Point mutation in the glycoprotein of lymphocytic choriomeningitis virus is necessary for receptor binding, dendritic cell infection, and long-term persistence

Brian M. Sullivan[a,1], Sébastien F. Emonet[a,1], Megan J. Welch[a], Andrew M. Lee[a], Kevin P. Campbell[b], Juan C. de la Torre[a], and Michael B. Oldstone[a,c,2]

[a]Viral-Immunobiology Laboratory, Department of Immunology and Microbial Science, and [c]Department of Infectology, The Scripps Research Institute, La Jolla, CA 92037; and [b]The Howard Hughes Medical Institute, Department of Molecular Physiology and Biophysics, Departments of Neurology and Internal Medicine, University of Iowa Roy J. and Lucille A. Carver College of Medicine, Iowa City, IA 52242

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Arenaviruses are a major cause of hemorrhagic fevers endemic to Sub-Saharan Africa and South America, and thus a major public health and medical concern. The prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) is widely used as a model system for studying persistent and acute infections, as well as for gaining an understanding of mammalian immune function. When originally characterized three decades ago, the LCMV isolate, Armstrong, which causes an acute infection in adult mice, was found to differ from the LCMV Clone 13 strain that causes a persistent infection by two amino acid changes, one within the virus surface glycoprotein (GP1: F260L) and the other within the virus L polymerase (K1076Q). Mutation F260L was considered solely responsible for the exceptionally strong binding affinity of Clone 13 (L at GP1 260) to its cellular receptor, α-dystroglycan, which among cells of the immune system is preferentially expressed on dendritic cells, and consequently, alters dendritic cell function leading to viral persistence. Recently, we noted a previously overlooked nucleotide difference between these two strains that results in an additional amino acid change in GP1, N176D. To investigate the potential contribution of this newly identified mutation to the Clone 13 phenotype, we used reverse-genetics approaches to generate recombinant LCM viruses with each of these individual mutations. Phenotypic characterization of these rLCMV showed that mutation F260L, but not N176D, in the GP1 of LCMV is essential for mediating the long-term persistence of Clone 13 infections. This work emphasizes the importance of subtle differences in viral strains that determine disease outcomes.

Viruses often modulate the host immune response to initiate and maintain persistent infection within their hosts. For more than three decades, the lymphocytic choriomeningitis virus (LCMV) model has been used to study the effects of viral persistence on host immunity (1). Findings with LCMV have led to insights on negative immune regulators (2–4) present in other persistent viral infections, such as HIV (5, 6) and hepatitis C virus (7–9).

LCMV Clone 13, originally isolated from the spleens of mice infected with the Armstrong strain of LCMV (10), was shown to differ from its parental strain by five nucleotides, two of which resulted in coding changes (11–13). A change from a lysine (K) to a glutamine (Q) at position 1079 in the L protein, an RNA-dependent RNA polymerase, selectively increased viral titers in macrophages, but not in other cell types studied (14). The mechanism as well as the contribution of this change to viral persistence remains unclear.

The second coding change occurs at position 260 in the viral spike protein GP1, where a leucine (L) in the Clone 13 strain replaces a phenylalanine (F) in the parental Armstrong strain (12, 13). A leucine or isoleucine in this position is conserved among LCMV isolates that bind at high affinity to α-dystroglycan (αDG), the receptor for the virus, infect dendritic cells (DCs), and that abort T-cell antiviral function, leading to persistent infection (15–17). Clone 13 but not Armstrong is able to displace extracellular matrix proteins, such as laminin from αDG, which share the same binding site as the virus, thereby facilitating virus–receptor interaction (18). In contrast, the 1079Q change in the polymerase of Clone 13 is not constant but variable in other LCMV variants that cause persistence (15).

The increased affinity of Clone 13 and other Old World arenaviruses for αDG is highly relevant, allowing the virus to infect and deregulate an essential component of the viral immune response, the DCs (16, 18–20). Among cells of the immune system, DCs preferentially express high levels of αDG and are subject to a high rate of infection by Clone 13 (20), leading to decreased amounts of costimulatory ligands and an inability to fully prime T cells, thus promoting a persistent infection (21). In addition, persistent LCMV infection of DCs also up-regulates immunosuppressive cytokines and, coupled with up-regulation of T-cell inhibitory receptors, cause cytotoxic $CD8^+$ T cells and $CD4^+$ T helper cells to become functionally exhausted (2, 3, 22–24). Thus, these negative regulators of the immune response impair the ability of $CD8^+$ T cells to clear LCMV infections because mice depleted of these cells never purge the virus (25). $CD4^+$ helper T cells assist virus specific $CD8^+$ T cells in clearance of persistent infection and provide help to B cells to make antibodies. During Clone 13 persistence, there is a pan immunosuppression of T-cell and B-cell responses (19). In addition, Clone 13 infection induces the release of immunosuppressive cytokines and the up-regulation of inhibitory receptors on $CD8^+$ T cells. These negative regulators and inhibitory receptors maintain the exhausted state of T cells during a persistent infection. Mice depleted of or deficient in immunosuppressive cytokines, such as IL-10, PD-L1, and TGF-β, fail to establish or maintain a persistent infection (2–4, 22). Blockade of inhibitory receptors PD-1, IL-10R, Tim-3, and LAG3 can resurrect cytotoxic $CD8^+$ T-cell function and lead to significant lowering of viral titers in Clone 13 infected mice (23, 24).

