Reactivity profiles of broadly neutralizing anti-HIV-1 antibodies are distinct from those of pathogenic autoantibodies

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Abstract

Objective—Broadly neutralizing antibodies (bNt Abs) against HIV-1 are rarely produced during natural infection, and efforts to induce such Abs by vaccination have been unsuccessful. Thus, elucidating the nature and cellular origins of bNt Abs is a high priority for vaccine research. As the bNt monoclonal Abs (MAbs) 2F5, 4E10 and 2G12 have been reported to bind select autoantigens, we investigated whether these MAbs display a broader range of autoreactivity and how their autoreactivity compares with that of pathogenic autoAbs.

Methods—An autoantigen microarray comprising 106 connective tissue disease-related autoantigens and control antigens was developed and used, in combination with ELISAs, to compare the reactivity profiles of MAbs 4E10, 2F5 and 2G12 to those of four pathogenic autoAbs derived from patients with antiphospholipid-syndrome (APS), and to serum from a patient with systemic lupus erythematosus (SLE).

Results—The APS MAbs and SLE serum reacted strongly with multiple autoantigens on the microarray, whereas anti-HIV-1 MAb reactivity was limited mainly to HIV-1-related antigens. The APS autoAbs reacted strongly with CL, yet only 4E10 bound CL at high concentrations; both 2F5 and 4E10 bound their HIV-1 epitopes with a 2–3-log higher apparent affinity than CL. Moreover, the polyreactivity of 4E10, but not CL15, could be blocked with dried milk.

Conclusion—The reactivity profiles of bNt anti-HIV-1 MAbs are fundamentally distinct from those of pathogenic autoAbs that arise from dysregulated tolerance mechanisms. This suggests that the limited polyreactivity observed for the bNt MAbs, and for HIV-1-Nt Abs in general, may arise through alternative mechanisms, such as extensive somatic mutation due to persistent antigen selection during chronic infection.

Keywords

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Introduction

Induction of rare broadly neutralizing (bNt) antibodies (Abs) able to block cellular infection by a variety of HIV-1 isolates remains the elusive goal of vaccine design. Currently, passive transfer with bNt Abs is the only means of conferring protection against viral challenge in animal models [1–4]. Six of the eight bNt monoclonal Abs (MAbs) identified to date have unusually long third complementarity-determining regions of the heavy chain (CDR-H3s) (b12, 2F5, 4E10, 447–52D, PG9/16 and HJ16 [5]; D. Corti, Bellinzona, Switzerland, personal communication), and all are highly mutated, prompting the question of whether these features are necessary for broad neutralization. MAbs 2F5 and 4E10 recognize adjacent linear epitopes in the membrane-proximal external region of gp41 (MPER; reviewed in [6]). Structural studies of these Abs in complex with peptides have revealed that their CDR-H3s make marginal contact, if any, with their epitopes [7,8]; yet, mutational studies have shown the hydrophobic tips of their CDR-H3s are required for viral neutralization [9–13].

In addition to their MPER epitopes, MAbs 2F5 and 4E10 have been reported to bind self antigens, including cardiolipin and other membrane lipids [9,14–18]. It has been proposed that 2F5 and 4E10 are polyreactive autoAbs, produced by plasma cells whose autoreactive precursors emerged due to a loss of tolerance checkpoints during development of the naive B-cell repertoire [19,20]. Evidence supporting this hypothesis includes the reactivity of 2F5 and 4E10 with self-antigens [14–19]; 4E10's weak lupus anticoagulant activity [14,21]; the long CDR-H3s of these Abs, given that tolerance checkpoints may play a role in restricting CDR-H3 length [22]; and the disruption of B-cell development in IgH knock-in mice bearing the 2F5 variable region [23]. More recently, it has been shown that the hydrophobic tip of 2F5's and 4E10's CDR-H3 is required for weak interaction with lipid [9–13], and that residues of synthetic MPER peptides that are critical for interaction with MAbs 2F5 and 4E10 face into detergent micelles and lipid bilayers [24,25], leading to a proposed binding mechanism in which membrane surfaces are 'scanned' for MPER residues, which are then extracted from the viral membrane [15,25–27]. Based on these studies, it has been suggested that polyreactive, lipid-binding Abs are initially selected from the naive repertoire and later recruited into the anti-HIV-1 response by viral antigens [28]. Furthermore, one study has shown that serum anti-cardiolipin Ab titers are strongly correlated in chronically infected patients with anti-MPER Ab titers and neutralization breadth [29]. The proposal that HIV-1-Nt Abs arise from polyreactive and/or autoreactive precursors that are normally absent in the naive repertoire has fueled speculation that conventional vaccination strategies are unlikely to elicit bNt Abs, as selection for polyreactive Abs [28] or disruption of immunological tolerance [20] would be required for their development.

Serum reactivity in clinical anti-cardiolipin assays is associated with antiphospholipid syndrome (APS), an autoimmune, thrombogenic condition and systemic lupus erythematosus (SLE) [30]. In contrast, the clinical significance of reactivity with cardiolipin and other phospholipids that is commonly observed for serum Abs produced during HIV-1 infection is unclear [30,31]. The antiphospholipid Abs that develop during infection by HIV-1 and other pathogens [32] differ in several respects from those arising in APS and SLE: they are often transient [31,32], do not cross-react with β2-glycoprotein I [30,31] and are not associated with lupus anticoagulant activity or thrombosis [31]. Despite their putative cardiolipin reactivity, sustained passive infusion of MAbs 2F5 and 4E10 is well tolerated, with no incidents of adverse effects or thrombotic complications [21,33], and an alternate hypothesis to explain the mechanism of lipid reactivity of 2F5 has been described [10,34]. Thus, to further clarify the polyreactive and autoimmune nature of bNt anti-HIV-1 MAbs, we compared their reactivity profiles on an extensive panel of autoantigens with

those of autoreactive, thrombogenic MAbs and serum produced by a patient with active SLE.

