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Public, private and non-specific antibodies induced by non-cytopathic viral infections

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Lymphocytic choriomeningitis virus (LCMV) represents a useful experimental model of murine infection with a non-cytopathic virus, bearing resemblance to HIV and hepatitis C virus (HCV) infections in humans. Recent data from the LCMV model indicate that the humoral immune response that is induced by non-cytopathic viruses is far more complex than previously appreciated. LCMV-induced IgG production is largely polyclonal, with more than 90% of the antibody repertoire constituting non-relevant specificities. A delayed virus-neutralizing antibody response is induced, including specificities directed not only against the parental LCMV-strain present in the host but also cross-specifically against LCMV-variants isolated from other hosts. These findings provide novel insights to aid our understanding of clinically relevant observations that are recorded following human infection with HIV, HCV and dengue viruses.

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Abbreviations

CTL	cytotoxic lymphocyte ($CD8^+$ T cell)
HCV	hepatitis C virus
LCMV	lymphocytic choriomeningitis virus
SAP	SLAM(signalling lymphocyte activation molecule)-associated protein

Introduction

Viruses can be broadly divided into those that are cytopathic to the host and those that are poorly or non-cytopathic. Cytopathic viruses — including poliovirus and vesicular stomatitis virus (VSV) — interfere with essential cellular processes, ultimately resulting in cellular death, and are capable of killing the host if the immune response cannot control viral replication in a timely fashion. This normally necessitates the rapid production of neutralizing antibodies because, although effective, cytolytic $CD8^+$ T cell responses normally occur too late to prevent viral spread [1,2]. Cytopathic viruses are often classified into relatively few serotypes according to the specificity of the host's antibody response for surface glycoprotein antigens. However, it is now clear that such definitions are overly simplistic in light of the finding that a greater genetic variability often exists at other loci within the viral genome [3,4].

Poorly or non-cytopathic viruses, including murine lymphocytic choriomeningitis virus (LCMV), hepatitis C virus (HCV), HIV and also dengue virus, have evolved to replicate within host cells without interfering with those processes that are essential for cellular survival [5–7]. Instead, disease is largely caused by the host's own immune response, including CTL (cytotoxic lymphocyte)-mediated lysis of virus-infected cells [8] and chronic immune activation [9•,10]. Clearance of poorly or non-cytopathic viruses is usually mediated by $CD8^+$ T cells, and is reliant upon the gain of lytic function by these cells, as demonstrated by the importance of molecules such as perforin [11], granzymes [12] and Fas ligand [13]. Yet many poorly or non-cytopathic viruses tend to persist, either as a consequence of their localisation in the periphery, as a consequence of the formation of CTL-escape viral mutants or as a result of viral-induced exhaustion of the CTL response [14–16]. Neutralizing antibodies, which are crucial for protection against cytopathic viruses, are usually detectable only at late time-points after infection with poorly or non-cytopathic viruses and are more prominent in situations of $CD8^+$ T-cell non-responsiveness [17,18•,19,20]. Nevertheless, should a poorly or non-cytopathic virus manage to escape CTL attack, the subsequent neutralizing antibody response becomes crucial for viral control. This can be demonstrated by the isolation of neutralizing antibody viral escape mutants at late time-points after infection of $CD8^{-/-}$ mice [21–23].

Poorly or non-cytopathic RNA viruses are normally classified into different biological strains, genetic subtypes or genetic clades [24–26]. Importantly, replicating virus within one host should be regarded as a so-called 'quasispecies', reflecting a dynamic set of genetically distinct viral subtypes [27,28]. As a general rule, a greater number of viral serotypes can be distinguished for poorly

or non-cytopathic viruses than for cytopathic viruses; however, correlations between genetic subtypes, biological behaviour and neutralizing serotypes remain equivocal [29].