Recently, we identified an additional, previously overlooked, amino acid substitution at position 176 in GP1 resulting in the replacement of an asparagine (N) encoded by Armstrong by an aspartic acid (D) encoded by Clone 13. Although not as pervasive as the leucine or isoleucine at position 260 found in >95% of cloned virus isolates shown to cause persistence, this newly identified N176D substitution in GP1 was found to be present in roughly half of persistent LCMV strains, suggesting it may have a possible role in viral persistence. We used arenavirus reverse

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[1]B.M.S. and S.F.E. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: mbaobo@scripps.edu.

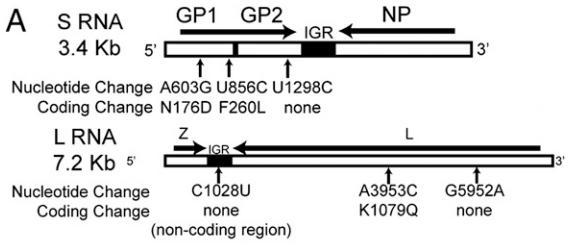
genetics (26) to examine the individual contribution of each mutation in the viral GP1 of LCMV to binding to its cognate receptor, ability to infect DCs and interfere with the generation and function of cytotoxic $CD8^+$ T cells, and persistance in its murine host.

Results

Identification of a Second Mutation in the Viral Glycoprotein of Clone 13. We recently identified a change at position 176 in the GP1 of current working stocks of Clone 13, which led us to revisit the sequence of early stocks of Clone 13. Sequencing of several independent early stocks of Clone 13 prepared in the early 1980s consistently showed the presence of this additional mutation in GP1 of Clone13. This mutation results in a coding change from the parental Armstrong codon of AAT to a GAT, changing the amino acid at position 176 of the GP1 protein from an asparagine to an aspartic acid (Fig. 1*A*). Previously, a leucine at position 260 was considered essential for high-affinity virus binding to the host receptor, αDG, which resulted in high levels of DC infection associated with establishment of the persistent infection by Clone 13 (15–17). Sequencing of a collection of persistently infecting LCMV strains show a high prevalence of a leucine or isoleucine coded at this position (15). To determine whether an aspartic acid (D) at position 176 is conserved in persistently infecting strains of LCMV, we sequenced several laboratory isolates. Although the aspartic acid at position 176 was not uniformly conserved (Fig. 1*B*), it loosely correlated with a higher viral titer at day 30 postinfection in mice infected with these viruses, suggesting that it may play a role in viral fitness in vivo.

To study the role of each of the GP1 mutations in binding of αDG and virus persistence, we used reverse genetics to generate genetically defined molecular clones containing individual mutations between Armstrong and Clone 13, as well as parental Armstrong and Clone 13 strains. This protocol ensured genetic homogeneity in our viral populations. Recombinant LCMV 260L retains both the leucine at position 260 of the GP1 and the glutamine present in the L protein of the Clone 13 strain, and also encodes the asparagine at position 176 of the GP1 shared by Armstrong. Recombinant LCMV 176D retains the aspartic acid at position 176 of GP1 as well as the glutamine in the L protein of Clone 13 (Fig. 2*A*) and also encodes a phenylalanine at position 260 of the GP1 shared by Armstrong. To assess the fitness of these viruses in cultured cells, we infected [multiplicity of infection (MOI) = 0.01] BHK-21 cells with each virus and, at the indicated times postinfection, the virus present in tissue culture supernatants were titered by immune focus assay (Fig. 2*B*). All viruses tested exhibited similar growth properties in BHK-21 cells.