Methods

Materials

The NIH AIDS Research and Reference Reagent Program provided 15-mer synthetic peptides derived from the lab-adapted HIV-1 strain, MN (>80% purity; sequences available online). Synthetic 2F5 peptide [NH_3^+-EQELLELDKWASLWSGK(Biotin)GC-$CONH_2$] and E4.6 peptide [$NH3^+$-LHEESMDKWSNLMQCCT AEGK(Biotin)-$CONH_2$] were from NeoMPS (San Diego, California, USA), synthetic 4E10 peptide [NH_3^+-SLWNWFDITNWLWYISGK(Biotin)GC-$CONH_2$] was from the University of British Columbia's NAPS Unit Peptide Synthesis Laboratory (Vancouver, Canada) and synthetic H3N6 peptide (NH_3^+-AEPAENNWFML TYFLAAEGC-$CONH_2$) was from EZBiolab Inc. (Westfield, Indiana, USA); all were more than 95% pure. MAbs CL1, CL15, IS2 and IS4 were provided by P. Chen (UCLA) and Abs 2F5, 4E10 and 2G12 by R. Kunert and H. Katinger (University of Agricultural Sciences, Vienna). Recombinant gp41 and gp120 envelope proteins (MN strain) were from ImmunoDiagnostics (Woburn, Massachusetts, USA). Cardiolipin, BSA, oval-bumin (OVA), p-nitrophenyl phosphate (pNPP) and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) were from Sigma-Aldrich (St Louis, Missouri, USA), nonfat dried milk from Bio-Rad (Hercules, California, USA) and Tween-20 from GE Healthcare (Piscataway, New Jersey, USA). Sources of microarray antigens are listed in Supplementary Table S1, http://links.lww.com/QAD/A142.

Titration ELISA

Protocols were adapted from an optimized procedure [14] (B.F. Haynes, Duke University, personal communication). Wells of 96-well microtiter plates (Corning Inc., Corning, New York, USA) were coated with 400 ng MN peptide in 50 µl PBS, pH 7.4 and dried at 55°C for 4 h; 2 µg cardiolipin in 50 µl methanol and dried at 55°C for 45 min; and protein [50 ng gp41 in 50 µl PBS, or 50 µl PBS containing 2% (w/v) BSA, dried milk, or OVA] with overnight incubation at 4°C. Wells were blocked at 37°C for 2 h with 50 µmol/l carbonate–bicarbonate buffer, pH 9.6, containing 3% BSA, then washed three times with PBS containing 0.05% (v/v) Tween-20 (PBST). Serial dilutions of 4E10, 2F5 and CL15 IgGs were prepared in Ab diluent [PBST containing 3% BSA and 2% (v/v) fetal calf serum (FCS)]; 50-µl aliquots were incubated in each well for 1 h at room temperature (RT). Wells were washed four times with PBST and then 50 µl alkaline phosphatase-conjugated goat antihuman IgG (Fab-specific; Pierce, Rockford, Illinois, USA), diluted 1 : 1000 in Ab diluent, was added for 1 h at RT. Wells were washed four times and developed using a pNPP tablet dissolved at 1 mg/ml in 100 µl 50 µmol/l carbonate–bicarbonate buffer containing 2 mmol/l $MgCl_2$. After 45 min, absorbance at 405 nm was measured using a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, California, USA).

Direct ELISA

Direct ELISAs were performed as described [35] with the following modifications. Wells were coated with 4E10, H3N6 or MN peptides (400 ng), autoantigens (1 µg), double-stranded DNA (2 µg) or cardiolipin (2 µg in 35 µl methanol) and dried at 55°C for 1 h and then other wells were coated overnight at 4°C with 50 ng gp41 or 2% BSA, dried milk or OVA. MAbs were diluted in TBS, pH 7.2, containing 0.1% Tween-20 and either 5% BSA, 5% dried milk, 50% FCS or 5% FCS as indicated.

Autoantigen microarrays

Purified autoantigens (0.2 mg/ml in PBS) were deposited in triplicate on nitrocellulose-coated FAST slides (Whatman, Florham Park, New Jersey, USA) using a VersArray ChipWriter Pro Robotic Arrayer (Bio-Rad). Arrays were blocked in dilution buffer (PBS, 5% FCS, 0.1% Tween-20) overnight at 4°C. Serum samples were diluted 1 : 300 in dilution buffer; MAbs were diluted to 35, 7 and 1.5 µg/ml (representing 50, 10 and 2% of total serum IgG, respectively, given a typical serum IgG concentration of 21 mg/ml) and then diluted 1 : 300; diluted Ab samples were incubated on slides for 1 h at 4°C. Arrays were washed twice in dilution buffer and then probed with a 1 : 2000 dilution of Cy-3-conjugated goat antihuman IgG/IgM (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) for 1 h at 4°C. After two washes in dilution buffer, followed by one in PBS and then in water, arrays were spun dry and imaged using a GenePix 4000B microarray scanner (Molecular Devices). Protocols are available at http://utzlab.stanford.edu/protocols/.

Data analysis and heat map generation

Scanned array images were analyzed using Axon GenePix Pro 6.0 software (Molecular Devices). Median fluorescence intensity (MFI) values of less than 10 digital fluorescence units (DFUs) were set to 10 DFUs exactly. MFI values minus background from three replicate spots were averaged for each antigen. Heat maps were generated in TMEV Multiple Experiment Viewer v4.5.1 software by setting the highest intensity value for a given Ab–antigen interaction as 1.0 and normalizing the intensity of other Abs to this value, generating a percentage scale.

Significance analysis of microarrays

Significance analysis of microarrays (SAM) was performed in TMEV Multiple Experiment Viewer v4.5.1 software to identify significant differences in array reactivities. Raw MFI values were log_2 transformed and two-class unpaired analyses were performed to compare each HIV-1 MAb to the APS MAb CL15 across all three dilutions tested. For each comparison, 20 permutations were calculated and threshold parameters were selected such that the false discovery rate was less than 0.05. Antigens revealed by SAM to be bound significantly differently were selected for cluster analysis. A hierarchical clustering algorithm using a Euclidian distance metric and complete linkage method was applied to order array features into clusters based on similarities in Ab–antigen reactivities.

Results

We first compared the cardiolipin reactivity of MAbs 2F5 and 4E10 with that of the pathogenic APS MAb, CL15 [36], using published ELISA conditions [14] (Fig. 1). In agreement with other works [21,37], cardiolipin binding by MAb 2F5 was undetectable, whereas binding by 4E10 occurred only at high concentrations; in this assay, 4E10 IgG bound cardiolipin and OVA approximately equally. There was more than a 2-log difference in the concentration of 4E10 MAb required to produce a given signal on 4E10 peptide (or gp41) and that required to produce the same signal on cardiolipin. In contrast, CL15 saturated all antigens tested, with the exception of BSA. Although MAbs 4E10 and CL15 bound the 4E10 peptide at similar half-maximal concentrations, only 4E10 neutralized HIV-1 strain HxB2 (Supplementary Fig. S1, http://links.lww.com/QAD/A142; provided by M.B. Zwick, The Scripps Research Institute). These data demonstrate that MAbs 2F5 and 4E10 prefer their cognate gp41 epitopes to cardiolipin by orders of magnitude, compared with the pathogenic and highly polyreactive autoAb CL15.

