isolated to date: the antibody IgG1b12 which recognizes an unique epitope overlapping the CD4BS, the carbohydrate specific antibody 2G12 which recognizes an equally unique mannose-dependent epitope within gp120, and the aforementioned MPER specific antibodies 2F5 and 4E10. In an intense effort epitope characteristics, modes of action and potency [38,40-43,48,49,52,54,103,105-107] as well as biochemical properties and structures [50,53,108] of these four antibodies have been unravelled, in the hope that they could function as prototypic models for vaccine design. Notably, while these four antibodies recognize distinct epitopes they share a striking similarity: They all have unusual structural characteristics [51-54]. Antibodies with similar characteristics and specificities appear to be highly uncommon in vivo [109,110] and have proven to be difficult to be induced by vaccination: None of the attempted vaccination strategies tested to date has been successful in eliciting antibodies of comparable specificity and activity [111,112]. Based on the knowledge gained over recent years development of novel scaffolds for epitope presentation or envelope structure mimetics that might overcome the limitations of previous antigens used for vaccination could become possible. Nevertheless, a new, concerted effort in dissecting antibody responses in natural HIV infection is urgently needed: Despite the fact that patients who mount a high neutralization response have been identified, it still remains largely unclear which epitopes these neutralizing serum antibodies recognize. Notably, antibodies that bind to HIV-1 gp120 preferentially (or only) after CD4
Background

engagement (CD4i) appear to be very common in vivo across genetic subtypes but their overall impact against autologous virus remains to be determined [82]. Delineating humoral immune responses in natural HIV infection and vaccines, paired with a concentrated effort to unravel potential new epitopes for neutralizing antibodies will therefore be a major task of coming years (Box 2).

Box 2: Next steps on the way to antibody mediated immune control

<table>
<thead>
<tr>
<th>Neutralization</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Fine map specificities of neutralization response elicited in vivo.</td>
</tr>
<tr>
<td>• What do we need to neutralize - free virus or cell-cell transmission?</td>
</tr>
<tr>
<td>• How much antibody is needed for protection and where? Evaluate antibody levels needed for mucosal and systemic effectiveness.</td>
</tr>
<tr>
<td>• New immunogens that elicit protective and broadly active neutralizing responses.</td>
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<table>
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<tr>
<th>Effector functions</th>
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<tbody>
<tr>
<td>• Should vaccines aim to trigger effector responses?</td>
</tr>
<tr>
<td>• Are ADCC, complement lysis and phagocytosis effective in HIV infection? Timing, specificity and magnitude of response are still largely unknown.</td>
</tr>
<tr>
<td>• Do neutralizing or non-neutralizing antibodies mediate effector functions?</td>
</tr>
<tr>
<td>• What is the role of Fc and complement receptor dependent enhancement in vivo? Does it occur? Can it be circumvented?</td>
</tr>
<tr>
<td>• Are non-neutralizing antibodies overall beneficial or detrimental? Should they be included or avoided in vaccine design?</td>
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</table>

Are neutralizing antibodies effective in vivo?
Neutralizing antibodies can have a pronounced effect in vitro, but their in vivo efficacy, although widely investigated, awaited confirmation for long. Low to moderate neutralization activity against autologous virus can be detected at virtually all disease stages while a direct impact on virus replication in HIV infected individuals was not readily evident. Until recently the decrease in viremia observed during the acute infection phase was thought to be solely prompted by the advent of cellular immunity to HIV as neutralizing antibodies were described to be largely absent during this period [6,7,9,26,31,113,114]. However, several recent studies confirmed that neutralization activity can develop within weeks of infection and thus may contribute to viral clearance earlier than previously assumed [8,27,115]. Nevertheless, both
quantity and quality of the response improves over time and broader neutralization activity against heterologous viral strains is predominantly found in chronic infection [7,8,11,26,56]. High neutralizing activity has been associated with long-term non-progression in some [7,116] but not all investigated cases [29,117-119].

Despite the comprehensively documented in vivo and in vitro activity of neutralizing antibodies, HIV is comparably refractory to neutralization as it has adopted several means to avert antibody recognition. With the distinctive spatial organization of the envelope trimer on the virus particle and ensuing reduced accessibility of antibody binding sites (masking of epitopes, oligomeric exclusion, steric interference), the heavy glycosylation of the envelope proteins and the high mutation rate of the virus that allows rapid evolution of variants during adaptation to selection pressures, HIV has assembled an array of mechanisms that allow it to persist in vivo and prevail over humoral and cellular immune responses [8,12,27,34,46,47,66,112,120-123].

It is evident from numerous studies that at all disease stages, neutralizing antibodies preferentially recognize and inhibit preceding but not the current autologous viral strains signifying that HIV rapidly escapes the antibody pressure [8,26-34]. This escape is continuous and to our current knowledge the virus in general stays ahead of the newly evolving antibody repertoire. Whether the selection pressure employed by neutralizing antibodies induces a higher viral diversity or in turn reduces variability as only a fraction of the viral quasispecies will tolerate the necessary mutations and survive the antibody selection, needs to be investigated in detail in coming years [124].

That HIV escapes the neutralizing antibody pressure has for long been the best documented evidence for the protective role of this immune response in vivo until the effect of neutralizing antibodies was formally demonstrated by passive immunization studies both in animal models and in humans [56-58,125-132]. However, the noted effects on viremia suppression in these in vivo studies were generally modest and subject to rapid escape [58,126,133,134]. Defining to what extent the course of HIV infection is shaped by the humoral immune response will be a challenge of coming years. As the emergence of escape variants appears to be inevitable, can we really expect that antibodies have lasting effects in vivo? Arguably, the magnitude of the impact cannot be conclusively answered to date. Nevertheless, recent years brought us the formal proof that antibody responses can actively drive viral escape in vivo which still must stand for the best indication of the antibody activity in natural infection [58,126]. In further support, it can be reasoned that the constant antibody pressure and the ensuing necessitated mutations could eventually restrict viral fitness which will lead to a decrease in viremia and
Background

aid long term control. Notably, evidence increases that neutralizing antibodies lead to a selection in viral transmission [59-61] arguing that at least partial protection of transmission could be conferred. A notion that is further substantiated by reports suggesting that high titers of neutralizing antibodies may prevent superinfection [62,63].

Quantity or quality?

Provided that the struggle to define relevant neutralization epitopes and induce respective antibody responses is successful, other complex issues need to be solved before HIV specific humoral immunity can be successfully exploited for treatment and prevention. Whereas assay systems developed over recent years allow a relatively robust estimation and comparison of in vitro measured neutralization activities [8,27,103,135,136], they not yet allow us to defer what quantities of antibody are needed to prevent or control infection in vivo. Recent passive immunization studies suggest that at least in the case of established HIV infection, in vivo antibody levels may need to exceed in vitro necessary activities by more than a log to show effect [58,126,133]. If indeed cell-to-cell spread plays an important role in vivo, this could explain to some extent the comparatively low efficacy of neutralizing antibodies in these studies. Defining which mechanisms - free virus or cell-to-cell transmission - dominates in vivo and what the consequences for the activity of the humoral immune response are, will therefore be one of the challenges of coming years (Box 2). Whether vaccination will be capable of eliciting the necessary high levels of antibodies remains to be seen. Broader and potentially synergistic responses are sought for as they may reduce quantities needed to protect. Of note, antibody concentrations required to prevent infection are expected to be lower as only a comparatively small viral inoculum will be encountered at a given time. Nevertheless, sustaining high antibody levels over prolonged time periods post vaccination and providing the necessary systemic and mucosal distribution will be critical for vaccines to show effect.

Focusing back on the molecular level: How many antibody molecules are needed to neutralize an HIV particle? Current estimates report that on average primary virions bear 14 intact spikes on the viral surface [68]. Recent studies disfavour a previous hypothesis of a one-hit model (one antibody per virion [74]) and suggest that even as little as one functional spike may be sufficient to promote virus entry [70,137]. Consequently all functional trimers on the virus surface would need to be bound by a neutralizing antibody for it to be effective.
Non-neutralizing antibodies – friend or foe?

It is well established that only a fraction of the elicited envelope specific response bears neutralizing activity. While binding monomeric envelope subunits with high avidity, the vast majority of these antibodies fails to interact with the functional envelope spikes on the virion and hence lack neutralizing activity [94,95,138]. Uncertainty prevails concerning the biological function of these antibodies. Despite the lack of direct neutralizing activities these antibodies could potentially activate several effector functions which are known to improve immune control in other viral diseases [14,16,18-22,139]. So could non-neutralizing antibodies contribute to viral clearance through phagocytosis of immune complexed virus, lysis of virions by complement and through lysis of infected cells via activation of killer cells (ADCC) or complement (Table 1 and Figure 2). Indirectly, activation of complement by opsonized virions likely supports also stimulation of immune responses as described in other diseases [140]. Others have proposed that certain non-neutralizing antibodies by induction of conformational changes render epitopes of neutralizing antibodies better accessible and hence augment their activity [141]. By contrast, adverse effects could prevail, as non-neutralizing antibodies may promote cell infection through complement or Fc receptor driven uptake as shown in vitro by several groups [11,142-150]. Which of these effects occur in vivo, if non-neutralizing antibodies are mere by-products or bear function, whether beneficial or detrimental effects dominate or hold each other the balance has been debated but not conclusively answered. All these mechanisms are mediated via Fc dependent interactions. Thus not only epitope specificity but also antibody isotype, subtype and ensuing affinities for Fc receptors and complement factors will steer the efficacy of the immune response (Table 1). Even though in principle effector functions can be mediated by both neutralizing and non-neutralizing antibodies, it is reasonable to think that in HIV infection non-neutralizing antibodies due to their abundance may outweigh neutralizing antibodies in this activity. However, whether or not this holds true and for which functions is currently uncertain. Of note, in other viral infections non-neutralizing antibodies were shown to have distinct beneficial effects [14,73,151].

As outlined below in more detail, diverse effector functions may be impaired in HIV infection allowing immune complexed/opsonized virions to retain their infectivity.

What is the fate of antibody (and complement) opsonized virus that it is not destructed? Is phagocytosis effective? Can uptake of immune complexed virions lead to infection of the Fc or complement receptor bearing cells? Or alternatively, promote transfer to susceptible target cells as described in several viral diseases [142]? In vitro studies with macrophages,
Background

erthrocytes and dendritic cells suggest that antibody mediated infection enhancement could also occur in HIV infection. Binding of the opsonized HIV-1 via Fc and complement receptors has at least in vitro been demonstrated to promote infection of specific cell types or allow subsequent transfer of infectious virus to target cells (see below and Table 1).

Although to our current knowledge the bulk of non-neutralizing antibodies is directed to epitopes on envelope monomers or trimers that are not relevant to the infection process and hence do not interfere with neutralizing antibody action, non-neutralizing antibodies which bind to overlapping or adjacent epitopes could potentially hinder efficient interaction of the neutralizing antibodies with their target. However, recent data suggest that this is commonly not the case [138]. Requirement for both infection inhibiting and enhancing effects of non-neutralizing antibodies is that they efficiently bind to virions or infected cells. Recent studies unravelled that antibodies directed to non-functional epitopes on the envelope proteins can indeed efficiently interact with HIV particles. As consequence of envelope shedding and/or incomplete formation of trimers, HIV-1 bears a high number of non-functional gp120/gp41 monomers and gp120-depleted gp41 stumps on its surface [96] that are readily accessible for antibody binding. Considering that the number of intact trimers on virions is relatively low (on average 14 spikes), non-functional envelope may outnumber functional proteins overall. If the latter holds true, this may explain why such a strong response to these proteins is elicited and how antibodies to these seemingly irrelevant or inaccessible epitopes can bind to virus particles [152].
Figure 2: How antibodies combat HIV-1.
A) Neutralization of free virus by antibodies, B) Complement-mediated lysis of free virus and infected cells triggered by antibodies, C) Opsonization of virus particles by antibodies and phagocytosis of virus particles via Fc- or complement-receptors, D) Antibody-dependent cellular cytotoxicity (ADCC) against infected cells.
Table 1: Potential functions of HIV-specific antibodies

<table>
<thead>
<tr>
<th>Antibody subtypes/isotypes</th>
<th>Inhibition Neutralization</th>
<th>Complement Lysis</th>
<th>ADCC</th>
<th>Phagocytosis</th>
<th>Enhancement Fe-ADE</th>
<th>C-ADE</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG (mostly IgG1 and IgG3) &gt;&gt; IgA, IgM</td>
<td>IgM &gt; IgG3, IgG1 &gt; IgG2</td>
<td>IgG1 &gt; IgG3, IgA</td>
<td>IgG, IgM, IgA</td>
<td>IgG, IgM, IgA</td>
<td>IgG, IgM</td>
<td></td>
</tr>
</tbody>
</table>

Effector cells
- None

Modes of action
- Inhibition of attachment and fusion by binding to free virions
- Activation of complement system through opsonized virus; lysis of virus
- Lysis of infected cells
- Uptake and destruction of antibody/complement opsonized virus particles
- FcR dependent enhancement of virus binding and uptake; Fe-dependent infection or transmission
- CR dependent enhancement of virus binding and uptake; CR-dependent infection or transmission

Evidence
- In vitro
- Ex vivo
- In vivo (SHIV and HIV)
- In vitro
- Ex vivo
- In vivo (SHIV)
- In vitro
- Ex vivo
- In vivo

Defects/evasion mechanisms in HIV infection
- Rapid escape
- Glycan shield
- Steric occlusion
- Cell-to-cell transmission
- Virus lysis restricted by complement control proteins
- Defects in NK, macrophage, neutrophil function
- Defects in macrophage, neutrophil and DC function

Legend: Table 1 gives a summary of the effects of the humoral immune system in HIV infection described in this review. Extensive referencing on these topics can be found in the respective sections in the text. Abbreviations: NK, Natural killer; FcR, Fc-receptor; CR, Complement receptor. In vitro: Studies with cell lines, monoclonal antibodies, Ex vivo: studies on clinical material. In vivo: clinical proof of concept in vivo in human (HIV) or animal studies (SHIV and SIV).
Background

Antibody-dependent cellular cytotoxicity
ADCC functions as potent immune effector mechanism in a wide variety of infectious diseases by combining components of the innate and the acquired immune system [18,153-156]. Thereby, antibody binding to pathogen antigens expressed on infected cells prompts recruitment of Fc receptor bearing effector cells. IgG and IgA can trigger ADCC in conjunction with several types of effector cells, most importantly natural killer (NK) cells (see Table 1).

The ensuing interaction between Fc domains of antibodies and corresponding receptors on the effector cells triggers a series of events that lead to the destruction of the infected cell through the release of the content of cytotoxic granules (perforin, granzymes), cytokines (TNF), chemokines, proteases, nitric oxide, reactive oxygen radicals or Fas/FasL interactions [157-162]. Despite substantial research efforts, which comprise the initial discovery of antibodies to HIV that mediate ADCC [11,43,126,163-171], the presence and activity of the response at different disease stages [172-176] and more recently, the potential protective effect of ADCC responses elicited by vaccination in animal models [177-179], the relevance of ADCC in HIV infection is not yet clear. In principle, the impact of this mechanism could indeed be high, since in contrast to neutralizing antibodies ADCC-mediating antibodies can eliminate HIV-1 infected cells and therefore reduce production of progeny, limit cell-to-cell spread and potentially also lower the pool of infected cells that revert to latency. Antibodies which mediate ADCC develop rapidly in the majority of patients with HIV infection and can be detected within few weeks after onset of symptoms of acute infection [32,163-165,171,175-177,180], and thus could potentially contribute to the decline in viremia during this phase. Epitope specificities of antibodies mediating ADCC have not been completely unravelled. Both neutralizing and non-neutralizing antibodies were found to elicit ADCC and various epitopes within gp120 and gp41 that serve as targets for ADCC have been identified over the years [43,166-170]. Notably, ADCC activity prevails at all disease stages [166] and titers are generally higher than neutralizing responses [177,181]. ADCC responses appear to be relatively broadly active against both autologous and heterologous HIV-1 strains [163,175,177,182,183] and have been suggested to positively influence disease progression [164,175,176,184-187]. Although no effect on perinatal transmission has so far been established, high ADCC activity in HIV-infected mothers and their newborns was found to be associated with better clinical stages of the infected children [174,188,189]. Of note, in animal models ADCC activity has been associated with delayed disease progression and
Background

Protection [177,190-193]. Most notably, vaccine-elicited ADCC responses reduced acute viremia in rhesus macaques challenged with SIVmac251 [178]. Concerns on the overall benefit of the ADCC activity have been raised when it became clear that free gp120 that binds to CD4 receptors on uninfected T cells renders these cells accessible for ADCC attack [194-196]. Ensuing depletion of uninfected CD4 T cells could then promote disease progression if this scenario holds true [197,198]. However, whether or not this can occur in vivo is unclear as free gp120 may not be present at high enough concentrations [199].

Central to the in vivo activity of ADCC are effector cells. However, functions of the major effectors, NK cells, deteriorate with disease progression which likely will limit the impact of ADCC later on in infection [200-206]. Equally, macrophages and neutrophils develop deficiencies upon progressive HIV infection which has been found to perturb several of their functions [207-209]. Despite the considerable information gathered over the years, the functionality of the ADCC response in HIV infection remains uncertain and a concentrated effort will be needed to decipher its impact in controlling and preventing HIV infection.

Activation of the complement system in HIV-1 infection

The complement system is a key component of the innate immune defence and a crucial effector mechanism of the humoral arm of the immune system [16,210,211]. Either directly, or through activation by virion bound antibodies, complement has been found effective in lysing various enveloped viruses, resulting in fragmentation of the outer membrane and disintegration of the nucleocapsid [16]. Beyond lysis activity the complement system has opsonizing, phagocytosis inducing, chemoattractant and immune stimulatory functions which shape the immune response to pathogens [16,210-213]. A considerable activation of the classical pathway and to a lesser extent the alternative and lectin pathway occurs during HIV-1 infection as evidenced by a decrease in the levels of complement components paired with an increase in complement activation products in patient sera [148,214,215]. A decline of complement receptor (CR) 1 expression on patient erythrocytes was found to be inversely correlated with severity of symptoms [216-218] which could be consequence of increased uptake of opsonized HIV through CR1 during periods of high viremia. Several studies have demonstrated activation of the classical pathway by monoclonal and serum derived HIV-specific antibodies upon binding to HIV-1 particles [148,215,219]. Notably, the envelope proteins gp41 and gp120 can in addition activate complement in an antibody-independent manner by binding C1q or mannose-binding lectin (MBL), respectively [220-224]. As discussed in detail below, in vitro studies suggest that the roles of complement in HIV
infection are multifaceted and comprise beneficial (virus lysis, opsonization, phagocytosis and immune activation) but also detrimental effects (infection enhancement through complement receptor dependent uptake).