F260L Mutation Is Responsible for Persistence of Clone 13 in Vivo. Despite Clone 13 and Armstrong displaying similar fitness in cultured cells, in mice Armstrong was readily cleared, whereas Clone 13 established a persistent infection lasting over 60 d (Fig. 2*C*, *Upper*) (10). We then determined the contribution of each mutation to the persistent phenotype of Clone 13. For this process, we infected C57/Bl6 mice with 2×10^6 pfus intravenously and determined serum titers at several time points up to 60 d postinfection. Mice injected with either Armstrong or 176D cleared the infection by 17 d postinfection. In contrast, mice injected with either Clone 13 or 260L displayed similar viremia that lasted longer than 60 d postinfection (Fig. 2*C*). Importantly, when resequenced, virus from the plasma of each infected mouse matched the input virus, indicating that no sig-



B

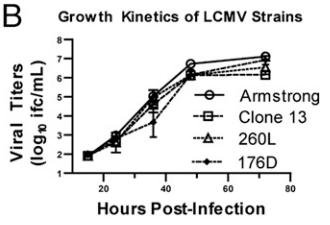
Virus Isolate	a.a.[1] at position 176 of GP	a.a. at position 260 of GP	a.a. at position 1079 of L protein
Arm 53b	N	F	K
Clone 13[2]	D	L	Q
Mac 13.3[2]	D	L	K
PBL 7.1[2]	D	L	K
PBL 36.2[2]	N	L	N
PBL 36.3[2]	N	L	N
PBL 66.1[2]	N	L	K
PBL 66.2[2]	N	L	K

[1]a.a. = amino acid
[2]LCMV strains that cause persistent infection when innoculated into adult mice

Fig. 1. Three amino acids differentiate acute and persistent infections. (*A*) Nucleotide changes are indicated comparing the parental Armstrong strain causing acute infection to the Clone 13 strain causing a persistent infection. Two coding mutations occur in the viral spike, GP1, on the S segment, whereas a single coding change occurs in the viral polymerase (L protein). (*B*) Amino acids at positions 176 and 260 of the viral GP and position 1079 of the L protein in different isolates of LCMV. Various strains of LCMV were sequenced and the occurrence of specific amino acids at the three positions where Armstrong and Clone 13 differ is noted. All strains listed except for Armstrong were previously shown to persist in mice for at least 30 d post infection (15).

A

	S Segment GP1		L Segment L
	176	260	1079
Armstrong3B	Asn	Phe	Lys
Clone 13	Asp	Leu	Gln
260L	Asn	Leu	Gln
176D	Asp	Phe	Gln



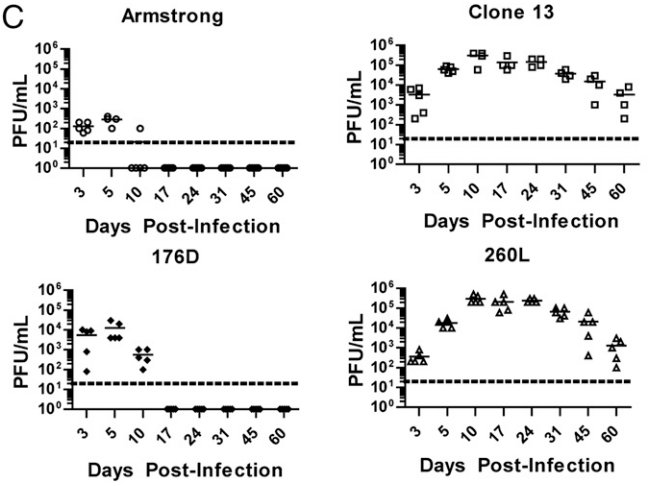


Fig. 2. Viral titers in vitro and in vivo of recombinant LCMV strains differing in the two amino acids under study in the viral glycoprotein. (*A*) Chart listing the strains used in experiments described in this manuscript and their distinctive properties. (*B*) Growth kinetics showing viral output from BHK-21 cells infected at an MOI of 0.001. Supernatant was collected at times indicated on the graph and titered on Vero cells using a fluorescent focus assay to count individual infected centers. (*C*) Serum titers of mice injected with 2×10^6 pfu of virus. Mice infected with each recombinant strain were bled at times indicated on the graph. Serum titers were determined by plaque assay on Vero cells. $n = 5$ group except for Clone 13 where one mouse died before day 10.

nificant mutations garnered in vivo contributed to persistence. Therefore, a leucine at position 260 of the GP1 is strictly necessary for Clone 13-type persistence.

N176D Mutation Does Not Contribute to Functional Exhaustion of T Cells. Persistent infection with Clone 13 was shown to be caused by functional exhaustion of virus-specific $CD8^+$ T cells, rendering the host's immune system incapable of clearing the virus (10, 27). We, therefore, used a chromium-51 (^{51}Cr) release assay to test splenocytes recovered at day 9 postinfection from mice infected with each recombinant virus for the ability to kill LCMV-infected H-2–matched target cells. Splenocytes from Armstrong- and 176D-infected mice effectively killed infected target cells, whereas splenocytes from Clone 13- or 260L-infected mice were severely restricted ($P < 0.0005$ in each case) from such killing (Fig. 3*A*, *Left*). Importantly, splenocytes from mice infected with any of the recombinant viruses could not kill infected H-2 mismatched target cells (Fig. 3*A*, *Right*), indicating that specific killing resulted from absolute MHC restriction for cytotoxic T lymphocytes (CTLs).

