Nonneutralizing antibodies binding to the surface glycoprotein of lymphocytic choriomeningitis virus reduce early virus spread

Lars Hangartner,1 Raphaël M. Zellweger,1 Mattia Giobbi,2 Jacqueline Weber,1 Bruno Eschli,1 Kathy D. McCoy,1 Nicola Harris,1 Mike Recher,1 Rolf M. Zinkernagel,1 and Hans Hengartner1

1Institute of Experimental Immunology, University Hospital Zürich, 8091 Zürich, Switzerland
2Mettler-Toledo (Schweiz) GmbH, 8606 Greifensee, Switzerland

The biological relevance of nonneutralizing antibodies elicited early after infection with noncytopathic persistence-prone viruses is unclear. We demonstrate that cytotoxic T lymphocyte–deficient TgH(KL25) mice, which are transgenic for the heavy chain of the lymphocytic choriomeningitis virus (LCMV)–neutralizing monoclonal antibody KL25, mount a focused neutralizing antibody response following LCMV infection, and that this results in the emergence of neutralization escape virus variants. Further investigation revealed that some of the escape variants that arose early after infection could still bind to the selecting antibody. In contrast, no antibody binding could be detected for late isolates, indicating that binding, but nonneutralizing, antibodies exerted a selective pressure on the virus. Infection of naive TgH(KL25) mice with distinct escape viruses differing in their antibody-binding properties revealed that nonneutralizing antibodies accelerated clearance of antibody-binding virus variants in a partly complement-dependent manner. Virus variants that did not bind antibodies were not affected. We therefore conclude that nonneutralizing antibodies binding to the same antigenic site as neutralizing antibodies are biologically relevant by limiting early viral spread.
RESULTS

Viral escape in CTL-deficient TgH(KL25) mice

Mice exhibiting diminished or absent CD8\(^+\) T cell numbers have difficulties in eliminating LCMV and can only transiently control viremia (28, 29). CTL-deficient mice are also prone to the selection of LCMV variants that are resistant against neutralization by the endogenous antibody response (29–31). We used mice deficient in transporter associated with antigen processing (TAP\(^{-/-}\)) but transgenic for the KL25 heavy chain (TgH(KL25)) (32) to select for KL25-neutralizing antibody escape variants. TgH(KL25)xTAP\(^{-/-}\) have very low CD8\(^+\) T cell numbers and are unable to mount antiviral CTL activity (33). However, because of the presence of the KL25 heavy chain, TgH(KL25)xTAP\(^{-/-}\) mice are able to mount a strong LCMV-WE–neutralizing antibody response by day 4 after infection.

TgH(KL25)xTAP\(^{-/-}\) mice were infected with 2 \(\times\) 10\(^6\) PFU LCMV-WE, and viremia as well as neutralizing antibody responses against the immunizing virus were monitored. As a control, the parental TAP\(^{-/-}\) or TgH(KL25) mouse strains were infected with the same dose of virus. As depicted in Fig. 1, TAP\(^{-/-}\) animals failed to control viremia and mounted a weak neutralizing antibody response between days 28 and 46 after infection. Concomitantly with the emergence of neutralizing antibodies, viremia was reduced or temporarily controlled to a point below the detection level (for two out of three animals at day 67 after infection). As expected (32), TgH(KL25) mice controlled viremia efficiently and mounted a strong neutralizing antibody response by day 4. In contrast, TgH(KL25)xTAP\(^{-/-}\) mice failed to control viremia despite the simultaneous presence of high titers of neutralizing antibodies, suggesting that the persisting viruses were not sensitive to neutralization by the endogenous antibody response. Indeed, virus isolates from these mice were not neutralized by 100 \(\mu\)g/ml KL25 in infectivity reduction assays (Fig. 2 A).

A low-dose (200 PFU) infection of TgH(KL25)xTAP\(^{-/-}\) mice also selected for antibody escape variants and resulted in a strong neutralizing antibody response by day 4 after infection.