To further test the specificity of these MAbs, their binding was studied in ELISA to a panel of overlapping 15-mer peptides derived from HIV-1 envelope protein isolate MN (Fig. 2).

When diluted in 5% BSA, MAb 2F5 bound specifically to peptides bearing its cognate HIV-1 epitope, and MAb 4E10 bound its cognate epitope better than the multiple peptides with which it weakly cross-reacted. In contrast, the APS autoAb CL15 bound promiscuously to more than half the assayed peptides and to OVA and dried milk. Dilution in 5% FCS or 5% dried milk abolished nonspecific binding of 4E10 but not of CL15. Linear regression analysis revealed that CL15 binding, but not 4E10 binding, was associated with a net negative charge on the peptides (Supplementary Fig. S2, http://links.lww.com/QAD/A142). These results are consistent with our previous studies in which both MAbs 2F5 and 4E10 selected peptides from phage-displayed libraries that share a core consensus sequence with their gp41 epitopes [38,39], whereas MAb CL15 selected peptides bearing completely unrelated sequences [40].

To compare the autoreactivity profiles of bNt anti-HIV-1 MAbs to those of pathogenic APS MAbs and SLE serum, we developed and validated an autoantigen microarray containing clinically relevant autoantigens targeted by Abs from patients with systemic autoimmune diseases [41], HIV-1 antigens and control antigens. For comparison, we analyzed the anti-MPER MAbs, 2F5 and 4E10, the anti-gp120 MAb 2G12 and four MAbs derived from APS patients: CL15, CL1 [36], IS2 [42] and IS4 [36]. In a previous study, cross-reactivity of MAbs 2F5, 4E10 and 2G12 against a panel of 400 mainly non-self, recombinant proteins was minimal [37]. However, we wanted to more comprehensively assess potential autoreactivity of these MAbs using an autoantigen microarray that had been previously validated with pathogenic autoantisera [43–49]. Figure 3a shows the reactivity profiles of MAbs 2F5, 4E10 and 2G12 against positive and negative control antigens, including synthetic peptides bearing the 2F5 and 4E10 epitopes, recombinant gp120 and gp41, overlapping MN peptides covering the gp41 MPER (MPER1-MPER6), cell lysates bearing gp41 fragments covering the MPER tethered to the cell surface by the gp41 transmembrane region [50], cell lysates bearing this gp41 fragment containing single Ala substitutions in the MPER sequence (mutants 3–5 and 11–13) and cell lysates alone. As expected, MAbs 2F5 and 4E10 each bound their cognate peptide epitopes [38,39], and 2G12 bound strongly to its carbohydrate epitope on gp120. As seen in Fig. 3a, MAb 2F5 reacted only with overlapping MPER peptides (MPER2 and MPER3) containing its core ELDKWA epitope (shown in bold). Similarly, MAb 4E10 bound only to peptide MPER5 containing its core NWF(D/N)ITepitope (shown in bold italics); no binding was observed to peptide MPER4, which contains the 4E10 core epitope but little additional C-terminal sequence, consistent with mapping studies [51]. In addition, both MAbs 2F5 and 4E10 bound to lysates of COS-7 cells transiently transfected with plasmids expressing MPER-TM1, a gp41 fragment covering part of the C-heptad repeat, the MPER, the transmembrane region and part of the cytoplasmic domain of HIV-1 JRCSF [50]. Binding was epitope-specific, as substitution of residues in either the 2F5 or 4E10 core epitope with Ala abrogated binding of the respective MAb (Fig. 3a). Thus, the specific binding patterns of anti-HIV-1 MAbs to their epitopes validated the sensitivity and specificity of the array platform.

As seen in Fig. 3b, the bNt MAbs 2F5, 4E10 and 2G12 bound their cognate HIV-1 epitopes but had limited reactivity with all autoantigens tested (shown in alphabetical order), whereas the APS autoAbs and SLE serum IgGs displayed extreme polyreactivity. Consistent with the ELISA results, binding of MAbs 2F5 and 4E10 to cardiolipin was minimal, and 4E10 reacted significantly with cardiolipin and other autoantigens only at the highest concentration tested (35 μg IgG/ml). In contrast, most APS MAbs tested (CL1, CL15 and IS4) displayed strong binding to cardiolipin even at lower concentrations. The cardiolipin reactivity of MAb 4E10 was not associated with binding to β2-glycoprotein I (a well characterized APS autoantigen), whereas two APS MAbs (CL1 and CL15) reacted with both antigens. Binding of MAb 2F5 to centromere protein B (CENP-B), whole histones or histone subunits was undetectable, conflicting with previous reports [14]; MAb CL15,

however, was particularly histone-reactive, and MAbs CL15 and IS4 bound CENP-B. MAbs 2F5 and 2G12 bound lysozyme at lower concentrations but not at higher ones, casting doubt upon this result. Together, these data provide further evidence that the bNt anti-HIV-1 MAbs 2F5 and 2G12 are not especially autoreactive or polyreactive, whereas MAb 4E10 exhibits modest polyreactivity at concentrations reflecting high levels of serum Ab (the equivalent of 50% of the average serum concentration of total IgG in healthy individuals).

We used SAM, a well validated algorithm [52] that we and others have used in comparing serum autoAb reactivities [43–49], to identify differences in the reactivity profiles of MAbs 2F5, 4E10, 2G12 and CL15. Table 1 lists antigens that were bound significantly differently ($q < 0.05$) by a given pair of MAbs. MAb CL15 bound significantly better than anti-HIV-1 MAbs to a variety of autoantigens such as cardiolipin, CENP-B and double-stranded DNA as well as control antigens such as OVA and BSA, and in some cases, even HIV-1 antigens. Importantly, the only antigens to which MAbs 2F5, 4E10 and 2G12 bound significantly better than CL15 were antigens bearing their cognate HIV-1 epitopes; the same was true for pairwise SAM comparisons between each HIV-1 MAb and the least polyreactive APS MAb, IS2 (data not shown). Furthermore, statistical comparison of MAbs 2F5 and 4E10 confirmed that the only antigens shown to be bound differently by these MAbs bore their respective cognate epitopes (Table 1). MAbs were also arranged by hierarchical clustering based on reactivity similarities [53]. In each case, MAbs 2F5, 4E10 and 2G12 belonged to a distinct clade from MAb CL15 and SLE serum (Supplementary Figs S3–S6, http://links.lww.com/QAD/A142); the same overall trend was observed when hierarchical clustering was conducted using all antigens (data not shown). Thus, the behavior of MAbs 2F5, 4E10 and 2G12 is unlike that of pathogenic APS autoAbs and polyclonal SLE sera, as demonstrated by their minimal reactivity with self antigens.