Next, we tested the ability of $CD8^+$ T cells isolated from mice infected with each recombinant virus to respond to known dominant viral peptide epitopes. Splenocytes isolated from mice at 9 d postinfection were incubated with IL-2, brefeldin A, and either GP33-41 or NP396-404 immunodominant CTL peptide epitopes. The percentage of Thy1.2, $CD8^+$ cells expressing IFN-γ and TNF-α was assessed by flow cytometry (Fig. 3*B*, *Left*). The percentage of functionally activated T cells was significantly lower in mice infected with Clone 13 and 260L than in their counterparts infected with either Armstrong or 176D ($P < 0.005$ in each case). In addition, percentages of T cells specific for either epitope, measured by staining with MHC tetramers specific for these T-cell clones, were similar in all groups (Fig. 3*B*, *Right*). Taken together, these data indicate that although $CD8^+$ T cells are present in mice infected with any of the LCMV strains studied here, the function of these T cells are severely impaired in mice infected with LCMV Clone 13 strain or recombinant LCMV 260L but not mice infected with LCMV Armstrong or recombinant 176D.

F260L Mutation Enhances GP1 Binding to Host Receptor, αDG. The ability of Clone 13 to persist in mice has been associated with high binding affinity for its host receptor, αDG. Clone 13 has been reported to bind αDG several orders of magnitude more strongly than Armstrong and can, therefore, displace cognate ligands of αDG, such as laminin, whereas Armstrong cannot (18). To identify the contribution of residues at positions 176 and 260 of the viral GP1 for αDG binding, we performed two distinct solid-phase binding assays using a soluble fragment of αDG, called DGFc4 (18, 28). This fragment contains the lectin binding domain that has been shown to bind LCMV virions. We purified each LCMV variant and incubated equivalent pfus of each with DGFc4 immobilized onto nitrocellulose. Binding was determined by probing with antibodies against GP1 and GP2 followed by HRP-conjugated secondary antibodies and substrate for visualization on film. Our results illustrate that Clone 13 and 260L robustly bound DGFc4 but, in contrast, Armstrong and 176 did not (Fig. 4*A*).

We obtained similar results with an ELISA-based assay. Here, equal amounts of purified viral variants were immobilized onto wells of an ELISA microtiter plate and various amounts of DGFc4 were added. Binding was determined by adding antibodies to the human Fc portion of DGFc4 and HRP-conjugated secondary antibodies, followed by addition of a colorimetric substrate. Clone13 and 260L bound strongly even at low DGFc4 concentrations compared with Armstrong and 176D (Fig. 4*B*). These data indicate that the leucine at position 260 of the viral GP1 is responsible for the strong binding of Clone 13 to its cognate host receptor, αDG.