Figure 1. Rapid selection of antibody escape variants after infection of CTL-deficient TgH(KL25) mice. Blood viremia (top) and neutralizing antibody responses (bottom) in TAP\(^{-/-}\), TgH(KL25), or TgH(KL25)xTAP\(^{-/-}\) mice after i.v. infection with 2 \(\times\) 10\(^6\) or 200 PFU LCMV-WE. The dashed line indicates the detection level for blood virus and neutralizing IgG titers. Error bars represent the mean ± SD \((n = 2–3\) mice each). One representative out of two independent experiments is shown.
in persistent infections, but this selection was delayed, with viremia only being detected after day 12 (Fig. 1).

These data demonstrate that increased LCMV replication in the absence of CTL was sufficient to select for variants that have escaped a concomitantly present oligoclonal neutralizing antibody response.

**Analysis of virus isolates resistant to KL25-mediated neutralization revealed differences in their ability to bind KL25**

Loss of sensitivity for KL25-mediated neutralization has previously been associated with a loss of antibody binding (30, 34). We therefore analyzed the ability of the neutralization-sensitive virus isolates from TAP−/− mice to bind to KL25 and compared these with neutralization-resistant isolates from TgH(KL25)xTAP−/− mice. The mAb KL25 was used for this analysis, as the neutralizing antibodies induced early in TgH(KL25) mice are known to be closely related to KL25 and are recognized by idiotypic antibodies specific for mAb KL25 (32).

Binding of KL25 to MC57G cells infected with different virus variants was determined by flow cytometry. We corrected for differing GP surface expression levels by normalizing flow cytometric measurements with parallel stainings using mAb WEN1.3, which binds equally well to the GP of either WT or KL25 escape variants (30, 34). Three categories of virus isolates could be identified: (a) strong-binding isolates, which were defined as those variants whose KL25 mean fluorescence intensity (MFI) was measured to be 50–100% of the MFI measured for WEN1.3; (b) low-binding isolates, defined as those whose MFI percentiles ranged from 10 to 50; and (c) nonbinding isolates, defined as those having <10% KL25 binding.

A total of 59 single-round subcloned virus isolates obtained from 8 TAP−/− and 55 single-round subcloned virus isolates obtained from 8 TgH(KL25)xTAP−/− mice were analyzed. As expected, all neutralization-sensitive isolates from TAP−/− mice retained strong binding to KL25 (Fig. 2 B, left), indicating that in TAP−/− mice the polyclonal antibody response did not exert a selective pressure against the epitope recognized by KL25. In contrast, several neutralization-resistant LCMV variants isolated from TgH(KL25)xTAP−/− mice had lost binding to KL25 (Fig. 2 B, right). Yet, we were able to isolate two neutralization-resistant variants from day 8–infected TgH(KL25)xTAP−/− mice that retained strong binding to KL25. In addition, a considerable fraction of the neutralization-resistant clones isolated from TgH(KL25)xTAP−/− mice on days 12 and 20 exhibited low binding to KL25. Thus, in TgH(KL25)xTAP−/− mice most of the virus isolates that had escaped KL25 neutralization by day 8; however, some antibody (KL25)–binding activity remained. More interestingly, the presence of such antibody–binding isolates was observed to diminish over time, indicating the presence of a selective pressure against such isolates.

**Characterization of four viral isolates**

Four escape variants, no longer susceptible to neutralization by KL25, were chosen for a more detailed analysis based either on their KL25 binding properties or on the mutations identified by sequencing (Table I). The variants chosen consisted of one strong-binding (CL2.1, Asn121Lys), two low-binding (20#8p2.1, Asn119Asp and 8#1p1.1, Asn171Asp), and one nonbinding (10.1.1, Asn190Ser) isolates. Infectivity of these isolates could eventually be decreased with increasing concentrations of KL25; however, the amount of KL25 required was at least 60-fold higher compared with WT LCMV-WE (Table I). These high ID50 scores likely indicate a drastically decreased affinity of KL25 for the functional GP multimers on the virion surface. All mutations observed involved replacement of a single Asn residue, which has previously been associated with antibody escape by LCMV (29–31). Moreover, the Asn171Asp mutation found in the isolate 8#1p1.1 abolished N-linked glycosylation at the same glycosylation site that is absent in Armstrong isolates sensitive to neutralization by gp-1D epitope–specific antibodies (35).
Table I. Overview of the LCMV isolates used

