1 **A single, improbable B cell receptor mutation confers potent neutralization against cytomegalovirus**

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14 **Abstract**

15 Cytomegalovirus (CMV) is a leading cause of infant hearing loss and neurodevelopmental delay, but
 16 vaccine candidates have faced challenges eliciting neutralizing antibodies. One of the most well-studied
 17 targets for CMV vaccines is the viral fusogen glycoprotein B (gB), which is required for viral entry into host
 18 cells. Within gB, antigenic domain 2 site 1 (AD-2S1) is a target of potentially neutralizing antibodies, but gB-
 19 based candidate vaccines have yet to elicit robust responses against this region. We mapped the
 20 genealogy of B cells encoding potentially neutralizing anti-gB AD-2S1 antibodies from their inferred unmutated
 21 common ancestor (UCA) and characterized the binding and function of early lineage ancestors.
 22 Remarkably, we found that the single amino acid heavy chain mutation A33N, an improbable mutation
 23 rarely generated by somatic hypermutation machinery, conferred broad CMV neutralization to the UCA
 24 antibody. Structural studies revealed that this mutation mediated key contacts with the gB AD-2S1 epitope.
 25 Collectively, these results provide insight into potentially neutralizing gB-directed antibody evolution and a
 26 foundation for designing next-generation CMV vaccines.

27

28 **One-sentence summary**

29 This manuscript identifies an early B cell lineage mutation that confers neutralizing function to antibodies
 30 targeting CMV fusogen gB.

31 Introduction

32 Human cytomegalovirus (CMV, human herpesvirus 5) is a pervasive viral pathogen and major cause
 33 of disease in infants and immunocompromised patients worldwide (1, 2). Although CMV infection is typically
 34 asymptomatic in healthy adults, CMV transmission *in utero* can cause permanent hearing loss, cognitive
 35 impairment, retinitis, and cerebral palsy in affected infants (3). Congenital CMV infection alone is
 36 responsible for nearly a quarter of all newborn hearing loss (4). CMV infection is also a major cause of
 37 morbidity and mortality in transplant recipients and persons with HIV (5). Accordingly, there have been
 38 many efforts to develop vaccines that will prevent infection and transmission with the goal of reducing the
 39 global CMV-related burden of disease (6). However, vaccine development has faced challenges identifying
 40 immunogens that can induce broad and potent immunity and confer sustained protection against CMV
 41 infection (7).

42 One of the leading targets for vaccine development is CMV glycoprotein B (gB), which is a viral
 43 envelope protein that mediates fusion with host-cell membranes and is required for viral entry into all known
 44 cell types (8, 9). Indeed, the most efficacious CMV vaccine trial to date was composed of a postfusion CMV
 45 gB subunit protein combined with an MF59 adjuvant (gB/MF59) that conferred approximately 50%
 46 protection from primary acquisition in multiple phase 2 clinical trials (10-13). Follow-up immunogenicity
 47 studies found that in CMV-seronegative vaccinees, gB/MF59 elicited antibody responses against three of
 48 the four total neutralizing antigenic domains of gB (14, 15) but not against gB antigenic domain-2 site 1 (AD-
 49 2S1), which is known to be the target of potentially neutralizing antibodies in natural infection (16).

50 CMV gB AD-2S1 is a linear epitope at the N-terminus of gB (amino acids 68–81), which is
 51 extracellular, and is highly conserved across clinical strains (17). Multiple studies have implicated antibodies
 52 against gB AD-2S1 in protection from CMV disease and vertical transmission. In CMV-seropositive renal
 53 transplant recipients, the presence of serum anti-gB AD-2S1 antibodies was associated with reduced risk of
 54 post-transplant CMV disease (18). In CMV-seropositive transplant recipients immunized with gB/MF59,
 55 vaccination boosted anti-gB AD-2S1 serum antibody titers in a subset of subjects with preexisting anti-gB
 56 AD-2S1 antibodies, and these titers were associated with protection from CMV viremia (19). We also found
 57 that in a study of *in utero* CMV transmission among HIV-infected mothers, the presence of maternal serum
 58 antibodies against gB AD-2S1 was associated with a reduced risk of vertical CMV transmission (20). As

elicited in the context of natural infection, antibodies targeting gB AD-2S1 are well recognized for their role in protection from CMV viremia and congenital transmission.

Based on its location at the extracellular N-terminus, gB AD-2S1 should be readily available for immune recognition. Yet, surprisingly, only ~50% of naturally CMV-infected individuals have detectable circulating anti-gB AD-2S1 antibodies (21). Vaccine candidates to date including gB/MF59 have failed to elicit antibodies targeting this region (14, 15), and direct immunization with gB AD-2S1 peptides in animal models has also failed to elicit neutralizing antibody responses (21). These findings indicate the presence of barriers, such as structural constraints within gB and host genetic restriction, that prevent the generation of neutralizing antibodies targeting gB AD-2S1. Indeed, there are two known glycosylation sites within gB AD-2S1 that may contribute to glycan shielding, and crystal structures of postfusion gB suggest that the nearby gB antigenic domain-1 may cloak the gB AD-2S1 region, blocking immune access to this epitope (17).

The host genetic restriction of germline B cells may also prevent the generation of neutralizing anti-gB AD-2S1 antibodies. Germline B cell receptors can have thousands of potential heavy (V_H) and light (V_L) pairings, yet all of the potently neutralizing gB AD-2S1 monoclonal antibodies (mAbs) isolated from naturally infected individuals to date are derived from the same V_H/V_L pairing: IGHV3-30 (or the related IGHV3-30-3) and IGKV3-11 (22-24). The use of a single pairing out of thousands of potential combinations reflects the incredibly small subset of germline B cells with complementarity-determining regions (CDRs) capable of recognizing gB AD-2S1. Thus, the elicitation of gB AD-2S1-specific antibodies by vaccination likely requires rational vaccine design that addresses potential germline restriction and lineage maturation.