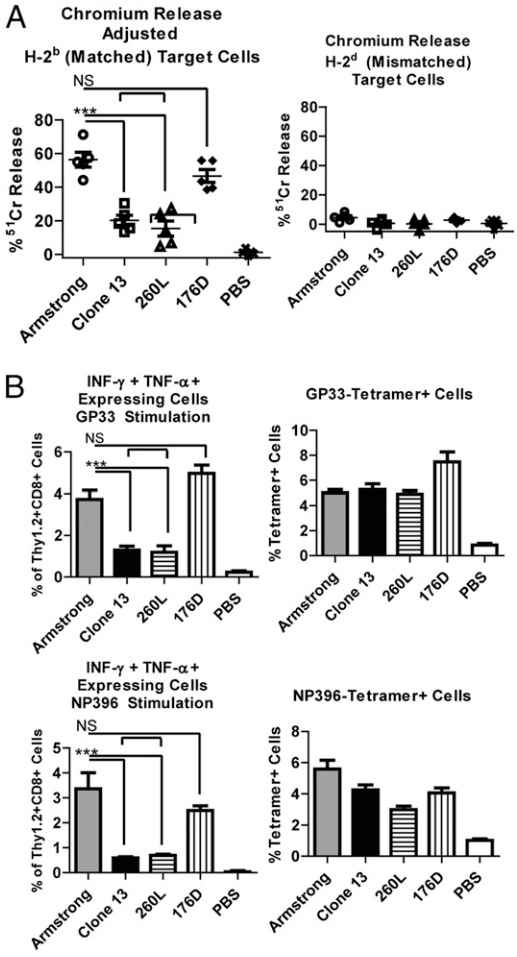


Fig. 3. LCMV strains that persist, Clone 13 and 260L, suppress CTL activity. C57BL/6 mice ($n = 5$ per group) injected with each recombinant strain were killed 9 d postinfection. NS, not significant; $^{**}P < 0.005$, $^{***}P < 0.0005$. (*A*) Isolated splenocytes were incubated with MHC matched MC57 target cells (*Left*) or MHC mismatched BalbC17 target cells (*Right*) at a ratio of (50:1). Target cells labeled with ^{51}Cr were infected 2 d earlier with the Armstrong strain of LCMV. ^{51}Cr release of splenocytes mixed with non infected MC57 cells was subtracted from ^{51}Cr release of splenocytes mixed with infected target cells (*Left*). (*B*) Splenocytes were collected and incubated for 5 h with the indicated peptide as well as 50 U/mL recombinant human IL-2 and 4 μg/mL Brefeldin A. Cells were stained with fluorescently conjugated Abs against Thy1.2, CD8, TNF-α, and IFN-γ (*Left*) or Abs against Thy1.2 and CD8 as well as APC-conjugated MHC tetramers specific for $CD8^+$ T-cell receptors clones recognizing NP396 or GP33 (*Right*).

Increased DC Infection and Germinal Center Disruption with Viruses Carrying a Leucine at Position 260 of the Viral GP1. In the immune system, DCs express the overwhelming majority of αDG (>99%) (15). LCMV infection of DCs is associated with decreased expression of the costimulatory and MHC molecules necessary for the activation of T cells (21). High percentages of infected DCs in mice inoculated with Clone 13 have been associated with the ability of this virus to persist (20, 21). Therefore, we investigated the percentage of DCs infected with each recombinant LCM virus to determine whether an increased ability to bind αDG was associated with increased infection rates of DCs. For this process, we infected mice with 2×10^6 pfus of each virus and, at various times postinfection, we collected splenocytes and surface-stained with antibodies against a DC marker, CD11c. Then, after intracellular staining with an antibody against the viral nucleoprotein, an epitope shared by all of the strains of virus, the numbers of infected DCs were determined. Consistent with

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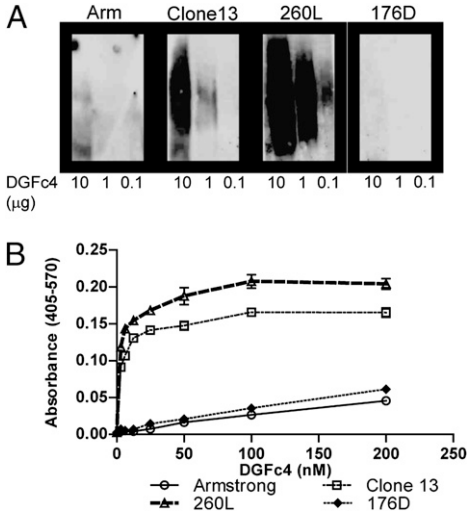

Fig. 4. Binding of recombinant viruses to a soluble αDG fragment, DGFc4. (*A*) DGFc4 was separated by SDS/PAGE on an 8% Tris-glycine gel and transferred to nitrocellulose. Blots were incubated overnight with 2×10^7 pfu of purified virus, washed, and incubated with GP1 and GP2 antibodies. Blots were developed with HRP-conjugated antibodies and appropriate substrates. (*B*) Next, 1×10^6 pfu of purified virus were bound to each well of a EIA/RIA high-bond microtiter plates overnight at 4 °C. DGFc4 was added at various concentrations, washed, and incubated with antibodies against human Fc receptors. Plates were developed with HRP-conjugated antibodies and appropriate colorimetric substrates.

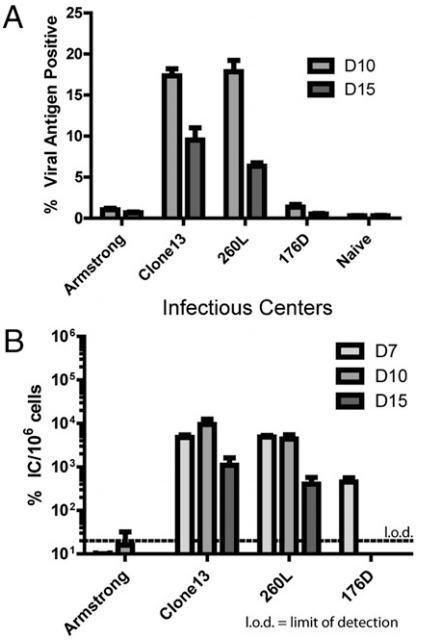

Fig. 5. Infection of DCs in vivo. Splenocytes of infected or naive mice ($n = 5$ per group) from the indicated time points postinfection were stained with antibodies against CD11c. (*A*) Cells were gated on CD11c high-expressing cells and viral antigen positivity was assessed by intracellular staining using antibodies against the viral nucleoprotein. (*B*) Cells were sorted for high CD11c expression and limiting dilutions of cells were made on confluent monolayers of Vero cells subsequently overlaid with agarose for plaque determination.