<table>
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<tr>
<th>LCMV isolate</th>
<th>Mutation</th>
<th>Neutralization by KL25†</th>
<th>Binding of KL25‡</th>
<th>ID50 (μg KL25/ml)†</th>
<th>Kd (KL25)/Kd (WEN3.1)§</th>
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<tr>
<td>WE</td>
<td>None</td>
<td>Yes</td>
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</tbody>
</table>

†Viruses isolated from blood of LCMV-WE–infected TgH(KL25)xTAP−/− mice on day 8 or 20 after infection.
‡Neutralization as determined by the ability of purified mAb KL25 [100 μg/ml] to reduce viral infectivity as described in the Materials and methods.
§According to flow cytometric measurement of infected cells stained with FITC-labeled KL25 at a concentration of 15–20 μg/ml.

To confirm that the flow cytometric data could be directly correlated with the detected mutations, and to determine the avidity of KL25 for dimeric mutant GP-Fc fusion proteins, the extracellular portion of GP from WT and/or mutant LCMV isolates was recombinantly expressed as a fusion protein with human immunoglobulin Fc. Equal amounts of recombinant proteins were then immobilized on anti–human-Fc–coated plates, and the binding of titrated amounts of KL25 assessed. As a control to ensure equal amounts of correctly folded fusion proteins were present, the binding of WEN3.1 (a different mAb that recognizes WT and mutant GP with equal efficiency) was also assessed (depicted as OD405 values in Fig. 3). All neutralization escape variants displayed comparable binding to WEN3.1. Consistent with the flow cytometric data, we observed no binding of KL25 to the GP fusion protein from the nonbinding isolate LCMV-10.1.1, but intermediate binding to GP fusion proteins constructed from isolates 20#8p2.1 and 8#1p1.1 (Fig. 3). Moreover, the GP fusion protein carrying the Asp121Lys mutation from the strong-binding LCMV isolate cl12.1 exhibited the highest degree of binding to KL25 (Fig. 3). Collectively, these data confirm that the mutations found in the GP of the KL25 escape variants were directly responsible for the different degrees of KL25 binding observed. These data also demonstrate that KL25 has a lower avidity for neutralization-resistant, compared with neutralization-sensitive, GP variants. These differences in avidity, however, were less than would have been predicted from the neutralization data. This may indicate that the escape mutations affect KL25 binding more severely in its functional multimeric form on the virion surface than for the recombinant form. Indeed, a similar phenomenon has been observed for gp120 of HIV (36).

Residual binding to KL25 correlated with accelerated virus clearance and immunogenicity in vivo

The observed disappearance of KL25–binding virus variants in TgH(KL25)xTAP−/− mice over time suggested that neutralization-resistant viruses that retain binding to KL25 have a selective disadvantage in these hosts (Fig. 2). To determine whether surface binding of nonneutralizing antibodies could contribute to viral clearance, C57BL/6 and TgH(KL25) mice were infected with 200 PFU of LCMV-WE, cl12.1, 8#1p1.1, 20#8p2.1, or 10.1.1 virus isolates.

Neutralizing antibody titers were then determined against both WT LCMV-WE and the infecting LCMV variants. As expected from previous studies demonstrating an original antigenic sinlike behavior of LCMV (29, 31), infection of TgH(KL25) mice with LCMV variants other than WE readily induced neutralizing antibodies against LCMV-WE (Fig. 4 A). This induction of WE–neutralizing antibodies was most efficient with the strong-binding isolate cl12.1 and the low-binding isolate 8#1p1.1, but was marginally delayed for the low-binding isolate 20#8p2.1. Interestingly, the nonbinding isolate 10.1.1 not only induced a delayed WE-specific.
IgM antibody response but also failed to induce a normal anti-LCMV-WE IgG neutralizing antibody response. Analysis of the antibody response against the infecting variants revealed that only isolates cl12.1 and 8#1p1.1 were able to induce low (1:40–80) neutralizing titers against themselves in TgH(KL25) mice (Fig. 4 A). The other two variants were unable to induce autologous neutralizing titers within the observation period of 28 d. Collectively, these data demonstrated that the early antibody response of TgH(KL25) mice against escape variants is largely constituted of binding, but nonneutralizing, antibodies.