Neutralizing antibody-escape variants

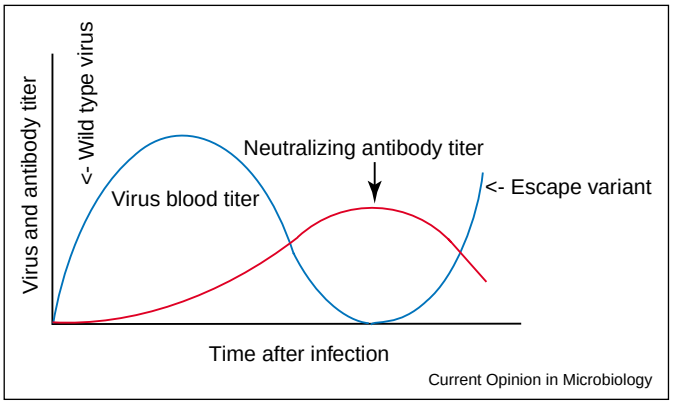
Despite their late appearance, neutralizing antibodies represent a very effective means of controlling persistent infections with poorly or non-cytopathic viruses. Indeed, studies of simian immunodeficiency virus (SIV) infection in macaques or LCMV infection of mice have demonstrated that the passive transfer of monoclonal neutralizing antibody before viral infection results in rapid viral clearance and protection from a productive infection [30,31]. Neutralizing antibodies can also act to prevent CTL exhaustion and the emergence of CTL-escape variants, by virtue of their ability to limit viral replication [32,33]. The formation of neutralizing antibody-escape variants can be demonstrated by the ability of the host serum to neutralize the parental viral strain (used to inoculate the host) but not virus recovered from the host at later time-points [21]. This finding presents a great concern for clinical diagnosis of chronic viral infections; most current technologies use monoclonal antibodies directed against the parental viral strain for viral detection, and therefore do not account for the possible emergence of antibody-escape variants [34].

The emergence of neutralizing antibody-escape variants has been most widely studied in LCMV infection of murine hosts. Using this model, experiments can be performed in which pressure on the virus to develop neutralizing antibody-escape variants is enhanced through infection of $CD8^{-/-}$ mice [21]. These mice exhibit a high initial rate of LCMV replication as a consequence of the absent CTL response; however, between days 40–60 post-infection, neutralizing antibodies are generated and blood virus titers drop, indicating viral control. In these mice, pressure on the virus to develop escape mutants is mainly provided by the neutralizing antibody response and not by CTL activity (because $CD8^{+}$ T cells are missing). Accordingly, by day 80 post-infection, neutralizing antibody-escape variants can be subcloned from the blood of $CD8^{-/-}$ mice, and these correlate with viral re-emergence (Figure 1). Such antibody-escape variants have been shown to possess acquired amino acid substitutions, clustered within three distinct regions of the surface glycoprotein, suggestive of a tertiary LCMV-glycoprotein structure in which these three regions combine to form one conformational antibody epitope [21]. However, this hypothesis awaits formal confirmation by the crystallization of the LCMV-glycoprotein. Recent evidence demonstrates that HIV uses similar strategies *in vivo* to escape the pressure of neutralizing antibodies [35••]. Here, mutations primarily involved changes in N-linked glycosylation, suggesting a 'glycan shield' mechanism of neutralization escape, whereby selected changes in glycan packing prevent antibody but not receptor binding [35••].