To validate reactivity patterns to selected autoantigens observed in the microarray, ELISAs were performed using antigens revealed by SAM to be bound differently by anti-HIV-1 MAbs and APS MAbs. Without exception, all differences between anti-HIV-1 and APS Abs observed by ELISA (Fig. 4a) were reflected in the microarray analysis (Fig. 4b), although the magnitudes of these differences were not always accurately replicated. In particular, two of the APS autoAbs, CL1 and IS4, appeared far more autoreactive in the ELISA compared to the microarray. However, overall binding trends indicated concordance of reactivity patterns, with the autoantigen microarray being more sensitive than ELISA in detecting differences in binding to a particular antigen.

Several ELISA experiments designed to gauge poly-reactivity and autoreactivity of bNt anti-HIV-1 MAbs have been performed using very high concentrations of Ab and no detergent [16–19,22,54–56]. Here, we tested several ELISA protocols to understand the effect of different assay conditions on the reactivity profiles of MAbs 2F5, 4E10 and CL15. We found that the modest polyreactivity of MAb 4E10 was markedly reduced or ablated by replacing BSA with FCS, and especially dried milk, in the Ab diluent; yet, this did not significantly affect binding to its cognate gp41 epitope (Fig. 2). This suggests that there may exist an Ab subspecies, perhaps in partially denatured form, whose activity is saturated in the presence of complex proteins and/or lipids existing in dried milk and serum. In contrast, dilution of MAb CL15 in FCS or dried milk only slightly reduced its polyreactivity. As their polyreactivity vanishes in assays that more closely approximate physiological conditions, we surmise that the polyreactivity of MAbs 2F5 and 4E10 is not biologically significant *in vivo*; this is supported by their lack of pathogenicity after passive infusion [21,33].

Discussion

Our data indicate that reactivity of MAbs 2F5 and 4E10 with cardiolipin is biologically insignificant compared with reactivity with their HIV-1 epitopes or with the cardiolipin reactivity of the pathogenic APS MAb, CL15. Moreover, the global reactivity patterns of MAbs 2F5, 4E10 and 2G12 with an extensive panel of clinically relevant autoantigens reveal that these Abs are not polyreactive autoAbs in the same sense as pathogenic APS or SLE autoAbs. These results support and extend those of Scherer *et al.* [37], who showed that MAbs 2F5 and 4E10 have very limited polyreactivity on a panel of antigens. The microarray data from these two experiments, assaying hundreds of autoantigens and unrelated proteins, have allowed us to draw strong conclusions regarding the polyreactivity and autoreactivity of bNt anti-HIV-1 MAbs. However, antigen microarray techniques have limitations in that a control antigen (gp120) was recognized by only one of its 'cognate' MAbs (2G12, but not b12) when printed on nitrocellulose slides; thus, b12, but not 2G12, was excluded from this analysis.

It is likely that the weak polyreactivity of bNt anti-HIV-1 Abs, in particular MAb 4E10, with self-antigens and nonself-antigens [14,15,21] is not evidence of a poly-reactive or autoreactive origin, but rather reflects normal, low level self-reactivity of Abs from the memory B-cell and plasma-cell repertoires [54]. Such reactivity could increase with chronic infection as a result of prolonged somatic mutation, and a lack of post-germinal center checkpoints for deleting weakly self-reactive or poly-reactive clones. Recently, Mouquet *et al.* [55] showed that somatic mutations and polyreactivity are high in anti-gp140 Abs cloned from the memory B cells of HIV-positive individuals, as compared with their non-gp140-binding Abs and with Abs from healthy controls. Although the in-vivo relevance of this polyreactivity –as measured by in-vitro assays in which Abs were diluted in PBS lacking blocking proteins or detergent [56] – is unclear, the increased polyreactivity of the anti-gp140 Abs is striking. Moreover, in only a few cases did reversion of somatic mutations to germline reduce polyreactivity, leading the authors to conclude that polyreactive precursors of these clones must have been present in the naive B-cell repertoire. However, as somatic mutations in CDR3, particularly in CDR-H3, are not easily identified, their contributions to polyreactivity may have been overlooked (N.B., this issue has been circumvented using TdT knockout mice, with the result that all Ab autoreactivity was abolished upon reversion to germline [57]). Similarly, the dysregulation of B-cell development observed when using highly mutated VH genes (such as MAb 2F5's [22]) for knock-in experiments can be attributed to disruption of the pre-B-cell receptor, and thus is not necessarily good proof of tolerance induction [58].

We propose that the polyreactivity and self-reactivity observed among anti-HIV-1 MAbs and sera are generated during chronic viral infection, which drives both repeated rounds of somatic mutation and persistent antigen selection. In this scenario, extensive mutation, including insertions that lengthen CDR-H3, would broaden the reactivity profiles of clones that continue to retain affinity for antigen, and therefore to be recruited into the Ab response. Consistent with this hypothesis, breadth of serum neutralization is significantly correlated with viral load [59,60], reflecting antigen persistence, and where anti-MPER Nt Abs have been observed, with cardiolipin polyreactivity [29]. Although the possibility that low-level polyreactivity may be a general feature of highly mutated Abs against viral pathogens in persistent or repeated infection [61] awaits further investigation, it is certainly conceivable, given the shared genetic features of these Abs [5] and the role of somatic hypermutation in generating polyreactive and self-reactive specificities [54,57].