**Complement-mediated lysis of HIV-1 virions**
Successful opsonization of virions alone has been suggested to increase the effects of neutralizing antibodies as the dense coating of the virion with antibodies and complement may obstruct efficient interaction with the target cells [225-227]. The most dramatic impact on infectivity, however, is expected through direct lysis of virions or infected cells upon antibody-mediated activation of the classical pathway. Only few studies on the effects of complement on HIV infected cells have been conducted [228-230], hence the impact of this mechanism remains unclear. By contrast, complement activity against the virus has been investigated in great depth: Several antibodies that induce complement lysis of HIV have been described [219,231]. *In vivo*, antibodies inducing complement lysis can be found already during acute infection. These responses increase during the chronic infection phase and appear to be maintained throughout the course of the disease [225,232-235]. Analysis of patient derived virus particles brought further evidence that complement lysis occurs *in vivo* [226,234]. Interestingly, as described for ADCC, antibody responses that mediate HIV lysis are generally less type specific than neutralization activities [225,234,235]. Epitope specificities of these responses still need to be determined but since antibody binding of all envelope structures (intact spikes, monomers, stumps) will trigger complement activation, it is expected that as for ADCC non-neutralizing antibodies dominate also in this mechanism [225,235]. Lysis activities described to date reached up to 90% virus destruction at the highest concentration of patient sera tested [234,235]. Precise titers of the complement lysis response have not been consistently measured in these studies but appear to be lower than ADCC responses (Huber M, Trkola A, unpublished observation). Whether this is simply due to differences in assay sensitivities, or reflects differences in the mechanisms is currently not known. So could number of antibody molecules required for complement lysis and cell killing by ADCC differ. Alternatively, specific antibody responses that vary in epitope recognition and quantity could be required for the execution of ADCC and complement lysis. That an effort in elucidating these mechanisms may be worthwhile is suggested by recent evaluations of *in vivo* responses which indicated that complement lysis impacts on viremia control during the acute infection phase [225,235].
**Protection of HIV-1 from complement mediated lysis**

Although functional, the overall lysis activity against HIV is arguably low compared to other viruses [148,234-236]. The underlying mechanisms of this increased resistance against complement destruction have been deciphered over the years. HIV can evade or diminish complement attack by incorporating the cellular complement regulatory proteins CD46, CD55, CD59 into its outer membrane which, by interference with the complement cascade, lead to its termination and hence reduced viral lysis [230,237-242]. Of note, the levels of these control proteins in vivo were suggested to decline during the course of the disease which could render both virus and infected cells again more sensitive to complement attack [228,243]. In addition to the membrane-bound control proteins, complement factor H (CFH), a negative regulator of complement activation, functions as potent inhibitor of HIV lysis by complement. Upon binding to gp120 and gp41, CFH down-regulates complement activation and prevents membrane attack complex formation [223,227,244-246].

**Phagocytosis**

Coating with antibody and complement renders virions subject to uptake and destruction by phagocytes. Most prominent in this function (and hence referred to as professional phagocytes) are mononuclear phagocytes (several subsets of macrophages and monocytes) and polymorphonuclear granulocytes (neutrophils), but also several other cell types including dendritic cells [247]. The phagocytic cells capture and internalize immune complexes via their Fc receptors which upon engagement trigger phagocytosis and subsequent degradation. In complement receptor (CR) mediated phagocytosis, C3b and iC3b (generated through the activities of plasma factors H and I) act as opsonins and initiate binding to the phagocyte through complement receptors [248]. The efficacy of the phagocytic process in HIV infection against both HIV and other opportunistic pathogens is not completely understood and awaits further investigation. Most importantly, defects in both complement and Fc receptor dependent phagocytosis of macrophages and neutrophils can occur during disease progression [207-209,249].

**Infection enhancing effects of HIV-specific antibodies**

What is the fate of phagocytosed virus that is not destructed? Evidence has accumulated that phagocytes can become either infected upon Fc or complement receptor uptake of HIV (e.g. macrophages), or function as reservoir that readily disseminates and transmits virus to other cells (e.g. dendritic cells [250]). Fc receptor mediated antibody dependent enhancement has
been demonstrated in several *in vitro* and *ex vivo* cell systems [144,150,251-259]. Recently, genetic polymorphisms in Fcγ IIa and IIIa receptors, which are linked to increased avidity of the receptors for immune complexes, have been suggested to increase risk of HIV infection in individuals receiving vaccination with recombinant gp120 in the VAX004 trial [260]. Nevertheless, this observation still awaits confirmation and it remains currently unclear which role FcR mediated enhancement plays *in vivo*. Of note, neither in human nor animal studies where high levels of monoclonal or polyclonal antibodies were infused, enhancing effects were apparent [58,125,133]. Whether both neutralizing and non-neutralizing antibodies (and of which specificities) mediate enhancement has not been conclusively determined. Productive infection of the phagocyte upon FcR uptake has been described in systems that were dependent or independent of CD4 mediated entry [253,255,261,262]. Notably though, where CD4 and coreceptor interactions were required, infection was consequently found to be sensitive to neutralizing antibodies [261,262]. Nevertheless, that neutralizing antibodies may equally promote enhancement at sub-neutralizing concentrations has been shown by several groups [256,262,263]. By contrast, a recent report suggests, that not all interactions of HIV-specific Ig with FcR may have adverse effects: Employment of FcγRI in macrophages and FcγRII in iDC by non-neutralizing HIV-specific antibodies was described to inhibit infection of the respective cells [264].

**Complement-mediated enhancement of HIV-1 infection**

Since HIV employs mechanisms that allow it to at least partially circumvent complement lysis, antibody and complement opsonized virions can remain infectious [116,148,227,240-242,265-267]. Uptake of opsonized virus via CR is not restricted to professional phagocytes and has been demonstrated for various cell types, which can either become infected themselves or promote viral infectivity and potently transmit virions to target cells [147-149,266,268-271]. As for Fc antibody-dependent enhancement (ADE) the *in vivo* relevance of this process (termed complement dependent ADE (C-ADE)) is not yet clear. Notably though, trapping of infectious HIV in human lymphoid tissues was found to be complement dependent [267]. Many aspects of C-ADE still need to be defined: Little is known on antibody specificities and levels required for this to take effect. Are the same types of antibodies that mediate lysis also involved in triggering enhancement? And if so, what decides whether destruction or enhancement occurs? Is it only the antibodies that decide which turn to take or are variations in complement concentration and control proteins the deciding factors? Likewise it is not known whether all CR bearing cell types are prone to this
effect *in vivo*. It will be pivotal to determine these factors to allow assessment of the consequences of antibody-driven enhancement *in vivo*.

**Concluding remarks**

Despite immense research efforts over the past 20 years we have not yet reached the ultimate goal: Exploiting humoral immunity for prevention and therapy. While considered by some even as impossible at times, recent research advances have substantially strengthened the possibilities of vaccine development and funnelled the field with new vigour. What more do we need to understand before we can effectively and safely utilize antibodies for immune control? The list is long indeed as summarized in Box 2, but worthwhile going for as only then we will have the chance to generate a broadly active and potent vaccine that tackles HIV.

**Conflict of interest statement**

The authors have no conflict of interest to declare.

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Background

Background


Background


Background


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4.2 Antibodies for HIV Treatment and Prevention: Window of Opportunity?

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Antibodies for HIV treatment and prevention: Window of opportunity?

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Introduction

Twenty five years into the HIV epidemic, antiretroviral therapy (ART), where available, succeeds in dramatically reducing mortality and morbidity and significantly lowers rates of mother-to-child transmission. However, although initiation of ART suppresses viral loads to undetectable levels for several years in most individuals (Gulick et al. 20061, hopes that HIV infection would eventually be cleared by ART have not yet been fulfilled. Consequently infected individuals may require life-long treatment, which can be problematic due to side effects of the drugs and evolution of viral resistance. Emergence of drug resistant strains has been reported for all currently licensed substances and necessitates the unremitting development of alternate therapies. As oral drugs are in general the preferred choice due to their ease of administration, parenterally administered antibodies that interfere with viral replication have been considered for therapeutic purposes in HIV infection.

To date polyclonal and monoclonal antibody therapeutics are routinely used in cancer therapy and diagnostics, in autoimmune disorders, as antitoxins and in treatment or prevention of viral, bacterial or parasitic infections (Keller et al. 2000; Sawyer 2000; Zeitlin et al. 2000;
Background

Brekke et al. 2003; Reichert et al. 2005; Schrama et al. 2006). Over 20 monoclonal antibodies (mAbs) and immunoglobulin Fc fusion proteins have received FDA marketing approval. In a number of instances, these products represent first-line therapy and the current standard of care. In viral infections, polyclonal and monoclonal antibodies are used for treatment and prevention of infections with hepatitis B virus, cytomegalovirus, varicella zoster virus, respiratory syncitial virus and rabies virus (Sawyer 2000; Brekke et al. 2003). For example, Synagis™ (palivizumab, MedImmune, Inc.), a humanized IgG1 mAb to the respiratory syncytial virus (RSV) fusion protein, remains the product of choice for prevention of serious lower respiratory tract disease caused by RSV in children despite intensive research to develop small molecule inhibitors (Ding et al. 1998; McKimm-Breschkin 2000; Huntley et al. 2002; Cianci et al. 2004).

What potential antibody based therapeutics bear in treatment and prevention of HIV infection has been zealously debated over the years. Do we have enough evidence that naturally occurring antibodies affect HIV infection to support this approach? Have clinical studies of investigational antibodies provided clear proof-of-concept? Which antibodies would we need to develop? In which clinical settings could antibody based therapeutics be of greatest use? In principle, a wide spectrum of antibodies with diverse specificities and modes of action could be envisioned for therapeutic purposes in HIV infection (see Fig. 1 and Table 1). Antibodies that are directed against both the virus and cellular receptors have demonstrated activity against HIV and can block its infectivity. In the following we will summarize knowledge gained on the functionality and feasibility of antibody therapeutics in HIV infection over the years and emphasize areas that await further investigation.
Virus
infected cell
neutralization
of free virus
complement-mediated lysis
complement-mediated lysis
antibody-dependent
cellular cytotoxicity
inhibition of receptor
interaction
complement components (green),
anti-HIV antibodies (blue), anti-cell antibodies (red), CD4 and co-receptors (violet), Fc receptors (black).

Virus
infected cell
complement complex C1
complement
proteins
antihiv antibodies
Fc receptor
perform
granulys
antibodies
co-receptors

Uninfected cell

Figure 1: Modes of action of therapeutic antibodies in prevention and therapy.
<table>
<thead>
<tr>
<th>Target</th>
<th>Virus</th>
<th>Infected cell</th>
<th>Uninfected cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epitope</td>
<td>HIV envelope proteins (gp120, gp41)</td>
<td>HIV envelope proteins (gp120, gp41)</td>
<td>HIV receptors (CD4, CCR5, CXCR4)</td>
</tr>
<tr>
<td></td>
<td>virus subtype specific</td>
<td>virus subtype specific</td>
<td>no subtype specificity</td>
</tr>
<tr>
<td>Mechanisms of action</td>
<td>Neutralization (inhibition of entry and fusion)</td>
<td>Antibody-dependent cellular cytotoxicity (ADCC)</td>
<td>Inhibition of receptor interaction/entry</td>
</tr>
<tr>
<td></td>
<td>Fc and CR-mediated phagocytosis</td>
<td>Complement-mediated lysis</td>
<td></td>
</tr>
<tr>
<td>Effector functions</td>
<td>Potentially advantageous</td>
<td>Potentially advantageous</td>
<td>Potentially detrimental</td>
</tr>
<tr>
<td>Antibody class</td>
<td>IgG (all subtypes), IgM, IgA</td>
<td>IgG (all subtypes), IgM, IgA</td>
<td>IgG2 and IgG4 preferred to limit effector functions</td>
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<tr>
<td>Potential use in prevention</td>
<td>Microbicide</td>
<td>Microbicide</td>
<td></td>
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<tr>
<td></td>
<td>Mother to child transmission</td>
<td>Mother to child transmission</td>
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<td></td>
<td>Active/passive immunisation</td>
<td>Post-exposure prophylaxis</td>
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<tr>
<td>Potential use in therapy</td>
<td>Combination therapy as part of ART regimen</td>
<td>Combination therapy as part of ART regimen</td>
<td>Combination therapy as part of ART regimen</td>
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<tr>
<td></td>
<td>Intermittent treatment during drug holiday</td>
<td>Combination therapy with early ART to reduce pool of infected cells</td>
<td>Intermittent treatment during drug holiday</td>
</tr>
<tr>
<td></td>
<td>Diagnostics</td>
<td>Immuntoxin (combined with other strategies to reactivate/eliminate latent reservoir)</td>
<td></td>
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<tr>
<td>Escape mechanisms</td>
<td>Rapid escape through mutations in viral proteins</td>
<td>Rapid escape through mutations in viral proteins</td>
<td>Escape (virus changes binding site on receptor)</td>
</tr>
<tr>
<td>Potential safety concerns</td>
<td>Antibody-dependent enhancement of infection</td>
<td></td>
<td>Interference with immune functions and cell depletion</td>
</tr>
<tr>
<td>Tested in humans</td>
<td>2G12 a)</td>
<td>Effector function of anti-HIV antibodies have not been verified in vivo</td>
<td>TNX-355 b)</td>
</tr>
<tr>
<td></td>
<td>2F5 b)</td>
<td></td>
<td>PRO 140 b)</td>
</tr>
<tr>
<td></td>
<td>4E10 c)</td>
<td></td>
<td>CCR5mAb004 e)</td>
</tr>
<tr>
<td></td>
<td>F105 d)</td>
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a) (Armbruster et al. 2002; Stiegler et al. 2002; Trkola et al. 2005)
b) (Armbruster et al. 2002; Stiegler et al. 2002; Armbruster et al. 2004; Trkola et al. 2005)
c) (Armbruster et al. 2002; Armbruster et al. 2004; Trkola et al. 2005)
d) (Wolfe et al. 1996; Cavacini et al. 1998)
e) (Jacobson et al. 2004b; Norris et al. 2006)
f) (Olson et al. 2006)
g) (Roschke et al. 2004)
Targeting the virus and infected cells

HIV infects host cells upon interaction of the viral envelope glycoprotein gp120 with the cellular receptor CD4 (Maddon et al. 1986) and a coreceptor (most commonly CCR5 or CXCR4) (Alkhatib et al. 1996; Deng et al. 1996; Dragic et al. 1996; Feng et al. 1996). Receptor binding induces conformational changes in gp120 that subsequently lead to rearrangements in gp41, the transmembrane unit of the envelope, and prompt fusion (Wyatt et al. 1998b; Pierson et al. 2003). Antibodies employ distinct modes of action to interfere with the life cycle of viruses. The initial steps in viral infection - receptor engagement and fusion mediated by the envelope glycoproteins - are primary targets for neutralizing antibodies (Klasse et al. 2002). All neutralizing antibodies against HIV described to date inhibit infection of target cells by blocking engagement of CD4 or coreceptor (e.g., CCR5 or CXCR4), or by binding to domains involved in subsequent steps of the fusion process (Trkola et al. 1996a; Wu et al. 1996; Wyatt et al. 1998b; Parren et al. 2001a; Xiang et al. 2002; Decker et al. 2005; Pantophlet et al. 2006). On large, the initial hopes of exploiting antibody based immunity for treatment and prevention were dampened once it became clear that most of the neutralizing activity elicited to HIV-1 in vivo is strain and subtype specific. Demands on therapeutic antibodies are high: they have to be safe, potent and broadly active against divergent HIV strains. To date, only a handful of monoclonal antibodies have been isolated that neutralize with a comparable broad cross-neutralizing activity: the antibody IgG1b12 which recognizes an unique epitope overlapping the CD4-binding site (Burton et al. 1994), the carbohydrate specific antibody 2G12 (Trkola et al. 1996b) which recognizes an equally unique mannose-dependent epitope within gp120 (Scanlan et al. 2003) and the antibodies 2F5 and 4E10, which bind to the membrane proximal external region (MPER) in gp41 (Muster et al. 1993; Stiegler et al. 2001; Zwick et al. 2001; Binley et al. 2004).

Modes of action of antiviral antibodies in HIV infection

Initial efforts to develop antibodies for therapeutic use focused on defining antibodies with neutralizing capacity, i.e. antibodies that bind virus and inhibit entry into target cells. As discussed above, neutralizing antibodies and their mechanisms of action have been intensively studied over the years. It is generally agreed that antibodies with neutralizing capacity will be important components of vaccine-induced immunity and thus have also been prime candidates for the development as therapeutics. All HIV-specific antibodies probed for in vivo efficacy
to date have neutralizing activities. Their characteristics and effects are discussed in detail below.

Whether neutralizing activity is the sole function these antibodies can or should fulfill in vivo has been increasingly debated. Besides neutralizing free viruses, antibodies could have significant impact on virus elimination by inducing phagocytosis or complement-dependent lysis of opsonized viral particles (Fig. 1). Activity of complement and antibody in controlling viral infection has been described in other viral diseases (Pincus et al. 1995; Blue et al. 2004; Hangartner et al. 2006). However, to what extent this mechanism is active against HIV remains uncertain: Antibodies against HIV that induce complement lysis of virions are common in HIV infection (Aasa-Chapman et al. 2005; Huber et al. 2006) and may contribute to viral control in vivo (Huber et al. 2006). Nevertheless, the overall lysis activity against HIV is low compared to other viruses (Marschang et al. 1993; Sullivan et al. 1996; Stoiber et al. 2001; Huber et al. 2006). HIV weakens complement attack by incorporating the cellular complement regulatory proteins CD46, CD55, and CD59 into its outer membrane. These proteins can terminate the complement cascade and rescue the virus from lysis (Montefiori et al. 1994; Marschang et al. 1995; Saifuddin et al. 1997). More so, antibody- and complement-coated virions could potentially enhance infection of cells that express Fc and complement receptors, a possibility which needs to be considered if antibodies that trigger effector functions are used for therapy (Robinson et al. 1988; Takeda et al. 1988; Montefiori 1997; Stoiber et al. 2001; Stoiber et al. 2005). Of note, none of the passive immunization studies conducted to date has given evidence of antibody driven enhancement in vivo. That is, treatment with antibody resulted in decreased or unchanged viral loads; increased viral burden was not observed (Gunthard et al. 1994; Mascola et al. 2000; Armbuster et al. 2004; Trkola et al. 2005). Nevertheless, whether or not vaccines or therapeutics should include antibodies that elicit complement activity needs to be carefully evaluated to assess potential benefits and risks of their activity.