B cell lineage-targeted vaccine design aims to direct germline B cells along favorable maturation pathways, such as those targeting gB AD-2S1. In this approach, the germline B cell precursor sequence of clonally related antibodies with desired neutralizing potency is inferred using phylogenetic methods, then the amino acid mutations associated with the development of neutralizing functions along lineage maturation are identified. Antibodies with or without these functional mutations can then be produced and used as templates for structure-based immunogen design or for the empiric screening of vaccine immunogens. B cell lineage-targeted vaccine design has been used to guide the development of vaccine candidates that are designed to generate broadly neutralizing antibodies against HIV-1 (25-29) and

86 influenza (30). However, this approach has not yet been applied to herpes viruses such as CMV, which
87 could be a boon for their vaccine development.

88 When considering structure-based vaccine design for engaging germline B cell receptors, we are
89 particularly interested in identifying function-enabling somatic hypermutations that may require high-affinity
90 antigenic stimulation. These mutations may occur infrequently during normal affinity maturation due to 1)
91 the location of these mutations in areas of the germline that are rarely targeted by activation-induced
92 deaminase (AID) during somatic hypermutation and/or 2) the requirement of multiple nucleotide mutations
93 within a codon to introduce an observed amino acid substitution (31). However, these functional,
94 “improbable” amino acid mutations could be targeted for high-affinity immunogen stimulation by structure-
95 based vaccine design.

96 Based on the low prevalence of gB AD-2S1 antibodies in CMV-seropositive individuals, we
97 hypothesized that potently neutralizing antibodies against CMV gB AD-2S1 are lineage-restricted through
98 the requirement of improbable AID mutations for binding and neutralizing functions. To address this
99 hypothesis, we examined the lineage maturation of a well-studied, potently neutralizing gB AD-2S1-specific
100 mAb called TRL345 (16, 32). TRL345 was isolated from single B cell clones from a CMV-seropositive donor
101 with high plasma CMV-neutralizing activity, and based on its potent, broad neutralization of CMV, this mAb
102 was pursued as a potential passive therapeutic to treat CMV viremia (16, 23, 32).

103 In this study, we mapped the clonal genealogy of TRL345 from its germline precursor and produced
104 antibodies from its clonal family including inferred ancestral antibodies as well as mature antibodies. Then,
105 using a computational program to estimate the probability of individual amino acid mutations along
106 maturation pathways, we identified a single improbable heavy chain mutation required for mAb binding and
107 neutralization, which could serve as the basis for CMV gB-based immunogen design to target potent CMV
108 neutralization.

109

110 **Results**

111 *Identification of the TRL345 clonal lineage*

112 We first reconstructed the clonal genealogy of the highly neutralizing gB AD-2S1 mAb TRL345 using a set
113 of previously published mature gB AD-2S1 mAb sequences isolated from the same donor (16, 32). Using

the antibody sequence analysis program Cloanalyzer, we grouped the 14 reported mature AD-2S1 mAb sequences into clones. The clone that included mAb TRL345 was comprised of 9 members and utilized aIGHV3-30*01 and IGKV3-11*01 pairing, which is consistent with the heavy and light chain V gene segment pairings identified for all previously published gB AD-2S1 mAbs (16, 22, 33-35). With the 9 TRL345 clonal sequences as input, we then used Cloanalyzer to infer the unmutated common ancestor (UCA) sequence representing the B cell receptor of the naïve B cell that gave rise to the TRL345 clone, as well as a maximum likelihood genealogical tree of its clonal lineage (**Fig. 1**).

High affinity gB AD-2 recognition was acquired early in the TRL345 lineage

After producing the 9 mature mAbs, 8 clonal ancestor intermediates, and UCA mAbs, we measured their binding to gB. The UCA had relatively low binding affinity to both gB AD-2S1 peptide and gB ectodomain (**Fig. 1, 2A, 2B; Table S1**). However, upon transition from the UCA to the first intermediate (intermediate 8, I8), ELISA binding magnitude for gB AD-2S1 peptide and gB ectodomain increased 151-fold and 37-fold, respectively. Binding affinity for the linear gB AD-2S1, as measured by surface plasmon resonance (SPR), remained unchanged in the UCA to I8 transition, but affinity for gB ectodomain increased 32-fold, to its binding plateau (**Fig. 1, 2B, 2C; Table S1**). Upon evolution of I8 to the second intermediate (I4) in the TRL345 lineage, affinity for the linear gB AD-2S1 increased 5-fold. The high binding magnitude and affinity for both gB AD-2S1 and ectodomain were retained from I4 throughout the rest of the lineage (**Fig. 1, 2A-C; Table S1**). Together, the data indicate that high affinity recognition of the AD2 epitope is mediated by the naïve TRL345 B cell and that affinity maturation plateaus by the first or second intermediates along the TRL345 lineage.

Because the gB ectodomain soluble protein may not adequately mimic either the prefusion or postfusion state of gB on the viral envelope or infected cells (17, 36, 37), we measured mAb binding to full-length gB as expressed on the surface of human epithelial cells, which is a desirable immunogenicity target given that IgG binding to gB expressed on the surface of a cell was identified as an immune correlate of protection against CMV (38). Of note, gB expression on the surface of a cell may represent a combination of prefusion and postfusion gB forms (17). Although the UCA had negligible binding to gB AD-2S1 peptide and soluble

142 gB ectodomain, the UCA showed moderate binding to cell-associated gB (AUC of a 3-point dilution series=
143 97.3 ± 21.2) (**Fig. 2D; S1; Table S1**). Binding magnitude for cell-associated gB improved after maturation
144 from UCA to I8 (AUC= 132.6 ± 33.3) then remained stable along the lineage maturation to TRL345 (AUC=
145 151.6 ± 20.8).