Regardless of their origins, our results support a fundamental distinction between bNt anti-HIV-1 MAbs and pathogenic autoAbs that arise in autoimmune states like APS or SLE. The

majority of the bNt anti-HIV-1 MAbs isolated to date have atypically long CDR-H3s and extensive somatic mutations, but so do anti-HIV-1 Abs and antiviral Abs produced during chronic infection in general [5]. Elucidating the conditions under which bNt Abs arise will require a better understanding of the Abs comprising the naive and memory B-cell repertoires and mechanisms for selecting Ab-secreting plasma cells during chronic HIV-1 infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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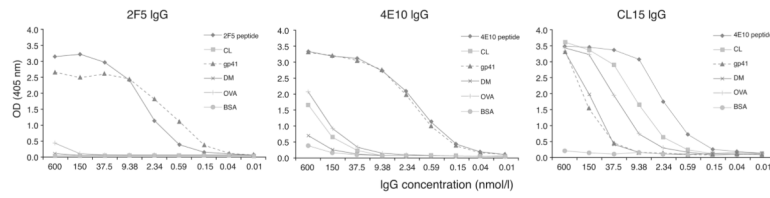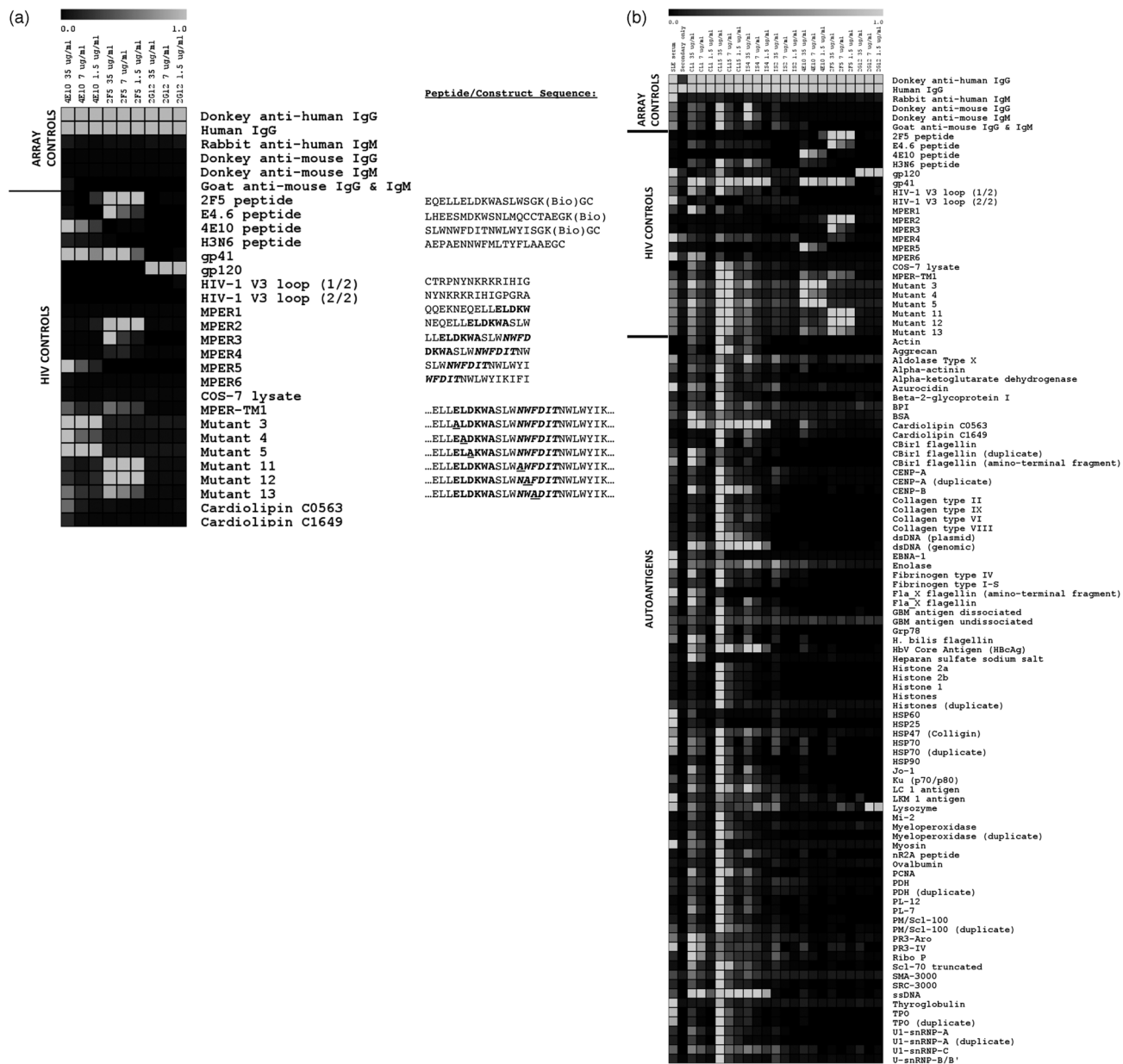
Fig. 1. Comparison of autoreactivity and polyreactivity of MAbs 2F5, 4E10 and CL15 and dependence on Ab concentration