Another major immune function of antiviral antibodies that needs to be evaluated is antibody-dependent cellular cytotoxicity (ADCC). If active in HIV infection, the impact of this mechanism could indeed be high, as ADCC-mediating antibodies would eliminate HIV-1 infected cells and thereby reduce production of progeny. Antibodies which mediate ADCC are omnipresent in HIV infected individuals, uncertainty prevails though on the functionality of the mechanism as effector cells (natural killer cells, neutrophils) may have decreased activity upon disease progression (Bender et al. 1988; Monari et al. 1999; Azzam et al. 2006). Evidence has accumulated that ADCC mediating antibodies may nevertheless function in
vivo, as they were described to positively influence disease progression (Baum et al. 1996) (Broliden et al. 1993; Ahmad et al. 2001; Forthal et al. 2001). Equally, in animal models ADCC activity has been associated with delayed disease progression and protection (Belo et al. 1991; Ferrari et al. 1994; Broliden et al. 1996; Banks et al. 2002). Antibody therapeutics that function through complement and/or ADCC are used in cancer treatment (Golay et al. 2003; Mimura et al. 2005; van Meerten et al. 2006) but have not yet been actively pursued for treatment of HIV infection. This approach may need reconsideration as the effect on HIV replication could be significant if ADCC could be successfully harnessed to destroy infected cells. Latent reservoirs of HIV infected cells with extremely long half lives have been identified (Finzi et al. 1997; Wong et al. 1997; Finzi et al. 1999) which exclude that HIV infection in a patient can be eradicated within a frame of several years as initially proposed (Perelson et al. 1997). Attempts to eliminate this latent reservoir in vivo through stimulation paired with ART have failed so far (Kulkosky et al. 2006). ADCC mediating antibodies could be important in such combined stimulation/elimination strategies, thus supporting their further investigation.

Antibodies that specifically target virus-infected cells could be further used for generation of immunotoxins, an approach that has proven successful in cancer treatment (Bross et al. 2001; Pastan et al. 2006; Schrama et al. 2006). Immunotoxins can be generated by linking antibodies to potent cytotoxins. Typically, the antibody serves to target the desired cell type and mediate internalization of an intracellularly active cytotoxin. Several immunotoxins that specifically eliminate HIV infected cells in vitro have been developed in previous years (Till et al. 1989; Pincus et al. 1990; Pincus et al. 1991; Pincus et al. 1993; Pincus 1996; Lueders et al. 2004). Clinical testing of the immunotoxin CD4(178)PE40, a fusion protein directed against the CD4 binding site of gp120, showed little effect, and this result was attributed to rapid clearance of the immunotoxin and the differential resistance of clinical HIV isolates (Davey et al. 1994; Ramachandran et al. 1994). Recent studies suggest that combination of antibodies of different specificities and other toxins could potentially improve efficacy and tolerability of immunotoxins (Johansson et al. 2006; Kennedy et al. 2006). Obviously, as with all medications, safety concerns are high for antibody therapeutics. As mentioned above, antibody and complement may lead to infection enhancement. Eliciting ADCC bears the potential of harming uninfected cells if antibodies are polyspecific or viral antigen bound to uninfected cells is recognized. The latter has been shown in vitro, where uninfected cells coated with gp120 were susceptible to ADCC (Lyerly et al. 1987; Ahmad et al. 1994; Hober et al. 1995). While in vivo free gp120 likely is not present at high enough
Background

concentrations for this to occur (Klasse et al. 2004), this scenario needs to be considered. Polyspecificity and autoreactivity of antibodies, and the potentially ensuing adverse effects, have been a recent focus when it was described that 2F5, 4E10 and to a lesser extent also IgG1b12 cross react with autoantigens (Haynes et al. 2005), prompting concern that these antibodies upon in vivo application may predispose patients for autoimmunity. Notably though, in the case of 2F5 and 4E10, prolonged treatment at high doses during phase I and II testing in adults showed no serious adverse effects (Trkola et al. 2005). Moreover, there was no signal of autoimmune disease despite high and sustained levels of these two antibodies. Nevertheless, considering the in vitro data available, monitoring of patients for autoimmune disease would be appropriate in future investigational trials with these antibodies.

While the main focus has been on defining neutralizing antibodies which limit transmission of free virus particles, the role free virions play in vivo compared to virus spread from cell-to-cell remains ambiguous. Notably, in vitro the capacity of neutralizing antibodies in limiting cell-to-cell spread was reported to be considerably lower than the activity against free virions (Pantaleo et al. 1995). Only recently it became evident that in close cellular contact HIV particles spread via so-called virological synapses from cell to cell (Sato et al. 1992; Bangham 2003; McDonald et al. 2003; Arrighi et al. 2004; Jolly et al. 2004a; Jolly et al. 2004b). Transmission through the synapse may render the virus less susceptible to neutralization; however, no change in susceptibility was observed for virus transmitted in trans across the synapse between dendritic cells and T cells (Ketas et al. 2003). Over the coming years, it will be important to determine the modes of viral transmission in vivo and whether these modes of transmission can be effectively blocked by neutralizing antibodies. This information may be important for designing effective vaccines and antibody therapeutics.

Efficacy of HIV-specific antibodies in vivo

Passive immunization studies in animal models conducted over recent years brought the confirmation that neutralizing antibodies function in vivo and can limit transmission and de novo infection when applied topically (Veazey et al. 2003) or systemically (Gauduin et al. 1997; Shibata et al. 1999; Baba et al. 2000; Mascola et al. 2000; Hofmann-Lehmann et al. 2001; Montefiori et al. 2001; Parren et al. 2001b; Ruprecht et al. 2001; Haigwood et al. 2004). Amongst the broadly neutralizing HIV antibodies, so far only 2G12, 2F5 and 4E10 have undergone clinical testing and will thus be discussed below in more detail. The discussion also includes PRO 542 (previously referred to as CD4-IgG2), a tetravalent CD4-immunoglobulin fusion protein that also broadly neutralizes HIV (Allaway et al. 1995). The
Background

first monoclonal antibody against HIV applied in passive immunization in vivo was MAb F105 which binds to the CD4-binding site (Posner et al. 1991). However this antibody has a comparatively restricted neutralization capacity and was not successful as a therapeutic (Wolfe et al. 1996; Cavacini et al. 1998).

The monoclonal antibodies 2G12, 2F5, 4E10, F105 and the immunoglobulin fusion protein PRO 542 have all been probed for efficacy in established HIV infection (Wolfe et al. 1996; Cavacini et al. 1998; Jacobson et al. 2000; Armbruster et al. 2004; Jacobson et al. 2004a; Nakowitsch et al. 2005; Trkola et al. 2005). These studies were limited in size and brought thus far no conclusive answer on the efficacy or potential of using HIV-specific antibodies as a therapeutic strategy (Gauduin et al. 1997; Mascola et al. 1999; Baba et al. 2000; Mascola et al. 2000; Hofmann-Lehmann et al. 2001; Parren et al. 2001b; Trkola et al. 2005). However, the trials provided important safety, pharmacokinetic and preliminary antiviral information.

All of these antibodies could be delivered at high doses, were tolerated without notable side effects and had half-lives in the range of other described antibodies in clinical use ranging from 4.3 to 21.8 days (Table 2). Notably though, despite their in vitro potency, the HIV-specific antibodies have demonstrated no or modest antiviral activity that was subject to viral escape (Armbruster et al. 2004; Nakowitsch et al. 2005; Trkola et al. 2005). Although comparatively limited, the antiviral effects observed in these studies provided the first direct proof of neutralizing antibody activity in humans. Yet, the outcome of these studies raised many questions that will need to be answered in order to drive development of vaccines and antibody therapeutics forward. A central issue is why HIV-specific antibodies were not more active in these trials. Possible reasons are multifaceted. Modes and kinetics of viral transmission may vary in vivo and in vitro in ways that are important for the action of this type of inhibitor. Distribution to relevant sites of viral replication may have not occurred or necessary doses may not have been reached. Collectively, these studies suggest that the quantities of HIV-specific antibody needed to control infection in vivo are higher than the in vitro effective doses, perhaps by 10-fold or more (Poignard et al. 1999; Veazey et al. 2003; Trkola et al. 2005). Further insight into these issues may be obtained from additional clinical trials both of HIV-specific antibodies and of antibodies that bind cellular receptors.
Table 2: Serum half-lives of antivirals in humans

**Monoclonal antibodies**

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>Serum half-lives (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-HIV</td>
<td></td>
</tr>
<tr>
<td>2G12</td>
<td>21.8</td>
</tr>
<tr>
<td>4E10</td>
<td>5.5</td>
</tr>
<tr>
<td>2F5</td>
<td>4.3</td>
</tr>
<tr>
<td>F105</td>
<td>13</td>
</tr>
<tr>
<td>anti-cell</td>
<td></td>
</tr>
<tr>
<td>TNX-355 (anti-CD4)</td>
<td>2.4</td>
</tr>
<tr>
<td>PRO 140 (anti-CCR5)</td>
<td>18</td>
</tr>
<tr>
<td>non-HIV licensed</td>
<td></td>
</tr>
<tr>
<td>synagis (anti-RSV)</td>
<td>20</td>
</tr>
<tr>
<td>hepatitis B-lg (polyclonal)</td>
<td>21</td>
</tr>
</tbody>
</table>

**Peptide/protein inhibitors**

<table>
<thead>
<tr>
<th>Peptide/protein inhibitors</th>
<th>Serum half-lives (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-HIV</td>
<td></td>
</tr>
<tr>
<td>CD4-IgG2 (PRO 542)</td>
<td>4.2-3.3</td>
</tr>
<tr>
<td>T-20 (enfuvirtide)</td>
<td>3.8</td>
</tr>
</tbody>
</table>

**Small molecule inhibitors**

<table>
<thead>
<tr>
<th>Small molecule inhibitors</th>
<th>Serum half-lives (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-HIV (NRTI)</td>
<td></td>
</tr>
<tr>
<td>AZT</td>
<td>1.1</td>
</tr>
<tr>
<td>3TC</td>
<td>5-7</td>
</tr>
<tr>
<td>abacavir</td>
<td>1.5</td>
</tr>
<tr>
<td>anti-HIV (NNRTI)</td>
<td></td>
</tr>
<tr>
<td>efavirenz</td>
<td>52-76</td>
</tr>
<tr>
<td>nevirapine</td>
<td>45</td>
</tr>
<tr>
<td>anti-HIV (protease inhibitors)</td>
<td></td>
</tr>
<tr>
<td>saquinavir</td>
<td>7</td>
</tr>
<tr>
<td>ritonavir</td>
<td>3-5</td>
</tr>
<tr>
<td>indinavir</td>
<td>1.8</td>
</tr>
<tr>
<td>anti-cell (anti-CCR5)</td>
<td></td>
</tr>
<tr>
<td>maraviroc (UK-427)</td>
<td>0.9-2.3 h&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>vicriviroc (SCH-D)</td>
<td>3.4 h&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>non-HIV licensed</td>
<td></td>
</tr>
<tr>
<td>oseltamivir</td>
<td>6-10</td>
</tr>
<tr>
<td>zanamivir</td>
<td>2.6-5.1</td>
</tr>
<tr>
<td>aciclovir</td>
<td>2.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> rat and dog; <sup>b</sup> rhesus monkey
Background

**Characteristics of clinically tested anti-HIV antibodies**

MAb 2G12

The antibody 2G12 recognizes a cluster of high mannose carbohydrates of N-linked-glycosylated amino acid residues on the immunologically silent face of gp120 (Trkola et al. 1996b; Wyatt et al. 1998a; Sanders et al. 2002; Scanlan et al. 2002) and has broad neutralizing activity in vitro against isolates from subtype B and to a lesser extent also against other subtypes (Burton et al. 1994; Binley et al. 2004; Trkola et al. 2005). The heavy and light chains of 2G12 are not associated in traditional “Y”-like manner but instead lie vertically and are adjacent to one another. This unique structure provides the antibody with the flexibility to undergo multivalent interactions with the gp120 oligomannose cluster (Calarese et al. 2003). Phase I and phase I/II studies with this antibody have been conducted (Armbruster et al. 2002; Stiegler et al. 2002; Trkola et al. 2005).

The MPER specific antibodies 2F5 and 4E10

The antibodies 2F5 (Muster et al. 1993; Purtscher et al. 1994; Trkola et al. 1995) and 4E10 (Stiegler et al. 2001; Zwick et al. 2001) bind to adjacent linear epitopes on the ectodomain of gp41 in close proximity to the viral membrane. This membrane proximal external region (MPER) of gp41 is accessible to neutralizing antibodies, as recently confirmed by cryoelectron microscopy tomography (Zhu et al. 2006). The core epitopes of 2F5 and 4E10 span amino acids 662-668 (ELDKWAS) and 671-676 (NWF(D/N)IT), respectively. Both antibodies were successfully tested in phase I studies (Armbruster et al. 2002; Armbruster et al. 2004).

In a recent clinical study we found that a combination of 2G12, 2F5, 4E10 was able to delay viral rebound in several patients after cessation of successful ART (Trkola et al. 2005). Notably, escape mutant analysis demonstrated that the activity of 2G12 was crucial for the in vivo effect of the neutralizing antibody cocktail in this trial (Trkola et al. 2005).

The tetravalent CD4-immunoglobulin fusion protein PRO 542

Gp120 binds the most amino-terminal of the four immunoglobulin-like domains of CD4 (Peterson et al. 1988; Arthos et al. 1989; Kwong et al. 1998), and antiviral activity has been demonstrated in CD4-based proteins that incorporate 1, 2 or 4 domains. PRO 542 (CD4-IgG2, Progenics Pharmaceuticals, Inc.) is a tetravalent CD4-immunoglobulin fusion protein that comprises the D1 and D2 domains of human CD4 genetically fused to the heavy and light chain constant regions of human IgG2,κ (Allaway et al. 1995). Compared to mono- and di-
valent CD4-based proteins, PRO 542 more broadly and potently neutralizes primary HIV-1 isolates independent of viral subtype and coreceptor usage in a variety of preclinical settings (Trkola et al. 1995; Gauduin et al. 1996; Gauduin et al. 1998; Trkola et al. 1998; Nagashima et al. 2001; Ketas et al. 2003; Rusert et al. 2005). The activity of PRO 542 compares favorably with that of the leading HIV-1 neutralizing mAbs and is preserved also against in vivo viral isolates, primary viruses that have not been cultured in vitro and thus have not acquired higher sensitivity to CD4 based inhibitors (Olson et al. 2003; Beaumont et al. 2004; Jacobson et al. 2004a; Pugach et al. 2004; Shearer et al. 2006). Due to its mechanism of action PRO 542 can act synergistically with other entry inhibitors as was demonstrated for enfuvirtide in vitro (Nagashima et al. 2001). Administration of single-dose PRO 542 to treatment-experienced HIV-infected adults at doses ranging from 0.2 to 25 mg/kg (Jacobson et al. 2000; Jacobson et al. 2004a) was generally well tolerated, with no dose-limiting toxicities observed. Mean HIV RNA reductions of approximately 0.5 log10 were observed at the higher dose levels, with a trend towards greater antiviral effects in patients with more advanced disease (Jacobson et al. 2000; Jacobson et al. 2004a). Overall similar results were observed in a pediatric study that examined four weekly doses of 10 mg/kg PRO 542 (Shearer et al. 2000).

Targeting the uninfected cell

Modes of action and efficacy of anti-cell antibodies

The cellular receptors for HIV, CD4 and the coreceptors CCR5 and CXCR4, have proven to be promising targets for entry inhibition and an array of small molecule inhibitors, antagonists and antibodies targeting these receptors has been developed over the years (Pierson et al. 2003). When used as short-term monotherapy, small-molecule CCR5 antagonists have resulted in 1.5 log10 mean reductions in HIV RNA in patients without target-related toxicities, providing proof-of-concept for targeting host receptors required for entry (Fatkenheuer et al. 2005; Lalezari et al. 2005).

MAbs to host receptors are attractive from an efficacy perspective, given the immutable nature of the target. For mAbs that bind virus, viral resistance can result from mutations that abrogate antibody binding. However, for mAbs to host receptors, viral resistance requires mutations that circumvent mAb binding, i.e. the virus must adapt to no longer require the host receptor, to utilize another site on the receptor, or to utilize the mAb-bound form of the receptor. These escape mechanism have been described for receptor-targeting drugs and may also occur in response to antibodies (Trkola et al. 2002; Kuhmann et al. 2004; Mosley et al.
Background

2006). Preliminary indications are, however, that these forms of viral resistance typically require multiple mutations and may be slower to develop.

Safety considerations are particularly important for the use of mAbs to host receptors, given the potential to activate or dysregulate immune functions or to deplete cells that express the host receptor as recent incidences have shown (Glass et al. 2006; Suntharalingam et al. 2006). To minimize the risk of unwanted cell destruction, IgG4 mAbs commonly are employed as these are amongst the least reactive in terms of Fc effector functions, therefore have a limited potential to eliminate cells by antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytolysis.

Despite the potential drawbacks, several receptor-targeting antibodies have been developed and have shown favorable tolerability in clinical application. MAbs to the first immunoglobulin-like domain (D1) of CD4 can inhibit HIV-1 entry but antibodies tested thus far were shown to induce immune suppression and depletion of CD4+ T cells, and therefore no such mAb has been pursued for therapeutic application in HIV infection. Similarly, development of CXCR4 mAbs for HIV-1 therapy has been complicated by the relatively broad tissue distribution of the receptor and its critical role in development and haematopoiesis (Murdoch 2000).

CCR5 and the second immunoglobulin-like domain (D2) of CD4 have proven to provide more viable targets for mAb therapy. A significant number of Caucasians lack a functional CCR5 gene due to naturally occurring mutations (Martinson et al. 1997). The observation that these individuals display no obvious phenotype but are highly resistant to infection by HIV-1 (Liu et al. 1996; Lederman et al. 2006), prompted a focus on developing CCR5 specific drugs and antibodies. Antibodies directed to CCR5 and CD4 have been identified which do not block the natural activity of these receptors in vitro, thereby increasing the chance that they will be well tolerated in vivo. As discussed below, both CCR5 and CD4 mAbs are currently progressing through clinical development.