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147 *Broad neutralization of CMV was acquired early in the TRL345 lineage*

148 We then quantified the neutralization activity of the TRL345 lineage mAbs against three CMV strains, each
149 of which expressed a different gB genotype. We measured the neutralization of CMV strains Towne (gB
150 genotype 1), AD169 repaired (AD169UL131-GFP, genotype 2), and Toledo (genotype 3) on MRC-5
151 fibroblasts and neutralization of AD169rUL131-GFP on ARPE epithelial cells. The UCA did not neutralize
152 any CMV strains on either fibroblasts (**Fig. 1, 2E; Table S1**) or epithelial cells (**Fig. 2F; Table S1**), but it
153 acquired broad neutralization activity in its first lineage branch at I8 (fibroblast neutralization IC_{50} of Towne,
154 AD169rUL131-GFP, and Toledo = $2.78 \pm 3.40 \mu\text{g/mL}$, $0.83 \pm 0.72 \mu\text{g/mL}$, and $0.83 \pm 0.10 \mu\text{g/mL}$,
155 respectively; Epithelial cell IC_{50} of AD169rUL131-GFP = $0.79 \pm 0.17 \mu\text{g/mL}$). Neutralizing activity further
156 improved along lineage maturation, with 4.8-fold and 2.2-fold increases in average CMV neutralization on
157 fibroblasts and epithelial cells, respectively, after the I8 maturation to I4. The most potently neutralizing
158 antibodies on fibroblasts ($IC_{50} < 0.25 \mu\text{g/mL}$) were TRL345, MAB310 (I3), and MAB313, and on epithelial
159 cells ($IC_{50} < 0.35 \mu\text{g/mL}$) were TRL345, MAB343, MAB310 (I3), and MAB313 (**Fig. 1; Table S1**).

160

161 *Early acquired amino acid mutations were improbable in the absence of B cell selection*

162 We next investigated the probability of each mutation occurring in the absence of B cell selection. During B
163 cell development in germinal centers, B cells undergo somatic hypermutation, wherein AID generates DNA
164 point mutations at immunoglobulin variable regions (39). B cells with mutations that improve antigen binding
165 avidity undergo subsequent selection. Due to codon degeneracy and biases in AID targeting, certain amino
166 acid changes occur more frequently than others during somatic hypermutation (40). The acquisition of
167 improbable mutations that contribute to neutralization function can act as rate-limiting steps in the
168 development of neutralizing antibodies (27) and represent high value targets for selection in lineage-based
169 vaccine design strategies (28). We used the ARMADiLLO computational program to simulate somatic

hypermuation and estimate the probability of each observed mutation in the TRL345 clone (27). Consistent with previous studies, we defined “improbable” mutations as those estimated to occur at <2% frequency in the absence of selection – a frequency corresponding to approximately one mutation per clone per germinal center (27). We found that all of the mutations acquired by the UCA during its evolution to I8, namely the V_H A33N and Y52aN and V_L S30G and N53D, were improbable in the absence of B cell selection (**Fig. 1; Table S2**). Throughout the lineage, of the total 62 heavy chain mutations observed, 7 occurred in CDR1 and 10 in CDR2 regions, with none observed in CDR3. Of the total 29 light chain mutations observed, 6 occurred in CDR1, 1 in CDR2, and 4 in CDR3 (**Fig. 1**). Of the heavy and light chain mutations in the lineage, 37% (23 of 62) and 59% (17 of 29) were estimated to be improbable in the absence of selection, respectively. In TRL345 alone, 6 of 12 total observed heavy chain mutations and 2 of 7 total light chain mutations were improbable (**Fig. 1; Table S2**).

Early, improbable mutations in the UCA were both necessary and sufficient for neutralization

To identify which early mutations conferred neutralizing activity to the UCA, we performed site-directed mutagenesis to either 1) revert the mutations in I8 back to germline or 2) introduce the mutations into the UCA. We produced 14 mutant mAbs with single heavy chain mutations A33N and Y52aN or light chain mutations S30G and N53D or combinations of these mutations, then we quantified mAb binding and neutralization. We found that reversion of single mutations V_H Y52aN, V_L S30G, or V_L N53D in I8 had negligible impact on mAb binding to gB AD-2S1, gB ectodomain, and cell-associated gB (**Fig. 3A, B; S2A, B**). However, reversion of V_H A33N in I8 decreased binding magnitude to gB AD-2S1, gB ectodomain, and cell-associated gB by 395-fold, 1.7-fold, and 1.4-fold, respectively.

Introduction of V_H A33N to the UCA conferred a 269-fold, 45-fold, and 2.5-fold increase in binding magnitude to the gB AD-2S1 linear epitope, gB ectodomain, and cell-associated gB, respectively (**Fig. 3A, B; S2A, B**). By contrast, introduction of V_L S30G conferred only 1.8-fold, 13.5-fold, and 1.2-fold increases in binding magnitude to the gB AD-2S1 linear epitope, gB ectodomain, and cell-associated gB, respectively. Introduction of the single mutations V_H Y52aN or V_L N53D conferred 2.0-fold and 8.7-fold increases in binding magnitude to gB ectodomain, respectively, but they had negligible effect on binding to gB AD-2S1

or cell-associated gB. Early, improbable mutations introduced in combination to the UCA demonstrated binding patterns consistent with the single mutants. Thus, mutant mAbs containing V_H A33N had increased binding to gB AD-2S1 linear domain, gB ectodomain, and cell-associated gB, and this pattern was not observed for any other early mutation (**Fig. 3A, B; S2A, B**).

We then determined the role of these early mutations in mAb neutralizing function. We found that reversion of V_H Y52aN in I8 had negligible impact on neutralization on both fibroblasts and epithelial cells (**Fig. 3C; S2C, D**). Reversion of V_L S30G and N53D moderately decreased neutralization of all strains, with the largest impacts on Towne neutralization as observed by decreases in neutralization potency of 9.0-fold and 2.5-fold, respectively. By contrast, reversion of V_H A33N abrogated neutralizing function of all strains on both fibroblasts and epithelial cells. Introduction of the single heavy chain Y52aN or light chain mutations S30G or N53D to the non-neutralizing UCA did not confer neutralization function. Only introduction of the heavy chain A33N mutation conferred neutralizing function, and this was observed for neutralization of all three CMV strains and on both cell types. Consistent with the binding patterns of these mutant mAbs, these neutralization results indicate that the improbable heavy chain A33N mutation was both necessary and sufficient for neutralization across multiple CMV strains.