MAbs 2F5, 4E10 and CL15 were titrated at the concentrations indicated in a direct ELISA against wells containing immobilized peptides bearing the 2F5 or 4E10 epitopes (400 ng each), recombinant gp41 (50 ng), cardiolipin (CL; 2 μg) or a nonspecific antigen, BSA, dried milk (DM) or ovalbumin (OVA) (2%). Data are representative of three independent experiments. MAb 2F5 does not bind CL or any nonspecific antigen significantly, whereas MAb 4E10 binds CL and OVA with apparent affinities that are at least two orders of magnitude smaller than that for gp41. MAb CL15 binds many antigens with high apparent affinity.
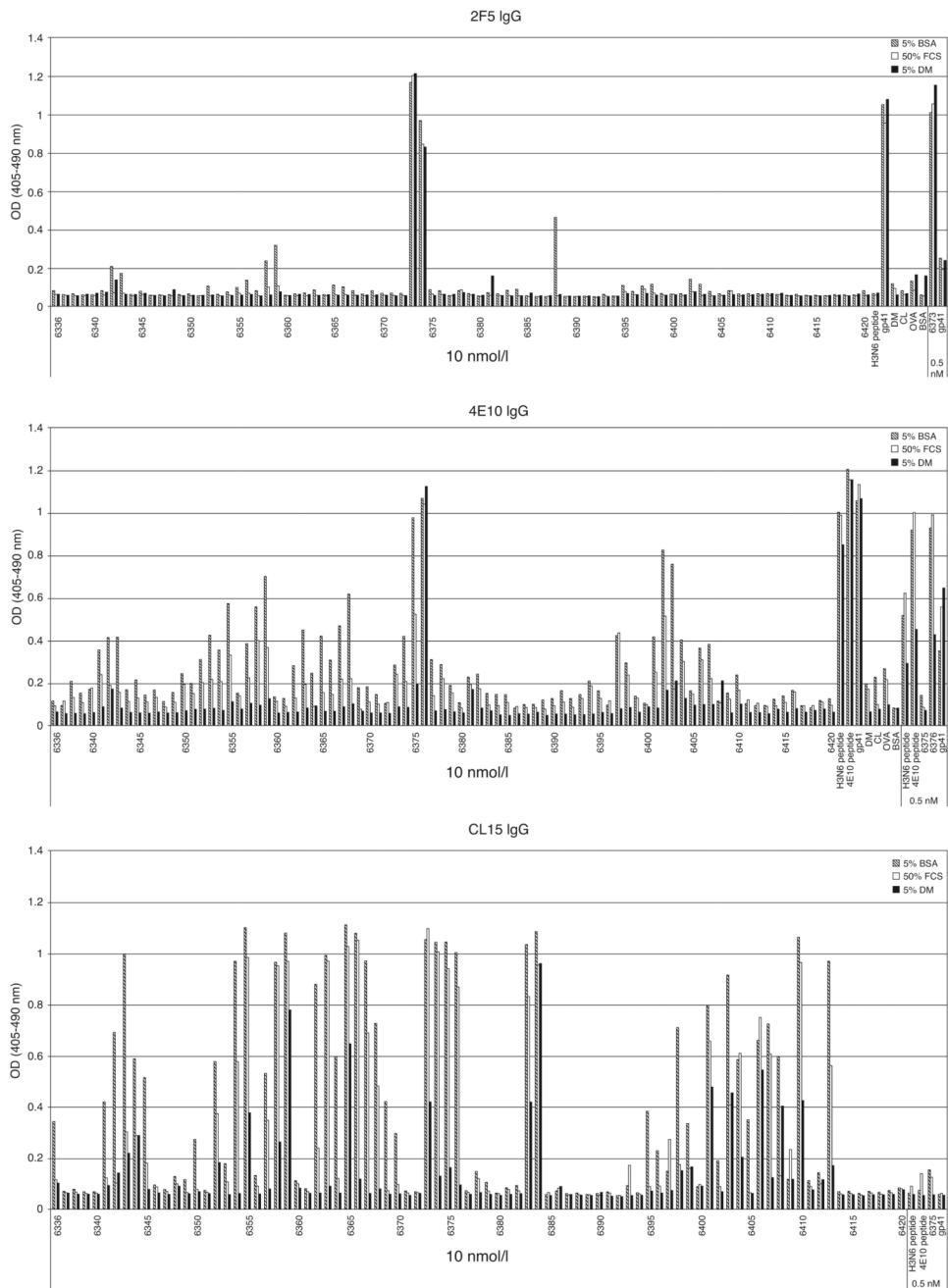
Fig. 2. Effects of complexity of Ab diluent on reactivity profiles of MAbs 2F5, 4E10 and CL15
MAbs 2F5, 4E10 or CL15 (10 nmol/l or 66.7 μg/ml IgG) were diluted in 5% BSA, 50% fetal calf serum (FCS) or 5% dried milk (DM) and assayed on wells containing immobilized overlapping HIV-1 MN peptides (400 ng), 4E10 peptide (400 ng), H3N6 peptide (400 ng), recombinant gp41 (50 ng), cardiolipin (CL; 2 μg) or BSA, DM or ovalbumin (OVA) (2%). Each MAb was also tested at a 0.5 nmol/l concentration against positive-control antigens to verify that binding remained unaffected by choice of diluent. Low-level polyreactivity of MAb 2F5 was abrogated in FCS or DM, and that of MAb 4E10 was reduced in FCS and abrogated in DM; in contrast, MAb CL15 retained a promiscuous binding pattern even in DM.

Peptide/Construct Sequence:

Antigen	Sequence
2F5 peptide	EQELLELDKWASLWSGK(Bio)GC
E4.6 peptide	LHEESMDKWSNLMQCCTAEGK(Bio)
4E10 peptide	SLWNWFDITNWLWYISGK(Bio)GC
H3N6 peptide	AEPAENNWFMLTYFLAAEGC
HIV-1 V3 loop (1/2)	CTRPNYNKRKRIHIG
HIV-1 V3 loop (2/2)	NYNKRKRIHIGPGRA
MPER1	QQEKNEQELL**ELDKW**
MPER2	NEQELL**ELDKW**ASLW
MPER3	LL**ELDKW**ASLW*NWFD*
MPER4	**DKW**ASLW*NWFDIT*NW
MPER5	SLW*NWFDIT*NWLWYI
MPER6	*WFDIT*NWLWYIKIFI
MPER-TM1	...ELL**ELDKW**ASLW*NWFDIT*NWLWYIK...
Mutant 3	...ELL_A_LDKWASLW*NWFDIT*NWLWYIK...
Mutant 4	...ELLE_A_DKWASLW*NWFDIT*NWLWYIK...
Mutant 5	...ELLEL_A_KWASLW*NWFDIT*NWLWYIK...
Mutant 11	...ELL**ELDKW**ASLW_A_*NFDIT*NWLWYIK...
Mutant 12	...ELL**ELDKW**ASLWN_A_*FDIT*NWLWYIK...
Mutant 13	...ELL**ELDKW**ASLWNW_A_*DIT*NWLWYIK...

Fig. 3. Autoantigen microarray analysis of reactivity profiles of bNt anti-HIV-1 MAbs, APS MAbs and SLE serum

The HIV-1 bNt MAbs 2F5, 4E10 and 2G12, APS MAbs IS2, IS4, CL1 and CL15 (at indicated concentrations) and an SLE serum were used to probe slides. (a) Validation of the microarray platform using a panel of HIV-1 antigens and array control antigens. Positive control Abs include antihuman IgG (as all MAbs tested are isotype IgG) and human IgG (bound by the secondary Ab), whereas antihuman IgM and antimouse IgG/IgM are negative controls. The bNt anti-HIV-1 MAbs 2F5, 4E10 and 2G12 bind their cognate epitopes in the microarray but not to closely related HIV-1 antigens. Sequences of peptides and constructs are shown, with the core 2F5 and 4E10 epitopes in bold and bold italics, respectively; Ala substitutions in these epitopes are underlined. Cardiolipin is included as a control antigen because of conflicting reports of binding by MAbs 2F5 and 4E10; here only MAb 4E10 bound cardiolipin, and only at high concentration (35 µg/ml). (b) Full heat-map

representation of binding patterns of all MAbs and sera to all array antigens, including the control antigens in part (a) for comparison. All data are representative of three independent experiments. Color versions of both figures are included as Supplemental Fig. S7a and b and raw log2-transformed MFI values are presented in Supplemental Fig. S8, http://links.lww.com/QAD/A142.