Characteristics of clinically tested anti-cell antibodies

CD4 specific: TNX-355

TNX-355 (Tanox, Inc.) is a humanized IgG4 version of the non-depleting murine mAb 5A8 generated against CD4 (Burkly et al. 1992; Moore et al. 1992; Burkly et al. 1995; Reimann et al. 1997; Reimann et al. 2002). Unlike most HIV-inhibitory CD4 mAbs, TNX-355 does not block gp120 binding to CD4 but rather blocks a post-attachment event in the entry cascade by binding the D2 domain of CD4. Notably, MAb binding to CD4 does not lead to T cell
Background

depletion or immunosuppression (Reimann et al. 1997; Boon et al. 2002; Jacobson et al. 2004b). TNX-355 potently inhibits HIV-1 in a coreceptor-independent manner and demonstrates antiviral synergy with enfuvirtide in vitro (Zhang et al. 2006). In vivo administration of single dose of TNX-355 to HIV-infected patients was well tolerated and led, despite the relatively rapid clearance of antibody (serum half-life 2.4 days, Table 2), to a decrease of HIV-1 RNA of 1-1.5 log10 measured 14 days after application (Jacobson et al. 2004b). This durability of the antiviral effect was correlated with prolonged coating of CD4 lymphocytes (Jacobson et al. 2004b). In a current Phase II trial, patients were randomized to receive TNX-355 or placebo plus optimized background therapy. TNX-355 was administered intravenously every two weeks at doses of 15 mg/kg or 10 mg/kg, with the 10 mg/kg group receiving the first 9 doses on a weekly basis. Forty-eight week data on this trial have been presented (Norris et al. 2006). Both dose levels were well tolerated and demonstrated significant antiviral activity. At 48 weeks, the mean HIV RNA reductions were 0.14, 0.96 and 0.71 log10 for the placebo, TNX-355 10 mg/kg and TNX-355 15 mg/kg groups, respectively.

**CCR5 specific: PRO 140**

PRO 140 (Progenics Pharmaceuticals, Inc.) is a humanized IgG4 CCR5 mAb derived from the murine mAb PA14 which potently and specifically blocks R5 HIV-1 entry in vitro (Olson et al. 1999). PRO 140 and/or PA14 have been shown to broadly inhibit primary R5 HIV-1 isolates independent of genetic subtype (Trkola et al. 2001; Cilliers et al. 2003), HIV-1 disease stage (Rusert et al. 2005), target cell type (Ketas et al. 2003) and resistance to existing antiretrovirals (Olson et al. 2003; Shearer et al. 2006).

Unlike the available small-molecule CCR5 inhibitors (Tagat et al. 2004; Dorr et al. 2005; Takashima et al. 2005; Watson et al. 2005), antiviral concentrations of PRO 140 do not block natural CCR5 function in vitro (Olson et al. 1999). Preliminary studies indicate that PRO 140 is active against viruses that are resistant to small-molecule CCR5 antagonists (Kuhmann et al. 2004; Marozsan et al. 2005), and shows antiviral synergy when combined with small-molecule CCR5 antagonists in vitro (Murga et al. 2006). These complementary properties may reflect the distinct differences in CCR5 binding: Small-molecule CCR5 antagonists bind a hydrophobic pocket formed by the transmembrane helices of CCR5 and inhibit HIV-1 via allosteric mechanisms (Dragic et al. 2000; Tsamis et al. 2003; Nishikawa et al. 2005; Watson et al. 2005), while PRO 140 binds an extracellular epitope on CCR5 and appears to act as a competitive inhibitor of virus binding (Olson et al. 1999). The synergies and complementary resistance profiles indicate that PRO 140 and small-molecule CCR5 antagonists may
represent distinct subclasses of CCR5 inhibitors. Phase I safety testing in healthy individuals examined doses ranging to 5 mg/kg. PRO140 was well tolerated in vivo, was non-immunogenic, and had a half-life of 18 days. Importantly, at the 5 mg/kg dose level, CCR5 lymphocytes were coated with PRO 140 for >60 days without cellular depletion (Olson et al. 2006). A phase Ib study is underway to examine the safety, pharmacokinetics and antiviral effects of single-dose intravenous PRO 140 in individuals with CCR5-tropic (R5) HIV-1 infection.

CCR5 specific: CCR5mAb004

CCR5mAb004 (Human Genome Sciences, Inc.) is a fully human IgG4 mAb to CCR5 that inhibits R5 isolates in a subtype-independent manner (Roschke et al. 2004). This mAb inhibits MIP-1ß binding to CCR5 and does not induce CCR5 signaling in the absence of chemokine. As expected for an IgG4 antibody, CCR5mAb004 does not mediate ADCC or CDC in vitro (Roschke et al. 2004).

A phase I study was performed in 63 HIV-1 patients with R5 virus who were randomized to receive a single intravenous infusion of placebo or CCR4mAb004 at doses ranging from 0.4 to 40 mg/kg (Lalezari et al. 2006). The antibody was well tolerated overall; however, infusion-related allergic reactions necessitated pre-medication with antihistamines at doses above 2 mg/kg. The serum half-life was 5-8 days, and >80% receptor occupancy was observed for 14-28 days with the higher dose cohorts. HIV RNA reductions of ≥1 log were observed at day 14 in 16 of 29 subjects treated with 8, 20 and 40 mg/kg, with corresponding mean HIV RNA reductions of 0.8-1.0 log10. The findings provide initial proof-of-concept for CCR5 mAb therapy of HIV-1 infection.

Potential advantages of therapeutic antibodies

Several factors make antibodies an attractive class of molecules for HIV-1 therapy. The most valuable feature of antibodies lies in their intrinsic nature: the immune system engineers them to recognize their target with high specificity and affinity. Polyclonal and more recently also monoclonal antibodies have been used for decades to treat a variety of conditions, with generally modest non-target toxicities (Sawyer 2000; Zeitlin et al. 2000; Reichert et al. 2005). Metabolic side effects, as observed with many small molecule drugs, are generally not seen due to the predictable catabolism of antibodies into naturally occurring amino acids. Additionally, unlike small-molecule drugs, mAbs do not passively diffuse across cellular membranes, reducing their potential for metabolic and other non-target toxicities. Therefore,
mAb therapy could be expected to offer an improved or at least non-overlapping side-effect profile compared to existing antiretrovirals. Unless selected to react with host moieties, mAbs with cross reactivity to host antigens are rare and usually can be excluded during pre-clinical development. Immunogenicity of human or humanized antibodies is commonly low allowing continued, high-dose application. Notably also, half-lives of humanized and human antibodies are in general considerably higher than those of small-molecule inhibitors (Table 2). All HIV-1 drugs currently in use have to be administered once to several times per day to maintain therapeutic levels. If drug adherence is not strict, resistant viral strains evolve rapidly. Antibodies, if provided as a component of ART, could be extremely beneficial in this regard. Due to their typically long serum half-lives, they could enable infrequent dosing that does not require daily vigilance on the part of the patient.

In addition, unlike mAbs, several of the currently used antiretrovirals (protease inhibitors, non-nucleoside reverse transcriptase inhibitors) are substrates or inducers of cytochrome P450 enzymes and thus can substantially perturb metabolic pathways (Cressey et al. 2006). Several of these drugs are associated with significant drug-drug interactions between antiretroviral drugs used in combination and between additional medications that the patient may require for other conditions (de Maat et al. 2003; Winston et al. 2005). Antibodies could offer advantages in this regard as they are not metabolized by cytochrome P450 enzymes, and could thus simplify the selection of combination treatment regimens. Lastly, mAbs may efficiently block protein-protein interactions or other targets that are not readily druggable with small molecules. For example, there presently are no licensed small-molecule drugs that target the CD4, CCR5, gp120 and gp41 epitopes recognized by the mAbs TNX-355, PRO140, 2G12, and 2F5/4E10, respectively. Such mAbs can be expected to inhibit viruses that are broadly resistant to the available antiretroviral therapies.

A potential drawback of antibody therapeutics is the cost of production. However, progressive advances in cell engineering and bioreactor operation have enabled these products to be manufactured efficiently in mammalian cells, and current processes often yield multi-gram per liter expression in chemically-defined medium (Butler 2005). A further limitation of mAb therapy is the need to deliver the product by injection for systemic application. However, infrequent, self-administered injections may provide an attractive alternative to daily pill regimens for many patients, and needleless delivery devices offer a means to further improve patient acceptance.
Theoretically, various clinical settings can be envisioned where a non-toxic, long-acting therapy could be beneficial (Box 1 and Table 1). Like other investigational drugs for HIV, antibody therapeutics are being developed for treatment of HIV-1 in combination with other antiretroviral agents as a component of ART, and antibody therapeutics have the potential to offer new treatment classes and additions to the armamentarium of HIV drugs. As with any new drug for HIV, antibody therapeutics initially may find the greatest use in treatment-experienced patients with fewer treatment options. However, as clinical experience increases, use of mAb therapies in earlier stage patients could be expected to increase as warranted by the safety and efficacy profile of the molecule.

Where available, ART of infected mothers has dramatically lowered transmission rates to offspring (De Cock et al. 2000; UNAIDS 2006). Antibody therapeutics have been suggested as a potential adjunct to ART therapy in mother-to-child-transmission (MTCT) as they could extend protection throughout the breast feeding period. Passive immunization to prevent MTCT has been considered for many years and may provide an option as not all HIV drugs are approved for pediatric use. In untreated mothers, transmission rates before and during birth are high and an almost equally high proportion of infant infections is thought to be acquired through breast feeding (De Cock et al. 2000; UNAIDS 2006). Although breast feeding bears the risk of HIV infection, abstaining from breast feeding in these settings is problematic as it can lead to malnutrition of the newborn and increase mortality due to other infections. Likewise, passive immunization or a combination of active and passive immunization as successfully employed against HBV infection (Kabir et al. 2006) could help to reduce transmission post partum.

Use of antibodies as topical microbicides may equally come in reach, particularly as technologies for the controlled release and local delivery of therapeutic antibodies have been developed and are in clinical use in other settings (Grainger 2004). As mentioned above, a potentially high impact of antibodies in therapy could be envisioned, if these antibodies target and destruct infected cells through activation of effector functions or by delivering immuno-toxins.
Box 1: Future perspective – What is needed to drive development of antibody therapeutics forward?

- Discovery of novel antibodies
  - Define new isolation and screening methods for HIV specific MAbs
  - Define new target epitopes
  - Isolate antibodies that neutralize divergent genetic subtypes with high activity
  - Define modes of action in vivo (neutralisation versus effector functions)
  - Define modes of transmission in vivo (free virus versus cell-to-cell)

- In vitro engineering of antibody characteristics to improve
  - Antiviral activity
  - Activation of effector functions
  - Stability and half-life
  - Immunotoxin design

- Probe antibody combinations
  - Multiple epitope specificities
  - Neutralizing and effector function inducing antibodies
  - Anti-viral and anti-cell antibodies

- Improve production, formulation, and delivery
  - Self-administered formulations
  - Needleless delivery
  - Controlled release and local delivery for topical and systemic application

- Probing clinical efficacy in relevant settings
  - In combination with optimized background ART
  - Mother to child transmission
  - Microbicide

Future perspectives
The current generation of antibodies has provided initial insights into modes and potential of antibody therapeutics and the accumulated knowledge provides a solid basis for further development (Box 1). Importantly, all antibodies tested to date have shown favorable safety profiles in man. In addition, compared to HIV-specific antibodies, antibodies to host receptors have shown more promising antiviral activity. CD4 and CCR5 mAbs currently are progressing through controlled clinical trials, and the results undoubtedly will add to our understanding of the potential role of antibody therapeutics in HIV.

To date, the antiviral effects seen for HIV-specific antibodies have been modest. For these to be effective, we will need novel antibodies with enhanced features (Box 1). The best characterized neutralizing antibodies, despite their comparatively broad activity, preferentially
Background

recognize subtype B isolates against which they originated. Novel scaffolds for epitope presentation or envelope structure mimetics that overcome the limitations of previous antigens used for vaccination are under development and are anticipated to foster the isolation of antibodies with novel specificities.

Impressive strides in antibody engineering have been made in recent years. Antibody affinity can be enhanced through methods of directed evolution (Carter 2006; Luginbuhl et al. 2006). Increased affinity may reduce the amount of antibody required and thereby improve the affordability and delivery of these products. For example, high potency may be a requirement for self-administerable, sub-cutaneous formulations of antibody. Directed evolution of antibody affinity is especially attractive for antibodies to invariant host receptors. Antibody engineering has been successful in enhancing effector functions by >2 orders of magnitude (Umana et al. 1999; Lazar et al. 2006) and in eliminating residual effector functions if desired (Hsu et al. 1999). Additional modifications can improve the serum half-life of antibodies and thereby reduce dose levels and intervals (Hinton et al. 2006). This tool-chest of technologies can be exploited to potentially optimize the efficacy, safety and convenience of antibody therapies for HIV.

Conclusion

Developing safe and effective antibody therapies for HIV infection is rich in challenge and opportunity. Any single approach to battling HIV bears a high risk of failure; however, this risk is spread amongst the diversity of targets and modalities for antibody therapy. Recent clinical trials have provided initial optimism for antibodies to host receptors and a foundation for further studies. The ultimate prospects for HIV-specific antibodies as therapeutic agents are less clear at present. But can we afford not to try this approach? At minimum, there is much that HIV-specific antibodies can teach us about vaccine design. By fully exploring the possibilities of antibody therapies for HIV, we might end up with both better vaccines and new therapeutics.

Acknowledgements

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Background


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Background


Background


Background


Background


Background


5. Results

5.1 Complement Lysis Activity in Autologous Plasma Is Associated with Lower Viral Loads during the Acute Phase of HIV-1 Infection


Complement Lysis Activity in Autologous Plasma Is Associated with Lower Viral Loads during the Acute Phase of HIV-1 Infection

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Abstract

Background

To explore the possibility that antibody-mediated complement lysis contributes to viremia control in HIV-1 infection, we measured the activity of patient plasma in mediating complement lysis of autologous primary virus.

Methods and Findings

Sera from two groups of patients—25 with acute HIV-1 infection and 31 with chronic infection—were used in this study. We developed a novel real time PCR based assay strategy that allows reliable and sensitive quantification of virus lysis by complement. Plasma derived at the time of virus isolation induced complement lysis of the autologous virus isolate in the
Results

majority of patients. Overall lysis activity against the autologous virus and the heterologous primary virus strain JR-FL was higher at chronic disease stages than during the acute phase. Most strikingly, we found that plasma virus load levels during the acute but not the chronic infection phase correlated inversely with the autologous complement lysis activity. Antibody reactivity to the envelope (Env) proteins gp120 and gp41 were positively correlated with the lysis activity against JR-FL, indicating that anti-Env responses mediated complement lysis. Neutralization and complement lysis activity against autologous viruses were not associated, suggesting that complement lysis is predominantly caused by non-neutralizing antibodies.

Conclusions
Collectively our data provide evidence that antibody-mediated complement virion lysis develops rapidly and is effective early in the course of infection; thus it should be considered a parameter that, in concert with other immune functions, steers viremia control in vivo.

Introduction
The humoral immune response to HIV-1 infection is elicited early in infection and is generally vigorous at later disease stages, but its efficacy and modes in controlling viremia in vivo have not yet been completely unraveled. While numerous studies suggest that neutralizing antibodies may impact viral replication in vivo [1-7], the effect of antibodies in mediating effector functions and in limiting viral spread via the complement system, phagocytic cells, or killer cells remains uncertain. A detailed characterization and quantification of the relative contribution of direct and effector-mediated antibody functions on virus containment in vivo will be of central importance in defining relevant immune responses and designing vaccines.

The complement system is a key component of innate immune defense, and it provides a link to the adaptive immune response [8-10]. Besides inducing direct lysis of pathogens, the complement system also has opsonizing, phagocytosis-inducing, chemoattractant, and immune stimulatory functions [8,11]. Specific antibody alone can efficiently neutralize many viruses, but complement activation can enhance the antiviral effects of antibodies by opsonizing virions or inducing lysis of the particles [11]. Complement is effective in lysing many enveloped viruses, resulting in fragmentation of the outer membrane and disintegration of the nucleocapsid [11].
Results

The role of complement in HIV pathogenesis appears to be multifaceted [12,13]. HIV-1 virions can activate complement via the classical pathway either by antibodies bound to the virus surface or, independent of antibody, by direct activation of complement through the viral envelope (Env) proteins gp41 and gp120 [14–17]. Direct lysis of HIV-1 upon antibody-mediated activation of the complement system has been demonstrated in vitro [18–20]. Complement has further been shown to boost the activity of neutralizing antibodies in vivo and in vitro [21,22], and non-neutralizing antibodies may play a role in containing viremia during the acute phase of infection by inducing direct complement-mediated viral lysis [23]. Opposing these observations, several reports have suggested that complement lysis of HIV-1 is limited in vivo by several host cell-derived complement control proteins incorporated by the HIV-1 virions [24–26]. Since antibody and complement efficiently lead to opsonization but not always to destruction of HIV-1, the virus may remain infectious [27]. In addition, opsonized virions were found to bind to complement receptor-expressing cells, which can enhance viral infectivity and transmission in vitro [12,13,28–31].

In the present study we sought to investigate the question of whether antibody-mediated complement lysis of HIV-1 contributes to virus containment in vivo and, if so, to quantify the relative contribution of this defense mechanism at different disease stages.

Methods

Patients and Virus Isolates

The study included 25 acutely infected and 31 chronically infected patients (Table 1). A control group of 11 healthy HIV-1 negative donors was recruited from volunteers affiliated with the University Hospital Zurich (Table S1). Patient demographics and selection and isolation of autologous virus have been described [32,33]. Written informed consent was approved by the ethics committee of the University Hospital Zurich and was obtained from all individuals (both infected and uninfected) according to the guidelines of the University Hospital Zurich.

Plasma

Patient blood was sampled in Vacutainer tubes containing EDTA (BD [http://www.bd.com]), and plasma was collected within 6 h and frozen in 1 ml aliquots at −75 °C. Plasma was heat inactivated (1 h at 56 °C) to destroy complement activity and centrifuged at 500g for 10 min.
before use to remove cell debris and lipids. Normal plasma and infected-patient plasma were treated identically.

**HIV-1 Virion Complement Lysis Assay**

A mix of sera from one to five healthy donors, stored at −75 °C, was used as source of complement.

HIV-1 virus stock (25 µl) was incubated with 20 µl of patient plasma (final dilution 1:5), 50 µl of complement (NHS final dilution 1:2), and 1 µl of RNase A (Qiagen, Valencia, California, United States) in RPMI 1640 (BioWhittaker [http://www.cambrex.com]) for a total volume of 100 µl; this mixture was incubated for 3 h at 37 °C (Figure 1). The reaction mixture was frozen at −20°C, thawed, and treated with RNase A (0.77 mg/ml, Qiagen) and DNase I (0.92 mg/ml, Roche [www.roche-applied-science.com]) for 1 h at 37°C in a thermoshaker (Eppendorf; 1,400 rpm). Samples were treated with protease (0.71 mg/ml, Qiagen) to remove RNase and DNase activity. Residual viral RNA in intact virions was extracted (RNeasy Mini Kit, Qiagen) and quantified by real-time PCR.