Heavy chain mutations A33N and A33G confer neutralizing activity in germline mAbs

All human gB AD-2S1-specific mAbs sequenced to date are derived from the IGHV3-30 (or related IGHV3-30-3) and IGKV3-11 germline pairing and are associated with either a V_H G33 or V_H D33 residue in the mature mAb sequence (41). We anticipated that the germline V_H mutations A33N and A33G might represent two alternative pathways to achieve neutralization potency.

To determine the functional role of the V_H mutation A33G, we introduced this mutation to the non-neutralizing TRL345 UCA and found that this mutation conferred approximately 15.0-fold and 14.2-fold increases in binding to gB AD-2S1 and gB ectodomain, respectively, with a minimal increase of 1.1-fold in binding to cell-associated gB (**Fig. 4A, B**). However, this mutation did not confer CMV neutralizing activity on fibroblasts or epithelial cells (**Fig. S3A**).

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We also evaluated the comparative advantages of the V_H A33N and V_H A33G mutations to the UCA of another donor. To identify a UCA with a CDR3 region capable of binding gB AD-2S1, we computationally inferred the UCA from the-mAb 3-25 (42). The 3-25 UCA was inferred to use the IGHV3-30*01 allele, which encodes an alanine at V_H residue 33 (**Fig. S4A, B**). We found that the 3-25 UCA had low binding to gB AD-2S1 (EC₅₀>10 µg/mL) and moderate binding to gB ectodomain (6.1 ± 1.2 µg/mL) and cell-associated gB (22.1 ± 1.8 µg/mL. **Fig. 4C, D**). Introduction of V_H A33N to the 3-25 UCA conferred binding to gB AD-2S1 (2.7 ± 2.5 µg/mL) and increased binding magnitude to gB ectodomain and cell-associated gB by 35.2-fold and 4.4-fold, respectively. By contrast, introduction of naturally observed mutation, V_H A33G, to the 3-25 UCA conferred minimal binding, with increases of 1.3-fold, 5.5-fold, and 1.4-fold to gB AD-2S1, gB ectodomain, and cell-associated gB, respectively (**Fig. 4C, D**). When introduced into the 3-25 mAb UCA, neither the V_H A33N mutation nor A33G mutation was sufficient to confer CMV neutralizing activity (**Fig. S4B**), suggesting that there may be other mutations required to develop this function for germline pairings different from the IGHV3-30*01 and IGKV3-11*01 used in TRL345.

Molecular determinants of gB AD-2S1 linear epitope binding and neutralizing potency

To obtain high-resolution information on the binding of the early intermediate mAb I8 to the gB AD-2S1 linear epitope, we conducted crystallographic studies of TRL345.I8 antigen-binding fragment (Fab) in complex with the linear gB AD-2S1 peptide (65-HRANETIYNTTLKYG-79). A crystal in the space group *P2₁2₁2₁* with one complex per asymmetric unit diffracted X-rays to a resolution of 1.8 Å. Following molecular replacement and manual building of the model, the structure was refined to an R_{work}/R_{free} of 15.0%/17.4% (**Table S3**). This high-resolution structure revealed hydrogen bonding between the side chain of Y78 of the gB AD-2S1 peptide and the side chains of V_H N33 and N52a (both the result of improbable mutations), anchoring the C-terminus of the peptide in a pocket formed by N33 of V_H CDR1, N52a and N57 of V_H CDR2, and the V_H CDR3 loop (**Fig. 5A, B**). The side chain of E69 of gB AD-2S1 hydrogen bonds with the side chain of Y32 of V_H CDR1, anchoring the N-terminus of the peptide, and the side chain of T74 of gB AD-2S1 forms hydrogen bonds with the sidechain S98 and the mainchain of V99 of V_H CDR3 (**Fig. 5B**).

We then compared the TRL345.I8 structure with the previously published structure of the potentially neutralizing gB AD-2S1-specific mature mAb 3-25 in complex with gB AD-2S1 (PDB ID: 6UOE), which also uses the IGHV3-30 and IGKV3-11 pairing (17, 35, 37, 43). The structures of the peptides in the two complexes are highly similar, with an RMSD of 0.9 Å for 11 C α atoms, despite numerous amino acid differences in the heavy chain at the binding interface (**Fig. 5B-D, S4A-B**). The structural similarity of the peptide suggests that this particular conformation of gB AD-2S1 may represent the conformation that this epitope adopts in the prefusion form of gB. Interactions between gB AD-2S1 and the V_L of 3-25 are recapitulated in the TRL345.I8 complex, with the side chain and main chain of N73 of gB AD-2S1 hydrogen bonding with the side chains of Y32 and R91 of the V_L CDR1 and CDR3, respectively, and the side chain of gB T75 hydrogen bonding with the side chain of V_L CDR3 W94 (**Fig. 5D**). Notably, these interactions recapitulated in both the TRL345.I8 and 3-25 complexes occur at shared germline-encoded amino acids (**Fig. 1, S4A-B**). Moreover, in both complexes, the main chain atoms of gB AD-2S1 T75, L76, and Y78 and the side chain of T75 form hydrogen bonds with main chain atoms of the V_H CDR3. However, whereas the side chain of gB AD-2S1 T74 hydrogen bonds with the side chain of S98 of the TRL345.I8 V_H CDR3, the side chain of T74 hydrogen bonds with the main chain of C98 of the 3-25 V_H CDR3, the side chain of which forms a disulfide bond with C100B, stabilizing the longer 3-25 mAb CDR3 loop (the 3-25 V_H CDR3 is one residue longer than TRL345.I8 V_H CDR3).