Public and private antibody specificities

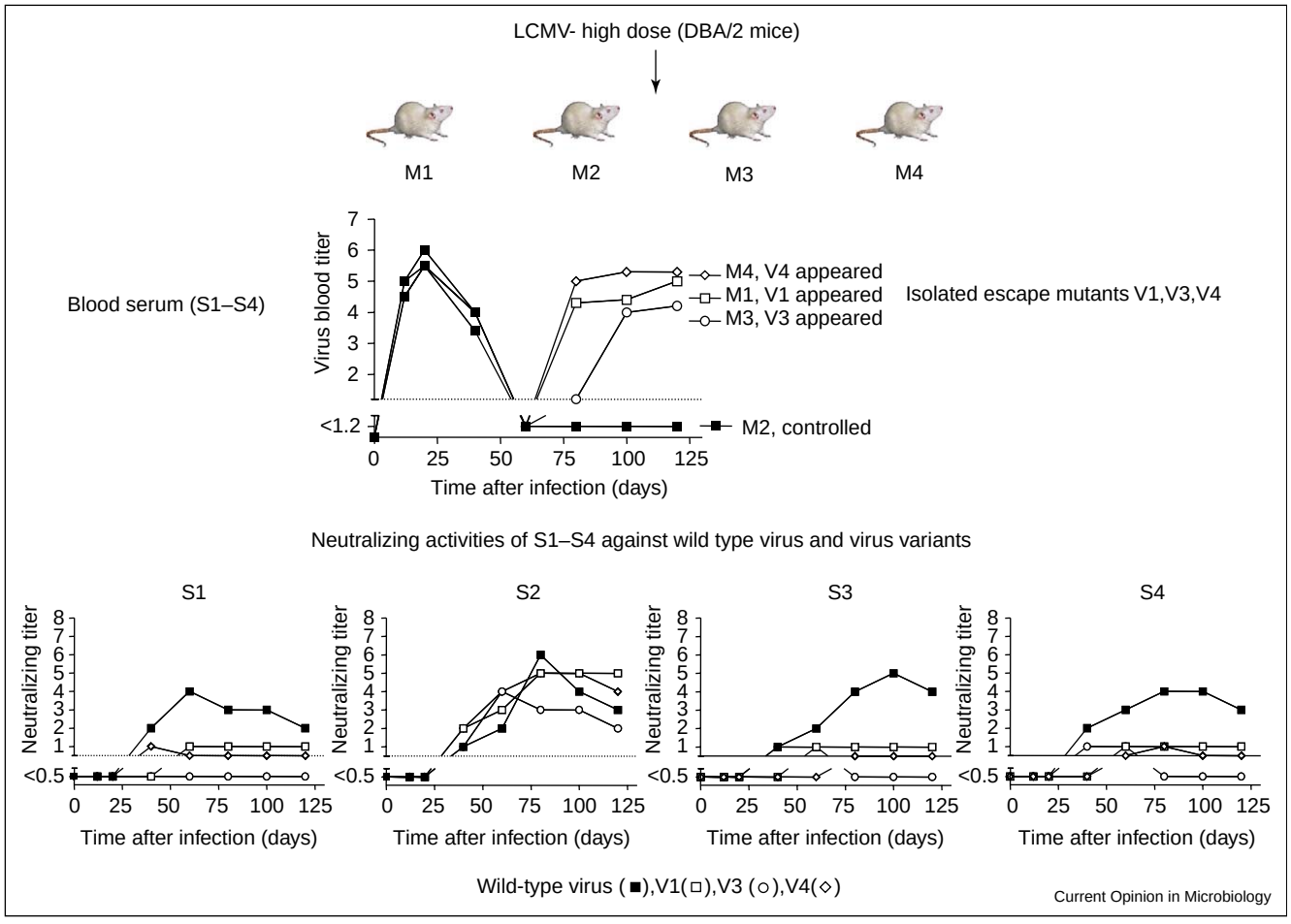
Neutralizing-escape viral variants can be isolated from $CD8^{-/-}$ hosts, subcloned and used for infection of new hosts. In this situation, the new host invariably develops a neutralizing antibody response against the variant glycoprotein and the virus is usually controlled in a manner similar to the control of the wild-type variant in its original host — possibly resulting in the generation of further escape variants [21,36•]. This suggests that, within the original host, a given viral escape mutant does not manage to escape the genetically possible B cell repertoire, but only the current neutralizing antibody response. Should a new host be infected with a neutralizing-escape variant, subcloned from a carrier mouse, the new host generates antibodies that not only exhibit neutralizing activity against the escape variant that was used for inoculation, but also against the parental virus (which this host has never seen). This cross-specific antibody response is

Figure 1



The *in vivo* generation of LCMV neutralizing antibody-escape mutants in $CD8^{+}$ T-cell-deficient mice.