The extraction efficiency was controlled by adding and quantifying synthetic murine prion protein (PrP) mRNA (7,866 copies/µl) as an internal standard. In each assay, samples were tested in triplicate. Complement-mediated lysis activity was expressed as the percentage of lysed HIV-1 RNA copies compared to control plasma treatment. A mixture of plasma from 1-5 HIV-1-negative donors was used as negative control (no lysis activity, 0% value).
Results

**Figure 1. Schematic Overview of the Virion Lysis Assay**

(A) Primary HIV-1 virions were incubated with autologous plasma and complement and freeze-thawed once, and viral RNA was digested by RNase and DNase. After inactivation of RNase and DNase by protease and addition of an internal standard (PrP RNA), RNA was extracted and quantified by real-time PCR.

(B) HIV-1 virions sensitive to antibody-mediated complement lysis were disrupted, making viral RNA accessible for degradation.

(C) Viral RNA of complement lysis-resistant virions remained intact. RNA was extracted and could be quantified by real-time PCR.

**Real-Time PCR**

HIV-1 virions were quantified using primers either to Gag or to the 5' end of HIV-RNA. For detection, dual-labeled fluorescent probes with a fluorescein (FAM) moiety at the 5' ends and a tetramethylrhodamine (TAMRA) moiety at the 3' end were used. HIV-Gag was measured using primers s5'-gag (upstream; 5'-CAAGCAGCCATGCAAATGTTAAAAGA-3'), boe2 (downstream), and boe3 (probe) [35] for amplification and detection. HIV-5' RNA was measured using primer mf86 (5'-CCACACTGACTAAAAGGGTCTGAGGATCT-3'), cr1 (5'-TCTCTGGCTAACAGGGATCCACTGCTT-3'), cr2 (5'-TGACTAAAAGGGTCTGAGGATCTCTAGTTACCAG-3'), and mf74tq (FAM-5'-agcactcaagcaagcgtttgtgagc-3'-TAMRA). PrP mRNA was measured using PCR primers as previously described [36], and using a fluorescent probe prpe2+tq.
Results

(FAM-5'-CAACCGAGCTGAAGCATTCTGCCT-3'-TAMRA). PCR was performed as described previously [37,38] in a single-tube system (Qiagen 1-step RT-PCR) with an additional “hot-start” using AmpliWax (Applied Biosystems, Foster City, California, United States) to separate cDNA synthesis and PCR amplification steps. cDNA synthesis and subsequent amplification were performed in duplicate in a real-time thermocycler (i-Cycler [BioRad, Hercules, California, United States]) as described [37,38].

Anti-gp120, Anti-gp41, and Anti-p24 Plasma Antibody Titer

Plasma IgG titers to recombinant gp120 from the JR-FL strain (kindly provided by W. Olson, Progenics, Tarrytown, New York, United States), recombinant gp41 (amino acids 541–682 of the HxB2 strain [Viral Therapeutics, Ithaca, New York, United States]) and recombinant p24 (IIIB [Aalto BioReagents, Dublin, Ireland]) were determined by ELISA as described [32].

Plasma IgG titers to recombinant gp41 were determined as described for the other two antigens using plates coated with 0.1 μg of gp41 per well (amino acids 541to 682 of the HxB2 strain; Viral Therapeutics). Maximal binding to gp41 was defined using the antibody 2F5 (kindly provided by H. Katinger) as a reference. Detection of bound antibody and calculation of midpoint titers were done as described [32].

IgG Antibody Depletion

IgG was depleted from patient plasma using Protein G Sepharose beads (Amersham Pharmacia [http://www.amershambiosciences.com]). Beads were washed with PBS and incubated with patient plasma at room temperature for 1 h. Beads were removed by centrifugation and IgG-depleted plasma was taken from the supernatant.

Neutralization Assays

Neutralization activity of patient plasma against replication competent autologous primary virus isolates was evaluated on peripheral blood mononuclear cells as described [39]. The plasma dilution over 1:40 causing 90% reduction (neutralization titer, NT90) in p24 production was determined by regression analysis.

Neutralization activity of patient plasma against the heterologous virus isolate JR-FL was evaluated on TZM-bl cells (National Institutes of Health AIDS Research and Reference Reagent Program) using JR-FL Env pseudotyped virus as described [4,40]. The plasma dilution over 1:20 causing 50% reduction (NT50) in luciferase reporter gene production was determined by regression analysis.
Results

Effects of Active Complement on Antibody-Mediated Inhibition of Viral Infectivity

Inhibitory activity of patient plasma in the presence of active or heat-inactivated complement was evaluated using JR-FL Env pseudotyped virus on TZM-bl cells [4,40]. Patient plasma (1:5 or 1:25 for acute and chronic patients, respectively) and complement in the same ratios as for the HIV-1 virion complement lysis assay were preincubated with virus (TCID_{50} = 1,000 ml^{-1}) for 3 h at 37 °C without a subsequent freeze-thaw cycle (total volume, 60 μl). Plasma and complement content of the virus control were adjusted to the corresponding sample dilution with uninfected control plasma and active or inactivated complement, respectively. After preincubation, inhibition was measured in duplicates on TZM-bl cells at a final plasma concentration of 1:40 or 1:200 for acute and chronic patients, respectively.

Statistical Analysis

Data analyses were performed using Prism version 4.03 for Windows (GraphPad Software, San Diego, California, United States) and Stata SE/9.2 for Windows (Stata Corporation, College Station, Texas, United States). The normality assumption was checked using the D’Agostino and Pearson omnibus normality test and had to be rejected for most variables (unpublished data). Hence, nonparametric methods were employed for testing of group differences (Mann-Whitney U test and Wilcoxon signed-rank test for unpaired and paired testing, respectively). Correlation analysis was performed using Spearman’s rank correlation. All tests of significance were two-tailed and the level of significance was set at 0.05. P values reported in the main text and figures refer to values obtained after singular testing. Since multiple testing was performed caution must be taken in evaluating significance. In sum, 47 tests of significance were performed in our study, thus our level of significance was set at \( p < 0.00106 \) after Bonferroni correction to adjust for multiple testing. This stringent approach does not alter any of our main conclusions.

Results

Antibodies in Patient Plasma Mediate Complement Lysis Activity against Autologous Viruses

The primary intent of this study was to evaluate the influence of antibody- and complement-mediated lysis on viremia control in vivo. To this end we utilized an assay strategy that allows reliable and sensitive quantification of virus lysis by complement. In order to probe complement lysis activity under in vivo-relevant conditions, virus preparations used in our
study were derived from infected primary peripheral blood mononuclear cells, because these
cells are known to incorporate high numbers of complement control proteins, rendering these
viruses less susceptible to complement lysis than those produced on immortalized cell lines
[24]. In our assay, incubation of HIV-1 isolates with autologous patient plasma and
complement is followed by one freeze-thaw cycle to completely disrupt the complement-
attacked virions (Figure 1). Released RNA from lysed virions is digested by RNase treatment,
and the RNase is inactivated by protease digestion. Viral RNA from the remaining intact
virions is then extracted and quantified by real-time PCR. Using this assay scheme we were
able to detect substantial complement lysis activity directed against the autologous virus in
patient sera (Figure 2A). Patient plasma sampled at the time of virus isolation from a
chronically infected individual (patient 106) induced lysis of 71% of the heterologous virus
JR-FL in the presence of active complement. In the absence of active complement or of
patient plasma, no efficient lysis was observed (lysis < 15%) (Figure 2A). Likewise, plasma
from uninfected donors did not show specific lysis activity in the presence of active
complement.

We further validated our assay and ensured that freeze thaw-cycles by themselves do not
disrupt virions. As previously described, we found that neither the single freeze-thaw cycle
used in our assay nor repeated cycles (2–4) led to disintegration of the virus in the absence of
patient sera (Figure 2B) [41,42]. Irrespective of the isolate or plasma tested, a minimum of
10% of the virions appeared to be resistant to lysis. This resistant fraction may consist of viral
particles carrying mutated, no, or low numbers of Env proteins, which render them
insufficiently recognized by antibodies, or it may represent virus populations carrying high
numbers of complement control proteins as suggested previously [24,43]. Neither increasing
the plasma concentration nor blocking the complement control protein CD59 rendered this
fraction of virions fully susceptible to lysis (unpublished data).

To determine if the observed virolysis activity depends on antibodies, we depleted plasma
from two patients of IgG using Protein G Sepharose beads prior to incubation with virus and
complement (Figure 2C). Undepleted plasma of patients 117 and 113 showed complement
lysis activity of 40 and 36%, respectively. Depletion of IgG led to a substantial decrease of
the observed lysis to 15% and 19% lysis for patients 117 and 113, respectively. The lysis
activity observed after protein G treatment likely resulted from IgM antibodies and/or residual
IgG. In summary, our data suggest that substantial complement-mediated lysis activity against
HIV-1 virions may be present in a patient’s autologous sera and that a considerable proportion
of this lysis activity depends on IgG antibodies in a patient’s plasma.
Figure 2. Virolysis by Antibody and Complement

(A) Plasma from HIV positive individuals specifically lyses HIV in presence of active complement. Virus isolate JR-FL was incubated with complement (+), without complement (−), or with inactivated complement (Ci) either in the absence of plasma (−) or with plasma from uninfected individuals (NHP) or from patient 106. One of three independent experiments is shown.

(B) Up to four freeze-thaw cycles do not destroy intact HIV-1 virions. Virus JR-FL was incubated in the presence of active complement with medium (no plasma), with plasma from uninfected persons (NHP), or with plasma from patient 106. Reaction mixtures were subjected to the indicated number of freeze-thaw cycles and the effect on virus disintegration measured. One of two independent experiments is shown.

(C) IgG depletion reduces lysis activity. Plasma of uninfected individuals (NHP) and of patients 117 and 113 were depleted of IgG with Protein G Sepharose beads (darkened bars) and lysis activity was compared to untreated plasma (open bars). Error bars indicate standard deviation of triplicate measurements. Groups were compared using Mann-Whitney U test.
Results

Complement Lysis Activity in Plasma of Acutely and Chronically HIV-1-Infected Individuals against Autologous Virus

To explore the impact of antibody-mediated complement virus lysis at different disease stages we measured patient plasma complement virolysis activity against autologous virus in a previously described cohort of 25 acutely and 31 chronically infected patients (Figure 3A and Table 1) [32,33]. Lysis activity against the autologous virus was measured in plasma samples derived at the time of virus isolation. The fact that the extent of lysis measured in our assay is independent of the amount of virus input (Figure S1) allowed us to compare the activities of multiple divergent virus isolates without normalizing virus input. The latter process can be prone to error, because absolute quantification of highly divergent virus stocks is subject to sequence variation and the resulting differences in RNA or p24 detection.

Virus input ranged from $4.8 \times 10^3$ to $6.3 \times 10^7$ viral RNA copies. In two patients with very high viral load (patients 018 and 022), virus detected in the plasma contributed over 1% to the total amount of HIV-1 copies measured in our assay. Therefore, patient plasma and heat-inactivated complement were used as negative control in these cases.

Autologous plasma induced complement lysis of the respective virus in the majority of infected patients (Figure 3A). Nineteen of 25 acutely infected and 30 of 31 chronically infected individuals showed specific lysis activity (over the 95% confidence interval of normal controls, i.e., more than 5.8% lysis). Complement lysis activity was highly variable between patients and ranged from 90% lysis to no lysis at a plasma dilution of 1:5. The median of complement lysis was almost twice as high during chronic disease stages (37.63% lysis) than during the acute phase (19.89% lysis, p = 0.001, Mann-Whitney U test) (Figure 3A and Table 2).
Table 1: Patient Characteristics, Complement Lysis Activity, Antibody Titers, and Neutralization Titers

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</tr>
<tr>
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<td>60</td>
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<td>293</td>
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\(^{a}\) Viral load measured as HIV-1 RNA copies per ml blood at the day of virus isolation or the geometric mean (indicated by b) of the two closest time points before and after are shown.

\(^{b}\) Heterologous lysis activity was assessed against the virus strain JR-FL.

\(^{c}\) Neutralization activity was assessed against the respective autologous primary isolate in a peripheral blood mononuclear cell-based assay.

\(^{d}\) Heterologous neutralization activity was assessed against the reporter gene virus pseudotyped with the envelope of the virus strain JR-FL in a cell line based assay.
### Table 2: Group comparisons of measured parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit of Analysis</th>
<th>Subgroup name</th>
<th>Subgroup 1</th>
<th>Subgroup 2</th>
<th>p-Valuea</th>
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<td></td>
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<td>n</td>
<td>Median</td>
<td>IQR</td>
<td>n</td>
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<td>Virolysis HIV⁺ (%) b</td>
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<td>19.89</td>
<td>4.76-36.61</td>
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<td>Heterologous</td>
<td>25</td>
<td>14.70</td>
<td>5.10-24.52</td>
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<td>-9.12-7.69</td>
<td>25</td>
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<td>19.89</td>
<td>4.76-36.61</td>
<td>25</td>
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<td>37.63</td>
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<tr>
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<td>Anti-gp41</td>
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<tr>
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<td>42.05</td>
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<td>Complement</td>
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1Mann-Whitney U test unless otherwise stated
2Figure 3
3Figure 3
4Wilcoxon signed-ranks test.
5Figure 6
6Figure 7

IQR, interquartile range; NA, not available
Results

Complement Lysis Activity in Plasma of Acutely and Chronically HIV-1-Infected Individuals against the Heterologous Strain JR-FL

Antibody responses to HIV-1, particularly during early disease stages, are thought to be predominantly strain specific. To investigate whether the antibodies that mediate complement lysis activity are specific for the autologous virus isolate or if cross-reactivity with heterologous isolates exists, we evaluated the capacity of our panel of patient plasmas to mediate lysis of the heterologous primary virus strain JR-FL. All plasma samples derived from chronically infected individuals, and 19 of 25 samples derived from acutely infected individuals mediated lysis of the virus strain JR-FL (Figure 3B). In agreement with the pattern seen for autologous virolysis, the median of lysis activity against JR-FL was almost three times as high in plasmas derived from chronically than from acutely infected patients (p < 0.0001, Mann-Whitney U test) (Table 2). In comparison, plasma from uninfected healthy controls (n = 11), induced no or only marginal lysis that was lower than lysis activity seen in the acute group (p = 0.003, Mann-Whitney U test) (Table 2), indicating that virolysis in infected patients is predominantly mediated by HIV-1-specific antibodies.

In a further analysis, autologous and heterologous lysis activities were assessed within each group and then separately in the combined cohort. In general, plasma lysis activity against heterologous and autologous viruses did not differ in the acutely or the chronically infected group. The analysis of the entire patient cohort also revealed no differences in lysis activity (Table 2). Although the overall pattern of reactivities against heterologous and autologous viruses was similar, with plasma from chronically infected patients inducing higher activities than did plasma from acutely infected individuals, we found no evidence for a correlation between the lysis activity against the autologous and heterologous virus strains (rho = 0.03, p = 0.87 for acute; rho = 0.19, p = 0.30 for chronic group) (Table 3), indicating that the type and specificities of antibodies mediating these activities may not completely overlap.
Table 3: Correlation Analyses

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<th>Parameter 2</th>
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<th>Rho^</th>
<th>p-Value</th>
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<td>Heterologous lysis chronic group</td>
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<td>Anti-gp120 titer versus</td>
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<td>0.76</td>
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</table>

^aSpearman's rank correlation
^bResults shown in Figure 4A.
^cResults shown in Figure 4B.
^dResults shown in Figure 4C.
^eResults shown in Figure 4D.
^fResults shown in Figure 8.
NA, not available
Results

Figure 3. Lysis Activity against Virus in Plasma of Acutely and Chronically Infected Patients

Complement-mediated lysis activity against autologous (A) and heterologous (JR-FL) (B) virus was compared between acutely (blue triangles) and chronically (green circles) infected individuals. HIV specific lysis activity is present at acute and chronic diseases stages but generally higher during chronic infection. Crosses denote uninfected plasma controls. Groups were compared using Mann-Whitney U test.

Analysis of the Anti-Env and Anti-Gag Responses

In the course of HIV-1 infection, commonly a strong, high-titered antibody response to the structural viral proteins (Env and Gag) is elicited [44–46]. Complement lysis of HIV virions depends on antibodies that interact with the viral surface, and thus most likely involve antibodies that recognize the viral envelope proteins gp120 and gp41. It has been shown that the majority of Env-specific antibodies recognize epitopes that are inaccessible in the native oligomeric, fusion-competent form of the virus envelope [47–52]. Antibodies that bear neutralizing activity bind only to selected epitopes on the viral envelope oligomer that are involved in the interaction with the viral receptors or fusion. In contrast, antibodies that
activate complement could be theoretically directed to any epitope accessible on the virion surface. To obtain an initial insight into which classes of anti-HIV antibodies mediate the complement lysis activity in patient plasma, we determined antibody responses to the viral core protein p24 and the Env proteins gp120 and gp41 with recombinant proteins derived from the viral strains JR-FL (gp120) and IIIB (p24 and gp41) (Table 1) [32]. As expected, all antibody responses were lower in acute than in chronic infection (Table 2) [32]. Since we had no recombinant gp41 from strain JR-FL available, we used the gp41 protein of the closely related IIIB strain (89.2% sequence homology at the amino acid level) to measure anti-gp41 responses in patient plasma. We found that anti-gp120 and anti-gp41 titers were associated in both the acute (rho = 0.53, p = 0.007) and the chronic patient groups (rho = 0.71, p < 0.0001) as well as when all patients were analyzed together (rho = 0.90, p < 0.0001), indicating that anti-gp41 and anti-gp120 responses may develop in parallel (Table 3 and Figure 4A). Anti-Env responses did not appear to be correlated with anti-p24 responses during the chronic phase (unpublished data), suggesting, as previously found [39], that anti-Env and anti-Gag responses are differentially regulated. During the acute phase, anti-gp120 and anti-p24 correlated significantly (rho = 0.76, p < 0.0001), probably reflecting the parallel maturation of the immune response against all epitopes during this stage.
Results

Figure 4. Correlation Analysis of Antibody Responses and Virolysis Activity

(A) Correlation analysis of anti-gp120 and anti-gp41 antibody titers in plasma samples from the acute and chronic patient groups shows that titers to the envelope glycoprotein correlate tightly independently of disease stage.