In assessing both the general similarity and key differences in the interactions between the early TRL345 intermediate and mature mAbs with gB AD-2S1, we have identified a structural basis for the increase in binding affinity following the transition from the UCA of TRL345 to I8 and suggest that there may be multiple pathways to developing potentially neutralizing responses against CMV.

Discussion

Advancements in the antibody-to-vaccine approach have successfully enabled the translation of antibody lineages and structures to effective vaccine immunogens. Recently, structure-based design was used to engineer a candidate vaccine against RSV by stabilizing the prefusion F conformation and preserving neutralization-sensitive epitopes on the vaccine antigen (44, 45). For CMV, structure-based vaccine design of CMV gB to elicit potent neutralization has faced challenges, partly due to the high flexibility of the gB AD-2S1 region, a target of broadly and potently neutralizing antibodies. Previous structural studies of the full-length gB protein, in both the prefusion and postfusion conformations, have not been able to resolve the gB AD-2S1 conformation (17, 35, 37, 43, 46). The recently solved prefusion structure, in which AD-2 remained unresolved, suggested that the N-terminal flexible region containing gB AD-2 may bind gH/gL to initiate fusion (46). In our study, we solved the atomic structure of a gB AD-2S1 peptide bound by an early B cell precursor mAb, which may represent the structure of the gB AD-2S1 linear region capable of eliciting potently neutralizing antibodies. Thus, stabilization of the gB AD-2S1 peptide in this conformation may be the next key step in the design of a next-generation gB candidate vaccine.

This study revealed that only a low level of mAb affinity maturation is required for gB AD-2S1-specific antibody precursors to achieve CMV neutralization, as a single mutation in the heavy chain of the B cell receptor germline was both necessary and sufficient to confer potent neutralizing function (**Fig. 1, 2**). Addition of this single, key mutation V_H A33N also conferred binding function to the UCA from another donor to an even greater magnitude than the naturally observed mutation V_H A33G. By comparison, broadly neutralizing antibodies against HIV-1 require multiple mutations and can lose neutralizing function along lineage maturation, posing significant challenges for mutation-guided vaccine design strategies (47). Thus, our study lays a foundation for attainable, rational CMV gB vaccine design.

We identified the structural basis of affinity maturation from the TRL345 UCA to early intermediate mAbs. In particular, the V_H A33N and Y52aN mutations enabled hydrogen bonding to the side chain of Y78 of the gB AD-2S1 peptide, anchoring the C-terminus of the peptide in a pocket (**Fig. 5B**). Notably, the mature TRL345 mAb contains an N52aI substitution, indicating that there may be key differences between the structures of early lineage and mature mAbs in binding to the flexible peptide. Moreover, there were

several differences in the interactions between gB AD-2S1 and either TRL345 I8 or the mature 3-25 mAb, suggesting that there may be multiple pathways to developing potentially neutralizing responses against CMV.

This study has several limitations. Due to the limited number of anti-gB AD-2S1 B cell clones sequenced to date, we were only able to investigate the anti-gB AD-2S1 B cell lineage of a single donor. Accordingly, we were not able to observe whether there may be parallel evolution of key mutations in other donors. Follow-up studies are needed to determine whether the V_H A33N mutation can confer gB AD-2S1 binding and neutralization function to germline mAbs in other donors that share genetic and structural characteristics. Moreover, the role of potential allelic variation at V_H residue 33 should be explored.

There are additional challenges facing this approach for vaccine design. This study does not address the level of antigen affinity required to target germline B cell precursors *in vivo*. Follow-up studies should be performed to create gB antigens with high binding affinity to B cell precursors of gB AD-2S1-binding mAbs and test them in animal models for their ability to elicit gB AD-2S1-specific antibodies. Ideally, these studies would be performed in models with human gene knock-ins that are representative of the germline repertoire of human B cells. Furthermore, there may be low inherent frequency of these B cell precursors across donors, which should be assessed in unbiased B cell repertoire sequencing studies. Additionally, the current versions of algorithms used in this study do not calculate the probability of insertions or deletions in the antibody clonal lineages, which may identify additional targets, and this vaccine strategy is limited in its ability to elicit these types of mutations.

Our combined approach of B cell receptor sequence analyses, computational modeling, x-ray crystallography, and functional assessments of CMV gB AD-2S1 mAbs enabled the identification of gB AD-2S1 peptide structure which may be capable of eliciting the maturation of neutralizing antibodies from non-neutralizing early lineage precursors. The gB AD-2S1-specific early antibody intermediates and gB AD-2S1 peptide structures could serve as novel, valuable tools to guide the design of the next-generation of CMV gB-based vaccines.

327 **Materials and Methods**

328 *Study design*

329 The objectives of this study were to assess whether potentially neutralizing antibodies against CMV gB AD-
 330 2S1 are lineage-restricted through the requirement of improbable AID mutations for binding and neutralizing
 331 functions. To address this hypothesis, we evaluated the following experimental units: 18 mAbs in the clonal
 332 genealogy of the neutralizing gB AD-2S1 mAb TRL345 (16, 32) including the TRL345.UCA, 14 mutated
 333 TRL345.UCA mAbs with single or combinations of early lineage mutations, the potentially neutralizing mAb 3-
 334 25 (42), the 3-25.UCA, and 2 mutated 3-25.UCA mAbs. In each experiment, each sample was run in
 335 duplicate, and each experiment was performed two or more times.

337 *Peptide production*

338 The gB AD-2S1 peptide was synthesized by ThermoFisher as the sequence HRANETIYNTTLKYG, which
 339 includes the underlined, minimal gB AD-2S1 epitope. The gB ectodomain protein was produced in-house.

341 *Production of lineage mAbs and mutant mAbs*

342 Antibody genes were synthesized by Genscript and recombinantly produced in a human IgG backbone.
 343 Single amino acid mutations were introduced or reverted by site-directed mutagenesis, using the
 344 QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent) according to manufacturer's protocol.
 345 Sequence efficiency was confirmed by Sanger sequencing.