Viral titers within the spleens of C57BL/6 and TgH(KL25) mice were determined at day 4 or 6 after infection with 200 PFU of LCMV-WE, cl12.1, 8#1p1.1, 20#8p2.1 or 10.1.1. In TgH(KL25) mice, the neutralization-sensitive WE strain of LCMV was the most susceptible to antibody-mediated suppression (Fig. 4 B). This was in clear contrast to the neutralization-resistant and KL25-nonbinding isolate 10.1.1, which displayed splenic virus titers that were comparable in C57BL/6 and TgH(KL25) mice on both days 4 and 6. The neutralization-resistant, but strong-binding, isolate cl12.1 behaved similarly to the WE strain. In TgH(KL25) mice, this isolate also exhibited reduced maximal virus titers on day 4 that became undetectable by day 6. In contrast, the range of viral titers in C57BL/6 mice at this time point was still $10^2$–$10^3$ PFU/organ. The low-binding isolates (8#1p1.1 and 20#8p2.1) displayed only minor differences in splenic organ virus titers between C57BL/6 and TgH(KL25) mice.

Collectively, these data demonstrate that viruses that are not sensitive to KL25-mediated neutralization in vitro, but retain the ability to bind to KL25, are more immunogenic in TgH(KL25) mice and more efficiently cleared than virus variants that display no residual antibody binding.

Accelerated viral clearance mediated by binding, but nonneutralizing, antibodies is partially dependent on complement

We next assessed the role of complement in accelerated virus clearance because complement has been shown to augment the antiviral activity of nonneutralizing antibodies (19, 21, 23). C57BL/6 and TgH(KL25) mice were infected with 200 PFU of cl12.1 and treated daily with 2 U of cobra venom factor (CVF) from *Naja naja* i.p. to deplete C3 and C5 complement components (37). Nontreated C57BL/6

Figure 4. Accelerated viral clearance by nonneutralizing antibodies. (A) Neutralizing antibody response of TgH(KL25) mice infected with 200 PFU of the indicated variants of LCMV. (top) Heterologous neutralizing antibody titers induced against WT LCMV-WE. (bottom) Autologous neutralizing antibody titers induced against the infecting viral variant. Error bars represent mean ± SD (n = 3 mice). Complement present in sera was not heat inactivated before the assay. (B) Viral titers in spleen of C57BL/6 and TgH(KL25) mice infected with 200 PFU of LCMV-WE or the indicated variants of LCMV. Each symbol represents the viral titer measured for a single mouse on the indicated day. The solid line indicates the mean of the experimental group, and the dashed line indicates the detection level of the assay.
Figure 5. Impact of complement on virus clearance of the high-binding isolate cl12.1 in TgH(KL25) mice. (A) Virus titers in spleen of TgH(KL25) mice infected with 200 PFU of the cl12.1 isolate. To deplete activity of the C3 and C5 complement components, animals received 2 U of CVF i.p. before infection and every 24 h thereafter while the control group remained nontreated. Results are representative of at least two separate experiments. (B) Virus titer in spleens of C57BL/6 or C3-deficient mice receiving $3 \times 10^7$ TgH(KL25) splenocytes 1 d before infection with 200 PFU cl12.1 i.v. Each symbol represents an individual animal. The solid line indicates the mean of the experimental group, and the dashed line indicates the detection level of the assay. Results are representative of a single experiment.