(B–D) Correlation analyses of heterologous lysis (JR-FL) with anti-gp120 (B) or anti-gp41 (C) revealed positive correlations, whereas anti-p24 (D) antibody titers did not associate with heterologous lysis. This suggests that lysis activity is driven by envelope specific antibodies.

Blue triangles denote acutely, green circles denote chronically infected patients. Spearman’s rank correlation coefficient (rho) and p-values are depicted for the entire cohort (all), the acute patients (a), and the chronic patients (c). If antibody titers were below 1, the value 1 was used for statistical analysis.

Impact of Anti-Env Responses on Complement Virolysis

In order to probe the impact of anti-Env antibody responses on viral lysis activity, we first analyzed the interdependency between lysis activity against JR-FL and antibody titers to the gp120 protein of this strain. We found a strong correlation between anti-gp120 titers and lysis activity when we analyzed the entire patient cohort (rho = 0.87, p < 0.0001) (Figure 4B and Table 3). This association between anti-gp120 titers and lysis activity was more pronounced in chronically infected individuals (rho = 0.69, p < 0.0001) than in acutely infected patients (rho = 0.41, p = 0.04). Likewise, we observed a strong association between JR-FL lysis...
activity and anti-gp41 antibody titers when all patients were analyzed together (rho = 0.83, p < 0.0001) (Figure 4C). When groups were analyzed individually, the association in the chronically infected patients alone was weaker (rho = 0.58, p = 0.0006) and no interdependency was evident in the acute infection group (rho = 0.38, p = 0.06). In contrast to the anti-Env responses, antibody titers to the core protein p24 exhibited no pronounced influence on the heterologous lysis activity induced during acute and chronic infection (rho = 0.37, p = 0.005 for the entire cohort) (Figure 4D). Taken together, our analysis strongly suggests that anti-Env responses are central in mediating complement lysis activity during both acute and chronic disease stages.

Antibody titers against the autologous virus strains could not be determined. Due to the heterogeneity of virus isolates in our panel and the resulting variable sequence divergence in the recombinant proteins used to determine antibody titers, it was unlikely that we would see the same degree of association between autologous lysis activity and binding activity to the recombinant proteins. Nevertheless, a trend for a positive association between gp120 binding titers and lysis activity was observed in acutely infected individuals (rho = 0.47, p = 0.02). However, no evidence for an association was seen in chronically infected individuals (rho = -0.09, p = 0.63). Equally, no association between lysis activity to the autologous strain and reactivity to gp41 was seen in either patient group (rho = 0.19, p = 0.37 and rho = -0.14, p = 0.46 for the acute and chronic group, respectively). In accordance with the interdependency of anti-Gag and anti-Env responses during the acute phase, we observed a trend for a weak correlation between p24 responses and the autologous lysis activity in acute patients (rho = 0.41, p = 0.04).

**Longitudinal Assessment of Autologous Complement Lysis Activity in Patient Plasma**

Our cross-sectional analysis suggested that complement lysis activity is mediated by anti-Env responses and may increase when the antibody response broadens. To investigate the development of the lysis activity more closely, we monitored antibody responses and complement autologous lysis activity over extended time periods in plasma of six acutely HIV infected patients (Figure 5). Of these six individuals one stayed treatment-naive (patient 022). The remaining five patients initiated antiretroviral treatment ART during the acute infection phase and subsequently stopped treatment after 12–31 months on successful antiretroviral treatment. Lysis activity was measured against the autologous strain derived at the first specimen collection. Antibody reactivity and lysis activity were subsequently measured solely during the treatment-free periods.
As expected, titers of antibody to gp120 and gp41 increased steadily in all patients, which was paralleled by an increase in lysis activity. In the five patients who underwent treatment interruption it is evident that along with viral load levels, antibody responses and lysis activity rose. A direct impact on in vivo viral load levels cannot be easily investigated in this setting, since upon rebound it takes several weeks to months before set points of viral load and immune responses are reached. Collectively, our observation strongly suggests that early antibody responses against the viral Env proteins gp41 and gp120 mediate lysis activity against the autologous virus strain.

**Figure 5. Longitudinal Assessment of Autologous Complement Lysis Activity in Patient Plasma**

Lysis activity against autologous virus (red circles) of plasma from six acutely HIV-1-infected patients was measured longitudinally and plotted against anti-gp120 titers (orange triangles), anti-gp41 titers (blue squares), and viral load (asterisks). At the first data point, patients were treatment-naïve and acutely HIV infected. All patients (except patient 022) subsequently went on antiretroviral therapy (ART) for the indicated time periods. Time point 0 was assigned to the date of treatment interruption and the remaining time points were calculated according to this time point. (A) Patient 022 (treatment-naïve), (B) patient 015, (C) patient 003, (D) patient 016, (E) patient 026, and (F) patient 002.

**Plasma Neutralization Activity**

Neutralizing antibodies directed against the autologous HIV strain can appear during the acute infection phase [4,5], but are in general more pronounced during chronic disease stages [1,6]. A central question in our analysis, therefore, was to investigate whether neutralizing
antibodies are elicited alongside complement activating antibodies or if the latter precede the neutralizing response. To this end we evaluated the neutralizing activity mediated against autologous and heterologous virus in our patient cohort.

Only six (24%) of the plasmas from the 25 acutely infected patients and 14 (45%) from the group of 31 chronic patients showed measurable autologous neutralization activity (NT90 > 40) (Figure 6A and 6B). A trend for a somewhat higher autologous lysis activity was observed among chronically infected patients whose plasma had neutralizing activity against the autologous viruses (p = 0.02, Mann-Whitney U test) No such difference was seen in the acute group (p = 0.13) (Table 2). Likewise, we observed no correlation between autologous lysis and neutralization activity in either group (Table 3). The latter could imply that non-neutralizing antibodies contribute predominantly to the lysis activity. However, we cannot exclude the possibility that neutralizing antibodies are present at concentrations below the detection level of the neutralization assay and that low concentrations of these antibodies could suffice to induce virolysis. Nevertheless, neutralizing activity per se is low at best in these patients and thus is unlikely to drive viremia control.

Although none of the patient plasmas from the acute group showed neutralizing activity against the heterologous virus strain JR-FL (Figure 6C), 15 (48%) of the chronically infected patients neutralized this virus (NT50 > 20) (Figure 6D). Heterologous lysis activity was again higher among chronically infected patients with neutralizing activity (p = 0.02, Mann-Whitney U test) (Table 2). A marginal correlation between heterologous lysis and neutralization activity was observed in the chronic group (rho = 0.49, p = 0.005) (Table 3), indicating that antibody reactivities involved in neutralization and lysis of heterologous HIV-1 virions may overlap to some extent.
Results

**Figure 6. Neutralizing Antibodies Are Not the Major Constituent of Complement Lysis-Inducing Antibodies**

Complement lysis activity of non-neutralizing and neutralizing patient plasmas was compared in the acute and the chronic infection cohorts. Autologous neutralization (A and B) and heterologous (JR-FL) (C and D) neutralization are shown, with blue triangles denoting acutely, green circles chronically infected patients. Each data point represents the mean of two or three independent experiments with plasma from the same patient. Groups were compared using Mann-Whitney U test.

**Complement Lysis Leads to Reduction in Viral Infectivity**

Since our virus lysis assay measures complement destruction under nonphysiological conditions (freeze-thaw cycle and RNA digestion) we investigated whether virion lysis occurs also under natural conditions and leads to a reduction of viral infectivity. To circumvent nonspecific inhibitory or enhancing effects of human plasma in our in vitro assay, we chose assay conditions in which controls contained the corresponding concentration of normal human plasma and complement. To be able to compare effects of antibodies in the absence of active complement (neutralization) and inhibition induced in the presence of active
Results

complement (neutralization and complement lysis), we chose plasma dilutions that allowed simultaneous evaluation of both effects. Thus, patient plasmas from the acute group, all of which had marginal neutralization activity against the isolate JR-FL, were studied at a dilution of 1:40; chronic patients, whose plasma generally had higher neutralization activity, were studied at a dilution of 1:200. Each patient group was assessed separately, which allowed us to use two different plasma dilutions. The latter was necessary, because otherwise neutralization activity would have dominated the readout in the chronic group. In the majority of patients the presence of active complement increased the inhibitory effect of the patient plasma (Figure 7). Median inhibition was markedly lower when complement was inactivated, both in the acute (p < 0.0001, Wilcoxon signed-rank test) (Table 2) and the chronic group (p = 0.0009), demonstrating that complement lysis reduces viral infectivity.

![Graph showing influence of complement on viral infectivity](image)

**Figure 7. Influence of Complement on Viral Infectivity In Vitro**

Inhibitory activity of patient plasma against the heterologous virus JR-FL was measured in presence of active (C+) or heat-inactivated (Ci) complement on TZM-bl cells. Blue triangles denote acutely, green circles chronically infected patients. Data points are means of two independent experiments with plasma from the same patient. Differences in inhibition between inactivated and active complement within the subgroups were compared using Wilcoxon signed-rank test. The results demonstrate that complement increases the inhibitory activity of HIV specific antibodies in vitro.
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Impact of Complement Lysis on Viremia Control In Vivo

Both HIV-1-specific T cell activity and somewhat delayed humoral immune responses develop early in HIV infection. The initial rise in cytotoxic T lymphocytes (CTL) responses is often associated with a decline in viremia shortly after infection [53]. Although neutralizing activity of antibodies in patient sera during the acute phase can be found [4,5], it is not clear to what extent these antibodies contribute to viral containment. Even less is known about the effects of complement lysis mediating antibodies in vivo. Since our analyses thus far had clearly shown that complement-activating antibodies are elicited soon after infection, we next investigated whether these antibodies have a clear impact on viremia control in vivo. To this end we probed whether interdependencies existed between autologous or heterologous lysis activities and the plasma viral load measured at the time of plasma and virus collection.

We found that lysis activity against autologous plasma inversely correlated with the in vivo viral load (RNA copies/ml of plasma) in the acute group (rho = -0.72, p < 0.0001), but not in the chronic group (rho = 0.18, p = 0.33) (Figure 8 and Table 3). Our assessment of the in vivo activity of the viral lysis activity solely focuses on the potential contribution of the HIV-specific antibodies in the patient plasma. In vivo lysis activity may further vary due to variations in complement activity influenced by different genetic backgrounds and disease progression. No interdependency between CD4 levels and virolysis activity or virus load existed in the acute cohort (unpublished data). In contrast to autologous lysis activity, heterologous lysis activity and viral load did not correlate, indicating that isolate-specific antibodies may dominate virolysis activity in vivo. Antibody titers to the viral proteins gp120, gp41, and p24 showed no correlation with the viral load in both the acute and the chronic cohorts (all rho values ≤ 0.34, p ≥ 0.09) (Table 3). Hence, the inverse association between autologous lysis activity and viral loads during the acute phase does not simply reflect the appearance of binding antibodies during this period in these patients. Taken together our data demonstrate that increased antibody-mediated complement lysis coincides with lower viral loads in the acute phase. Therefore, complement lysis could potentially function as an early immune defense mechanism against HIV-1 that impacts on viremia control in the acute infection phase, during which the adaptive immune response has not yet fully matured.
Results

Figure 8. Correlation Analysis of Complement Virolysis and In Vivo Viremia Levels
In vitro-determined autologous virolysis activity was correlated with in vivo HIV-1 RNA copies measured per milliliter of plasma in the acutely infected group (A) and the chronically infected group (B). Correlations were evaluated using Spearman’s rank correlation. Our data demonstrate that increased antibody-mediated complement lysis coincides with lower viral loads in the acute phase. No evidence was found for a similar correlation in the chronically infected group.

Discussion
In recent years substantial effort has been put into investigating the humoral immune response to HIV-1. While neutralizing antibodies are considered a correlate of protection against HIV-1 and a necessary component of vaccine-induced immune responses, the role of effector mechanisms mediated by anti-HIV antibodies in immune control remains largely unclear. In the present study we investigated the efficacy of the humoral immune response elicited during acute and chronic disease stages in inducing complement-dependent lysis of HIV virions. Evidence obtained through a novel complement virion lysis assay suggests that antibody-mediated complement lysis in the plasma of HIV-1-infected individuals has been underestimated in the past. Previously used virolysis assays mostly relied on the measurement of reverse transcriptase [54] and p24 antigen released from lysed virions [24,25,55], which limited their use in measuring complement lysis activity directly in patient plasma because antibodies specific for reverse transcriptase and p24 can interfere with the detection of these proteins following virolysis. The development of a novel, highly sensitive and quantifiable assay for virolysis-mediating antibody responses in plasma samples was thus key for the current study, and it allowed us to investigate complement lysis in patient samples directly by quantifying viral RNA.
Results

We found that, in most patients, antibodies are elicited very early after infection (< 3 mo) that induce complement-mediated lysis of the autologous virus and thus could contribute to viremia control during the acute phase of HIV-1 infection. The latter is signified in our study by a tight inverse correlation between lysis activity and in vivo-measured viral loads. Our results corroborate the findings by Aasa-Chapman and coworkers, who recently reported complement lysis activity in sera of some acutely infected individuals [23]. In our study, lysis activity was in general higher during chronic infection, probably reflecting the elevated antiviral antibody responses in later stages of the infection. Unlike in acute infection, however, no association between complement lysis activity and the viral set point was seen in chronically infected patients. Virolysis, if active in vivo, should destroy virions in both disease stages and even more so during chronic infection, as the in vitro-measured activity is higher then. A possible explanation of these contrasting observations in acute and chronic infection is that complement lysis-activating antibodies may be important only early in infection, when other adaptive immune responses have not yet fully matured. In chronic infection the overall influence of these complement-activating antibodies may be still present, but their impact lower or negligible because neutralizing antibody and cellular immune responses have matured. Thus the effect of virolysis on viral load levels may only be measurable during the acute phase, because during later stages of the infection other immune functions have developed that are more powerful and therefore determine the viral set point. Virolysis activity, while still present, would then have a comparatively small effect on in vivo virus loads.

In support of the differences between acute and chronic group we found that, particularly during the acute phase, complement lysis appeared to be predominantly mediated by non-neutralizing antibodies. Lysis activity against the heterologous strain JR-FL was observed despite the absence of neutralizing antibodies against this virus in acutely infected patients. Likewise, lysis and neutralization activity against the autologous viruses showed no interdependency. Although in the chronic cohort higher lysis activity was found in patients that harbored neutralizing activity, we observed no direct relationship of these reactivities against the autologous virus, and only a minor influence in the heterologous system. Nevertheless, we cannot exclude the possibility that neutralizing antibodies at concentrations lower than the detection limit of the neutralization assay are present and contribute to virolysis activity.

The role of complement in HIV pathogenesis has been a matter of debate for many years. While several reports have proposed that complement-dependent virus lysis occurs in vivo
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[43,56], and that complement can boost the effect of neutralizing antibodies both in vivo and in vitro [21–23], other groups have demonstrated a pronounced resistance of HIV to complement lysis in vitro [24–26,55,57,58]. Moreover, opsonization of virus with antibodies and complement has been shown to enhance viral infectivity and transmission in vitro [12,13,28–31]. Which of these complement activities occur in vivo and what their relative contributions are in inhibiting or enhancing viral replication, remain currently unknown. Whether complement lysis activity is outweighed by more specific adapted immune responses in chronic infection, as suggested above or whether at later disease stages enhancing effects of antibodies and complement emerge and reduce the impact of lysis activity are questions bearing further investigation.

The factors that direct complement action to either lysis or enhancement have not been fully defined. While our study demonstrates inhibitory effects of complement and antibody in early HIV infection, complement-dependent enhancement of HIV infection of complement receptor-bearing cells likely occurs as well in vivo. Complement concentration may vary at different disease stages [43,59–62]; thus, in vivo, individual lysis activity could differ due to fluctuations in complement supply.

Moreover, complement concentrations in tissue are only in the range of 10%–20% of the levels in serum [63–65]. Thus the relative contribution of virus lysis and enhancement in tissue and in the periphery may differ and needs to be investigated. Further studies will be needed to unravel whether the beneficial or the detrimental effects of complement and antibody dominate in vivo.

It is currently also not known whether antibodies that mediate complement lysis and enhancement are directed to the same epitopes. Nevertheless, it seems feasible that their reactivities overlap to some extent. Our data indicate that if enhancing activity impacts on virus replication in vivo, it appears not to be a direct consequence of high antibody titers, as we observed no notable interdependency between binding antibody titers to HIV and viral loads. We demonstrated, however, that also under culture conditions, complement in concert with HIV-1-specific antibodies reduces viral infectivity, which further supports a role for virus lysis in vivo.

We determined in our study that complement lysis activity is mediated by anti-Env antibodies, as demonstrated by the tight correlation between lysis activity and antibody titers to gp120 and gp41. While neutralizing antibodies are known to recognize native viral Env oligomer epitopes that are involved in receptor binding and fusion, complement-activating antibodies are not limited to these sites. Additionally, non-neutralizing antibodies binding to
the oligomer, or antibodies reacting with gp120 monomers or with epitopes on gp41 that are exposed after shedding of gp120, could potentially activate complement if bound to the virion in sufficient densities.

A more detailed characterization of the antibodies mediating lysis activity will be particularly important if complement-activating antibodies are to be considered a component of effective vaccines.

Our finding that HIV-1 is susceptible to lysis mediated by specific antibodies in patient sera and complement is in agreement with a previous study by Sullivan and coworkers, who showed that virus derived from patient plasma can be lysed in the presence of complement due to virion-bound antibodies that activate the complement system [43]. While this study provided initial evidence that complement lysis of HIV-1 may occur in vivo, it was limited to the analysis of patient samples with high viral load due to the inherent insensitivity of the assay used. The range of lysis activity found in this ex vivo analysis (14%-86% lysis) corresponds closely with the values obtained in our study. The profound complement lysis seen in our study and, most importantly, the inverse correlation between lysis activity and in vivo viral loads during the acute phase strongly suggest that antibody-mediated complement lysis could contribute to viremia control and may therefore be a defense mechanism in vivo.

Several recent reports have emphasized that complement activation boosts humoral and cellular immune responses [8]. Consequently, complement-stimulating antibodies, besides mediating direct lysis of virions, might have an important function in aiding the development of immune responses to HIV both in natural infection and in responses to vaccines.