347 *mAb binding to soluble peptides and proteins by ELISA*

348 mAb binding to gB AD-2S1 peptide and gB ectodomain were measured by 384-well plate ELISA. Plates
 349 were coated overnight at 4°C with 45 ng gB AD-2S1 or gB ectodomain per well then blocked in assay
 350 diluent (1× PBS pH 7.4 containing 4% whey, 15% normal goat serum, and 0.5% Tween-20). mAbs were
 351 plated in a 12-point 3-fold serial dilution at a starting concentration of 1 µg/mL (1 µg/mL to 5.7*10⁻⁶ µg/mL),
 352 in duplicate. Binding was detected by goat-anti human HRP-conjugated IgG secondary (Jackson
 353 ImmunoResearch). Plates were developed using the SureBlue Reserve tetramethylbenzidine (TMB)

peroxidase substrate (KPL). Data are reported as the half-maximal effective concentration (EC_{50}). Each mAb was run in two or more independent experiments.

mAb binding to soluble peptides and proteins by surface plasmon resonance (SPR)

The kinetics and affinity of the binding interactions between monoclonal antibodies and the CMV gB ectodomain protein and the CMV gB AD-2S1 peptide were assessed by surface plasmon resonance (SPR) on a Biacore T200 platform (Cytiva) at 25°C in HBS-EP+ (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% v/v Surfactant P20, pH 7.4) running buffer. Monoclonal antibodies (10 µg/mL) were non-covalently captured on the surface of a Series S Sensor Chip Protein A (Cytiva) by injection for 60 seconds at a flow rate of 5 µL/min. Single-cycle kinetic titration analyses were performed by sequential 180 second injections of 5 antigen concentrations, followed by a 1200 second dissociation phase, at 30 µL/min. The CMV gB ectodomain protein was assayed in a two-fold dilution series from 1.25-20 µg/mL, and the CMV gB AD-2S1 peptide was assayed in a two-fold dilution series from 6.25-100 ng/mL. The Protein A surface was regenerated after each antigen injection with a 30 second injection of 10 mM glycine-HCl, pH 2.0, at a flow rate of 30 µL/min, allowing for subsequent analysis of all mAb-antigen pairs. Binding data was analyzed using the Biacore T200 Evaluation software (v2.0, Cytiva). Binding profiles were reference subtracted using a negative control Synagis mAb (anti-flu) surface and running buffer injections. Curve fitting analysis was performed using either a 1:1 Langmuir model or a heterogeneous ligand model. For interactions fit with the heterogeneous ligand model, affinities were calculated using the fast kinetic components of the model fit. Some interactions reported dissociation rate constants (k_d) beyond the limit of detection of the software ($<1.00 \times 10^{-5} \text{ s}^{-1}$). In such cases, the values were recorded as 1.00×10^{-5} and affinity calculations are reported as the upper limit of the respective value.

mAb binding to cell-associated gB

HEK293T cells at 50% confluency in a T75 flask were cotransfected using the Effectine Transfection Reagent (Qiagen) with DNA plasmids expressing GFP (gift of Maria Blasi, Duke University) with or without plasmids expressing the full-length gB ORF from the autologous Towne strain (SinoBiological). After incubation for 2 days at 37°C and 5% CO₂, transfected cells were washed with Dulbecco's PBS (DPBS) pH

7.4 (Gibco) then removed from the flask by gently rinsing with Trypsin-EDTA 0.05% with phenol red (ThermoFisher). Cells were resuspended in wash buffer [DPBS pH 7.4 + 1% fetal bovine serum (FBS)] then manually enumerated for count and viability using trypan blue (ThermoFisher). Cells were plated in 96-well V-bottom plates (Corning) at 100,000 live cells/well, then centrifuged at 1200 *g* for 5 minutes. Supernatant was discarded. Cells were co-incubated with gB mAbs at a 3-point, 10x-fold serial dilution starting at 10 µg/mL (10 µg/mL to 0.1 µg/mL), in duplicate for 2 hours at 37°C and 5% CO₂. Cells were washed and resuspended in live/dead Near-IR or Aqua cell stain (ThermoFisher) diluted to 1:1000 for 20 minutes incubation at room temperature. Cells were washed then coincubated with PE-conjugated mouse anti-human IgG Fc (Southern Biotech) diluted to 1:200 for 30 minutes at 4°C. Cells were washed twice and fixed with 1% formalin for 15 minutes. Cells were washed twice then resuspended in PBS pH 7.4. Events were immediately acquired on an LSR II (BD Biosciences) using a high-throughput sampler (HTS). The threshold for PE positivity was defined as 99% of the PE binding by the anti-CMV pentameric complex mAb TRL310 at 10 µg/mL. The % of PE-positive cells was calculated from the live, GFP-positive cell population and reported as the average for each sample run in duplicate. Each sample was run in two or more independent experiments.

Neutralization

Neutralization was measured by high-throughput Cellomics bioimaging and fluorescence as previously described (48). In brief, MRC-5 fibroblasts were seeded in 384-well clear, flat-bottom plates then incubated at 37°C and 5% CO₂ until ~90% confluent. Human CMV virus strains Towne (gB genotype 1), BadrUL131-GFP (genotype 2), or Toledo (genotype 3) at an MOI=2 were coincubated with mAbs in an 8-point, 3-fold serial dilution in cell media (RPMI + 10% FBS) for 2 hours at 37°C. All mAbs were coincubated at a starting concentration of 50 µg/mL (50 µg/mL to 22.9 ng/mL), except the 3-25 mutant mAbs which were coincubated at a starting concentration of 500 µg/mL (500 µg/mL to 228.6 ng/mL). Virus and antibody mixtures were added to cells and allowed to incubate for 16 to 24 hours at 37°C and 5% CO₂. Then, cells were fixed in 3.7% formaldehyde for 10 minutes at room temperature. Plates with Towne virus were stained with mouse anti-human CMV IE1 (MAB810, Millipore) then goat anti-mouse IgG-AF488 (Millipore). Nuclear staining was performed in all plates by DAPI (ThermoFischer Scientific). Plates were imaged using a Cellomics

CellInsight CX5 fluorescent reader, and the number of total cells and infected cells was enumerated by the number of cells expressing DAPI and GFP, respectively. The 50% inhibitory concentration (IC₅₀) of each mAb was calculated according to the Reed and Muench method, wherein the IC₅₀ was defined as the mAb concentration at which there was 50% maximal infection, based on wells containing cells and virus only. This calculation was performed in GraphPad Prism version 9.0 using the non-linear regression 4-point sigmoidal function. Each sample was run in two or more independent experiments.