Accelerated viral clearance mediated by binding, but nonneutralizing, antibodies does not require the presence of specific B cells

To investigate whether the accelerated virus clearance only requires antibodies, or whether specific B cells need to be present, passive immunization experiments were performed. Splenic viral titers were assessed in three groups of C57BL/6 mice at days 4 and 6 after infection with 200 PFU of LCMV cl12.1 i.v. To investigate the role of increased natural antibody titers, a first group received 750 μl of pooled naive TgH(KL25) serum 20 min before infection. For analysis of the role of the early nonneutralizing antibodies, a second group of mice received 200 μg of purified mAb KL25 1 d after infection, whereas a third group of mice remained nontreated. The amount of passively transferred antibody was chosen according to the maximal volume suitable for i.v. injection (naive serum), or as determined by previous experiments (6).

As depicted in Fig. 6, transfer of naive TgH(KL25) serum or mAb KL25 decreased splenic virus titers on days 4 and 6. This reduction was more prominent after transfer of mAb KL25 compared with transfer of TgH(KL25) serum, probably as a result of neutralizing antibody dose and half-life. However, both sets of data demonstrate that accelerated clearance can be mediated by nonneutralizing antibodies alone and does not require the presence of LCMV-GP–specific B cells.

DISCUSSION

We have assessed the biological function of antibodies that exhibit binding, but not neutralizing, activity against the major neutralizing antigenic site (39) of LCMV-GP. For this purpose we used LCMV isolates selected in CTL-compromised TgH(KL25) mice. Two distinct groups of escape variants were identified among the neutralization resistant isolates. One group had lost both the ability to bind to the selecting antibody and sensitivity to antibody-mediated neutralization. The second group of escape variants was highly resistant to neutralization in vitro but still displayed antibody binding. Antibody-binding virus variants were only isolated at early time points after infection and were not found in later blood samples obtained from the same mice. This indicated that these binding antibodies were able to exert selective pressure on LCMV. Indeed, antibody-binding virus variants were
cleared more rapidly than nonbinding variants. This enhanced clearance was partially dependent on complement.

For all isolates exhibiting KL25 binding but not neutralization, KL25 was eventually able to decrease infectivity in vitro; however, this required very high concentrations of antibody. Yet, such antibody-mediated reduction of viral infectivity would not be expected to contribute to the observed biological effects in vivo, as the serum concentration of KL25 on day 4 after infection is ≤10% of the concentration required for in vitro neutralization. Moreover, passive transfer of 200 μg KL25 into C57BL/6 mice exhibited a clear biological activity against the cl12.1 isolate despite a calculated final serum antibody concentration well below that required for the in vitro effect.

Interestingly, avidity of KL25 for mutant GP multimers on virions was more affected than for the multimeric form present in the Ig fusion proteins. This conclusion was drawn from the observations that the ID50 values measured for the WT or escape mutation viruses showed greater differences than did KL25-binding affinities (as detected by ELISA) for the same mutations present in GP-Fc fusion proteins (Table I). Moreover, because we observed binding of KL25 to cells infected with the antibody-binding escape variants, it is possible that the observed biological activity of KL25 against neutralization-resistant variants is mediated by antibody binding to unnatural multimerized GP variants present on infected cells. Subsequent complement activation might therefore be responsible for a considerable proportion of the observed antiviral activity. The residual antiviral effects may be attributed to antibody-dependent cellular cytotoxicity. Based on other reports investigating the role of natural antibodies in viral clearance (40), we assume that this acceleration is also improved by increased removal of virus in the marginal zone of the spleen and accelerated priming of the adaptive immune system. However, from our data it cannot be excluded that complement-mediated lysis of virions and infected cells was also involved. Indeed, complement-dependent in vitro inactivation of HIV virions has been demonstrated for nonneutralizing sera obtained from acute phase HIV patients (23). In addition, complement-deprived rhesus monkeys infected with the mac251 strain of simian immunodeficiency virus were found to have higher virus titers during the acute phase of infection and an accelerated clinical course of disease compared with monkeys with a functional complement system (41).