While our study demonstrates that presence and magnitude of autologous antibody-mediated complement lysis of HIV-1 coincide with increased viremia control during the acute infection phase, direct associations cannot be formally proven. Immune functions and the timing of their appearance during the course of early HIV-1 are intertwined, and it is therefore difficult to ascertain direct relationships. Activities of cytotoxic T cells, neutralizing antibodies, antibodies that mediate antibody dependent cellular toxicity (ADCC), opsonization, aggregation, phagocytosis and—as our current report suggests—antibodies that mediate virus lysis via activation of the complement system will impact on viral spread in vivo. Total or neutralizing antibody titers or CTL activity, however, have not been proven to reliably predict viremia levels in the past [39,66–69]. While cellular immune responses were not assessed in our study, the fact that virolysis activity induced by complement and antibody inversely correlated with in vivo viral loads, but not with binding antibody titers per se nor with neutralizing activities, leaves room for a scenario in which virolysis has an impact on viral
load levels in vivo. Nevertheless, subsequent studies will be required to unravel the exact associations between these diverse immune functions and to assess their individual impact. Based on our current study, we hypothesize that complement lysis activity induced by specific antibodies may be an additional player in the network of immune function countering HIV replication in vivo and that, therefore, the impact of these antibodies in vivo should be further evaluated, as they could be a critical component of vaccine-induced immunity to HIV-1.

Supporting Information
Figure S1: The Extent of Lysis Is Independent of the Amount of Virus Input
The varying amounts of RNA input into the autologous assays were correlated with measured autologous virolysis activities. Correlation was evaluated using Spearman’s rank correlation (n = 56, rho = −0.09, p = 0.53, two-tailed).

Table S1: Control Group Demographics

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Author contributions
MH, MF, HFG, and AT designed the study. MH, MF, VvW, and AT analyzed the data. RW and HFG enrolled patients. MH, MF, AM, HK, RW, VvW, HFG, and AT contributed to writing the paper. BM developed the assay for the determination of GP41 antibody titers, determined all the antibody titers (anti-gp41, anti-gp120, and anti-p24), and analyzed these data. AM provided an experimental contribution to assays. HK performed virus isolations and neutralization assays and determined plasma IgG titers. BN performed RNA extractions and real-time PCRs. RW contributed to the design of the clinical studies and collection of patients’ data. HFG designed and conducted the clinical studies forming the base for this laboratory work and was significantly involved in the overall design of this investigation.

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Editors' Summary

Background.
If untreated, most people who become infected with the human immunodeficiency virus (HIV) eventually develop acquired immunodeficiency syndrome (AIDS). Over time, HIV infects and kills their CD4 T lymphocytes—immune system cells that stimulate B lymphocytes to make antibodies (proteins that recognize and destroy infectious agents) and that help CD8 T lymphocytes to kill cells that contain viruses and bacteria. The loss of CD4 T lymphocytes—a central player in “adapted immunity”—leaves patients very susceptible to infections. However, the immune system does not die quietly. It does its best to fight HIV infection by mounting a cell-mediated immune response in which T lymphocytes attack HIV-infected cells. It also mounts a “humoral” immune response in which antibodies that recognize HIV are made. Some of these are neutralizing antibodies, which prevent HIV entering its host cells and replicating. Other antibodies may limit viral spread by inducing destruction of the virus. One way they can do this is by activating another part of the immune system called the complement system, which can break open and kill viruses (this is known as antibody-mediated complement lysis). In addition, antibodies and complement can coat the HIV virus particles so that phagocytes (for instance macrophages - yet another type of immune system cell) engulf and destroy the virus.

Why Was This Study Done?
The role that humoral immunity plays in fighting HIV infection is complex and poorly understood. In particular, it is not clear whether the complement system helps to stop the spread of HIV or whether it inadvertently helps it to spread by facilitating its entry into host cells. It is important to understand as much as possible about the humoral immune response to HIV infection so that vaccines can be designed to provide maximum protection against HIV. In this study, the researchers have investigated whether antibody-mediated complement lysis controls the amount of virus in the blood of patients infected with HIV.
Results

What Did the Researchers Do and Find?
The researchers collected plasma (the liquid part of blood that contains circulating antibodies) from patients recently infected with HIV (acute infection) and patients who had been infected for some time (chronically infected). They also isolated HIV from each of the patients—so-called autologous virus. They then used a sensitive molecular biology assay to test each plasma sample for its ability to lyse the autologous virus (and also a standard virus) when supplied with complement from a healthy donor. Most of the plasma samples were able to lyse HIV, although the samples taken from chronically infected patients generally caused more lysis than those from acutely infected patients. In the chronically infected patients, the level of lysis induced was not related to the amount of virus in the patients’ blood (viremia). However, plasma taken from acutely infected patients with higher viral loads was less active in the lysis assay than plasma taken from patients with lower viral loads. Finally, the researchers showed that the levels of antibodies in the various plasma samples to the two envelope proteins of HIV correlated strongly with the ability of each sample to lyse the standard virus and that these antibodies were mainly non-neutralizing antibodies.

What Do These Findings Mean?
By showing that antibody-mediated complement lysis of HIV in the laboratory is inversely related to the patients’ viral loads during acute infection, these findings suggest (but do not prove) that antibody-mediated complement lysis of HIV contributes to the control of viremia early in HIV infections. But, the importance of this form of humoral immunity in combating HIV infections remains uncertain, since complement has the potential to enhance as well as block viral spread. Further work is needed to unravel which of these effects is dominant in patients and to characterize fully the antibodies that activate complement. Nevertheless, the results of this study suggest that complement-activating antibodies should be considered in future attempts to design an effective HIV vaccine.

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5.2 Potent HIV-Neutralizing and Complement Lysis Activity of Antibodies Are Not Obligatorily Linked


Potent HIV-Neutralizing and Complement Lysis Activity of Antibodies Are Not Obligatorily Linked

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Abstract

To evaluate the potential contribution of complement-mediated lysis to the \textit{in vivo} activity of neutralizing antibodies, we analyzed complement activity in patient plasma collected during passive immunization with the neutralizing monoclonal antibodies 2G12, 2F5 and 4E10, which were infused upon cessation of ART in these patients. We found that administration of monoclonal antibodies led to an immediate, high activation of the complement system already upon the first application in the absence of viremia. We previously determined that the monoclonal antibody 2G12 had an impact on delaying or suppressing viral rebound in a
subset of the 14 patients. Analysis of complement activity revealed neither an impact of complement lysis nor complement-dependent enhancement on determining the viral set points in responding and non-responding patients. Overall complement lysis activity increased upon viral rebound in parallel with the boost of the autologous antibody response. No evidence of an in vivo effect of the neutralizing antibodies on lysis activity was found as lysis activity remained elevated after washout and no association between in vivo levels of the monoclonal antibodies and virolysis was detected. To verify our ex vivo analysis we performed in vitro lysis assays with the neutralizing antibodies 2G12, 2F5 and 4E10. Strong neutralization activity of the monoclonals did not predict complement lysis activity against different patient viruses and the heterologous virus JR-FL supporting the notion that the in vivo activity of 2G12, 2F5 and 4E10 is likely due to direct neutralization or FcR-mediated mechanisms such as phagocytosis and antibody-dependent cellular cytotoxicity.

Introduction
Strong evidence has accumulated that neutralizing antibodies may impact on HIV-1 replication in vivo. Foremost led by observations that the antibody responses is subject to rapid viral escape [1-4] and by passive immunization studies [5-7]. Despite the knowledge gained on their activity, the exact modes of action of antibodies in vivo remain unclear. In viral defense, besides portraying direct neutralization activity, antibodies can also function through mediation of effector functions by inducing phagocytosis, antibody-dependent cellular cytotoxicity (ADCC) and activation of the complement system [8-10]. Which of these antibody functions contribute to viral containment in HIV infection and thus should be elicited by protective vaccines has not been unraveled. Likewise unknown remains to what extent HIV-specific non-neutralizing monoclonal antibodies may contribute to mediating effector activities and thus could also have a potential in containing HIV infection in vivo.

Direct lysis of HIV-1 upon antibody-mediated activation of the complement system has been demonstrated in vitro [11-13] and ex vivo [14-16]. Lysis activities described to date may reach up to 90% at the highest concentration of patient sera tested [15,16]. In vivo, antibodies inducing complement lysis were found to impact on viremia control during the acute phase of infection [16]. These responses increase during the chronic infection phase and appear to be maintained throughout the course of the disease [12-16]. Previous studies suggested that non-
neutralizing antibodies dominate in this mechanism [14,16]. Nevertheless, complement has also been found to boost the activity of neutralizing antibodies in vitro and in vivo [16-18].

Opposing these observations, several reports have suggested that complement-mediated lysis of HIV-1 is limited due to the action of several host cell-derived complement control proteins incorporated into the HIV-1 virions [19-21] and that opsonized virions bind to complement receptor-expressing cells promoting enhanced viral infectivity and transmission [22-27]. It remains currently unknown whether lysis or enhancement activities dominate in vivo and if and how the balance between beneficial and detrimental antibody functions is maintained during disease progression.

In the present study, we sought to define if antibody-mediated complement lysis of HIV-1 virions contributes to the in vivo activity of neutralizing antibodies and if so what the relative contribution of this defense mechanism at different disease stages is. We addressed this question in a retrospective analysis of a passive immunization study with the neutralizing antibodies 2G12, 2F5 and 4E10. A mix of these three monoclonal antibodies delayed viral rebound after cessation of therapy [7]. Here we analyzed patient plasma collected throughout the trial for complement activation, lysis activity and neutralization capacity against the autologous isolate and the heterologous virus strain JR-FL. In addition, to verify our ex vivo findings, we investigated the complement lysis activity of the monoclonal antibodies 2G12, 2F5 and 4E10 individually in vitro.

Materials and Methods

Patients, virus isolates and plasma

Plasma samples form six acutely and eight chronically HIV-1 infected patients derived during a passive immunization trial with the neutralizing antibodies 2G12, 2F5 and 4E10 [7] were studied. Patient demographics and isolation of autologous virus have been described previously [7,28].

Patient blood was sampled in EDTA vacutainers (BD) and plasma collected within 6 h after blood draw and frozen in 1 ml aliquots at -75 °C. Plasma was heat-inactivated (1 h at 56°C to inactivate autologous complement activity) and centrifuged at 500 g for 10 min before use to remove cell debris and lipids. Normal human plasma used as control was treated identically as patient plasma.
Results

Written informed consent was obtained from all patients and HIV-1 negative blood donors according to the guidelines of the local ethics committee.

Measurement of complement activation

Plasma levels of complement components C3 [29] and TCC [30,31] were measured by ELISA as described. Levels of C3a were measured by a commercial ELISA (Quidel) as recommended by the manufacturer.

Neutralization assays

Neutralization activity of patient plasma against the heterologous virus isolate JR-FL was evaluated using Env pseudotyped virus as described [32]. Briefly, 4000 TCID<sub>50</sub>/ml of the virus were pre-incubated for 1 h at 37° C with serial dilutions of plasma. The reciprocal plasma dilution causing 70% (NT70) reduction in luciferase reporter gene expression on TZM-bl cells (NIH AIDS Research and Reference Reagent Program) at day 2 was determined by regression analysis.

Neutralization activity of monoclonal antibodies 2G12, 2F5 and 4E10 against pseudotyped virus carrying JR-FL or patient derived Env glycoprotein (derived before initiation of the passive immunization trial [7,28], Manrique et al. submitted, Kuster et al. in preparation) was evaluated as described [32]. Briefly, 4000 TCID<sub>50</sub>/ml of the virus were pre-incubated with serial dilutions of the antibodies. The antibody concentration causing 70% (IC70) reduction in luciferase reporter gene expression on TZM-bl cells was determined by regression analysis.

HIV-1 virion complement lysis assay

Complement-mediated lysis activity was measured by a real-time PCR based assay as described [16]. A mix of 4-5 human healthy donor sera stored at -75° C was used as source of complement. Briefly, HIV-1 virus was incubated with patient plasma (final dilution 1:5), complement (final dilution 1:2), RNase A (Qiagen) and RPMI 1640 (BioWhitaker) in a total volume of 100 µl for 3 h at 37° C. Then the reaction mixture was subjected to a freeze-thaw cycle and treated with RNase A (Qiagen) and DNase I (Roche) for 1 h at 37° C. Residual viral RNA was extracted from intact virions (MagNa Pure LC, Roche) and quantified by real-time PCR.

In each individual assay, samples were tested in triplicates. Complement-mediated lysis activity was expressed as the percentage of lyzed HIV-1 RNA copies compared to control.
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plasma treatment. A mix of five HIV-1 negative healthy donor plasmas was used as negative control (no lysis activity, 0% value).

Lysis activity of monoclonal antibodies was measured analogously using 25 μg/ml of each antibody in RPMI instead of patient plasma and medium alone was used as negative control.

Real-time PCR

HIV-1 virions were quantified using primers to the 5' end of HIV-RNA as described previously (mf86, cr1 and cr2) [16]. For detection a dual-labeled fluorescent probe with a fluorescein (FAM) moiety at its 5’ end and a tetramethylrhodamine (TAMRA) moiety at its 3’ end was used (mf74tq) [16]. PCR was performed as described previously [33,34] using a single-tube system (QIAGEN 1-step RT-PCR) and an additional “hot-start” using Paraplast (Fluka) to separate cDNA synthesis and PCR amplification. cDNA synthesis and subsequent amplification were performed in duplicates in a real time thermocycler (i-Cycler, BioRad) as described [33,34].

Statistical analysis

Data analyses were performed using Prism version 4.03 for Windows (GraphPad Software). Non-parametric methods were employed for testing of group differences (Mann-Whitney U test and Wilcoxon signed-rank test for unpaired and paired testing, respectively). Correlation analysis was performed using Spearman’s rank correlation. All tests of significance were two-tailed. P values refer to values obtained after singular testing.

Results

Passively administered monoclonal antibodies activate complement system

To estimate the degree of complement system activation during passive immunization we quantified plasma levels of the complement components C3, C3a and the terminal complement complex (TCC). Blood was collected before and after infusion of the three monoclonal antibodies. The time window between initiation of the first infusion and completion of the last was usually 90 min (30 min per antibody infusion). Activation of the complement system is known to occur within minutes [35,36] and thus could be monitored in the investigated time frame. We found that administration of the antibodies induced a decrease of complement component C3 plasma levels while those of the cleavage product C3a
and the terminal complement complex (TCC) increased, indicating that the three monoclonals induced strong complement activation (Figure 1A). Notably though, activation occurred already at the first time point (week 0) in the absence of any viremia when patients were still on ART (Figure 1A). We also observed no further changes in complement activation upon rebound of viral load (Figure 1B). Neither was complement activation associated with the *in vivo* plasma levels of the monoclonals with the exception of 2G12 and the increase of C3a ($r = 0.33$, $P < 0.0001$). Taken together these observations indicate that the monoclonal antibodies 2G12, 2F5 and 4E10 strongly activated the complement system *in vivo* independently of viral replication.

![Figure 1: Complement is activated during passive immunization independent of viremia.](image)

**A**) Plasma levels of complement components were measured before and after the infusion of the monoclonal antibodies 2G12, 2F5 and 4E10. Levels at week 0 are shown in closed red circles, later time points in open grey circles. $P$ values are shown for time point 0. Groups were compared using Wilcoxon signed-rank test. Error bars show interquartile ranges.

**B**) Changes in complement level before (open symbols) and after (closed symbols) viral rebound (threshold level 100 copies/ml). Groups were compared using Wilcoxon signed-rank test. Error bars show interquartile ranges.
Results

Activation of complement by monoclonal antibodies has neither decreasing nor enhancing effect on viral set point in non-controlling patients. Pre-treatment viral set point is only available for chronic patients. Patient NAB 06 stopped the passive immunization study after week 12 and went back on ART. Acute patients are shown as blue triangles, chronic patients as green circles. Open symbols represent non-responders, closed symbols responders. Groups were compared using Wilcoxon signed-rank test.

**Figure 2: No evidence for in vivo complement dependent enhancement**

No evidence for complement dependent enhancement in vivo

A major concern in respect to antibody mediated effector mechanisms in vivo remains the possibility that Fc receptor (FcR) and complement receptor (CR) interactions may lead to enhancement of infection as demonstrated under certain in vitro conditions [24,26,37,38]. During the investigated passive immunization trial patient virus rebounded upon escape to antibody 2G12 [7]. Antibody treatment was continued for the entire study period, irrespective of when the antibodies had lost the capacity to control viremia and had rebound occurred. However, if induced by these monoclonal antibodies in vivo, enhancing mechanisms may still take place. To assess this, we compared viral set points reached after viral rebound in presence and absence of monoclonal antibodies. Within the eight patients of the chronic group the viral load before the last anti-retroviral therapy (ART) was comparable with the viral set point manifested after passive immunization indicating that monoclonal antibodies had no influence on viral set point (P = 0.95, Figure 2). When we analyzed the effects on viral load upon wash-out of the monoclonal antibodies, no differences in virus load in presence (week
Results

12) or absence (week 24) of antibodies was observed (P = 0.50 for entire cohort, P = 0.69 for non-responders only, Figure 2) indicating that activation of complement by monoclonal antibodies has neither decreasing nor enhancing effect on viral set point in controlling and non-controlling patients, respectively. Thus our analysis does not provide evidence that, in absence of inhibitory activity, these three monoclonals induced enhancement in vivo.

Longitudinal analysis of autologous complement mediated lysis activity

To assess if the passively administered antibodies elicited complement lysis of virions complement lysis activity was measured in patient plasma before antibody infusion (week 0), after the last infusion when antibody levels peaked in most patients (week 12) and after wash-out of the antibodies (week 24). We observed complement lysis activity in 12 out of 14 individuals before passive immunization and antibody infusion lead to a notable increase in this activity. Of note, virus lysis activity was found to increase in both acute and chronic patients upon viral rebound paralleling the boost of the autologous antibody response (Table 1, Figure 3A).

After wash-out of the antibodies lysis activity dropped to pre-immunization level amongst most chronically HIV infected individuals (Figure 4A) indicating that the monoclonals contributed to the elevated lysis activity observed at week 12 (Table 1, Figures 3A and 4A/B). Interestingly, patients with acute HIV infection (Figure 4A) preserved a heightened lysis activity even after washout of the monoclonal antibodies. In this group of individuals viral rebound occurred later than in chronically infected antibodies. In this group of individuals viral rebound occurred later than in chronically infected patients (acute: week 5 to >24, chronic: week 2 to 18 [7]). Hence, the boosts of autologous antibody responses following viral rebound will have occurred in most acute patients after week 12 which would explain why in these patients lysis activity after wash out of antibodies was still higher than at pre-treatment (Table 1, Figures 3A and 4A). When we stratified patients into responders and non-responders based on whether or not monoclonal antibody treatment lead to a delay in viral rebound, we observed a similar pattern (Figure 4B). The latter is not unexpected as the majority of non-responders were individuals with chronic infection (75%) and responders were mostly found amongst patients with acute infection (66%, Table 1). We also verified if an association between in vivo levels of the monoclonal antibodies and ex vivo measured virolysis exists. A correlation analysis revealed no association (data not shown) suggesting that the overall contribution of the monoclonals to lysis activity must be low.
Longitudinal analysis of heterologous (JR-FL) complement mediated lysis activity

Complement-mediated lysis activity was also measured against the heterologous virus isolate JR-FL (Table 1 and Figure 3B). Longitudinal patterns of lysis activities were similar as in the autologous assay and showed an increase in activity concomitantly with viral rebound. However, differences between acute/responder and chronic/non-responder patients were less pronounced (Figure 4C/D). No association between in vivo levels of the monoclonal antibodies and heterologous virolysis was detected (data not shown).