Crystallization and structure determination

The I8 Fab was generated by digesting I8 IgG at 1 mg/mL in PBS pH 7.4 with Lys-C protease at a ratio of 1:4000 (w/w) overnight at 37 °C. The reaction was quenched with a Roche EDTA protein inhibitor tablet (Sigma-Aldrich) at 1X concentration and the solution was then passed over a CaptureSelect™ IgG-CH1 column (ThermoFischer Scientific) to separate the Fab fragment from the Fc portion. The elution was concentrated in a 10 kDa molecular weight cutoff Amicon Ultra Centrifugal filter (Millipore Sigma) and purified by size-exclusion chromatography with a Superdex 200 Increase 10/300 column (GE Healthcare) in 2 mM Tris-Cl pH 8.0, 200 mM NaCl, 0.02% NaN₃ buffer. Purified I8 Fab was concentrated to 12.0 mg/mL in the aforementioned buffer, mixed with a 2.5-fold molar excess of 1.0 mg/mL gB AD-2S1 peptide (65-HRANETIYNTTLKYG-79) dissolved in DMSO, and incubated for 30 minutes at 4 °C. Crystallization screens were then performed using sitting-drop vapor diffusion with 200 nL drop volumes in either 1:1 or 1:2 protein:reservoir mixtures. Within a few days, diffraction-quality crystals formed in a drop composed of a 1:1 ratio of protein:reservoir solution containing 0.2 M magnesium chloride hexahydrate, 25% (w/v) PEG 3350, and 0.1 M Bis-Tris pH 5.5. Crystals were soaked in reservoir solution supplemented with 20% (v/v) glycerol before they were plunge frozen in liquid nitrogen. X-ray diffraction data were collected remotely at the 19ID beamline (Advanced Photon Source, Argonne National Laboratory). Data were indexed and integrated in iMOSFLM (45) and then merged and scaled to a resolution of 1.80 Å using AIMLESS (46). A Fab homology model generated from PDB IDs 6ZFO and 6UOE and the peptide model from PDB 6UOE were used with PHASER (47) to find a molecular replacement solution. The structure was then iteratively refined in PHENIX (48) and manually built in Coot (49). All crystallographic software programs used in this project were compiled and configured by SBGrid (50).

438

439 *Antibody sequence analysis*

440 The clonal membership of TRL345 was determined by partitioning the sequences of 14 mature anti-gB AD-
441 2S1 mAbs (Patent # US10,030,069B2) into clones using Cloanalyst, version 2007
442 (<https://www.bu.edu/computationalimmunology/cloanalyst/>). With the 9 observed mature TRL345
443 sequences as input, we also used Cloanalyst to reconstruct the TRL345 genealogical tree which included
444 inference of the TRL345 UCA sequence (PMID:24555054). To estimate the probability of antibody
445 mutations prior to antigenic selection, we used the ARMADiLLO program (<https://armadillo.dhvi.duke.edu/>),
446 which computationally simulates somatic hypermutation (27).

447

448 *Software*

449 Phylogenetic analysis was performed using Cloanalyst version 7. Mutation analysis was performed using
450 ARMADiLLO. Statistical tests were performed in GraphPad Prism version 9.0. Figures were created in
451 GraphPad Prism version 9.0. Figure 4 was created in PyMOL version 2.4.1 (Schödinger, LLC).

452

453 *Statistical analyses*

454 Statistical tests were performed as described in the figure legends, where applicable. Nonlinear regression
455 curve fitting was performed to calculate the EC₅₀ and IC₅₀ values. The relative contribution of individual
456 amino acid mutations to mAb neutralization function was calculated by linear regression. Statistical
457 significance was calculated using a nonparametric two-tailed Wilcoxon matched-pairs signed-rank test. All
458 tests were performed in GraphPad Prism version 9.0.

459 **Supplementary materials**

460 Fig. S1. Gating strategy to determine the % of binding to cell-associated gB.

461 Fig. S2. The VH A33N mutation was both necessary and sufficient for high gB AD-2 binding, binding to cell-
462 associated gB, and neutralization function.

463 Fig. S3. Introduction of the VH A33G mutation to the UCA of either the TRL345 or 3-25 lineages did not
464 confer neutralizing function.

465 Fig. S4. Sequence alignment of anti-gB AD-2S1 mAbs from the TRL345 and 3-25 lineages.

466 Table S1. Compiled binding and neutralization responses for TRL345 lineage mAbs and 3-25 mature mAb.

467 Table S2. Mutational probability analysis using ARMADiLLO algorithm.

468 Table S3. X-ray crystallographic data collection and refinement statistics.

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576

577

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594 **Author contributions**

595 Study aims and design were conceived by J.A.J. under the mentorship of K.W. and S.R.P. Funding was
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597 design and production were performed by J.A.J., A.K., and J.T. Antibody binding and neutralization
598 functions were measured by J.A.J. and S.A. Structural studies were performed by M.R.S., D.W., and J.S.M.
599 This study was performed under supervision of K.W., J.S.M., and S.R.P.

601 **Competing interests**

602 J.A.J. has been a paid invited speaker by Moderna x Popsugar. S.R.P. serves as a consultant for Moderna,
603 Merck, Dynavax, Pfizer, and Hookipa CMV vaccine programs and has a sponsored research program on
604 CMV vaccine immunogenicity with Moderna and Merck.