The nonneutralizing antibodies mediating accelerated virus clearance in this study belonged to the group of nonneutralizing antibodies that recognize the neutralizing epitope of LCMV-WE (GP-1A) (39) with low affinity. Nonneutralizing antibodies specific for other epitopes on the LCMV-GP (e.g., GP-1B or GP1C) (39) may also mediate some biological activity, although this could not be addressed in the presented models. However, our data does suggest that affinities close to the neutralization threshold of 5 × 10^7 M⁻¹ (12) are required for antiviral function of nonneutralizing antibodies. Indeed, we demonstrate that the biological effectiveness of nonneutralizing antibodies rapidly decreases with decreasing affinity of the LCMV-GP–antibody interaction (clearance of cl12.1 vs. 8#1p1.1 and 20#8p2.1). In contrast, even very high-affinity antibodies from the second group of debris-specific nonneutralizing antibodies may not exhibit such antiviral activity because their epitopes are unlikely to be present on infectious virions (17).

Sequence analysis of the GP gene of the isolated virus variants revealed that all neutralization-resistant viruses had single substitutions of Asn residues previously described to be involved in antibody escape of LCMV (29–31). Alteration of the glycosylation of viral surface proteins, as observed in variant 8#1p1.1, has been described previously as a mechanism to escape antibody recognition (35, 42, 43). Because we have no evidence that KL25 directly contacts glycans and because it has been described that complete glycosylation is crucial for the correct folding of LCMV-GP (35), we believe that loss of glycosylation at Asn171 induced a slight conformational change in the protein backbone, resulting in impaired KL25 binding and neutralization. All other mutations affected Asn19 or amino acids in close proximity. We therefore suspect that these amino acids are exposed within, or close to, the area contacted by KL25.

Antibody-binding escape variants like cl12 were only rarely isolated. This may result from LCMV cl12.1 having a decreased fitness as compared with the other escape variants or the parental LCMV-WE strain. Indeed, LCMV cl12.1 propagated less well in vitro (not depicted) and in vivo (splenic virus titers in C57BL/6 mice; Fig. 4 B), and was more efficiently controlled by nonneutralizing antibodies than the low- or nonbinding isolates (Fig. 4 B). Thus, it is not clear why such variants should arise. One might speculate that the mutation found in cl12.1 reflects a preference of the RNA-dependent RNA polymerase for certain substitutions, yet available sequence data provide no evidence for any particular sequence motif or preferred substitution (29–31). A two-phase model for the selection of escape variants may help to explain selection of viruses like LCMV-cl12.1. First, viral variants that have liberated the receptor-binding site of GP from the neutralizing activity of antibodies would be selected. These viruses would propagate locally by infecting neighboring cells, with their exposure to antibodies consequently being very limited. Such early variants could therefore afford to exhibit decreased viral fitness and to bind antibodies. However, during a second and later phase, viruses that propagate well and can disseminate via blood without being captured by binding antibodies and complement would be selected.

We do not believe that the findings presented in this study represent an isolated phenomenon specific for the transgenic mouse model used. First, all mutations found have previously been associated with antibody escape of LCMV in WT mice. Second, there are other reports that nonneutralizing antibodies may have a beneficial role in other viral infections (19–24). However, nonneutralizing antibodies may only be important for the clearance of viruses that induce
delayed and weak neutralizing antibody responses, such as HIV, hepatitis C virus, or hepatitis B virus. For viruses eliciting an early and strong neutralizing antibody response, the presence of binding, but nonneutralizing, antibodies is unlikely to play a substantial role in viral clearance.

To date, the biological function of nonneutralizing antibodies specific for the LCMV-GP has been poorly understood. Yet, circumstantial evidence that such antibodies may be of biological relevance has come from studies using B cell-deficient mice that exhibit impaired control of high-dose LCMV (5, 10, 11). We now provide direct in vivo evidence that antibodies that bind to the neutralizing epitope of LCMV, but do not mediate viral neutralization, are able to accelerate virus clearance in a complement-dependent manner. In summary, we demonstrate that antibodies that arise early during the immune response and exhibit binding activity against viral surface proteins can play an important biological role by reducing early spread.