results from the αDG binding experiments, mice infected with either Clone 13 or 260L had higher rates of DC infection than those infected with Armstrong or 176D (Fig. 5*A*). We also performed infectious center assays of purified populations of DCs sorted by FACS on cells expressing high levels of CD11c on their surface. For this process, we incubated purified DCs with Vero cells and, 6 d later, plaques formed by virus-producing DCs were counted. DCs from mice infected with either Clone 13 or 260L produced significantly more plaques than DCs from mice infected with Armstrong or 176D (Fig. 5*B*). The results clearly show a strong correlation between the ability of LCMV to bind its cognate receptor, αDG, and the infection of a critical cell type necessary for priming a robust immune response.

Proper architecture of follicles from secondary lymphoid organs, such as the spleen, is essential in mounting an immune response. Previous articles from our laboratory and others have shown that persistent strains of LCMV, such as Clone 13, cause a disintegration of follicular structure by day 6 postinfection in contrast to spleens from mice infected with Armstrong, which show normal splenic architecture (20, 29). We found that mice infected with recombinant LCMV strains encoding a leucine at position 260 of GP1 (Clone 13 and 260L) completely disrupt secondary follicle structure by day 7 postinfection (Fig. 6, *Right*). In stark contrast, germinal centers are apparent in spleens from mice infected with either LCMV Armstrong or 176D (Fig. 6, *Left*), the latter showing only moderate disruption in splenic architecture, perhaps because of the presence of the 1079Q polymerase mutation present in this recombinant strain.

Discussion

This study provides compelling evidence that of the two mutations found in the GP1 of LCMV Clone 13, only the F260L mutation is essential for Clone 13 persistence, whereas neither an asparagine nor aspartic at position 176 influence Clone 13's ability to persist. Consistent with previous studies, the ability of Clone 13 to bind its cognate host receptor, αDG, was associated with the F260L mutation, which also correlated with the virus's ability to persist in mice (15, 16). Although mutation F260L does not affect virus growth in vitro, it leads to high rates of DC infection, suppression of the cytotoxic T-cell response, and disruption of secondary lymphoid follicles in infected mice, events that contribute to Clone 13 persistence in the infected host.

The structural basis for LCMV binding to αDG has not been elucidated, but O-linked glycosylation stemming from stretches of serines and threonines present in the amino-terminal mucin-like domain of αDG is essential for interaction with the viral GP1 (18), as well as for its cognate host ligands in the extracellular

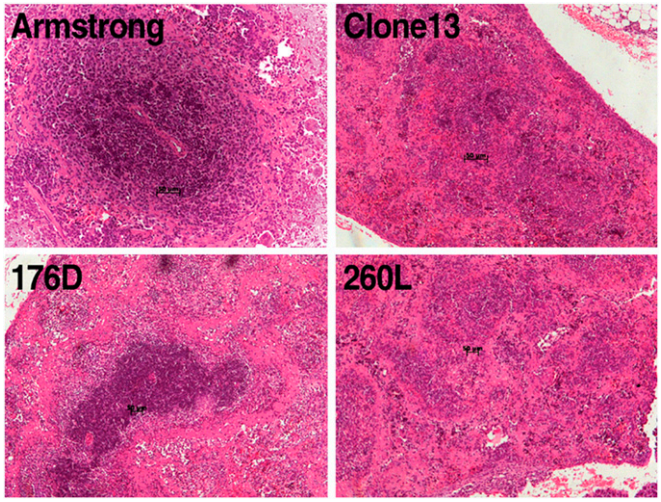

Fig. 6. Germinal center architecture at day 7 postinfection. Dark areas indicate B-cell zones of the germinal center. Representative H&E-stained sections of spleens from mice infected with recombinant LCMV strains are shown at 100× (*Upper*) or 200× (*Lower*) ($n = 3$).

matrix (30). One of these ligands, laminin, binds the O-mannosylated sugars of dystroglycan through stretches of basic residues (31, 32). Because the viral GP1 binding site overlaps with that of laminin (18), it is not surprising that mutation N176D involving the substitution of an uncharged asparagine for a negatively charged aspartic acid does not contribute to αDG binding.

The conservation of a leucine at position 260 in isolates of LCMV persistent strains and the importance of αDG binding established in this work indicate that this amino acid is a major contributor to the interaction between virus and host receptor. Because leucine is a nonpolar residue, it is likely that the contribution of this amino acid may lie more in the conformation of GP1, which may be disrupted by a bulky aromatic side chain, such as phenylalanine, rather than direct interaction with the O-linked mannose sugars present in αDG. Because threading predictions of viral class I glycoproteins can be misleading (33), high-resolution crystallographic structures of Old World arenavirus glycoproteins would be necessary for a detailed evaluation of this data.