**Figure 3: Complement virolysis activity increases upon viral rebound**

Longitudinal analysis of autologous (A) and heterologous (B) complement-mediated lysis activity. Legend of symbols see Figure 2. Groups were compared using Wilcoxon signed-rank test. Error bars show standard deviation of triplicate measurements.
Figure 4: Passively administered antibodies 2G12, 2F5 and 4E10 can contribute to \textit{in vivo} complement lysis but activity is low.

Autologous (A and B) and heterologous (C and D) lysis activity is compared between acute and chronic (A and C), as well as responders and non-responders (B and D) patients. Blue triangles denote acute patients, green circles chronic patients, closed squares responders, open squares non-responders. Groups were compared using Wilcoxon signed-rank test. Error bars show interquartile ranges.
Results

Table 1: Autologous and heterologous complement-mediated lysis activity during passive immunization

<table>
<thead>
<tr>
<th>code</th>
<th>infection phase</th>
<th>viremia control</th>
<th>autologous virolysis [%]</th>
<th>week 0</th>
<th>1 week</th>
<th>12 week</th>
<th>24 week</th>
<th>week 0</th>
<th>1 week</th>
<th>12 week</th>
<th>24 week</th>
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<tr>
<td>NAB 01</td>
<td>chronic no</td>
<td>71.9 83.4 86.6</td>
<td>6.5 9.8 43.3 35.2</td>
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<tr>
<td>NAB 02</td>
<td>chronic no</td>
<td>51.9 38.6 57.7</td>
<td>18.2 -7.9 49.7 36.3</td>
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<td></td>
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<tr>
<td>NAB 03</td>
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<td>31.6 42.6 67.6</td>
<td>40.7 40.7 48.1 65.0</td>
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<tr>
<td>NAB 04</td>
<td>chronic yes</td>
<td>21.2 28.6 31.6</td>
<td>68.6 63.7 73.3 72.4</td>
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<tr>
<td>NAB 05</td>
<td>chronic no</td>
<td>55.1 48.0 63.5</td>
<td>61.9 42.3 56.4 50.5</td>
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<td>NAB 06</td>
<td>chronic no</td>
<td>89.6 82.5 n.d.</td>
<td>n.d. n.d. n.d. n.a.</td>
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<tr>
<td>NAB 07</td>
<td>chronic no</td>
<td>10.5 36.4 72.9</td>
<td>13.8 11.8 42.2 10.2</td>
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<tr>
<td>NAB 08</td>
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<td>38.2 17.2 44.3 54.8</td>
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<tr>
<td>NAB 09</td>
<td>chronic no</td>
<td>38.7 37.7 63.0</td>
<td>24.4 31.3 63.1 36.5</td>
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<td>-0.6 -27.4 56.6 36.6</td>
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<td>NAB 11</td>
<td>acute no</td>
<td>8.9 7.3 58.5</td>
<td>51.7 49.3 50.7 68.9</td>
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<tr>
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<td>acute yes</td>
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<td>5.8 30.5 14.8 40.9</td>
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<tr>
<td>NAB 14</td>
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<td>52.7 36.4 54.6</td>
<td>53.5 45.4 43.1 33.1</td>
<td></td>
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<td></td>
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</table>

1 before last ART
2 before antibody infusion
3 at highest antibody level
4 after wash-out of the antibodies
5 patient NAB 06 stopped the passive immunization study after week 12 and went back on ART

Heterologous neutralization activity during passive immunization is predominantly mediated by 2G12, 2F5 and 4E10

To investigate whether the activity of the monoclonal antibodies was in vivo preserved we performed neutralization assays against the heterologous virus isolate JR-FL using patient plasma derived during passive immunization. Neutralization activity against JR-FL was high in all patients as soon as immunization started and decreased upon wash-out of the antibodies (Figure 5), indicating that neutralization activity was predominantly mediated by the administered monoclonal antibodies.
Results

Figure 5: Heterologous neutralization activity during passive immunization is predominantly mediated by 2G12, 2F5 and 4E10

Plasma neutralization activities against the heterologous isolate JR-FL. Legend of symbols see Figure 2.

*Neutralization activity of 2G12, 2F5 and 4E10 does not predict complement lysis activity*

To verify our *ex vivo* analysis we performed *in vitro* lysis assays with the antibodies 2G12, 2F5, and 4E10 and with a mixture of all three monoclonal antibodies. While the three monoclonal antibodies potently neutralized JR-FL, they failed to lyze this isolate (Figure 6B). Activity against the virus isolates from patients that participated in the passive immunization trial showed a range of reactivities but was overall low (Figure 6B) although most of the monoclonal antibodies potently neutralized these isolates (Figure 6A). For each of the monoclonal antibodies lysis activity differed dependent on the virus isolate. We found neither a common pattern of antibody lysis and neutralization activity against the tested virus isolates nor interdependency between these activities. Our analysis thus strongly suggests that complement lysis activity cannot be predicted by neutralization activity.
Results

Figure 6: Neutralization activity does not predict complement lysis activity
Neutralization (A) and lysis activity (B) of the monoclonal antibodies 2G12 (dashed), 2F5 (crossed) and 4E10 (diagonally crossed) against three selected patient virus isolates and JR-FL do not show a common pattern.

Discussion
It is generally accepted that neutralizing antibodies are a key immune defense mechanism against HIV as evidenced by a compelling number of in vitro and in vivo studies [1-7]. However, whether antibodies act solely by directly binding and neutralizing the virus particle or in addition also employ effector functions of the immune system remains currently unknown [39]. In this study we addressed this question by assessing if complement-mediated lysis contributes to the in vivo effect of neutralizing antibodies. It is known that the complement system can be activated by direct binding of complement components to HIV or more potently, via antibodies bound to the particle [11,22,40]. We found that complement
was highly activated \textit{in vivo} by passive immunization. Notably, complement already was activated even in absence of viremia (Figure 1) indicating that activation was mediated by the monoclonal antibodies 2G12, 2F5 and 4E10 alone and does not provide an indication of virus lysis. Antibody preparations used for passive immunization were controlled to ensure that no or only low aggregates were contained. Nevertheless, the high concentration of antibodies administered is conceivably sufficient to induce complement activation [41,42].

In all patients complement-mediated lysis activity increased during the trial which can be attributed to both activity of the monoclonal antibodies and a boost of the autologous response known to occur upon viral rebound. The latter may explain the observation that lysis activity levels remained high even after washout of the monoclonal antibodies amongst acutely HIV infected patients (Figure 4A/C). Since these patients are on the transition between the acute and chronic phase of infection their antibody response is expected to mature and increase. This appears to be the case also in our cohort: while pre-treatment lysis activity was notably lower in the acute, no difference between acute and chronically infected individuals was observed upon completion of the trial (week 24). Amongst chronic patients, whose antibody responses is more matured and expected to more rapidly reach a steady state level upon rebound [43] we observed a notable impact of the monoclonal antibodies on the overall lysis activity. It is likely that the monoclonal antibodies had the same impact in the acutely infected individuals. However, since there the rise in autologous responses occurred simultaneously the effects of passively administered and own responses cannot be dissected apart.

Increases and decreases in lysis activity observed in chronic infected patients during administration and wash-out of monoclonal antibodies provide clear evidence that the monoclonals are capable of inducing complement lysis either by themselves or in combination with the autologous response (Figure 4A). However, we failed to observe a measurable effect of this lysis activity on viral set points (Figure 2) nor is there clear evidence that this effect of the monoclonal antibodies contributed to the success of the treatment. This is particularly notable for chronically infected individuals as most of these patients failed to show response to the monoclonal antibodies treatment (Table 1 and [7]). This finding is supported by a previous study [16] where – in contrast to acute infection – the more effective lysis in chronic infection was not associated with lower viral set point.

Overall lysis activity mediated by the monoclonal antibodies \textit{in vivo} was low. We therefore conclude that the \textit{in vivo} activity of neutralizing antibodies 2G12, 2F5 and 4E10 likely is
dominated by neutralization of free virus or FcR-mediated mechanisms such as phagocytosis and ADCC.

The fact that some of the antibodies harbor neutralization but no measurable lysis activity is puzzling and asks for further assessment of the underlying mechanisms (Figure 6). This lack of lysis activity strongly suggests that binding sites (i.e. functional spikes) of these neutralizing antibodies on virions are not dense enough to get the classical pathway started. A threshold for neutralization, however, may be achieved already with a single bound antibody according to a model by Yang et al. [44], whereas for activation of complement several IgG molecules or a pentameric IgM have to associate. This hypothesis is supported by the fact that only few of the tested monoclonal antibodies induced complement lysis while antibodies in patient sera mediate significant lysis activity, indicating that polyclonal responses may be needed to reach the threshold (Figure 6 and [16]) [11]. In previous studies [15,16] lysis activity plateaued at about 90%. The remaining 10% of virions seem to be protected from virolysis which in a similar way could miss sufficient binding sites for antibodies to induce complement-mediated lysis.

In contrast we also found that antibodies show lysis but not neutralization activity [16]. The likely explanation for this scenario lies in the fact that in order to neutralize antibodies have to bind the functional trimer [45] whereas lysis-inducing antibodies could theoretically bind anywhere on the virion, i.e. to non-functional trimers, monomers, uncleaved gp160 and gp41 stumps [46] without interfering with binding sites that are required for viral entry.

Heterologous plasma neutralization is clearly mediated by the three monoclonal antibodies (Figure 5) in contrast to lysis activity (Figure 3) where an autologous and polyclonal response is mainly responsible. To what extent neutralizing and/or non-neutralizing antibodies mediate antibody effector functions, how the activities of neutralizing and lysis-inducing antibodies overlap and which epitopes are recognized by these antibodies requires further investigation.

While we found that the contribution of complement lysis to the total inhibitory effect of monoclonal antibodies in vivo appears to be low, further studies are needed on the impact of ADCC and phagocytosis, to determine to what extent antibodies exert their antiviral potential via effector functions. As neutralization affects only the virus particle, antibody-mediated effector functions targeting infected cells may be of particular importance in combating latent HIV-1 infection. A detailed characterization and quantification of the relative contribution of
Results

direct and effector-mediated antibody functions on virus containment \textit{in vivo} will be of central importance for the definition of relevant immune responses and the future of vaccine design.

Acknowledgements

We thank the participating patients for their commitment and the clinical staff for excellent patient care. Support was provided by the Swiss National Science Foundation (Grant No PP00B-102647 to A.T. and Grant No 3100A0-103748 to H.F.G. and A.T.), the Gebert Rüf Foundation and the UBS Foundation. A.T. is an Elizabeth Glaser Scientist supported by the Elizabeth Glaser Pediatric AIDS Foundation.

References

Results


6. Discussion

Over the last years more and more evidence accumulated that the humoral immune system has great potential to contain HIV-1 infection. Neutralizing antibodies can delay viral rebound \textit{in vivo}, the complement system is activated and may contribute to viremia control during the acute phase of HIV-1 infection and also other antibody effector functions like ADCC and phagocytosis are thought to play a role. However, which of these components, at what time point of infection and to what relative contribution are necessary for a successful containment of HIV-1 viremia remains unknown. Surely neutralizing antibodies that directly bind to free virus are crucial. However, only five broadly neutralizing monoclonals are known so far (Burton DR, 2005, PNAS 102, 14943). The virus is poorly immunogenic and has a high diversity, high titres are needed for protection, rapid viral escape occurs (Richman DD, 2003, PNAS 100, 4144; Wei X, 2003, Nature 422, 307) and all attempts to elicit similar neutralizing antibodies by vaccination \textit{in vivo} failed so far (Burton DR, 2004, Nat Immunol 5, 233). Unfortunately it is unlikely that neutralizing antibodies alone will succeed in forming an effective vaccine.

We showed that complement-mediated lysis can contribute to viremia control during the acute phase of HIV-1 infection. However, we found no evidence for a role of complement-mediated lysis in chronic infection or during passive immunization. The \textit{in vivo} activity of the monoclonal antibodies was therefore likely dominated by direct neutralization or Fc dependent mechanisms (ADCC, phagocytosis). These results may look disappointing in terms of the function of the passively administered antibodies, however, in our \textit{ex vivo} measurements we detected strong complement lysis mediated by the autologous antibody response both in acute and chronic infection. Moreover, we demonstrated that active complement contributed to inhibition of viral infectivity \textit{in vitro}. These observations confirm that complement lysis is effective \textit{in vivo} and may have the potential to further be exploited in prevention and therapy.

Further studies are needed to determine if antibody effectors functions contribute to viral containment \textit{in vivo}, and if they do, to what extent. So far it remains uncertain if
Discussion

complement-mediated lysis, ADCC and phagocytosis are potent enough to control HIV-1 infection. To unravel these effects, it will be important to define which antibody specificities and in what concentrations or combinations are needed to optimally activate the complement system and induce viral lysis. Vaccination strategies that induce also antibodies capable of complement-mediated lysis could then be considered. Engineering antibodies' Fc parts to enhance binding of complement and receptors would be another possibility to take better advantage of antibody effector functions, should they be used in passive immunization (Lazar GA, 2006, PNAS 103, 4005).

While neutralizing antibodies can only target free virus, ADCC and complement-mediated lysis may potentially also be effective against infected cells. Targeting infected cells is a prerequisite to eradicate the latent infection. However, latent infected cells need to be activated to express viral proteins that make them recognizable for the immune system. Therapeutic strategies to attempt this are considered (Kulkosky J, 2006, Curr HIV Res 4, 199). Antibodies in conjunction with effector functions could be envisioned as a means to target infected cells in these regimens.

Besides the inhibiting effects of antibodies bound to virions, coating of HIV-1 with antibodies could also have detrimental effects. Virions opsonized with antibodies and complement fragments can bind to Fc or complement-receptor bearing cells thereby enhancing attachment and entry of HIV-1. This was demonstrated in vitro but likely occurs as well in vivo (Stoiber H, 2001, Immunol Rev 180, 168). Possible beneficial and detrimental modes of action of both neutralizing and non-neutralizing antibodies are summarized in Figure 1. It is unclear what the role of Fc and complement dependent enhancement in vivo is and what consequences or risks this may bear for antibody-based therapies. It still needs to be determined where the balance between beneficial and detrimental effects of antibodies lies. In vitro, upon dilution antibodies loose their neutralizing or complement-inducing activity. They still bind to virions at sub-neutralizing concentrations but are unable to block all infectious units on a virion or do not reach quantities of antibodies to set of the threshold for complement activation. Bound antibody in this case is, however, still capable of enhancing infection (Banki Z, 2005, Aids 19, 481).
The contribution of complement lysis activity may be outnumbered by more specific adaptive immune responses in chronic infection as well as there might be a shift in balance between inhibiting and enhancing effects of antibodies and complement. Therefore, vaccine induced antibody response must be strong and likely polyclonal to avoid this scenario and further studies are needed to unravel if the beneficial or the detrimental effects of complement and antibody dominate in vivo.

Figure 1: Beneficial and detrimental effects of neutralizing and non-neutralizing antibodies in HIV-1 infection. Neutralizing antibodies are shown in red, non-neutralizing antibodies in blue, complement components in green.
Discussion

While it has been shown by numerous groups that HIV-1 escapes neutralizing antibodies rapidly (Richman DD, 2003, PNAS 100, 4144; Wei X, 2003, Nature 422, 307), it remains unknown whether or not an adaptation of virus to a strong effector-mediated response occurs. It is feasible that HIV-1 also escapes against complement-lysis inducing antibodies. During progression of the disease, following viral evolution there is further a constant change in the antibody repertoire.

The above-discussed topics all effect on future vaccine design. To find and understand the correlates of protection is a major goal in HIV-1 research. It remains a challenging task to define and characterize the immune responses needed for viral containment and to induce these responses by a successful vaccine.
7. Acknowledgements

First, I would like to thank Alexandra Trkola for mentoring my PhD thesis, her support and continuous stimulus. I was given a lot of freedom and trust to perform my research projects. Her cheerful Austrian character contributed a lot to the excellent atmosphere in our group.

I am grateful to Sebastian Bonhoeffer and Hans Hengartner for the supervision of my external PhD thesis.

I thank Huldrych Günthard for his generous support and all the insights he gave us to the clinics. I would like to thank Marek Fischer and Beda Joos, with whom I shared the office for an exciting time, for their tolerant attitude and abundant knowledge (not only in science).

A lot of thank goes to all the members of our group for their instruction, assistance and friendship during all the hours we spent together in the lab: Amapola Manrique, who became a very good friend; Peter Rusert, the last man standing in an increasingly women-dominated lab world; Christine Leemann, with who I nearly share the fascination for snowboarding; Barbara Niederöst and Herbert Kuster, for their spontaneous help and the enormous amount of work they did I always could profit from; Andreas Schweizer, Claudia Ruprecht, Philipp Kaiser, Livia Berlinger, Martina Ackermann, and last but not least Viktor von Wyl, our statistician.

I wish you all the best and good luck for your future research, as there remain one or two unresolved questions...
Appendix

8. Appendix

8.1 Curriculum vitae

Name: Huber
First name: Michael
Date of birth: 11. May 1977
Place of birth: St. Gallen
Citizenship: Hedingen ZH and St. Gallen SG

1990 - 1997: Lateingymnasium, Kantonsschule am Burggraben, St. Gallen

January 1997: Matura Typus B

1997 - 2002: Studies of Biochemistry at the University of Zurich
Subsidiary subject: Immunology

2001 - 2002: Diploma thesis at the Institute of Biochemistry, University of Zurich, supervised by Prof. Philipp Christen, "Induction of antibodies against Aβ1-42 cross-linked with DnaK; Kinetic and thermodynamic studies on wild-type and mutant DnaK"

July 2002: Dipl. biochem. University of Zurich

since 2003: PhD thesis at the University Hospital Zurich, Division of Infectious Diseases, supervised by Prof. Alexandra Trkola, "The effect of adaptive and innate immune response on HIV-1 entry and replication"
8.2 Publications


**Huber M**, Olson WC, and Trkola A. Antibodies for HIV treatment and prevention: Window of opportunity? (2007, in press Current Topics in Microbiology and Immunology)