**MATERIALS AND METHODS**

**Viruses.** LCMV strain WE was originally obtained from F. Lehmann-Grube (Heinrich-Pette-Institut, Hamburg, Germany) and propagated on L929 cells.

For isolation of mutant viruses from the blood of infected animals, blood was diluted 1:10 in BSS containing 0.02% (vol/vol) heparin and pre-amplified on 10^6 MC57G cells infected with 50 μl of the BSS-diluted blood. Supernatants were harvested after 48 and 72 h, and stored at 70°C for further analysis (polyclonal virus preparations).

For subcloning, preamplified virus was serially diluted 1:10 in MEM containing 2% FCS and added to 4–8 × 10^6 MC57G cells. After incubation of cells under an overlay of 3 ml DMEM containing 5% FCS and 1% low-temperature analysis (polyclonal virus preparations).

Flow cytometric analysis of KL25 binding to the surface of infected cells. To assess binding of KL25 to LCMV-GP expressed on infected cells, the method described by Seiler et al. was used (30). In brief, cells were infected at a multiplicity of infection of 0.001–0.01, incubated for 48 h at 37°C/5%CO2, and harvested using 1 mM EDTA in PBS as a cell-detaching reagent. For each virus, one sample was stained with purified WEN3.1 at a concentration of 15–20 μg/ml to determine LCMV-GP surface expression, and one was stained with purified KL25 at a concentration of 15–20 μg/ml to determine KL25 binding. The MFI of stained but noninfected samples was used to determine background staining, which was then subtracted from the corresponding MFI measured for the infected samples.

**LCMV infectious focus formation, neutralization assays, and determination of ID50 of KL25.** Virus and neutralizing antibody titers were determined as described previously (14, 32, 44). For determination of ID50 values, twofold serial dilutions of 11 μg/ml purified KL25 or PBS were prepared in MEM containing 2% FCS and mixed with the same volume of medium containing 50–150 PFU of the LCMV variant. The concentration of antibody reducing the number of plaques by half was determined as ID50.

**Recombinant expression of mutant LCMV-GP.** A detailed description of the construction and expression of recombinant LCMV-GP will be published elsewhere. In brief, for the construction of the mutant LCMV-GP/human Fc fusion protein, a PCR-based approach was chosen using a codon-optimized GP–open reading frame as a template (provided by D. von Laer, Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie, Hamburg, Germany). To prevent posttranslational processing of the fusion proteins into GP-1 and GP-2–Fcε portions, Arg 262 was replaced by Ala as described by Beyer et al. (45). Proteins were expressed in the supernatant of human 293T cells and purified on protein A columns. Protein concentration was determined by an anti-human-Fcγ1–specific ELISA using purified human IgG1 as a standard.

**Analysis of KL25 binding by ELISA.** WT and mutant recombinant GP was immobilized on goat F(ab)2 anti–human-Fcγ-coated plates (1:800; Jackson Immunoresearch Laboratories) at 1.6 ng/ml. Threefold serial dilutions of biotinylated KL25 or unlabeled WEN3.1 were prepared starting at 6 μg/ml and added to the immobilized fusion proteins. Bound KL25 was detected using peroxidase–labeled streptavidin (1:1,000; Jackson Immunoresearch Laboratories), whereas binding of WEN3.1 was revealed by horseradish peroxidase–labeled goat anti–mouse IgG (1:1,000; Sigma-Aldrich).

**Infectivity reduction assay.** Virus was incubated with 5 μg KL25, or the same volume of PBS, for 1 h at 37°C/5% CO2. After preparing twofold serial dilutions, virus was transferred to MC57G cells. Infectious foci were revealed by immunohistological staining after 2 d of incubation under a 1% methylcellulose overlay. Those titration steps giving >20 plaques were determined, and the delta value (Δ) was calculated as titer value of the mock-treated virus minus the value of the KL25-treated virus. Viruses were considered as neutralization resistant if Δ = 1 or 0.

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neuron disease by non-neutralizing antiviral antibodies without interference with virus replication.