Here we have used recombinant LCMV to measure the contribution of the glycoprotein to persistence. This approach minimizes the presence of viral mutants arising from multiple passages of viral strains, ensuring that the contribution of each viral mutant came from single amino acid differences we engineered in each strain.

As we were submitting this article, an article by Bergthaler et al. (34) was published detailing the importance of the 1079Q mutation in the L protein for viral persistence using reverse LCMV genetics. The majority of mice infected with recombinant LCMV encoding the 1079Q mutation but lacking a leucine at position 260, cleared the virus between 13 and 22 d postinfection: longer than mice infected with viruses lacking the 1079Q mutation but substantially shorter than the 60+ days for clearance of virus containing both 1079Q and 260L mutations (34). Published data from our laboratory have shown isoleucine or leucine at position 260 in the viral GP1 (22/22) was consistently found in virus isolates that cause persistent infection (serum titers >200 pfu at day 30 postinfection). In contrast, the 1079Q mutation was only found in 3 of the 16 (19%) persistent isolates examined at this position, although K and R were present at this position in 8 and 5, respectively, of the 16 persistent isolates examined (15). These findings suggest that a variety of L variants, not specifically but including 1079Q, are capable to confer the virus with a persistent phenotype via mechanisms that remain to be determined. In the Clone 13 system, both avidity to αDG conferred by the 260L mutation and the cell-specific replicative advantage conferred by the 1079Q mutation are necessary to establish and maintain persistence. The former phenotype is highly correlated with persistent isolates but replicative advantage of the latter phenotype in other persistent isolates is unclear. However, the inability, so far, of having an in vitro polymerase assay for LCMV or other arenaviruses has hampered investigation in this area.

The phenotypic differences seen between two strains of LCMV differing by a single amino acid are highly relevant. A particularly important note is that the Armstrong strain, although cleared early in infected mice, can persist when transmitted vertically (35, 36) (most often the natural route of infection) or under conditions of immunosuppression (25), suggesting that there is no inherent defect in viral fitness when immune pressure remains low. The difference between these strains, however, is the ability of Clone 13 to avoid clearance and persist despite the pressure exerted by an intact and robust immune response through infection of a key mediator of the immune response, the DC.

Although the seroprevalence of Old World arenaviruses is high among endemic populations, morbidity and mortality remain relatively low. Recently, articles examining population genetics in West Africa determined that host genetic changes important in the biosynthesis of αDG are linked with geographic areas where Lassa Fever is endemic, suggesting that this virus has likely exerted an evolutionary pressure on the population (37, 38). In that case, clinical outcomes of Lassa virus may be highly correlated with host genetics. In this article, we show that viral genetics of a related Old World arenavirus, LCMV, also play a significant role in clinical outcomes of Old World arenavirus infection. Clinical isolates are often collected only from patients that show significant morbidity and mortality but are rarely compared with isolates that may not cause disease. Complicating diagnosis, the morbidity and mortality of Old World arenavirus infections may lie at the interface between the genetics of both the virus and the host. Only with a clear understanding of both these variables can one begin to predict disease progression and design appropriate therapies for the victims of infection.

Materials and Methods

Cell Lines, Antibodies, and Reagents. BHK-21 cells were grown in DMEM supplemented with 10% FBS, 100 μg/mL penicillin-streptomycin (Gibco), 2 mM L-glutamine (Gibco), 0.56% glucose (wt/vol), and 7% tryptose phosphate broth solution (Sigma). Vero cells were propagated in Eagle's MEM supplemented with 7% FBS, 100 μg/mL penicillin-streptomycin, and 2 mM L-glutamine. MC57 and BALB/Cl7 cells were propagated in DMEM supplemented with 10% FBS, 100 μg/mL penicillin-streptomycin, and 2 mM L-glutamine. APC- and FITC-conjugated CD8α (53-6.7), PE-conjugated IFN-γ (XMG1.2), and FITC-conjugated TNF-α (MP6-XT22) anti-mouse Abs were purchased from BD Pharmingen. PE-Cy7-conjugated CD11b (M1/70), APC-conjugated CD11c (N418), PE-Cy7-conjugated CD90.2 (53-2.1), and CD16/32 Fc block anti-mouse Abs were purchased from eBiosciences. HRP-conjugated goat anti-mouse IgG and mouse anti-human IgGFc Abs were purchased from Jackson Laboratories. Fluorescent conjugation of anti-NP113 Abs was performed using an Alexa Fluor-488 Labeling Kit (Invitrogen). Recombinant human IL-2 was purchased from BD Biosciences and brefeldin A was purchased from Sigma.