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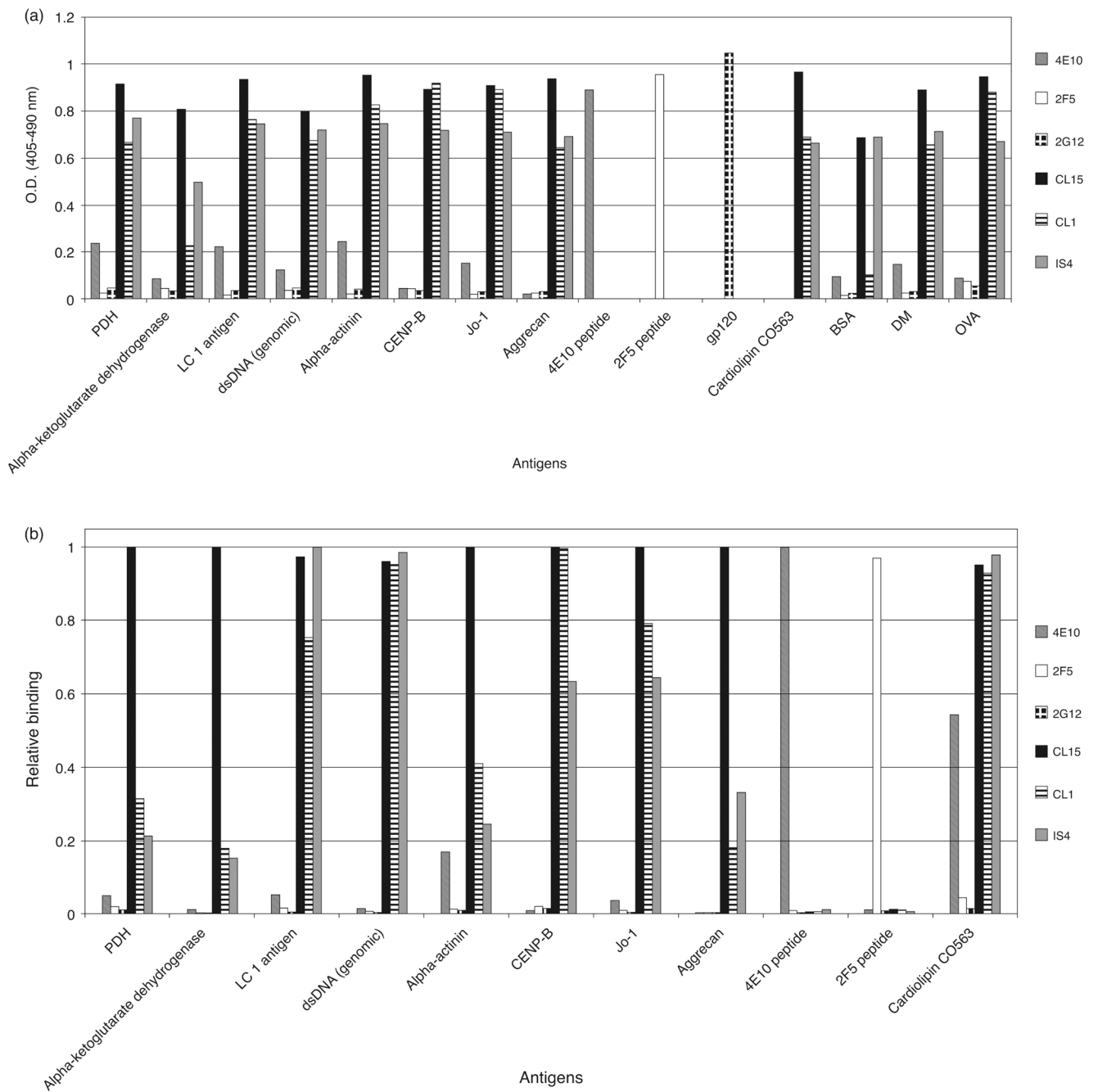
Fig. 4. Validation of the autoantigen microarray for detecting differences between HIV-1 bNt Abs and APS autoAbs

Side-by-side comparison of ELISA and microarray results for antigens that were bound significantly differently by anti-HIV-1 MAbs vs. APS MAbs. (a) MAbs 2F5, 4E10, 2G12, IS4, CL1 and CL15 (100 nmol/l) were diluted in 5% (v/v) fetal calf serum (FCS) and assayed against 1–2 μg of the indicated autoantigens immobilized in microplate wells. (b) Relative microarray binding of MAbs 2F5, 4E10, 2G12, IS4, CL1 and CL15 to selected autoantigens. For each antigen, the median fluorescence intensity (minus background) from the highest binding Ab in the microarray was set to 1.0, and all other Abs normalized to generate a scale based on the best-binding MAb.

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Table 1

List of antigens shown by significance analysis of microarrays to be bound differently by indicated monoclonal antibodies.