605

606 **Data and materials availability**

607 All data and materials used in the analysis are available in the main text or the supplementary materials.

617 lineage tree for the heavy and light chains, respectively, and amino acids are numbered according to the
618 Kabat scheme. The probability of a given mutation occurring in the absence of B cell selection was
619 calculated using the ARMADiLLO algorithm, which computationally simulates hypermutation. The
620 mutational probabilities are shown from high probability (>10%, dark green) to moderate (2-10%, light
621 green) to low (<2%, yellow to red text).

Fig. 2. The TRL345 UCA acquired high binding to gB AD-2S1 and broad CMV neutralization function early in lineage development. We produced the 17 clonally related mAbs of the TRL345 lineage and quantified antigen binding and neutralization functions. Shown here are the responses of the lineage ancestors of TRL345:

- (A) Binding magnitude to gB AD-2S1 peptide and gB ectodomain by ELISA.
- (B) Binding to and avidity for gB AD-2S1 peptide and gB ectodomain by SPR.
- (C) Binding coefficients for gB AD-2S1 peptide and gB ectodomain by SPR. K_d (nM) was calculated as k_d/k_a .
- (D) Binding to cell-associated gB. Binding of mAbs was determined by coincubating mAbs in a serial dilution with HEK293T epithelial cells-transfected with full-length gB and GFP. The % binding was calculated as the % of GFP-expressing cells bound by the anti-gB AD-2 mAb, detected by flow cytometry.
- (E) Neutralization of CMV strains Towne, AD169rUL131-GFP (AD169r), and Toledo on MRC-5 fibroblasts.
- (F) Neutralization of AD169rUL131-GFP on ARPE epithelial cells.

Each experiment was run with the negative control anti-CMV pentameric complex (PC) mAb TRL310 and positive control anti-CMV gB AD-2 mAb 3-25. For each figure, data are shown from one experiment, as the mean of two samples run in duplicate. Each figure is representative of results from two or more independent experiments.

Fig. 3. The V_H A33N mutation was both necessary and sufficient for high gB AD-2 binding, binding to cell-associated gB, and neutralization function. We produced 14 mutant mAbs wherein we introduced single early, improbable mutations or combinations of mutations to the UCA V_H and V_L chains then measured the following:

- Binding magnitude of mutant mAbs to gB AD-2S1 peptide and gB ectodomain by ELISA. Data are reported as the mean ELISA EC₅₀ (ng/mL) of two or more independent experiments, and the heatmap is colored from high (<50.0 ng/mL, dark green) to moderate (100.0 ng/mL, yellow) to low (>250.0 ng/mL, white) binding.
- Binding magnitude to cell-associated gB. Binding to HEK293T epithelial cells-transfected with full-length gB was measured in a three-point, 10-fold mAb serial dilution starting at 0.1 µg/mL, to gB-transfected cells and was reported as the area-under-the-curve (AUC). Data are reported as the mean binding AUC of two or more independent experiments, and the heatmap is colored from high (>300 AUC, red) to moderate (200 AUC, orange) to low (<100 AUC, white) binding.
- Neutralization of CMV strains Towne, AD169rUL131-GFP (AD169r), and Toledo on MRC-5 fibroblasts and AD169r on ARPE epithelial cells. Data are reported as the mean neutralization IC₅₀ of two or more independent experiments. The average neutralization on MRC-5's was calculated as the mean neutralization of the average Towne, AD169r, and Toledo neutralization IC₅₀'s; the average neutralization on ARPE's was reported as the mean neutralization of the AD169r strain. The heatmap is colored from high (<1.0 µg/mL, blue) to moderate (20.0 µg/mL, purple) to no (>50.0 µg/mL white) neutralization potency.

Fig. 4. Introduction of V_H A33N, as compared with V_H A33G, conferred higher binding to gB AD-2S1, gB ectodomain, and cell-associated gB for both the TRL345 UCA and 3-25 UCA antibodies. We produced 4 mutant mAbs wherein we introduced V_H A33N or A33G mutations to the TRL345 UCA V_H or 3-25 UCA V_H and measured the following:

- (A) Binding magnitude of TRL345 mutant mAbs to gB AD-2S1 peptide and gB ectodomain by ELISA.
- (B) Binding of TRL345 mutant mAbs to cell-associated gB. Binding of mAbs was determined by coinubating mAbs in a serial dilution with HEK293T epithelial cells-transfected with full-length gB and GFP. The % binding was calculated as the % of GFP-expressing cells bound by the anti-gB AD-2 mAb, detected by flow cytometry.
- (C) Binding magnitude of 3-25 mutant mAbs to gB AD-2S1 peptide and gB ectodomain by ELISA.
- (D) Binding to 3-25 mutant mAbs to cell-associated gB.

Fig. 5. Crystal structure of anti-CMV gB AD-2 Fab bound to gB AD-2S1 peptide.

- (A) In the TRL345 lineage, the I8 Fab is shown as a molecular surface, with the heavy chain in blue and light chain in white. The gB AD-2S1 peptide is shown as light-green sticks, with residues that form hydrophobic contacts with the TRL345 I8 Fab shown as transparent light green surfaces.
- (B) The TRL345 I8 Fab is shown as a cartoon with residues that contact the gB AD-2S1 peptide shown as sticks. Hydrogen bonds and salt bridges are shown as black dotted lines.
- (C) As a comparison between the TRL345 I8 structure and the previously published 3-25 structure, the two Fabs are overlaid. The TRL345 I8 heavy and light chains are shown in blue and white, respectively, bound to the gB AD-2S1 peptide in light green. The 3-25 heavy and light chains are shown in orange and gray, respectively, bound to the gB AD-2S1 peptide in dark green.
- (D) The previously published 3-25 structure is shown as a cartoon with residues that contact the gB AD-2S1 peptide shown as sticks.