Figure 2



Neutralizing antibody-escape mutants can also be induced in some inbred $CD8^+$ T-cell-competent mice, if high viral doses of LCMV (strain WE) are used. In contrast to $CD8^{-/-}$ mice, neutralizing-escape mutants are only induced in 75% of the cases. In mice with long-term controlled LCMV, serum activity has a broader cross-neutralizing activity (tested against virus-escape mutants arising in other mice) than in mice where virus re-emerges. Together, cross-specific neutralizing antibodies define a more 'public' or general neutralizing serotype. By contrast, neutralizing activity of a host that is specific for the inoculated strain defines the 'private' serotype (adapted from [36]).

usually of a lower titer and reflects a more 'general' or 'public' response. By contrast, the initial neutralizing antibody response against the inoculated strain is of a high-titer and reflects a more private antibody specificity (Figure 2). Public cross-reactive antibody specificities have also been described recently for HIV [37•].

Although all $CD8^{-/-}$ mice develop a 'carrier' status following LCMV infection, a proportion of DBA/2 mice (which are CTL-competent, see later) that are infected with parental virus do not allow the development of neutralizing antibody-escape-variants (Figure 2). These mice invariably generate an antibody response that exhibits some neutralizing activity, directed not only against the parental virus, but also against viral strains isolated from littermates in whom neutralizing-escape variants did emerge. This cross-neutralizing response, reflecting public specificity, was typically lower in DBA/2 mice, where escape variants emerged, indicating a crucial role of public specificities in long-term virus control. One possible explanation for this phenomenon is that, in a portion of LCMV infected hosts, the rapidly replicating virus acquires mutations and generates an array of quasi-species over time, with each new clone inducing a specific neutralizing antibody response. This possibility is supported by the observation that viral polymerases act in an error-prone manner, due to the absence of fidelity-editing functions, and thus generate many mutants over a relatively short time-period [38]. However, cross-neutralizing or 'public' antibody specificities often appear with the same kinetics as the so-called 'private' neutralizing antibody response [36•], indicating that the virus would have to be mutating at a rapid rate from the very beginning of the infection. But, in the absence of pressure from a neutralizing antibody response, the LCMV-glycoprotein appears to be resistant to mutations [36•]. Genetic

reversions of acquired mutations back to wild-type conformations have been observed following the removal of immunological pressure for both LCMV and HIV [36•,39••], suggesting that the parental virus strain represents a state of optimal replication fitness and is likely to be resistant to the acquisition of 'unnecessary' mutations. One also has to bear in mind that every mutational event can potentially decrease the replication fitness of the virus, and might therefore be undesirable. Indeed, experiments in which the rate of viral mutation was dramatically increased by the co-administration of a chemical mutagen led to a loss of replication-competent LCMV *in vitro* [40] and *in vivo* [41•], a situation termed 'error catastrophe'.

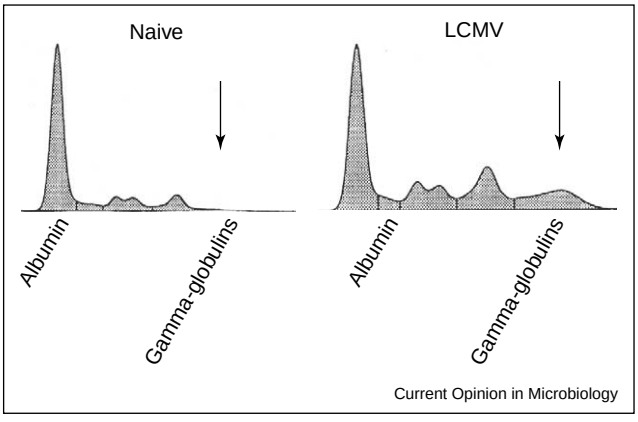
The rate of viral replication may also influence development of a 'public' neutralizing antibody response if, for instance, the presence of high antigen doses favours the induction of a B cell response [42] generating antibody cross-reactivity against both parental and escape-mutant viral strains. In $CD8^{-/-}$ mice, both low- and high-dose LCMV infection results in high viral replication at early time-points, whereas, wild-type C57BL/6 mice mount such an effective CTL response that both low- and high-dose LCMV infection is rapidly controlled. Thus, a new model was required to directly investigate the influence of viral replication on the neutralizing antibody response.