Virus Strains, Purification, and in Vitro Infection. Viruses were made using reverse genetic technology described previously (26). Virus stocks were generated by serial passage in BHK-21 cells and sequenced to ensure that no major genetic changes had occurred during passage of the virus. Purified virus stocks were generated by incubating infected tissue culture supernatant with polyethylene glycol at 6.5% wt/vol on an overhead shaker overnight at 4 °C. Supernatant was spun in a Sorvall GSA rotor at 11,000 × *g* for 30 min and the resulting pellet was dissolved in TNE (10 mM Tris-HCl pH 7.5/100 mM NaCl/1 mM EDTA). Dissolved viral pellets were layered over a discontinuous renografin gradient in TNE (50/40/10%) and subjected to ultracentrifugation in a Beckman SW41 rotor at 35 krpm for 90 min. The interface between the 10 and 40% layers was collected, diluted 1:2 in TNE, and overlaid on a second gradient and collected as above. Concentrated virus stocks were dialyzed against PBS overnight. Virus titers were assessed by immune focus on Vero cells as described previously (39).

Mice and in Vivo Infection. C57BL/6J mice were obtained through the Rodent Breeding Colony at the Scripps Research Institute (TSRI) and housed under pathogen-free conditions. All handling of mice conformed to training and guidelines set forth by the National Institutes of Health and the Department of Animal Resources at TSRI. Mice were infected intravenously with 2×10^6 focus-forming units (FFU) of virus.

^{51}Cr Release Assay. Erythrocyte-depleted splenocytes from day 9 infected and mock-infected C57BL/6J (H-2^b) mice were used in a standard ^{51}Cr release assay, as described (13, 20, 40). Briefly, matched MC57 (H-2^b) and unmatched BALB/Cl7 (H-2^d) cells were either uninfected or infected with LCMV Armstrong for 24 h at a MOI of 1 and labeled with ^{51}Cr. Splenocyte:target ratios were 50:1 and 25:1 in triplicate with $n = 5$ mice per group.

Peptide Stimulation. Experiments were performed as previously described (2). Erythrocyte-depleted splenocytes from infected and mock-infected C57BL/6J mice were incubated with H-2^b MHC class I restricted epitopes LCMV-$NP_{396–404}$ and $GP_{33–41}$, 50 U/mL IL-2 and 1 mg/mL brefeldin A.

Virus-Overlay Protein Binding Assay. DGFc4 was purified from the supernatant of expressing cell lines as described previously (18, 31). DGFc4 was resolved by SDS/PAGE and transferred to nitrocellulose membrane, subsequently incubated with purified LCMV overnight at 4 °C, and washed in PBS-Tween (0.1%). Hybridoma supernatants containing Abs against GP1 and GP2 were used as the primary antibody with HRP-conjugated goat anti-mouse Abs as

the secondary antibody. Virus binding was detected by chemiluminescence using the SuperSignal West Pico substrate (Pierce).

Virus-Receptor Binding in ELISA Format. Experiments were performed as previously described (41). Briefly 5×10^5 FFU of purified virus was bound to wells of an EIA/RIA high-bond plate (Costar) overnight at 4 °C and washed with PBS. Plates were blocked with PBS/1%BSA (wt/vol) for 1 h at room temperature. Dilutions of DGFc4 were incubated in virus-bound plates for 2 h at room temperature, washed with PBS, and bound DGFc4 was detected using mouse anti-human IgGFc followed by HRP-conjugated goat anti-mouse IgG. Binding was visualized by using ABTS tablets (Sigma). $OD_{405-570}$ was recorded and background corrected for BSA. Efficiency of virus binding was assessed by serially dilution onto EIA/RIA plates using Abs against LCMV GP2 to assess the relative amounts of virus bound. Only viruses demonstrating a titratable curve were used.

Flow Cytometric Analysis. All flow cytometry was performed on a LSR-II flow cytometer (BD Biosciences). Cells were surface-stained in PBS/1%FBS and intracellular staining was achieved by fixation and permeabilization using a BD Cytofix/Cytoperm kit. All splenocytes were incubated with Fc Block before staining with Abs.

Infectious Centers. Splenocytes were stained with APC-conjugated Abs against $CD11c^+$. Cells expressing high levels of CD11c were sorted to a final purity of 95% or greater on a FACS Vantage DiVa II. Fivefold dilutions of sorted cells were added to confluent layers of Vero cells and overlaid with 1% agarose/EMEM solution and incubated at 37 °C, 5% CO_2 for 6 d before staining with crystal violet solution.

Histology. Spleens from mice inoculated with 2×10^6 FFU of LCMV were collected at day seven postinfection, sectioned, and stained with H&E.

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