| Comparison: 2F5 vs. CL15 | | Comparison: 4E10 vs. CL15 | | Comparison: 2G12 vs. CL15 | | Comparison: 2FS vs. 4E10 | |
| CL15 >2F5 | | CL15 >4E10 | | CL15 >2G12 | | 4E10 >2F5 | |
Antigen	q-value	Antigen	q-value	Antigen	q-value	Antigen	q-value
Goat anti-mouse IgG and IgM	<0.001	Donkey anti-mouse IgM	<0.001	Goat anti-mouse IgG and IgM	<0.001	4E10 peptide	<0.001
H3N6 peptide	<0.001	MPER1	<0.001	E4.6 peptide	<0.001	H3N6 peptide	<0.001
gp120	<0.001	MPER4	<0.001	H3N6 peptide	<0.001	MPER5	<0.001
HIV-1 V3 loop (1/2)	<0.001	gp120	<0.001	MPER4	<0.001	Mutant 3	<0.001
COS-7 lysate	<0.001	HIV-1 V3 loop (1/2)	<0.001	gp41	<0.001	Mutant 4	<0.001
Actin	<0.001	COS-7 lysate	<0.001	HIV-1 V3 loop (1/2)	<0.001	Mutant 5	<0.001
Aggrecan	<0.001	Mutant 11	<0.001	COS-7 lysate	<0.001		
Alpha-actinin	<0.001	Actin	<0.001	MPER-TM1	<0.001		
Alpha-ketoglutarate dehydrogenase	<0.001	Aggrecan	<0.001	Mutant 12	<0.001		
Beta-2-glycoprotein I	<0.001	Alpha-ketoglutarate dehydrogenase	<0.001	Mutant 13	<0.001		
CBir1 flagellin (duplicate)	<0.001	Beta-2-glycoprotein I	<0.001	Actin	<0.001		
Cardiolipin C0563	<0.001	CBir1 flagellin (duplicate)	<0.001	Aggrecan	<0.001		
CENP-A	<0.001	CENP-A	<0.001	Alpha-actinin	<0.001		
CENP-A (duplicate)	<0.001	CENP-A (duplicate)	<0.001	Alpha-ketoglutarate dehydrogenase	<0.001		
CENP-B	<0.001	CENP-B	<0.001	CBir1 flagellin (duplicate)	<0.001		
Collagen type II	<0.001	Collagen type II	<0.001	Cardiolipin C0563	<0.001		
Collagen type VI	<0.001	Collagen type VI	<0.001	CENP-B	<0.001		
Collagen type VIII	<0.001	Collagen type VIII	<0.001	Collagen type II	<0.001		
Collagen type IX	<0.001	Collagen type IX	<0.001	Collagen type VI	<0.001		
dsDNA (plasmid)	<0.001	dsDNA (plasmid)	<0.001	Collagen type VIII	<0.001		
dsDNA (genomic)	<0.001	dsDNA (genomic)	<0.001	Collagen type IX	<0.001		
Fibrinogen type I-S	<0.001	Fibrinogen type I-S	<0.001	dsDNA (genomic)	<0.001		
Fibrinogen type IV	<0.001	GBM antigen dissociated	<0.001	dsDNA (plasmid)	<0.001		
GBM antigen dissociated	<0.001	HbV Core Antigen (HBcAg)	<0.001	Fibrinogen type I-S	<0.001		
Grp78	<0.001	Histone 2a	<0.001	Fibrinogen type IV	<0.001		
H. bilis flagellin	<0.001	Histone 2b	<0.001	GBM antigen dissociated	<0.001		

| Comparison: 2F5 vs. CL15 | | Comparison: 4E10 vs. CL15 | | Comparison: 2G12 vs. CL15 | | Comparison: 2F5 vs. 4E10 | |
| CL15 >2F5 | | CL15 >4E10 | | CL15 >2G12 | | 4E10 >2F5 | |
Antigen	*q*-value	Antigen	*q*-value	Antigen	*q*-value	Antigen	*q*-value
HbV Core Antigen (HBcAg)	<0.001	Histone 1	<0.001	HbV Core Antigen (HBcAg)	<0.001		
Histone 2a	<0.001	Histones	<0.001	Histone 2a	<0.001		
Histone 2 b	<0.001	HSP47 (Colligin)	<0.001	Histone 2b	<0.001		
Histone 1	<0.001	Ku (p70/p80)	<0.001	Histone 1	<0.001		
Histones	<0.001	LC 1 antigen	<0.001	Histones	<0.001		
HSP90	<0.001	Lysozyme	<0.001	HSP47 (Colligin)	<0.001		
Jo-1	<0.001	Mi-2	<0.001	HSP90	<0.001		
Ku (p70/p80)	<0.001	Myeloperoxidase	<0.001	Jo-1	<0.001		
LC 1 antigen	<0.001	Myeloperoxidase (duplicate)	<0.001	Ku (p70/p80)	<0.001		
Mi-2	<0.001	Ovalbumin	<0.001	LC 1 antigen	<0.001		
Myeloperoxidase (duplicate)	<0.001	PCNA	<0.001	Mi-2	<0.001		
nR2A peptide	<0.001	PDH (duplicate)	<0.001	Myeloperoxidase (duplicate)	<0.001		
PCNA	<0.001	PM/Scl-100	<0.001	nR2A peptide	<0.001		
PDH (duplicate)	<0.001	PM/Scl-100 (duplicate)	<0.001	PCNA	<0.001		
PL-12	<0.001	PR3-Aro	<0.001	PDH (duplicate)	<0.001		
PL-7	<0.001	Scl-70 truncated	<0.001	PL-7	<0.001		
PM/Scl-100	<0.001	SRC-3000	<0.001	PL-12	<0.001		
PM/Scl-100 (duplicate)	<0.001	ssDNA	<0.001	PM/Scl-100	<0.001		
Ribo P	<0.001	TPO	<0.001	PM/Scl-100 (duplicate)	<0.001		
Scl-70 truncated	<0.001	TPO (duplicate)	<0.001	Scl-70 truncated	<0.001		
SRC-3000	<0.001	U1-snRNP-A	<0.001	SRC-3000	<0.001		
ssDNA	<0.001	U1-snRNP-A (duplicate)	<0.001	ssDNA	<0.001		
TPO	<0.001			TPO	<0.001		
TPO (duplicate)	<0.001			TPO (duplicate)	<0.001		
U1-snRNP-A	<0.001			U1-snRNP-A	<0.001		
U1-snRNP-A (duplicate)	<0.001			U1-snRNP-A (duplicate)	<0.001		
U1-snRNP-C	<0.001			U1-snRNP-C	<0.001		

2F5 >CL15		4E10 >CL15		2G12 >CL15		2F5 >4E10	
Antigen	**q-value**	**Antigen**	**q-value**	**Antigen**	**q-value**	**Antigen**	**q-value**
2F5 peptide	<0.001	4E10 peptide	<0.001			2F5 peptide	<0.001
E4.6 peptide	<0.001	MPER5	<0.001			E4.6 peptide	<0.001
MPER2	<0.001					MPER2	<0.001
MPER3	<0.001					MPER3	<0.001
						MPER4	<0.001
						Mutant 11	<0.001
						Mutant 12	<0.001