DBA/2 mice contain $CD8^+$ T cells but mount a relatively weak CTL response, which, in practical terms, means that low-dose infection of DBA/2 mice is followed by limited viral replication, whereas high-dose LCMV infection quickly exhausts CTL function, resulting in a high level of viral replication [36•]. High-dose LCMV infection of DBA/2 mice results in a phenotype similar to that observed in $CD8^{-/-}$ mice, with virus control occurring between days 40–60 post-infection and correlating with the appearance of neutralizing antibodies (Figure 2). In contrast to $CD8^{-/-}$ mice, where neutralizing-escape variants develop in 100% of mice, only 75% of DBA/2 hosts allow re-emergence of virus in the form of escape variants. Escape variants from these DBA/2 hosts exhibited amino acid substitutions in the same region of the viral glycoprotein as described for variants isolated from $CD8^{-/-}$ mice [36•]. The remaining 25% of DBA/2 hosts achieved long-term control of viral replication and developed an initial neutralizing antibody response that was of a more 'public' nature (Figure 2 and [36•]). This finding indicated that the cross-neutralizing or 'public' nature of the initial antibody response prevented the development — or sufficient replication of — escape variants. Interestingly, serum taken from a subset of human patients infected with HIV also exhibits broad, or cross-reactive, neutralizing activity when tested against viral isolates obtained from other patients [18•]. Because the DBA/2 experimental model of LCMV infection uses genetically identical hosts, challenged with the same dose and strain of virus, differences that are exhibited by individual mice (in terms of the repertoire of neutralizing antibodies produced) suggest that the development of an antibody response underlies stochastic mechanisms. This is reminiscent of the process of affinity maturation, which has also been reported to be, at least partially, a stochastic process [43,44]. Thus, it is likely that the development of a neutralizing antibody response by individual hosts not only varies considerably [21], but perhaps also reflects a process of affinity maturation that involves somatic hypermutation. As mentioned previously, low-dose LCMV infection of DBA/2 mice results in the development of an effective $CD8^+$ T cell response [36•], which limits viral replication. Strikingly, DBA/2 mice infected with a low dose of LCMV were found to exhibit a more restricted or 'private' neutralizing antibody response [36•]. Together, these observations indicate that the development of a 'public' or 'private' neutralizing antibody response can be directly correlated to the level of virus replication.

Hypergammaglobulinemia

As discussed previously, hosts that exhibit long-term control of LCMV also appear to develop a more cross-specific or 'public' neutralizing antibody response. The common failure of cross-neutralising antibody formation can be partially explained by the finding that LCMV and HIV-specific $CD4^+$ T cell responses (required for the production of neutralizing IgG [45]) are rapidly energised in the presence of massive virus replication [46–48]. $CD4^+$ T-cell function might also determine the nature of the antibody response in another way. LCMV infection is characterised by an early polyclonal, replication-dependent and $CD4^+$ T cell-dependent, hypergammaglobulinemia [49••,50]. By day 12 post-LCMV infection, total IgG levels are elevated 6–10-fold, and appear as a broad gammaglobulin peak in serum electrophoresis, suggesting a polyclonal nature (Figure 3). A similar hypergammaglobulinemia can be found associated with other

Figure 3



Serum electrophoresis of naïve serum and serum 12 days post-LCMV infection. The gamma-globulin fraction is built of immunoglobulins.

persisting infections, including chronic tuberculosis [51], malaria [52], HIV [53•] and HCV [54].

Cytopathic viral infections are not typically associated with an increased level of total serum immunoglobulins, although B-cell responses might be somewhat polyclonal in nature [49••,55•]. By contrast, LCMV infection results in the production of an IgG response in which more than 90% of the total IgG can be said to be non-specific. The dramatic polyclonal nature of the early IgG response that is induced by LCMV infection is completely dependent on the presence of virus-specific $CD4^+$ T cells [50,56•]. It was recently demonstrated that these virus-specific $CD4^+$ T cells recognize LCMV-derived peptides presented by MHC class II molecules that are present on the surface of the B cell, despite the majority of stimulated B cells exhibiting a non-relevant receptor specificity [49••].

The biological consequences of this apparent cognate T helper (Th) cell-dependent polyclonal B-cell response remain unclear [55•]. Antibodies that are protective against other viral species, for example, VSV, are not detectable at the peak of the LCMV-induced hypergammaglobulinemia (author's own unpublished data), nor are antibodies that are capable of neutralizing LCMV. By contrast, IgG specificities that are directed against certain auto-antigens and non-related pathogens are detectable by ELISA [49••]. Nevertheless, apparent autoimmune disease is rarely induced following LCMV infections, arguing against a direct pathophysiological role for those autoantibodies detected. $CD4^+$ T cells are required for development of both the early polyclonal hypergammaglobulinemia and the later neutralizing antibody response, however, it remains to be determined whether subtle alterations in $CD4^+$ T-cell function promote one type of antibody response over another. Interestingly, infection of mice lacking SAP (SLAM (signalling lymphocyte activation molecule)-associated protein, which is involved in X-linked lymphoproliferative disease), among other immunological alterations observed, resulted in increased activation of $CD4^+$ T cells, correlating with an impaired LCMV-specific antibody response [57].

Cross-specific antibodies: protection or disease enhancement?

As discussed, in LCMV infection, the neutralizing antibody response can be classified as 'private' or 'public'; the generation of a public response clearly requiring a high level of viral replication and appearing to be a stochastic process. This classification system might be also be relevant to clinically important infections, such as dengue virus, where cross-specific antibodies have been found associated with severe hemorrhagic disease following secondary infection [57,58]. Although severe disease after secondary dengue virus infection is a complex process, it is usually associated with enhanced viremia [59]. Interestingly, dengue cross-specific antibodies can either be cross-protective (early after primary infection [57,60•]) or disease-enhancing, possibly depending on cross-neutralizing affinity. If cross-neutralizing titers are high enough, secondary dengue viremia is expected to be lower and disease milder [60•]. However, low cross-neutralizing titers may enhance dengue virus titers after secondary infection, due to better virus delivery to macrophages or endothelial cells via Fc-Receptors (receptor binding IgG antibodies via the constant domain). Antibody-enhanced virus-replication, depending on the particular virus studied, seems to involve not only accelerated delivery via Fc-receptors but also complement-components, as well as suppression of cellular antiviral genes by the replication of viruses entering cells via antibodies [61]. Low cross-neutralizing antibody titers could also allow the *in vivo* formation of dengue neutralizing antibody-escape mutants [57].

In addition, cross-neutralizing antibodies could potentially provide protection against unrelated pathogens [62••]. Indeed, antibody responses induced by challenge with *Escherichia coli* have been shown to provide protection against *Haemophilus influenzae*-induced meningitis [63]. For potential antibody-based HIV vaccines, it will be important to understand the mechanisms that result in the formation of broad or cross-neutralizing antibodies, especially with regard to the rapidly changing neutralizing epitopes [18•]. Conversely, polyclonal B-cell activation might generate a potentially harmful repertoire of IgG specificities that could be potentially auto-reactive, thereby enhancing the risk of auto-immunity or immune complex disease [64,65]. Autoimmune thrombocytopenia is a common complication of HIV infection in humans [66], and antibody-dependent autoimmune haemolytic anemia has been described following LCMV infection of some mouse strains [67]. However, autoantibodies following HIV infection were not found to be associated with clinical autoimmune manifestations [68], indicating that auto-reactive antibodies may often be of a low affinity or avidity. Another potential biological consequence of polyclonal B-cell activation is that non-specific B cells might compete with virus-specific B cells for space, survival factors or access to T-cell help. Recent data obtained from HIV infected patients [53•], as well as data obtained from ongoing LCMV experiments in our laboratory, demonstrate that competition might well occur between B cells that bear unrelated or virus-specific specificities.

Conclusions

Dissecting the complex nature of the antibody response induced by poorly or non-cytopathic viruses is crucial to our understanding of how to manipulate this response for the benefit of the host. The exact nature and determinates of the antibody response — including any requirement for $CD4^+$ T cells in regulating the 'private' versus 'public' nature of the response, and the biological consequence of viral-induced hypergammaglobulinemia — might reveal novel mechanisms that are used by viruses for immune

evasion. A full understanding of such mechanisms will be particularly important for the generation of new HIV vaccines that are capable of inducing both protective CTL and neutralizing-antibody responses.

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